10-27-2021

# The Multifaceted Role of CCAR-1 in the Alternative Splicing and Germline Regulation in *Caenorhabditis elegans*

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# The Multifaceted Role of CCAR-1 in the Alternative Splicing and Germline Regulation in *Caenorhabditis elegans*.

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
with a concentration in Cellular and Molecular Biology
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Date of Approval:
October 23rd, 2021

Keywords: RNA, heat shock, stress response, UAF-1

## DEDICATION

I would like to dedicate this dissertation to my parents, the late Hudson Lugano and Emily Lugano. Thank you for always working hard to support my dreams, for loving and believing in me. I also dedicate this to my life partner, Mathews Wakhungu, and our kids Ryan and Natutti. Thank you, Mathews, for pushing me to greater heights and supporting my ambitions. Your advice, love, encouragement, and support have been my rock during this time. I dedicate this dissertation to my extended family, Derrick and Becky; you both have a special place in my heart, Diana, for the love and support. Lastly, thank you to my friends Sylvia and Sherine for their emotional and mental support.

## ACKNOWLEDGMENTS

I would like to acknowledge my advisor Dr. Sandy Westerheide, for the encouragement, advice, and mentorship during my graduate studies. Thank you for the freedom to think, design, create experiments and your continued encouragement. I want to acknowledge my committee, Dr. Margaret Park, Dr. Meera Nanjundan, Dr. Younghoon Kee, and Dr. Eric Guisbert. I thank you for the feedback during committee meetings and the willingness to help. I especially want to thank Dr. Margaret Park for introducing me to alternative splicing and Bioinformatics. These techniques have shaped my graduate career, and I am immensely thankful. Next, I acknowledge my colleagues Andrew Deonarine and Lindsey Barrett. For teaching and sharing ideas with me, Andrew, I will never forget your contributions to my graduate studies. Lindsey, thank you for the assistance with experiments and your constructive criticism. Lastly, I acknowledge my graduate school colleagues turned to friends, Veena Subramanian and Ravneet Chhabra.

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## LIST OF ACRONYMS

CCAR-1- Cell cycle and apoptosis regulator 1
CCAR2/DBC1- The cell cycle and apoptosis regulator 2/ Deleted in Breast Cancer
*C.elegans – Caenorhabditis elegans*
*Ce*CCAR-1- *C. elegans* CCAR-1
LZ- Leucine Zipper
NLS- Nuclear Localization Sequence
ATM/ATR kinases- Ataxia telangiectasia mutated/ ATM and RAD-3 related kinases
SUMO- Small Ubiquitin Like Modifier
SENPs- SUMO Specific Proteases
SIRT1- Sirtuin 1
AROS- A Regulator of Sirtuin
AS- Alternative splicing
HSR- Heat Shock Response
HDAC- Histone Deacetylase
NAD+- Nicotinamide Adenine Dinucleotide
ADP-Ribose- Adenine Diphosphate Ribose
HSF-1 Heat Shock factor 1
HSP- Heat Shock Protein
HS- Heat Shock
HSE- Heat Shock Elements
PEPCK/PCK- Phosphoenolpyruvate Carboxykinase
HFD- High Fat Diet
ER- Estrogen Response
AR- Androgen Response
PARP1- Poly (ADP-Ribose) Polymerase 1
NHEJ- Non-homologous End Joining
HR- Homologous Recombination
DSB- Double-Stranded Breaks
L1- Larval Stage 1
L2- Larval Stage 2
L3- Larval Stage 3
L4- Larval Stage 4
YA- Young Adult
GA- Gravid Adult
GFP- Green Florescent Protein
RFP- Red Florescent Protein
YFP- Yellow Florescent Protein

EV- Empty Vector
RNAi- RNA interference
qRT-PCR- Quantitative Reverse Transcriptase PCR
NGM- Nematode Growth media
RNASeq- RNA Sequencing
DAVID- Database For Annotation, Visualization, and Integrated Discovery

## ABSTRACT

The Cell Division Cycle and Apoptosis Regulator (CCAR) family members are an enigmatic family of proteins regulating metabolism, cancer, apoptosis, DNA damage, and stress. Mammals have CCAR family members, CCAR1 and CCAR2/DBC1, which evolved from the founding family member CCAR-1/LST-3 expressed in *Caenorhabditis elegans*. Several studies have shown the importance of understanding these proteins' function in standard and altered physiological processes. Our studies aim to understand the genome-wide alternative splicing and germline regulation of *Caenorhabditis elegans* CCAR-1 in normal and heat shock conditions. Recently, mammalian CCAR family member CCAR2/DBC1 regulates the alternative splicing by forming a complex with ZNF326. This complex bind both RNA polymerase II and the ribonucleoprotein particle, integrating alternative splicing with RNA polymerase II elongation in A+T regions of the DNA. Additionally, *C. elegans* CCAR-1 affects the perlecan gene *unc-52* alternative splicing by allowing the excision of exon 17 during development. However, the CCAR-1 genome-wide alternative splicing regulation *in vivo* and during stress remains unknown. Also, the mechanism of action of this regulation is yet to be understood. Using RNA sequencing, we identify new alternative splicing targets with the deletion of CCAR-1. Furthermore, through mass spectrometry, we show the interaction of CCAR-1 with splicing factors of the spliceosome UAF-1 and UAF-2, unraveling a potential mechanism of action of CCAR-1. Also, our whole transcriptome RNA sequencing identifies CCAR-1's role in the gene expression of *Caenorhabditis elegans.* Our analysis shows that CCAR-1 regulates germline transcription, reproduction, lifespan, and DNA-damage-induced apoptosis, a role that is

previously unknown to the CCAR family. Overall, this work increases our knowledge of the CCAR family's role in alternative splicing and identifies a new function in the *C. elegans* germline.

# CHAPTER 1. INTRODUCTION

## The Discovery of the CCAR family of proteins

The human Cell Division Cycle Apoptosis Regulator (CCAR2) protein is essential in regulating metabolism, cancer progression, aging, apoptosis, and stress response pathways [1-8]. This gene was found on chromosome 8p21 in humans and, at the outset, gained interest due to being homozygously deleted in breast cancer [9, 10]. CCAR2 was previously named DBC1; however, it is now referred to as CCAR2 to distinguish it from the unrelated protein DBC1 (deleted in bladder cancer). Here, we will discuss the members of the CCAR family, their structure, domains, and the diverse molecular functions they regulate in the cell.

### *Members of the CCAR protein family*

In mammalian cells, CCAR2/DBC1 has a paralog CCAR1 (Fig 1.1). The phylogenetic analysis predicts that CCAR2 and CCAR1 evolved from a common ancestor, Caenorhabditis elegans CCAR-1, previously known as LST-3 (Fig 1.1) [9].

Mammalian CCAR2 and *C. elegans* CCAR-1 have 30% sequence similarity and share multiple conserved domains. *C. elegans* CCAR-1 (1150 AA) differs from CCAR2 (923 AA) due to the presence of a SAP domain and an extra coiled-coiled segment adjacent to the SAP domain (Fig 1.1). However, the SAP domain is present in the CCAR2 homolog, *C. elegans* CCAR-1 [9]. Most CCAR2 protein-protein interactions are in the N-terminal (1-264 AA) [11, 12]. The occurrence of conserved domains of the CCAR family allows the dynamic nature of the CCAR family of proteins [9]. These domains include the S1-like and nuclear localization signal (NLS) domains on the N terminus. The leucine zipper (LZ), nudix, and EF-hand domain

in the central region of the protein and the coiled-coiled domains in the C-terminus [11, 12 {Brunquell, 2014 #1]}.

The S1-like domain possesses RNA-binding capability; however, investigations on whether it binds to RNA are still underway [9]. In addition, this is next to a presumed nuclear localization domain (NLS) that plays an essential role in regulating post-translational modifications [8, 13].

Central to the protein structure are the LZ domain and the Nudix domain. The LZ domain regulates various cellular pathways, including the NFκB family members, RelB, and p52 [5]. The Nudix domain acts as a sensor for CCAR2, allowing the binding of NAD+ metabolites and the regulation of SIRT1 [9]. In addition, this domain is a catalytically inactive holdase [1].

On the C terminus, there is the EF-hand domain and the coiled-coiled domain. The EF-hand domain acts as an inactive variant of the calcium-dependent regulation of multiple cellular processes [14]. The coiled-coiled domain is responsible for protein-protein interactions [4, 9]. Lastly, The SAP domain is involved in transcriptional regulation, DNA repair, RNA processing, and apoptotic chromatin degradation of nuclear proteins. Several DNA damage repair genes such as PARP-1, Ku70, RAD18, and UVS2 have SAP domains [15-17]. The occurrence of a SAP domain suggests a potential role of the CCAR family in DNA repair, specifically mammalian CCAR1 and *C. elegans* CCAR-1, which both contain this domain (Fig 1.1).

CCAR proteins are intrinsically disordered, allowing structural flexibility. Mammalian CCAR2 and CCAR1 have 41% disordered residues, while *C. elegans* CCAR-1 has 61% of disordered residues [9]. These intrinsically disordered regions are sites of protein interactions,

modifications, and substitution rates [9, 11]. A further structural analysis predicts that mammalian CCAR2 and *C. elegans* CCAR1 disordered regions facilitate many protein-protein interactions [9]. Considering the similarities of the CCAR family structure in mammals and in *C. elegans*, it is expected that there are further similarities in molecular function.

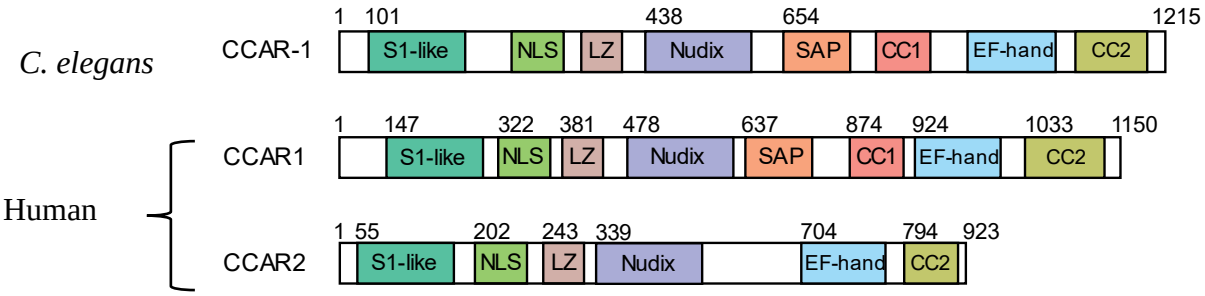


**Figure 1.1: A schematic of CCAR family member structures in various species.** Human CCAR2 and CCAR1 show conserved domains to C. elegans CCAR-1. These include the S1-like domain, the nuclear localization sequence, LZ domains, nudix domain, EF-hand, and coiled-coiled domains. Human CCAR-1 and C. elegans CCAR-1 share a common SAP domain.

***The Posttranslational modifications of the CCAR family***

CCAR family of proteins are subject to various post-translational modifications (PTMs). These PTMs include acetylation, phosphorylation, and sumoylation.

Acetylation is an esterification process that facilitates the adding an acetyl group to a protein, affecting its synthesis, stability, and localization. Most proteins are acetylated, affecting critical physiological processes such as apoptosis, metabolism, and cancer [18, 19]. CCAR2 is a binding partner to acetyltransferase CBP using multidimensional protein identification analysis [20]. The CCAR2 N terminus interacts with both the N and C terminus of CBP, regulating its E4 ligase activity.

Phosphorylation is the adding a phosphate group to a protein, which primarily activates it [21]. For example, CCAR2 phosphorylation occurs through the ATM/ATR pathway at Thr454, which works through its target Chk2 kinase and REGγ proteasome activator to induce p53-mediated apoptosis [22].

Another PTM is sumoylation, which involves the conjugation of a small ubiquitin-like modifier (SUMO) to proteins. This modification involves a three-step enzymatic process involving E1 activating enzyme, E2 conjugating enzyme, and E3 ligases. A family of SUMO-specific proteases (SENPs) can reverse this sumoylation.

CCAR2 sumoylation by SUMO2/3 and PIAS3 E3 ligase occurs during genotoxic stress [23]. CCAR2's sumoylation leads to the increase in transactivation of p53. However, an increase in the expression of desumoylase SENP1 can reverse this process [24].

Understanding the post-translational modifications of the CCAR family is needed to appreciate its function in changing physiological conditions.

**The molecular functions of the CCAR-1 family of proteins**

The molecular functions of the CCAR family members are an area of active research. These genes facilitate several molecular functions ranging from metabolism, cancer, and stress response pathways. Here, we will discuss the various roles of the CCAR family in regulating these physiological processes.

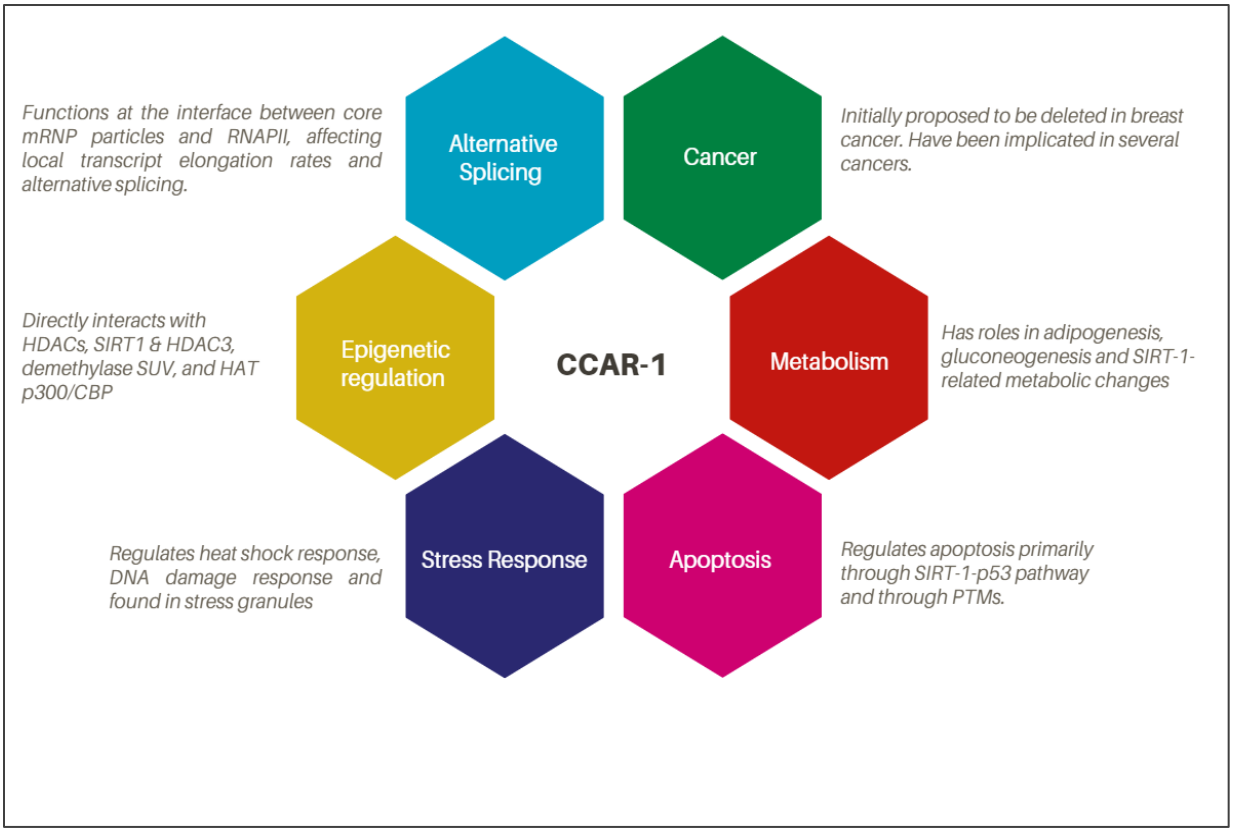


**Figure 1.2: A diagram of the molecular functions of CCAR family members.** CCAR family members regulate multiple cellular processes. These processes include cancer, metabolism, apoptosis, stress response, epigenetic regulation, and alternative splicing.

## The role of CCAR in epigenetic regulation

CCAR family members regulate the cell's epigenetics by working with chromatin modifiers SIRT1, HDAC3, CBP1/p300, and SUV39H1. Here we will discuss the interaction of each of these proteins with CCAR family members and its effect on the cell.

### *The role of CCAR in regulating deacetylase SIRT1*

The CCAR family's most studied function is its interaction with SIRT1, an NAD+ dependent histone and non-histone deacetylase that regulates processes such as apoptosis, DNA damage, stress response, metabolism aging, longevity, and cancer [10, 25]. SIRT1's regulation is mainly through transcription factors p53, FOXO and PGC1α. CCAR2 binds to the

catalytic domain of SIRT1, forming a stable complex that inhibits SIRT1 deacetylase activity and functions both *in vivo* and *in vitro [25]*.

Protein Kinase A, an AMP-activated protein kinase, increases the dissociation of CCAR2 and SIRT1 in an NAD+ independent manner [26]. CCAR2 regulates SIRT1's activity by sensing soluble products or substrates of the NAD-dependent deacetylation reaction [1]. An example of these soluble products is ADP-ribose. This binding of the ADP-ribose derivatives to the SIRT1-CCAR2 complex is through sensing the presence of SIRT1 and specifically downregulating it. The proximity of the SIRT1 to the coiled-coiled domains and inactive nudix domain on CCAR2 supports this process [1].

This interaction with sirtuin is conserved in *C. elegans*. *C .elegans* CCAR-1 negatively regulates the heat shock response through interaction with *SIR-2.1*, with CCAR-1 knockdown promoting stress resistance, motility, and longevity in a *SIR-2.1* dependent manner [2].

Overall, CCAR family members have a crucial role in regulating SIRT1 function, affecting its physiological processes.

***The role of CCAR in regulating histone acetyltransferase CBP1-p300***

The CREB-binding protein (CBP) is a histone acetyltransferase that regulates its target genes by activating the promoter region and transcriptional activation [27]. CBP1 has both histone and non-histone targets and is known to be a paralog of p300. CBP1/p300 plays a dual role in tumor suppressor p53 regulation through promoting p53 polyubiquitination and degradation in normal conditions [28]. However, changes in cellular conditions can promote p53 stability and transactivation.

A multidimensional protein identification analysis identified CCAR2's binding to CBP1 [20]. The CCAR2 N terminus interacts with both the N and C terminus of CBP1,

regulating its E4 ligase activity. Due to this binding, CCAR2 suppresses nuclear p53 ubiquitination in the nucleus and increases p53 apoptotic responses. These findings show that CCAR2 interacts with a histone acetylase CBP1/p300, crucial in regulating genotoxic-induced p53 functions [20].

***The role of CCAR in regulating deacetylase HDAC3***

Histone deacetylases (HDACs) are involved in the deacetylation of histone and non-histone proteins such as transcription factors, structural proteins, and enzymes. Therefore, these enzymes have a central role in facilitating chromatin structure and gene regulation, metabolism, and cancer. Furthermore, HDACs are known to have overlapping functions in the cell. For example, sirtuins are members of class III HDACs and have overlapping functions with HDAC3. Furthermore, HDAC3 also binds to CCAR2, forming a complex that affects its deacetylase function [3].

***The role of CCAR in regulating methyltransferase SUV39H1***

CCAR2 interacts with SUV39H1, a histone H3K9-specific methyltransferase essential for heterochromatin formation, gene expression regulation, and senescence induction in premalignant cells [7]. SUV39H1 forms a complex with SIRT1 disrupted by CCAR2 binding to its catalytic domain, inhibiting its ability to methylate histone H3.

Altogether, we see a crucial role of CCAR proteins in epigenetic regulation, mainly through binding with chromatin modifiers and affecting their activities.

***The role of CCAR in stress responses***

The CCAR-1 family has a crucial role in stress response. This section explores the various stress responses in which CCAR is involved and the role these family members play during stress.

***The role of CCAR in regulating the heat shock response***

CCAR2 regulates the heat shock response [2, 29]. The heat shock response (HSR) is a conserved pathway in bacteria, yeast, plants, and animals that are critical for maintaining the protein homeostasis of the cell. Exposure to proteotoxic stressors, such as elevated temperature, heavy metals, chemical toxicants, and oxidative stress, leads to the activation of the heat shock transcription factor 1 (HSF1). This transcription factor trimerizes and localizes to the nucleus to induce the expression of molecular chaperones. These molecular chaperones include HSP90, HSP70, HSP40/DNAJ, chaperonin HSP60, and small heat shock proteins and aid in the refolding or degradation of damaged proteins to maintain the proteome [30].

SIRT1 deacetylates HSF1 at its DNA binding domain and allows for prolonged DNA binding and expression of its molecular chaperone targets [31]. CCAR2 forms a protein-protein interaction with SIRT1 inhibiting its deacetylation of HSF1 [29]. As a result, CCAR2's binding to SIRT1 also decreases HSF1's binding to its target molecular chaperone genes [29].

This interaction is conserved in *C. elegans*, where CCAR-1 regulates the HSR in a *SIR-2.1*- (SIRT1 *C. elegans* homolog) dependent manner. CCAR-1 also affects HSF-1 (HSF1 *C. elegans* homolog) acetylation and binding to its target gene promoters [2]. Knockdown of CCAR-1 works through *SIR-2.1* to promote stress resistance, motility, longevity, and proteostasis at the whole organism level [2].

***The role of CCAR in regulating the DNA damage***

CCAR2 is a key player in the DNA damage response (Fig 1.3). DNA damage frequently occurs in the cell following many types of stressors, including UV damage, genotoxic stress, replication errors, or toxic metabolites like reactive oxygen species [22, 23, 32, 33]. To prevent this DNA damage from being replicated into the genome, the DNA damage

response (DDR) is activated. This activation includes many signaling pathways that combat the damage the DNA accumulates. Of these types of DNA damage, double-strand breaks (DSBs) in the DNA, where both strands of the DNA break, are the most toxic and can have a wide array of adverse effects for the cell. The DDR contains two pathways to resolve DSBs: nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ directly ligates the DNA ends of the broken DNA using endonucleases, whereas HR requires a homologous DNA strand as a template to synthesize the small section of DNA around the DSB [34].

CCAR2 is required for HR, as silencing CCAR2 reduces the efficiency of the HR pathway after DNA damage [35]. However, CCAR2 is recruitment to DNA damage sites limits the extent of DNA end resection of HR [36]. Also, CCAR2 works to repair heterochromatin DNA lesions [22] and inhibits poly (ADP) ribose polymerase (PARP1), an enzyme critical to multiple DNA repair pathways. When bound to CCAR2, PARP1 activity decreases, leading to DNA damage accumulation [37].

Several DNA damage repair genes such as PARP-1, Ku70, RAD18, and UVS2 contain a SAP domain [15, 16]. This domain again suggests a potential role of the CCAR family in DNA repair, specifically mammalian CCAR1 and *C. elegans* CCAR-1, which both contain this domain (Fig 1.1).

Overall, CCAR2 has complex roles in the DNA damage response pathway.

***The role of CCAR in regulating other stress responses***

The CCAR family has been implicated in other stress responses as well. CCAR1 localizes to and regulates the formation of RNA stress granules which contain subsets of sequestered mRNAs after stress for translational silencing [38]. Similarly, CCAR2 regulates

mitochondrial stress by binding to the heat shock protein HSP60 (Fig 1.3). Upon mitochondrial stress, CCAR2 localizes to the mitochondria, where it binds to HSP60 and promotes cell survival [39]. While CCAR1 and CCAR2 have a role in RNA stress granules and mitochondrial stress, the mechanisms underlying this regulation the following stress are still largely unknown.

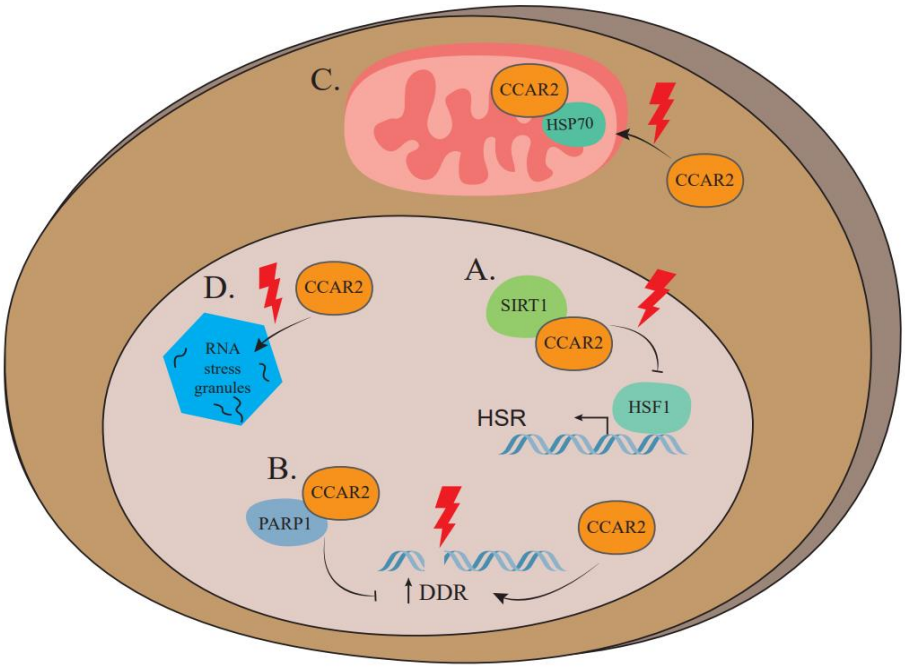


**Figure 1.3: The involvement of the CCAR family in various stress response pathways.** This figure shows how CCAR family member CCAR2 regulates various stress responses. A. CCAR2 regulates the heat shock response in a SIRT1-dependent manner, affecting HSF-1 acetylation and binding to HSP promoters. B. CCAR2 regulates the DNA-damage response by negatively regulating PARP-1. C. CCAR2 regulates mitochondrial stress through binding to HSP60, promoting cell survival. D. CCAR1 localizes and regulates the formation of RNA stress granules, which contain sequestered RNA and proteins after stress.

***The role of CCAR in cancer***

CCAR2 initially gained interest due to being homozygously deleted in breast cancer cells, giving it the initial name Deleted in Breast Cancer 1 (DBC1). However, irregular CCAR2 expression occurs in several cancers, including breast, gastric, colorectal, hepatocellular carcinoma, and leukemia [6]. Due to these irregularities, the correlation between CCAR2 and cancer progression and prognosis remains unclear [40].

CCAR2's role in cancer is primarily associated with its interaction with the deacetylase SIRT1. As previously stated, CCAR2 is a negative regulator of SIRT1 through binding to the catalytic domain and inhibiting its ability to deacetylate its targets. SIRT1's inhibition of p53-mediated apoptosis is key to cell growth and survival of cancer cells. CCAR2 acts as a tumor suppressor through the binding of the catalytic domain of SIRT. This binding leads to the inhibition of the deacetylase activity on p53. The relationship between CCAR2 and SIRT1 is essential in the prognosis of several cancers, including adenocarcinoma [41]. In a study of breast cancer tissue from 28 patients, the overall expression of CCAR2 and SIRT1 increased compared to normal tissues. However, the correlation between SIRT1/CCAR2 expression was weaker in tumor tissues compared to normal tissue. This study suggests that the correlation between the two genes can be a prognosis indicator in breast cancer [42].

CCAR2's relationship with estrogen and androgen receptors shows its ability to act as a tumor promoter. Estrogen receptor alpha (ERα) has a vital role in breast cancer progression and treatment. In breast cancer, CCAR2 enhances the transcriptional activities of ERα, hence playing a role in breast cancer progression. Several factors act to support this interaction between CCAR2 and ERα, including Ajuba. This nuclear export factor recruits CBP/p300 and CCAR2 forming a tertiary complex that increases the acetylation of ERα, hence enhancing its

target gene expression and breast cancer cell progression [43]. Additionally, CCAR2 inhibits SIRT1-mediated deacetylation and restores optimum recruitment of ERα to target gene promoters [44]. Several drug targets such as tamoxifen, anastrozole, and letrozole target the estrogen signaling pathway in cancer [45].

In contrast to the pro-apoptotic CCAR2/SIRT1 interaction during cancer, CCAR2 is pro-survival through its interaction with mitochondrial HSP60, allowing the survival of neuroblastoma cells following rotenone-induced mitochondrial stress [46]. CCAR2 forms a complex with HSP60 through the pro-survival protein survivin, which mediates anti-apoptotic effects by binding apoptosis regulating factors and inhibiting caspase activities [46]. Further exploration of the role of this family of genes in cancer is key to understanding its implications.

***The role of CCAR in regulating metabolism***

***<u>The role of CCAR in regulating metabolism through SIRT1</u>***

The cell's metabolic state regulates SIRT1's activity during aging and metabolic conditions [3, 26, 29]. SIRT1 activity decreases with metabolic diseases such as liver steatosis and high caloric diets and increases during starvation [3]. CCAR2 binds to the catalytic domain of SIRT1, preventing substrate binding to SIRT1 and, hence inhibiting its activity [10, 25]. Therefore, CCAR2 acts as a negative regulator of SIRT1 during various metabolic conditions.

The CCAR2-SIRT1 interaction is regulated during high-fat diet (HFD) feeding and starvation, where changes in these metabolic conditions are associated with changes in the CCAR2-SIRT1 interaction [3]. Knockdown of CCAR2 showed increased SIRT1 protection against the development of experimental liver steatosis and inflammation in mice liver samples. Furthermore, the knockdown of CCAR2 also showed beneficial effects on the development of cellular lipid accumulation in a SIRT1-dependent manner. Thus, this CCAR2-

SIRT1 interaction is a potential target for metabolic diseases such as liver steatosis and diabetes [4].

***The role of CCAR in regulating gluconeogenesis***

CCAR2 is a novel regulator of gluconeogenesis. Liver gluconeogenesis is a critical energy-providing process during fasting, maintaining systemic glucose levels in health and disease. This process, however, needs tight regulation because it could lead to glucose intolerance.

Phosphoenolpyruvate carboxylase (PEPCK) expression plays a critical role in the modulation of gluconeogenesis. The regulation of gluconeogenesis mainly targets the expression of the enzyme PEPCK/PCK1. This enzyme catalyzes a committed step in the gluconeogenic pathways. PEPCK regulation is by the glucagon-cAMP and insulin-AKT pathways, which are centrally controlled by nuclear receptors Reverbs. Specifically, Rev-erbα is a transcriptional repressor of PEPCK in liver cells and has a key role in hepatic metabolism. CCAR2, as previously mentioned, regulates Rev-erbα through stabilizing the protein and preventing degradation. In addition, CCAR2 also regulates circadian rhythms dependent on Rev-erbα. Interestingly, CCAR2 binds to both Reverb-α and SIRT1making it a candidate in the regulation of PEPCK. This work shows that CCAR2 is a novel reg of PEPCK expression and gluconeogenesis by a mechanism that involves Rev-erbα and SIRT1 [3].

***The role of CCAR in regulating adipogenesis***

Adipogenesis is a multiple-step process that leads to the formation of adipocytes. CCAR2 interacts with STAT5A (signal transducer and activator of transcription 5A), a transcription factor with a vital role in adipocyte development and function. CCAR2 regulates STAT5A's function by decreasing TNFα-induced lipolysis. With reduced CCAR2 expression,

free fatty acids are released from adipocytes and the knockdown of CCAR2 increases Glut4 expression [47]. Consequently, the loss of CCAR2 promotes metabolic health. These roles show that CCAR2 is also involved in adipocyte inflammation and is a possible marker of human adipose tissue senescence [48].

***The role of CCAR in transcriptional regulation***

***<u>The role of CCAR in the regulation of chromatin remodeling enzymes.</u>***

Chromatin remodeling enzymes SIRT1, HDAC3, and SUV39H1 act as transcriptional regulators to the FOXO family, PGC1α, p53, MEF2D, and NF-κB [8, 10, 25, 31, 44, 49].

The most widely studied chromatin remodelers that interact with CCAR2 are the class II histone deacetylase, SIRT1 [10, 25]. CCAR2 forms a stable complex with SIRT1 through binding to its catalytic domain, leading to the negative regulation of its transcriptional activity. This change in transcriptional activity affects the FOXO family, PGC1α, metabolic pathways, and p53-associated apoptosis pathways [50].

Other than SIRT1, CCAR2 negatively regulates histone deacetylase HDAC3, affecting its localization and transcriptional activity. This change in transcriptional activity, like SIRT1, affects p53, MEF2D, and NF-kB transcriptional pathways. In addition, CCAR2 interacts with acetyl transferase p300/CBP, impacting numerous gene acetylation and transcription landscapes [3].

Lastly, CCAR2 interacts with the H3K9-specific methyltransferase SUV39H1, which forms a complex with SIRT1, affecting SIRT transcriptional activity [7].

This section shows CCAR2's interaction with various chromatin remodeling enzymes, which eventually affects the transcriptional outlook of the cell.

***The role of CCAR in regulating Nuclear Receptor Complexes***

Nuclear receptor family proteins such as the estrogen receptor, androgen receptor, and Reverb-α are involved in the transcriptional genes that regulate metabolism, development, and proliferation [51, 52]. These receptors often work with co-activators that act as a bridging factor between the receptor and the basal transcriptional machinery. Several studies identify CCAR2 as an interaction partner and co-activator of nuclear receptor family proteins, affecting their transcriptional functions [44, 53, 54].

Androgen receptors (AR) mediate several roles of androgens, including the development and maintenance of male reproductive functions and the biology of prostate cancer. Coactivators and corepressors mediate the transcriptional regulation of AR, similar to other nuclear receptors. For example, CCAR2 is an AR coactivator in a ligand-stimulated manner and facilitates AR transcription. This interaction is through the ligand-binding domain of AR and CCAR2's N-terminal region [55].

To begin with, CCAR2 ensures optimal transcriptional activation of AR target genes in LNCaP cells and the binding of AR to a PSA enhancer [56]. Also, recombinant CCAR2 facilitated AR DNA binding activity in gel mobility shift assay in an *in vitro* study. Additionally, concomitant expression of CCAR2 and AR in advanced human malignant tumors has been reported [50, 56, 57].

Estrogen is the native ligand to estrogen receptors (ERα & ERβ) and has diverse roles in developing and maintaining the female reproductive system and the landscape of breast cancer [45, 49, 58]. CCAR1 interacts with ERα through the p160 co-activator and a mediator complex [49]. CCAR1 associates with components of the mediator complex and facilitates the recruitment of these complexes to the promoters of target genes. This binding provides a

physical link between the p160 co-activator and the mediator complex. Interestingly, CCAR2 associates and cooperates synergistically with CCAR1 to enhance ERα function and is required to express a subset of ERα target genes and estrogen-dependent growth of breast cancer cells [54].

Additionally, CCAR2 inhibits SIRT1-mediated deacetylation and restores optimum recruitment of ERα to target gene promoters [49]. SIRT1 represses the co-activator synergy between CCAR1 and CCAR2 by competing with CCAR1 for binding to CCAR2 and thereby disrupts the CCAR2-CCAR1 interaction. Usually, CBP/p300 acetylates ERα at K266/268 and K302/303, enhancing DNA binding and transcriptional activity [58]. SIRT1 deacetylates ERα inhibiting these functions [49].

Another way CCAR2 regulates the ERα receptor is by forming a tertiary complex with nuclear export factor ABUJA and with CBP-1/p300 in breast cancer cells. This binding increases the acetylation of ERα and enhances its target gene expression and cancer progression [43].

Lastly, CCAR2 has a role in the circadian rhythm by binding to the receptor Reverb-α both *in vivo* and *in vitro*. The binding of CCAR2 enhances Reverb-α stability by preventing its ubiquitination and degradation [53].

This section highlights the crucial role of the CCAR family in the nuclear receptor family function, which in turn affects their transcriptional abilities.

***The role of CCAR in regulating alternative splicing and RNA binding***

An emerging function of the CCAR family is its role in alternative splicing. Alternative splicing is a conserved mechanism that is responsible for protein diversity in the cell. This process allows the formation of alternate forms of messenger RNA with the help of the

spliceosome machinery and splicing factors [59, 60]. Here, I highlight findings of the CCAR family in mammals and *C. elegans* and discuss the advantages of using the worm to study the CCAR protein family's role in RNA binding.

***The role of CCAR in regulating alternative splicing***

The mammalian member of the CCAR family, CCAR2, has been implicated in the alternative splicing of several genes. CCAR2 forms a complex with zinc factor ZNF326 called the DBIRD complex. This complex binds to both RNA polymerase II and an RNP particle [61], hence integrating both alternative splicing with RNA polymerase II elongation in A+ T-rich regions of the DNA. Interestingly, this process is independent of SIRT1 [61].

In *C. elegans*, CCAR-1 affects hemidesmosome biogenesis through the alternative splicing of *unc-52*/perlecan. This UNC-52/perlecan protein is an extracellular matrix (ECM) ligand, which CCAR-1 regulates its exon 17 excision during development. To do this, CCAR-1 physically interacts with a splicing factor HRP-2/hnRNP R that mediates the alternative splicing of the *unc-52 gene* [62]. However, the role of CCAR-1 in the alternative splicing in *C. elegans* is only just emerging and remains largely unstudied. Further studies are examining how CCAR-1 functions in global alternative splicing would aid in understanding CCAR-1's functions in RNA regulation.

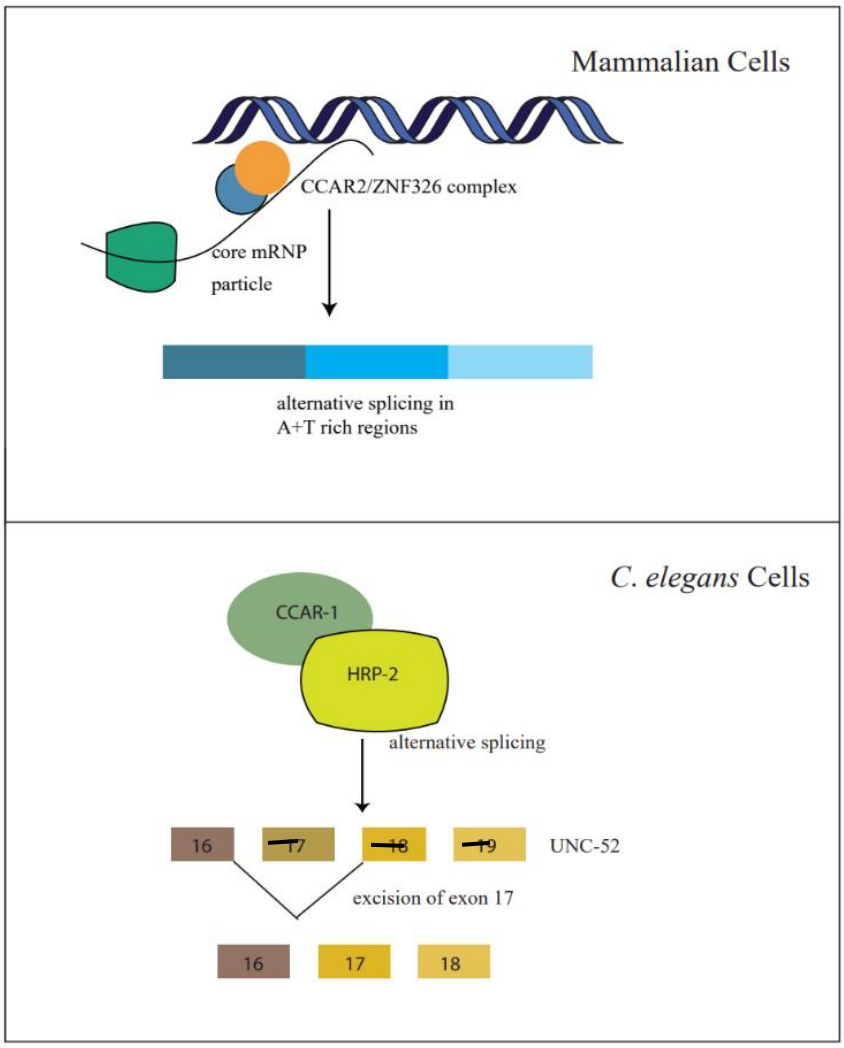


**Figure 1.4: The CCAR protein family's role in alternative splicing of genes.** A. In mammalian cells, CCAR2 binds to zinc factor ZNF326, which acts as an interface between RNA polymerase II and the core RNP particle. This binding affects transcription elongation rate and alternative splicing in A+T rich regions of the genes. B. *C. elegans* CCAR-1 regulates the alternative splicing of the perlecan gene, UNC-52, regulating the excision of exon 17.

***The role of CCAR in binding to RNA***

While CCAR2 regulates the alternative splicing of genes, it is still largely unclear if it binds directly to RNA and the domains involved. The first study to examine CCAR-RNA binding was a quantitative proteomics analysis used to characterize the protein interactome of a long non-coding RNA (lincRNA), MALAT1. MALAT1 is a widely expressed lincRNA that

regulates RNA processing and gene transcription [37]. MALAT1 physically interacts with CCAR2 in the N-terminus but not necessarily through the S1-like domain [9]. This binding of MALAT1 to CCAR2 affects the interaction and activity of SIRT1 and p53 during cell proliferation and apoptosis [37].

In another study, the CCAR1 5' UTR binds to the micro-RNA miR-1254 in a miRNA:mRNA host relationship [63]. Micro-RNAs bind to miRNA binding sites on their target RNAs, leading to a mutual regulation of expression. Specific base-pairing interactions between the 5' end of the miRNA (seed region) and the miRNA response elements within the coding region or untranslated regions (UTRs) mRNAs mediate the miRNA:mRNA targeting process. This targeting leads to mRNA destabilization/translational inhibition. The regulation of miRNAs:mRNA regulates various processes, including cancer progression. CCAR1 5'UTR binding to miRNA-1254 overrides tamoxifen resistance, an estrogen receptor modulator used in Erα-positive breast cancer patients in clinical trials [63].

Another study used photoreactive nucleoside UV cross-linking and oligo dT affinity purification to identify the mRNA-bound proteome and globally map the protein-RNA interactions sites in mammalian cells. This quantitative mass spectrometry identified close to 800 proteins, a large number being neither previously annotated nor predicated by computational methods to interact with RNA [64]. They validated 19 of these proteins, with one of them being CCAR2. This study further suggests the ability of CCAR2 to bind to RNA.

Furthermore, CCAR2 indirectly interacts with circular RNA, circZKSCAN1, in hepatocellular carcinomas (HCC) [65]. CircZKSCAN1 suppressed cell stems in HCC by regulating the function of the RBP fragile X mental retardation protein (FMRP), whose downstream target and co-activator is CCAR1. Further studies on CCAR1 and CCAR2

interaction with RNA and RNA binding proteins are required to understand if the primary role in alternative splicing is direct binding to RNA.

***The role of CCAR in regulating apoptosis***

Apoptosis is an essential physiological process that causes cell death due to stress, DNA damage, and lack of nutrients. The CCAR family regulates apoptosis mainly through the tumor suppressor gene p53. This section highlights the role of the CCAR family in p53-mediated apoptosis, specifically through changes in post-translational modifications.

***The role in regulating p53-mediated apoptosis through phosphorylation.***

p53 is a tumor suppressor gene known to regulate cell function and play a key role during cancer progression [66, 67]. P53 stability is regulated by post-translation modifications such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and neddylation [68].

To begin with, CCAR2 inhibits SIRT1, hindering its deacetylation activity on p53 and thus increasing apoptosis after DNA damage. CCAR2's phosphorylation by ATM/ATR enhances its binding to SIRT1, promoting p53 activation and apoptosis. The ATM/ATR pathway works through its target Chk2 kinase and REGγ proteasome activator to induce SIRT1 inhibition by CCAR2 and hence induce p53-mediated apoptosis [22].

Additionally, CCAR2 binds to casein kinase 2 (CK2α) in gastric cancer cells [69]. CK2α is a highly conserved serine/threonine kinase that has diverse roles in tumorigenesis. These roles are mainly through enhancing tumor-promoting signals such as the Wnt/Beta-catenin pathway, AKT pathway, NFκB, c-Myc, and suppressing tumor suppressor genes such as PTEN and p53. CK2α binds to phosphorylated CCAR2 in human gastric cells, promoting

the progression of gastric carcinoma and shortening the survival of gastric carcinoma patients [69]. This interaction also affects gastric cancer cells migration and proliferation.

***The role of CCAR in regulating p53-mediated apoptosis through sumoylation***

Sumoylation of CCAR2 increases p53-mediated apoptosis [23]. During genotoxic stress, CCAR2 sumoylation is by SUMO2/3 and PIAS3 E3 ligase. This sumoylation increased CCAR2's affinity and binding to SIRT1, inhibiting its function [23]. Thus, CCAR2 sumoylation dramatically blocks p53 interaction with SIRT1, which leads to increased acetylation of p53 and apoptosis. An increase in the expression of desumoylase SENP1 can counteract this process [23].

Interestingly, the ATM/ATR pathway's phosphorylation of CCAR2 promotes its sumoylation and inhibition of SIRT1 during genotoxic stress conditions [22, 23, 33]. The proposed model for this interaction is that in normal physiological conditions, CCAR2 is bound to SENP1 and hence desumoylated.

However, during genotoxic stress, CCAR2 is phosphorylated at Thr454 by the ATM/ATR kinases. This phosphorylation switches binding partners from SENP1 to SUMO2/3 and PIAS3, leading to sumoylation. CCAR2's sumoylation increases affinity and binding to SIRT1, displacing p53 and allowing more acetylation. In addition, the binding of CCAR to SIRT1 improves its transactivation and induction of apoptosis [23]. Remarkably, SENP1 desumoylates both SIRT1 and CCAR2; however, the cell fates in these circumstances are entirely different. CCAR2 sequesters SENP-1 from SIRT1 in normal physiological conditions, maintaining its sumoylated state for p53 deacetylation [23]. However, during genotoxic stress, CCAR2 is sumoylated by PIAS3, leaving SENP1 available to desumoylate SIRT1.

The regulation of CCAR2 by SENP1 occurs in high glucose conditions of bovine retinal pericytes. The sumoylation of CCAR2 was through the inhibition of SENP1, which contributed to apoptosis [24]

This section highlights the key role those post-translational modifications have on CCAR family members' role in apoptosis. This section shows the importance of PTMs on a protein, ensuring a mature protein and changing its function, localization, and stability.

## Caenorhabditis elegans

*Caenorhabditis elegans* was proposed as a model organism by Sydney Brenner to study development and the nervous system [70]. In this section, I will discuss the advantages, lifecycle, and impact on genetics that studies of the worm have contributed. Additionally, I will highlight the relevance to normal and disease state pathways that relate to humans.

### *Introduction to Caenorhabditis elegans and advantages as a model system for genetic studies*

*Caenorhabditis elegans* is a free-living nematode used in genetics studies over decades. The worm is key to discovering pathways such as the nervous system, apoptosis, and RNA interference, just to name a few, due to the numerous advantages compared to other genetics models [70-72].

First, *C. elegans* was the first multicellular organism to be fully sequenced in 1998. The sequencing revealed that 60-80 % of human genes have homologs in the worm [71, 72]. Hence, this model could study organelle structure and function, transcription, metabolism, stress response, and apoptosis. Consequently, the sequencing of the *C. elegans* genome has allowed the accessibility of a wealth of information on online platforms such as WormBase, WormBook, WormMethods, and Worm Breeders Gazette [71-75]. These platforms allow the worm community to share reagents and information to all areas of the world.

Secondly, *C. elegans* has ease of growth, maintenance, and a rapid lifecycle. The worms grow to adulthood between 3-3.5 days, depending on the temperature, and can then produce their eggs [70, 71, 73]. Remarkably, they can be frozen and recovered, allowing the preservation of several strains without accumulating genetic changes. A key discovered by Craig Mello and colleagues was RNA interference (RNAi), which uses double-stranded RNA (dsRNA) to reduce gene activity through soaking, injection, or feeding.[76, 77]. This technology has led to the formation of RNAi libraries that are readily available in bacterial stocks. RNAi has made genetic experiments using *C. elegans* easy to manipulate [76, 77].

Thirdly, *C. elegans* is transparent, enabling the ease of both labeling and observance of cells and tissues during microscopy. The transparency of the worm allows genetic screens of tagged proteins to identify mutants of various processes more straightforward to conduct [71, 72, 75].

Lastly, there are key discoveries in this model organism due to the high homology of genes between *C. elegans* and humans. These discoveries include aspects of Notch signaling [72], the complete wiring diagram of the nervous system [78], apoptosis genes discovery [79-81], the regulation of lifespan by the insulin pathway [82, 83], the first description of the role of RAS signaling [84-86], the first micro-RNA (*lin-4*) and its mRNA target (*lin-14*) [87], the introduction of GFP as a biological marker [88, 89], the first metazoan gene sequence [71, 73, 75], the discovery of RNA interference [76, 77], the discovery of the transgenerational inheritance of piRNAs [90, 91] and the discovery of the first nematode viruses [90, 91]. Also, studying the cellular fate of each cell in *C. elegans* has allowed the shape of all 302 neurons of an adult hermaphrodite to be determined, leading to discoveries in sexual dimorphism [78].

A few limitations of *C. elegans* as a research model is the lack of homologs of some human genes, lack of cell culture lines, and its small size, making it hard to manipulate tissues [72].

***The Caenorhabditis elegans lifecycle***

*C. elegans* worms exist as hermaphrodites and males; however, males exist at a very low frequency making most of the population hermaphrodites [72]. As previously mentioned, one of the main advantages of *C. elegans* is its rapid lifecycle. After fertilization, embryogenesis occurs between a sperm and an egg, leading to an impermeable eggshell that allows the embryo to be completely independent of the hermaphrodite [71, 72]. This embryo hatches to the first larval stage with 588 nuclei after 16 hours at 20˚C, and through the feeding of bacteria, it grows through larval stages L1-L4. Larval stage 1 (L1) is 16 hours long, and the other larval stages (L2, L3, and L4) are 12 hours long each. A period of sleep-like inactivity occurs after each larval stage, leading to a new outer collagenous layer. Twelve hours after the L4 molt, the newly formed adult hermaphrodite reproduces for 2-3 days. Interestingly, the mating of the hermaphrodites with males can produce offspring [71, 73].

During stressful growing conditions such as crowding or depletion of bacteria, L2 larva can undertake an alternative L3 lifecycle called the “Dauer,” which plugs its mouth and forms a cuticle surrounding the worm, preventing both eating and development [71, 72]. This stage ensures the survival of the worm for several months, specifically through enhancing resistance to chemicals and protection against environmental stresses. The reversal of the “Dauer” stage is by feeding the animals bacteria, allowing them to unplug their mouth and molt into L4 and adulthood [71, 73].

***The Caenorhabditis elegans genetics***

The *C. elegans* genome contains 20444 protein-coding genes in five chromosomes (I, II, III, IV & V) and the X chromosome [72, 75, 92]. *C. elegans* have no Y chromosome; hence the female is XX, and the male is XO. Compared to vertebrates, *C. elegans'* average gene size is smaller (approx. 3kb per gene) due to the occurrence of small introns [72, 75]. In addition, its chromosomes do not contain centromeres, and during mitosis, their spindles attach to more than one position of the chromosome. However, transcription, translation, chromatin remodeling, and posttranslational modifications are conserved, allowing critical discoveries in the *C. elegans* model [71, 72, 75].

The discovery of high throughput techniques such as microarrays and genome-wide sequencing has allowed the understanding of global genetic changes to be discovered [92]. These techniques have allowed understanding the transcriptional and post-transcriptional changes that are key to the proper specification, proliferation, and differentiation of cells. Additionally, it has permitted the understanding of global trends that provide a molecular framework to identify individual genes involved in specific functions. For example, *C. elegans* can identify specific gene expression changes using temperature-sensitive mutants and changes in expression during development [72, 93].

***The Caenorhabditis elegans germline***

As mentioned before, *C. elegans* has two sexual forms, hermaphrodite and males. These two sexes show anatomical differences in the gonad, where hermaphrodites have two mirror-image U-shaped gonad tubes and males have a single U-shaped gonad tube [71, 74, 94]. This gonad is where oocytes and sperm develop.

The hermaphrodite is a female whose gonads produce both sperm and oocytes. In terms of secondary features, hermaphrodites contain a vulva while males have a fan-shaped tail, allowing physical differentiation between the two [71, 74, 94]. The vulva acts as an outlet for egg-laying and sperm entry during mating with a male. The males appear thinner than females; however, they share the same signaling pathways in germline development. Hermaphrodites can produce 300 offspring; however, mating with males can produce 1000 offspring [71, 74, 94].

During early embryogenesis, P4 is the germline founder cell that gives rise exclusively to germ cells. This cell produces the primordial germ cells (Z2 and Z3) flanked by somatic gonad cells Z1 and Z4. These Z cells are inactive until larval stage 1, where both the somatic gonad cells (Z1 and Z4) and germline (Z2 and Z3) develop simultaneously [72, 73, 94]. The somatic gonad cells (Z1 and Z4) proliferate to 12 cells at the end of L1 that undergo rearrangement in late L2 to form the hermaphrodite somatic gonad primordium. Though, during L4 and young adulthood, there is up to four-fold amplification of germ cells [72, 73, 94].

Spermatogenesis, producing sperm, occurs during larval stage 4 and oogenesis during the adult stage. *C. elegans* spermatozoa use a crawling mechanism by utilizing key sperm proteins instead of actin. During oogenesis, there is a tremendous increase in the cytoplasmic volume of the oocyte. This increase in cytoplasmic volume allows a maternally provisioned cell that can support early embryogenesis. During this process, several germ cells undergo apoptosis in the loop region of the gonad by engulfing the gonad sheath cells. This process continues the growth of the oocytes. [72, 73, 94].

***The C. elegans germline survival and apoptosis***

C. *elegans* has separate germline and somatic apoptosis, which differ in regulation but share common apoptotic machinery proteins, CED-3 and CED-4 (Fig 1.5) [72, 95, 96]. Somatic apoptosis is developmentally programmed, whereas germline apoptosis is part of the oogenesis program. Furthermore, most apoptotic genes are conserved in *C. elegans*, making it an excellent model to elucidate apoptosis more broadly [95, 96].

The characteristics of the *C. elegans* germ cells, like other metazoans, include pluripotency, immortality, the ability of stem cells to produce mature gametes, and apoptosis that is not controlled by invariant cell lineage [95]. The *C. elegans* germline apoptosis occurs as physiological germline apoptosis independent of the BH3-only apoptotic regulator EGL-1 and stress-induced-germline apoptosis, triggered by genomic integrity checkpoint proteins [72, 95].

Physiological germline apoptosis occurs under normal conditions in adult hermaphrodites. This apoptosis is observed in the gonadal loop region, where developing oocytes leave the pachytene stage of meiotic prophase and transition into diplotene. Interestingly, in this late pachytene region, germ cells are connected by cytoplasmic "bridges" open to a common cytoplasm called the rachis [72, 95]. During physiological apoptosis, the targeted germ cell nucleus is centralized and contains very little cytoplasm, leaving most cytoplasm in the rachis [95]. The dying cell produces a corpse that is engulfed with neighboring somatic sheath cells. Evidence supports a "nursing model" where several germ cells in the region of the development of oocytes have physiological apoptosis, which increases the amount of cytoplasm available for the enlarging oocyte. When this apoptosis is absent, animals produce abnormal, smaller oocytes [72, 95]. Physiological apoptosis does not require

oogenesis and occurs in the absence of stress through initiation by core apoptotic machinery CED-3/CED-4. Also, this apoptosis does not rely on BH3-only protein EGL-1 or cell death inhibitory protein CED-9 [95, 96].

Interestingly, many of the germ cell apoptosis genes encode RNA-binding proteins found in P granules. These proteins physically and functionally interact and seem to regulate the translational of mRNA sequestered in oocytes and other cells [95, 96]. Thus, this localization in the P granules suggests a role of this organelle in germ cell apoptosis.

Germ cells can undergo DNA-damage-induced apoptosis, unlike somatic cells. Like physiological apoptosis, DNA-damage-induced apoptosis occurs in the late pachytene stage germ cells of hermaphrodites is not observed in the male germline [95, 96]. Stress-induced apoptosis requires the core apoptotic machinery CED-9, CED-4, and CED-3; however, it also needs the additional BH3-only proteins EGL-1 and CED-13. These genes indicate that stress-induced apoptosis regulation occurs by a different pathway [80] (Fig.1.5). Other factors such as SIR-2.1 regulate this pathway through CED-4 (Fig 1.5) [97]. Additionally, the mammalian CEP-1/p53 homolog has a key role in DNA-damage-induced apoptosis, where the knockdown of this gene during stress highly impacts the apoptotic machinery [95, 97].

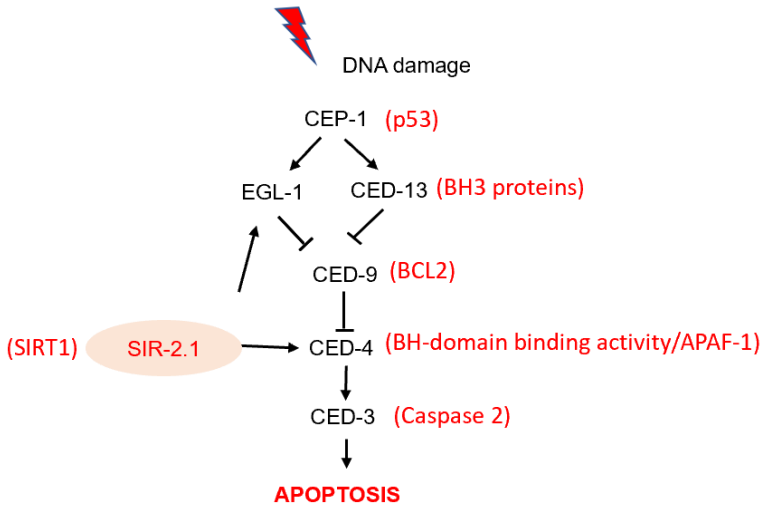


**Figure 1.5: A schematic of the canonical DNA damage-induced apoptotic signaling pathway.**

***The C. elegans PIWI argonaute and piRNA pathway***

RNA interference pathways are regulatory systems that include a single-stranded RNA and a member of the argonaute protein family binding together and leaving nucleotides accessible for base pairing with a target sequence [98]. These pathways target sequences by producing double-stranded RNA (dsRNA) processed by Dicer ribonuclease into 21-24 nucleotide short-interfering RNAs (siRNAs). The siRNAs then interact with members of the argonaute protein family. Argonaute proteins have three different classes: AGO clade, WAGO clade, and PIWI clade. AGO clade and WAGO clade proteins associate with siRNAs, while PIWI clade proteins associate with piRNAs [98]. Furthermore, these argonaute proteins have distinct protein domains: PAZ, MID, and PIWI. This section reviews PIWI argonaute proteins and their interactions with piRNAs to regulate various physiological functions.

piRNAs are small RNAs that interact with PIWI clade argonautes and regulate several processes in the germline. piRNA expression occurs mainly in the gonads; however, there is evidence of its evolution from soma and germline cells. PiRNAs in the soma are evident in cnidarians and *Macrostomum lignano* [99, 100]. piRNAs have a significant role in maintaining germline integrity and germ cells. However, other functions of piRNAs are viral defense in mosquitos, sex determination in silkworms [101, 102], and intriguingly, they can function in somatic cells under certain conditions. For example, deficiencies in piwi and piwi-like genes in *Drosophila*, *Arabidopsis*, and humans cause male sterility showing their importance in fertility [98].

***piRNA structure, expression, and biogenesis***

Unlike other small RNAs that form double-stranded (ds) RNAs processed by the Dicer, piRNAs lack secondary features that act as precursors [90, 91, 98, 103]. This lack of secondary structures could allow any sequence to be converted in piRNAs, depending on the tissue, developmental stage, or species; however, the selection of piRNAs is still not understood [98].

piRNAs occur in specific gene loci, known as piRNA clusters which are either uni-strand or dual-strand. These categories are based on the ability to be generated from one or both strands of genomic DNA [98]. Uni-strand clusters are the most prevalent form of piRNA hotspots, which appear indistinguishable from normal transcription units. These clusters have defined promoters that carry histone three lysine four demethylation marks (H3K9me3). They are also transcribed by RNA polymerase II and have canonical RNA processing ( capping, splicing, and polyadenylation) [98]. Dual strand clusters can be found in *Drosophila* germ cells but lack canonical transcription processes such as H3K4me2 marks on putative promoters or

lack promoters altogether [104]. They are, however, transcribed by RNA polymerase II but skip splicing and polyadenylation RNA processing steps.

Interestingly, piRNA precursors offer genetic memory of past invasions, allowing the piRNA pathways to act like an immune system that can distinguish between self and non-self [98, 101]. This genetic memory allows adaptation to new transposon challenges or invasion by viruses [101]. Maternal piRNAs have a crucial role in acting as carriers of epigenetic information and enable offspring to distinguish self from non-self and consequently silence transposons.

piRNA biogenesis is in two categories: 1. Primary biogenesis through conserved endonuclease Zuc or 2. Through the ping-pong cycle that is directed towards transposon mRNAs [98]. Zuc/MitoPLD is a conserved endonuclease that is key to piRNA biogenesis and cleaves piRNAs with a bias for the 5' end uridine (U1 bias). Zuc/MitoPLD is localized in the mitochondria showing the importance of this organelle in piRNA biogenesis. However, this mechanism requires other factors that support the process. The ping-pong cycle is where RNAs regulated by piRNAs produce substrates for additional piRNA production. These piRNAs predominantly function in transposon silencing and generate the abundance of piRNAs capable of targeting active transposons [98].

***PIWI: piRNA pathway in C. elegans germline***

PIWI proteins are members of the AGO family that work with piwi-related RNAs (piRNAs) and ensure genome integrity, cell immortality, and gene regulation [98, 103]. This pathway is conserved in many species, including *C. elegans* [90, 91, 98, 103, 105]. In early studies on *Drosophila*, these small RNAs with functions in transposon silencing were known as rasi-RNAs. However, these regions are now known to be a significant piRNA source [104].

The piRNAs in *C. elegans* are known as 21U-RNAs, due to the overwhelming bias for 5’ uracil and a length of 21 nucleotides. Like other species, these piRNAs are resistant to periodate degradation due to the occurrence of several uracil bases, a monophosphate, and modifications at the 3’ end [98, 103]. Nevertheless, piRNAs in *C. elegans* are unique in that they are shorter than other species, including humans, and have a different genomic arrangement.

*C. elegans* has over 15,000 unique 21U-RNAs clustered on chromosome IV and are predominantly expressed in the germline between L4 to gravid adulthood, in both males and hermaphrodites [98, 103]. Transcription by RNA polymerase II in piRNA clusters generates a 28-29 nucleotide precursor sequence from individual promoters that carry a Ruby motif controlled by Forkhead transcription factors as well as PRDE (piRNA silencing-defective) [106, 107]. Two classes of piRNAs occur in *C.elegans*: repeat-associated piRNAs that target transposons and a type of abundant function-unknown piRNAs that lack obvious targets, leaving some functions of piRNAs in *C. elegans* to be indescribable [98].

*C.elegans* have two PIWI proteins, PRG-1 and PRG-2, interacting with piRNAs [98, 105]. PRG-1 physically binds to 21U-RNAs forming an RNP complex required for proper germline development [98, 105]. The interaction between PRG-1 and 21U-RNAS is dicer independent and mainly occurs in the *C. elegans* germline. PRG-1 expression occurs in the germline p granules; a membranelles organelles found the germline required for 21U-RNA accumulation [98, 105]. PRG-1 mutants show significant germline defects, including reduced fertility and increased germline apoptosis. In addition, these mutants accumulate transgenerational stress, eventually triggering sterility [90, 91, 103, 105].

The transgenerational fertility defects in PRG-1 mutants can be suppressed by intermittent or constitutive reduction of IGF- stress response pathways DAF-2/DAF-16 [91]. This discovery showed a strategic link between an intervention that represses somatic aging and promotes stress resistance with transgenerational fertility defects caused by piRNA deficiency [90, 91]. Furthermore, H3K4 demethylases epigenetic signals and small RNAs are required for reduced insulin signaling. This reduced signaling promotes the fertility of the PRG-1 mutants/piRNA pathway [90, 91]. Also, PRG-1:21U-RNA complex works with WAGO family members to silence a single transposon family, Tc3 [108].

Intriguingly, *C. elegans* piRNAs: PRG-1 complex can also elicit the generation of complementary 22G-siRNAs, which engage in silencing non-self-elements in the nucleus [98, 109]. In addition, this complex can recruit RNA-dependent RNA polymerases (RdRP) that target RNA as a template for producing small secondary RNAs, achieving diversification of antisense RNA triggers [98, 109]. Notably, once piRNAs have silenced the transgene, they remain silent permanently and can act dominantly to silence other homologous transgenes forever [98].

***C.elegans P granules***

P granules are RNA-rich germ cell organelles essential for germline development [72, 94]. Thus, they have critical functions in delivering maternal proteins and RNA to the nascent germline. In addition, p granules contain RNA and RNA-binding proteins related to translational control [94].

RNA binding protein families in the P-granule include the PGL-family, GCH-1, IFE, GLH family, Sm proteins PIE-1, and CCCH finger proteins [94, 110, 111]. These protein families have roles in alternative splicing. PGL-1 and GLH proteins are the only ones known to

be in the granules through all developmental stages and are essential in the assembly/stability of the p granules. Offspring with mutations in these proteins show germline proliferation and gametogenesis defects and develop into sterile adults [98, 110, 111].

## The Heat Shock Response

### *Discovery of the HSR*

The heat shock response is a stress response pathway conserved in unicellular bacteria, yeast, and multicellular plants and animals [112, 113]. This response is critical for maintaining protein homeostasis by producing the molecular chaperones in stress conditions such as elevated temperature, heavy metals, chemical toxicants, and oxidative stress [113]. Molecular chaperones, also known as heat shock proteins (HSPs), include HSP90, HSP70, HSP40/DNAJ, chaperonin HSP60, and small heat shock proteins [30]. A transcription factor regulates HSPs, heat shock factor 1 (HSF1), which undergoes rapid homo-trimerization causing its translocation to the nucleus. Once the homo-trimer is in the nucleus, it binds to the heat shock elements (HSE) on the promoter regions of *hsp* genes, inducing their transcription [30]. However, through acetylation and feedback regulation by certain HSPs, HSF1 loses its DNA binding ability.

### *SIRT-1/SIR2.1 regulation of HSF-1 and the HSR*

HSF1 is acetylated by p300/CBP1, decreasing its binding to HSP promoters and the transcription of HSPs. Nevertheless, a deacetylase SIRT1 reverses the effects of HSF1 acetylation. This modification allows HSF1 binding to HSEs and restores the benefits of the heat shock response in the cell [29, 31]. SIRT1's protein-protein interaction with CCAR2 and AROS regulates its function [29]. AROS positively regulates SIRT1 increasing the

deacetylation of HSF-1. However, CCAR-2 negatively regulates SIRT1 attenuating its effects on the deacetylation of HSF-1 [1, 10, 29].

Experimentally, our lab has shown the interaction of CCAR2 on SIRT1 and HSF-1 in both human cells and in the *C. elegans* model [2, 29]. SIRT1 regulates the heat shock response in mammalian cells by deacetylation of heat shock factor 1 (HSF1) [31]. Depleting SIRT1 by either a small molecule inhibitor or a siRNA significantly reduced the hsp70 mRNA produced and HSF1 recruitment to HSP promoters. Furthermore, this interaction between SIRT1 and HSF1 affects its acetylation status in specific lysine's [29]. In a follow-up study, we showed that interactions between SIRT1 and HSF1 directly affect the HSR.

AROS, a positive regulator of SIRT1, was identified from a yeast-2-hybrid screen, while the negative regulator CCAR2 was found in a co-purification assay of proteins increasing p53 acetylation and p21 gene expression [29]. We show the impact that AROS and CCAR2 have on the HSR in a SIRT-1 dependent manner. Further, the overexpression and depletion of AROS and CCAR2 affects *hsp70* mRNA production, HSF1 acetylation, and HSF1 binding to the HSP promoters.

To understand this mechanism in a whole model organism, we used *C. elegans* to analyze the regulation of the HSR by CCAR2, as *C. elegans* does not have a homolog for AROS. As previously mentioned, *C. elegans* CCAR-1 shares up to 30% sequence similarity with human CCAR2. The high similarity between their structures and domains suggests that both genes share common molecular functionality [9]. We show that the negative regulation of the HSR by mammalian CCAR2 is conserved in *C. elegans* and is mediated by CCAR2 ortholog CCAR-1, in a SIRT1/SIR-2.1 dependent manner. CCAR-1 affects HSF-1 acetylation, and binding to the *hsp70* promoter and knockdown of CCAR-1 increased HSF1 binding

significantly in both heat shock and no heat shock conditions [2]. Using the advantages of the worm model, we tested the effects of CCAR-1 on proteostasis. We show that CCAR1 promotes stress resistance, motility, and longevity in a SIR-2.1 dependent manner. Interestingly, CCAR-1 RNAi promotes proteostasis in a *C. elegans* Huntington's disease model, showing its role in neurogenerative diseases [2].

**Alternative splicing**

The human genome contains less than 30,000 genes but requires about 80,000 to 400,000 proteins [59, 114-117]. To overcome this impediment, alternative precursor mRNA splicing/alternative splicing increases the proteome diversity in the cell. Alternative splicing (AS) expands the proteome and regulates gene expressions after transcription [59], which leads to subtle or dramatic changes in the amino acid sequence. These alternative splicing can be tissue-specific, hormone-responsive, developmental, cell cycle, and stress-responsive signals. Various types of alternative splicing occur, including mutually exclusive exons, cassette exons, alternative 5' and 3' splice sites, intron retention, alternative promoters, and alternative polyadenylation [59, 60]. These types of conserved alternative splicing patterns occur between species.

To coordinate AS the spliceosome and other splicing proteins ensure proper splicing. The spliceosome consists of five small ribonucleoprotein particles (snRNPs), U1, U2, U4, U5, and U6. These snRNPs work with approximately 100 splicing factors/trans-acting factors to mediate the splicing process [59, 114, 118, 119].

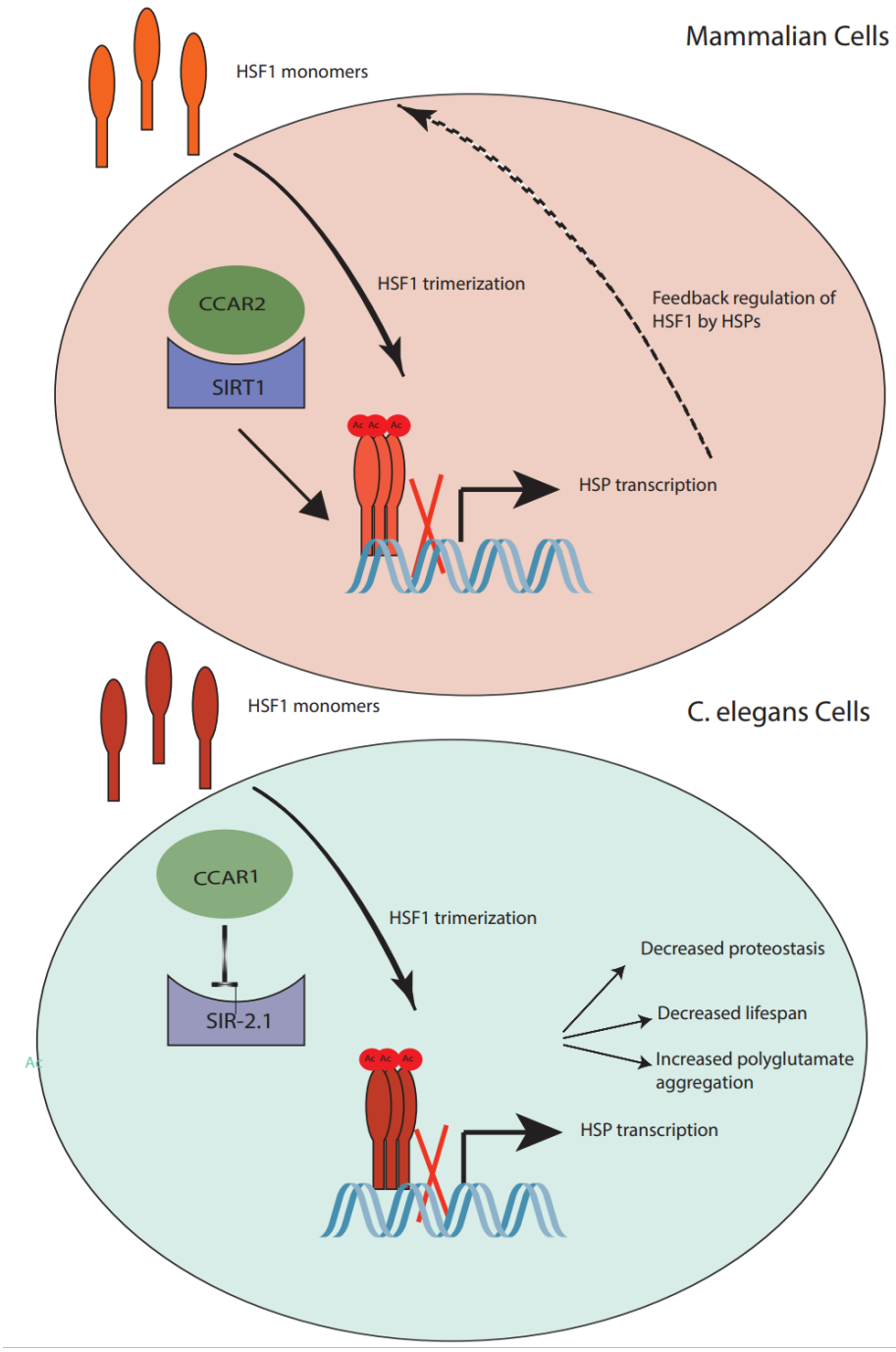


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**Figure 1.6: The role of mammalian and *C. elegans* CCAR-1 in the heat shock response.** A. Upon stress, HSF-1 trimerizes, leading to the localization from the cytoplasm to the nucleus. Mammalian CCAR2 negatively regulates the heat shock response in a SIRT-1-dependent manner, decreasing HSF-1 binding to promoter regions of HSPs and eventual HSP transcription. This regulation affects feedback regulation by HSPs to HSF-1. B. *C. elegans* CCAR-1 has a similar role in the heat shock response, where it negatively regulates HSF-1 in a SIR-2.1-dependent manner. Using the worm, we show that through the HSR, CCAR-1 regulates lifespan, proteostasis, and polyglutamate regulation.

For proper splice site selection on pre-mRNA, protein-RNA complexes bind weakly to elements found on introns and exons known as *cis-elements* [59, 60, 120]. These complexes allow the binding of members of the spliceosome snRNPs to the 3' and 5' end of the introns/exons allowing further excision. The formation of this protein: RNA complexes have several advantages, which change depending on the concentration of the regulatory proteins in various tissues and developmental stages [59, 114, 116, 120]. Other factors that can affect protein: RNA complex formation are changes in intracellular localization, enzymatic activity, post-translational modifications, relative abundance, and stability of the proteins [116]. Therefore, it is vital to understand AS in various conditions such as stress and diseases and the implications on protein-RNA complexes [116, 120].

Additionally, AS can introduce frameshift mutations by adding stop codons to the transcript, leading to degradation by a process called non-sense mediated decay, this process can be a way to switch of production of transcripts [121, 122].

The establishment of sequencing techniques shows that approximately 70 % of human genes undergo alternative splicing. Remarkably, all protein-coding genes on chromosomes 21 and 22 can undergo alternative splicing [59, 60, 123].

***Alternative splicing in Caenorhabditis elegans***

Alternative splicing is conserved between humans and *C. elegans*, with many discoveries in the field being made in the latter [59, 60, 123].

*C.elegans* is an excellent model to study alternative splicing due to the ability to conduct tissue-specific, developmental, age-specific studies and the availability of viable mutants of splicing factors [59, 60, 124, 125]. Also, the worm has conserved splicing machinery, and scientists have developed elegant fluorescent reporter constructs for genetic

screens to visualize these splicing changes [126-128]. Intriguingly, 20% of *C. elegans* genes alternative splicing forms alternative promoter sequences that provide unique first exons with a common second exon [59, 60] [125, 129].

Nonetheless, *C. elegans* is a powerful tool in increasing the knowledge of cis-elements and trans-acting factors in the study of AS. The structure of introns and exons is conserved between *C. elegans* and higher eukaryotes; however, the worm has an average of 6.4 exons with an average exon size of 218 nucleotides compared to 9.7 exons for humans with an average of 145 nucleotides of humans [59, 60]. However, *C. elegans* is an intron-rich organism, with much shorter introns (47 nucleotides) than 1023 nucleotides in humans.

Furthermore, Bioinformatic tools such as Wormbase, *C. elegans* splice browser, and Intronerator have been vital to expanding the knowledge on alternative splicing in *C. elegans*. The *C. elegans* splice browser is used to mine AS data. The Intronerator shows full-length expressed sequence tags (EST) aligned to full-length cDNA from Gene bank, allowing easier identification of splicing patterns[125, 129]. Lastly, Worm Base visualizes all isoforms of a gene identified.

***Types of alternative splicing in C. elegans***

Fascinatingly, *C. elegans* can go through both cis- and trans-splicing. Cis- splicing is the commonly known method of intron removal. In contrast, trans-splicing utilizes a 22-nucleotide splicing leader (SL) sequence not associated with the gene and a 100 nucleotide SL RNA [59, 60, 130]. The SL RNAs exist as snRNPs and have a distinct structure that binds to Sm proteins, playing a key role in snRNP biogenesis and structure [131, 132]. SL RNAs contain trimethyl guanosine (TMG) caps like U snRNAs and their 5’ splice site and the site of SL addition bind to the trans-splice site on the 3’ splice site of the mRNA on an intron-like

sequence known as the outron [130, 133]. The outron signals trans-splicing at the 5' end of mRNA with no functional splice site upstream.

Trans-splicing reaction occurs in a branched intermediate like the formation of a lariat in *cis* splicing. During *cis* splicing, the first step involves cleaving the 5' exon, the SL, and forming an intermediate with the 5' splices site of the SL RNA branched to an A on the outron. The splicing of the SL to the first exon of the pre-mRNA is the second step. Thus, genes that undergo trans-splicing can be distinguished by the presence of an outron; however, trans-splicing is as efficient as *cis*- splicing making it hard to identify outron-containing pre-mRNAs. The trans-splicing process catalysis occurs by members of the spliceosome, U2, U4, U5, and U6 snRNPs but not U1 [130, 133].

The prominent spliced leader in nematodes is SL1; however, a second spliced leader, SL2, is trans-spliced in *C. elegans* [130]. This splicing occurs mainly in polycistronic pre-mRNAs that occur in operons. Interestingly, about 15% of all *C. elegans* genes are present in the form of operons. Thus, the majority (70%) of *c. elegans* genes undergo trans-splicing. In cases where there is no trans-splicing, the promoter is at the 5' end of the first exon [130, 133].

***The Spliceosome***

The spliceosome is highly conserved machinery vital to the AS of pre-mRNA in cells [59, 134]. Uridine-rich small nuclear ribonucleoprotein complexes (U snRNPs) assemble to pre-mRNA via an ordered binding of the subunits. These steps are key to the proper functioning of pre-mRNA splicing. Furthermore, the spliceosome assembly is guided by RNA-RNA interactions between U snRNPs and precursor mRNA and the U snRNAs to each other [59, 134]. The members of the spliceosome include U1, U2, U4, U5, U6, and U2 auxiliary factors, U2AF65 and U2AF35 [134].

U1 binds to pre-mRNA guided by base-pairing interactions between its 5’ end and the 5’ splice site [134]. U2 binds by base-pairing interactions with a branchpoint sequence found in the intron [118, 119, 134, 135]. Subsequently, the U2 auxiliary factors (U2AF), which is composed of 35 and 65 kDa subunits, are responsible for binding to the polypyrimidine tract (U2AF65) and recognizing an AG nucleotide at the 3’ end of the intron (U2AF35), promoting U2 snRNA interactions with the intron branchpoint sequence [118, 119, 135]. This binding is followed by U4/U6-U5 tri-snRNP recruitment to the spliceosome. There is evidence that spliceosome components can be pre-assembled in a Penta-snRNP complex [134].

Other factors are required to assist the spliceosome assembly and alternative splicing. These factors interact with pre-mRNA, other splicing factors, and with protein components of snRNPs [118, 119, 124, 127, 135-142]. These factors include a family of serine-rich proteins (SR proteins) required for the earliest interaction of U1 snRNP and with pre-mRNA, allowing the continuation to subsequent steps [118, 135, 138-142]. U2AF, U1snRNP, and SR proteins assemble onto precursor mRNA in an ATP-dependent manner to form the E complex, which identifies both the 5’ and 3’ splices sites allowing further spliceosome assembly [134]. This identification of the splice sites cooperates with an increase of snRNPs to the splice sites and promotes RNA-RNA interactions significant for spliceosome assembly.

Weak consensus sequences found at the 5’ and 3’ends of introns and exons are targeted for splicing. This targeting further facilitates the splicing process. These sequences act as additional signals recognized by the spliceosome to aid in AS. *Cis*-acting pre-mRNA sequences are found in introns and exons and used as enhancers or silencers of splice site usage [59, 134, 143]. Trans-acting/splicing factors are proteins identified in several genes interacting with the high-affinity *cis*-elements in exons/introns [134, 143]. Trans-acting factors include SR

proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) A/B family [137, 138]. SR proteins and hnRNPs act in opposing manners where hnRNPs act as antagonists to SR proteins by blocking their binding to cis sequences on exons.

Additionally, the 3' splice site contains a branchpoint sequence usually bound by SF1/BBP protein and U2 snRNP, the polypyrimidine tract attached to U2AF65, and the splice site AG/R by the U2AF35 subunit [118]. In mammals, these sequences are separated by 10-30 nucleotides, with loose branchpoint consensus sequences and tight binding of U2AF65 to the polypyrimidine tract [118, 119]. Species with introns with a short polypyrimidine tract compensate by having the AG nearby, which enables interactions of U2AF35 and are called AG-dependent introns. Interestingly, yeast has no U2AF35 and a polypyrimidine, so all introns are AG independent [144]. Their splicing is dependent on BBP/SF1 protein [144].

*C. elegans* have no consensus sequence for the branchpoint sequence but have a conserved BBP/SF1. Their introns have a highly conserved octamer sequence UUUUCAG/R at their 3' splice sites [118]. *C.elegans* U2AF binds to a highly conserved octamer sequence at the 3' splice site, UUUUCAG/R, where U2AF65 binds to the polypyrimidine tract U2AF35 binds to the splice site AG/R [118]. *C.elegans* U2AF has a solid affinity for this octamer sequence and is responsible for the high conservation. The *C. elegans* homologs of U2AF65 (UAF-1) and U2AF35 (UAF-2) cause genome-wide changes in the 3' splice site [118, 119, 135], and UAF-1 mutants have defects on embryonic viability and motility [136]. Additionally, UAF-1 interacts with the survivor motor neuron (*smn-1*) gene, which affects the assembly of the members of the spliceosome [136].

### *Splicing Factors*

Over a hundred factors are needed to assist the spliceosome with AS. These factors can vary depending on tissues, stress signals, and cell cycle changes [134]. As previously mentioned, *trans*-acting factors interact with high-affinity *cis*-elements in exons and can act as enhancers or silencers of splicing. Trans-acting factors include SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) A/B family. SR proteins and hnRNPs act in opposing manners where hnRNPs block SR proteins from binding to *cis* sequences on exons.

#### ***SR proteins***

SR proteins are *cis*-elements binding splicing factors that act as enhancers to constitutive splicing and influence AS [138], whose homology is conserved from metazoans to plants. *C.elegans* have seven conserved members of SR proteins called *rsp* genes [138]. These genes expression changes with various stages of development, stress signals and tissues, to promote specific alternative splice site usage [60].

*C. elegans* SR proteins and SR protein-related homologs identified include SF2/ASF (*rsp-3*) , SRp20 (*rsp-6*), SC35 (*rsp-4*), SRp40 (*rsp-2*), SRp75 (*rsp-1*), SC35-2 (*rsp-5*) and p54 (*rsp-7*), and SR protein-related factors (SRrsps) such as Tra-2β (*rsp-8*) and SPRK (*rsk-1*), the only SR protein kinase [138, 139, 142]. Most of the functions of *rsp* genes overlap, except *rsp-3*, which causes changes in phenotype due to RNAi. Interestingly, the conservation between family members is stronger across species than other family members in the same species [138].

SR proteins have a modular structure containing one or two RNA recognition motif (RRM) domains with key AS functions [139]. Additionally, they contain a C-terminal domain rich in arginine and serine residues, known as the RS domain. These domains are functional

modules that coordinately function to act as activators of splicing and are functionally interchangeable. The RS domain does not contribute to AS but can activate it when bound to the RRM domain [139].

SR proteins' importance occurs in several species. Their concentrations vary by tissue or development specificities; for example, SF2/ASF and its antagonist hnRNPA1 molar ratio vary between species [145]. Hence, the balance between SR proteins and their antagonist hnRNP is key to regulating the AS. Additionally, different SR proteins show unique functions in splicing due to the ability to commit to different pre-mRNA in the splicing pathways.

It suggests that there is functional redundancy between SR proteins [138]. To address this issue, *C. elegans* was used to knock down the conserved versions of SR proteins with injection RNA interference. This work shows that RNAi on individual SR proteins did not lead to apparent phenotypes, suggesting functional redundancy in *C. elegans* [138]. However, lethality and other severe phenotypes were observed with simultaneous knockdown of two or more SR proteins in various combinations. This observation suggests that the knockdown of SR proteins that participate in a similar splicing event together could lead to an obvious phenotype [138]. The only SR protein that did not show functional redundancy was SF2/ASF (*rsp-3*), which is highly conserved and displays 73.8% similarity and 66% identity to human proteins, especially in the RNP-2 and RNP-1 sub-motifs. RNAi on this SR protein led to embryonic lethality, suggesting a key role in *C. elegans* development. Additionally, RNAi on SRPK (*rsk-1*), the SR protein-specific kinase resulting in early embryonic lethality, indicates that phosphorylation of SR proteins is essential for development in *C. elegans*. SR proteins are fundamental for the earliest interaction of U1 snRNP with pre-mRNA allowing the continuation to subsequent steps [138].

***hnRNPs***

SR protein function is important in alternative splicing regulation. These proteins are antagonized by heterogeneous ribonucleoproteins (hnRNPs) A/B family of proteins. This antagonism includes competition in splice site selection which promotes AS inhibition through binding to exonic splicing silencers [59, 134, 137, 143]. hnRNPs have several roles, including RNA metabolism, mRNA export, and telomere biogenesis.

*C.elegans* have conserved hnRNPs, including hnRNPs A1 (*hrp-1*), essential for the worm's viability. Additionally, hnRNPs Q/R (*hrp-2*) binds to the UCUAUC sequence in the nematode to regulate AS of two genes, *unc-52* and *lin-10* [137].

***Alternative splicing during stress and disease***

***Alternative splicing during stress***

During genotoxic stress, the cell initiates strategies that allow increases in survival [116]. The shutdown of pre-mRNA splicing is one of the best-characterized changes in the cell in response to stress. Several studies show reduced fidelity of pre-mRNA splicing during genotoxic stress affecting the production of proteins. Genotoxic stresses such as heat shock, radiation, chemo- toxic agents affect splicing through changes. These effects include changes in trans-acting factors post-translational modifications, subcellular localization of splicing machinery, production of non-productive splice variants targeted by non-sense mediated decay, and decreased gene expression levels of trans-acting factors [114, 116, 117, 146].

Genotoxic stresses cause varying changes in AS of genes. For example, during the addition of chemotherapeutics, Topoisomerase1's interaction with various splicing factors is affected [117]. Also, apoptosis genes, caspase, and Bcl2 families form different splice isoforms during genotoxic stress. Furthermore, several AS changes are observed during heat shock,

including producing unproductive splice variants of numerous genes targeted for NMD [147]. Nonetheless, polyadenylation effects on mRNA reduce with UV exposure [117].

There are various mechanisms by which genotoxic stress affects alternative splicing, which I will discuss in this section.

First, stress can affect transcription by increasing/ decreasing gene expression, which could play a role in the later phases of the stress response [116, 117]. For example, the E2F1 transcription factor stimulates the expression of the trans-acting factor SRSF2 in response to cisplatin [148, 149]. Alternative splicing can occur concurrently with transcription, although AS can continue post-transcription. Transcription can also influence AS by affecting the transcriptional speed, which involves selecting splice sites and recruitment of splicing machinery [117]. Stress can also affect the alternative splicing of trans-acting factors or inhibit transcriptional elongation. Depending on the trans-acting factor at hand, it can also cause the dissociation of the transcriptional machinery, increasing/decreasing exon inclusion. Intriguingly, chromatin modifications shown to regulate AS are affected by DNA damage [117].

Secondly, stress could affect the localization of splicing factors and members of the spliceosome [116, 117]. These changes could include alterations in subcellular localization; for example, stress causes the delocalization of trans-acting factors into the cytoplasm or membrane-less organelles known as nuclear stress bodies [116]. Trans-acting factors can also delocalize to nuclear compartments such as the nucleoli during stress. For example, Sam68 localizes to nuclear stress bodies in response to heat shock [150, 151]. The delocalization of these factors during stress could be enabling their participation in specific activities or sequestering them to influence post-transcriptional gene expression. For example, the

subcellular delocalization of hnRNPA1, which antagonizes SR proteins, highly impacts mRNA regulation [117, 152].

Thirdly, stress can affect the post-translational modifications of *trans*-acting factors, changing their subcellular localization, protein-protein interactions, and AS activity [116, 117]. For example, heat shock causes the dephosphorylation of SRp38, switching its role from a splicing activator to a repressor. However, SRp38's phosphorylation state recovers after stress returning to its role as an enhancer of splicing. Intriguingly, HSP 27, a member of the heat shock family, aids in the recovery of SRp38 to its phosphorylated state after heat shock stress [153]. Also, cisplatin treatments affect TIP60-dependent acetylation and SRPK2-dependent phosphorylation [154], and Protein kinase AKT promotes cell survival by increasing an anti-apoptotic caspase b isoform through the phosphorylation of SRSF1/ASF/SF2 [155].

Finally, stress can cause the formation of membrane-less organelles known as nuclear stress bodies, sites of accumulation of pre-mRNA processing factors, and long tandem arrays of satellite II repeats [116, 117]. These satellite repeats are induced during stress forming puncta in the nucleus. It is proposed that the NSB activity is to sequester pre-mRNA splicing factors and a select set of genes that bind to satellite II repeats. SR proteins, SR/ASF and SRp30c, and hnRNPs are recruited in these organelles [117, 150].

Therefore, we see the huge effect that stress has on AS using the above mechanisms. Further studies are needed to understand the production of specific splice variants and *trans*-acting factors during stress. These studies will enable us to understand the post-transcriptional landscape of many pathological conditions, where stress has a role in cell/tissue malignancy.

***AS during disease***

Highlighting the importance of understanding genes' AS, several diseases cause abnormal splicing patterns [115, 116, 120]. These variations can include alterations in the structure or function of *cis*-elements and *trans*-acting factors. Changes in *cis*-elements include mutations on splice-sites, silencer and enhancer sequences, and new binding sites in triplet repeat extensions [115]. In *trans*-acting factors, these changes include alteration in the concentration and ratio of *trans*-acting factors and mutations, which affect their binding and localization. This interaction can lead to the sequestration of trans-acting factors to different compartments/ organelles in the cell. Alteration on *trans*-acting factors during disease can also include post-translational modifications such as phosphorylation, which affect the interactions between regulatory proteins and their binding to exonic sequences [115, 116, 120].

During diseases, there is a positive correlation between the progression of the disease and the occurrence of abnormal splicing patterns. Mutations on *cis*-acting elements that occur in 10% of human gene mutations are classified into four types, Type I, II, III, and IV. Type I occurs at splice sites and alters exon usage. Type II creates novel splice sites that cause the inclusion of novel exons, and Type III and IV are rarely identified but also cause changes in exons and introns and alter exon usage [115].

As previously mentioned, several disease states affect AS. Two examples include cystic fibrosis and spinal muscular dystrophy. Cystic fibrosis is caused by the loss of function of cystic fibrosis transmembrane conductance regulator (CFTR). One thousand three hundred eighty-eight mutations have been identified, with 185 splicing mutations where 20 are in exons and the rest in introns [115]. Spinal muscular dystrophy is a neurogenerative disorder that causes progressive paralysis caused by losing alpha motor neurons in the spinal cord. Spinal

muscular dystrophy is caused by the loss of the SMN gene required for regulating the snRNP assembly. This function of assembly of the snRNPs is absent from all cells of SMA patients [115]. Several diseases are affected by alterations in the trans-factors. In Schizophrenia, alternative splicing of γ-amino butyrate type A receptor, N-methyl-D-aspartate (NMDA) R1 receptor, and the neuronal cell adhesion were altered. Also, the AS of tau proteins, exon ten, is changed during sporadic Alzheimer's disease. Several tumors such as breast cancer, melanoma, prostate cancer, and Wilm's tumor have reported changes in alternative splicing genes. Additionally, changes in localization and concentration of splicing factors are frequently observed in tumorigenesis; for example, SC35, ASF/SF2, and Tra2-β1 are altered in ovarian cancer[115] .

***AS a therapeutic tool***

Since alternative splicing can affect various disease models, both constitutive and regulated forms can be targeted by conventional and molecular therapies. For example, antisense oligonucleotides can be employed to promote the skipping of an exon, block an inappropriately activated exon, or stimulate binding sites weakened by mutations which could eventually lead to the treatment of various ailments [156].

***Nonsense Mediated Decay***

Nonsense-mediated decay (NMD) is a conserved mechanism from bacterium to mammals that aims at eliminating mRNA with premature stop codons (PTCs) [121, 122, 157]. This mechanism is coupled with both AS and translation [157]. NMD acts as a form of surveillance that targets truncated proteins that could produce toxic effects in the cell and cause dominant-negative effects. This surveillance includes targeting mRNA with PTCs due to

mutations, errors in transcription, or RNA processing. NMD can also be seen as a mechanism to proofread transcription and AS [121, 122].

Proper function of NMD requires various trans-acting factors and active mRNA translation. These factors include UPF-1, UPF-2, and UPF-3, evolutionarily conserved and have key roles in NMD [122]. These proteins can form a complex that links premature translation termination to mRNA degradation. They are also thought to bind to premature termination ribosomes. *C.elegans* has been a key model organism for the study of NMD. Additional factors linked to NMD have been identified in c. elegans, including SMG-1, SMG-5, SMG-6, and SMG-7, which are identified as allele-specific suppressors of mutations in various c. elegans genes [121, 122]. From c. elegans, homologs of UPF-1 (smg-2), UPF-2 (smg-3) and UPF-3 (smg-4) have been identified. SMG-1 is a protein kinase for UPF-1 and SMG-7 is also thought to regulate the phosphorylation of UPF1, which are key post-translational modifications for interactions with mRNA decay factors. Some NMD genes are essential for viability, i.e., UPF-1 is essential for mouse embryonic development, and UPF-2 deletion causes loss of hematopoietic stem and progenitor populations [122].

# CHAPTER 2: CCAR-1 HAS A NOVEL ROLE IN REGULATING THE CAENORHABDITIS ELEGANS GERMLINE.


## Abstract

The Cell Division Cycle and Apoptosis Regulator (CCAR) protein family members are putative transcription regulators characterized for modulating the cell cycle, apoptosis, metabolism, and the heat shock response. Mammals have CCAR family members, CCAR1 and CCAR2/DBC1, which evolved from the founding family member CCAR-1 expressed in *Caenorhabditis elegans*. Mammalian CCAR2, the most well-studied family member, has been shown to regulate genes involved in metabolism in cultured cells. However, the regulation of gene expression by CCAR family members at an organismal level is unknown. Here, we use whole transcriptome RNA sequencing to examine the effects of CCAR-1 on gene expression in *Caenorhabditis elegans.* We show that CCAR-1 regulates germline transcription, reproduction, lifespan, and DNA-damage-induced apoptosis. This study shows the role of CCAR-1 in vital physiological functions in the *C. elegans* germline that are previously unknown.


## Introduction

The Cell Division Cycle and Apoptosis Regulator (CCAR) proteins CCAR1 and CCAR2 regulate various physiological processes [158]. Mammalian CCAR1 and CCAR2 are predicted through phylogenetic analysis to have evolved from the *Caenorhabditis elegans* CCAR-1 [9]. Like mammalian CCAR proteins, *C. elegans* CCAR-1 regulates various functions, including epigenetic modifications, stress responses, and RNA splicing [9, 62, 159].

Mammalian and *C. elegans* CCAR proteins have 30% sequence similarity and share similar domains [9]. CCAR family domains of note include an S1-like putative RNA binding domain, a nuclear localization sequence, a leucine zipper domain that may allow for protein-protein and protein-DNA interaction, a Nudix domain that is predicted to bind to metabolites, an EF-hand domain, and coiled-coil domains that may allow for protein-protein interaction. Additionally, mammalian CCAR2 and *C. elegans* CCAR-1 have a SAP domain predicted to allow DNA binding.

These functional domains, along with the fact that these proteins have been localized to the nucleus, support the hypothesis that CCAR proteins may, directly and indirectly, regulate gene expression. CCAR proteins may bind to DNA directly and function as transcription factors. CCAR proteins may also affect transcription through the modulation of transcription factors. For example, human CCAR2 directly interacts with steroid hormone receptors, affecting their transcriptional functions [55]. CCAR proteins are also known to regulate transcription epigenetically through effects on the deacetylase SIRT1 [10, 25].

Transcriptome-wide RNA sequencing studies in mammalian tissue culture studies have shown that CCAR family members regulate gene expression of genes involved in metabolism. Studies using RNAi against CCAR1 or CCAR2 in A549 human lung cancer cells showed that this protein affected the expression of steroid hormone receptors, many of which are involved in cell metabolism. These findings make sense, as CCAR proteins interact with steroid hormone receptors [160]. Additional studies with CCAR2 RNAi performed in HEK 293 human embryonic kidney cells confirmed the involvement of CCAR2 in regulating genes involved in metabolism [61, 161].

Are metabolic genes the only gene category affected by CCAR proteins? To answer this question, we employed *C. elegans* as a whole animal model organism. We chose the worm as a model for our studies as there is only one CCAR family member, a CCAR-1 deletion strain is available, and we can assess gene expression in multiple tissues. Through RNA-sequencing, we demonstrate that the deletion of CCAR-1 affects the regulation of many gene categories. As expected, a top category of regulated genes is metabolism genes from the insulin signaling-related pathways. However, using our *C. elegans* model, we have uncovered an additional category of CCAR-1-regulated genes: germline genes.

Interestingly, we find that CCAR-1 RNAi increases stress-induced apoptosis in the germline and decreases progeny numbers. Additionally, we find that CCAR-1 RNAi increases lifespan, an effect that is dependent on the ability of CCAR-1 to regulate the germline. Thus, we have identified germline genes as a novel category of genes regulated by CCAR-1, providing a mechanism by which CCAR-1 can regulate lifespan in *C. elegans*.

## Methods

### *C. elegans strains and maintenance*

The following strains were used in the study: Wild-type Bristol N2, VC1029 *ccar-1 (gk433)* IV, MT7019 *ccar-1(n2667) IV,* DM1153 *ccar-1(ra14)* IV, DM1154 *ccar-1(ra5)* IV, WM161 *prg-1(tm872)* I, WS2170 *(unc-119(ed-3))* III; opls110 IV, CF1903 *glp-1(e2144)* III, and SDW080 (*peft::CCAR-1::GFP::3xflag*). VC1029 was outcrossed three times to our N2 wild-type strain to generate the SDW040 strain. N2, VC1029, MT7019, DM1153, DM1154, WM161, WS2170, and SDW080 strains were maintained at 20 °C on standard NGM plates seeded with *Escherichia coli* OP50-1. Synchronous populations of nematodes were obtained by bleach synchronization and plating for 19 hrs on NGM plates without food. CF1903 was

maintained at 16˚C until bleach synchronization where the L1s were then split and grown at either 16˚C or 25˚C.

***RNA preparation for RNA sequencing***

RNA was isolated from day 1 adults, at the transition to adulthood. Samples were prepared using Trizol reagent (Ambion, Inc.). The samples were sent to the Brigham Young University DNA sequencing center. RNA libraries were prepared using the TruSeq Total RNA kit (Illumina, Inc.) with a Ribo-zero gold kit (Illumina, Inc.) to remove rRNA and mitochondrial RNA transcripts. Samples were not size excluded.

***RNA-sequencing analysis***

HISAT and STRING-Tie were used in the alignment and assembly of raw FASTQ files from the RNA sequencing. The R program, Ballgown package, was used to calculate reads per kilobase of the transcript, per million mapped reads. The same package was used to calculate the data set's statistics, including the p-value, the q value, which has Benjamini-Hochberg correction for multiple testing, and the log2 fold change of genes.

We used the Database of Annotation, Visualization and Integrated Discovery (DAVID) to identify enriched gene classes as well as analysis of individual WormBase IDs of genes from our Ballgown analysis on R to identify the germline genes.

***Heat map generation***

The heatmaps were generated using R Studio. The Bioconductor package with the gplot library and the heatmap.2 function was utilized.

***Venn Diagram analysis***

Bioinformatics and genomics website http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl and LucidChart were used to construct Venn diagrams.

Venn diagrams were generated for significantly altered mRNA for each condition tested (p-value <0.05).

***Brooding Assay***

The wild-type Bristol N2, VC1029 ccar-1 (gk433) IV, MT7019 ccar-1 (n2667) IV, DM1153 ccar-1 (ra14) IV, DM1154 ccar-1 (ra5) IV were used in the brood assay. Synchronized animals were grown on OP50 plates until day 1 of adulthood at 20˚C. Individual worms were transferred to 12 well plates to assess daily brood amounts. The worms were moved to a new well each day, and the number of progeny was recorded. The experiments were repeated in triplicate with n=6 for each trial. The same procedure was repeated for all the strains in EV and *sir-2.1* RNAi plates.

***qRT-PCR***

Worms were collected, and RNA extraction was done using Trizol reagent (Ambion) followed by cDNA synthesis using the SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. cDNA was diluted to 50ng/µl and used as a template for qRT-PCR. qRT-PCR and the normalization was done as previously described [162]

***Fluorescence imaging***

WS2170 (unc-119(ed-3)) III; opls110 IV was synchronized and fed with control (EV), CEP-1, CED-4, or CCAR-1 RNAi from the L1 to day 1 of adulthood. Worms were irradiated at day 1 with 400uJ/$m^3$ UV using a Stratalinker. Treated worms were imaged for the presence/absence of act-5::YFP 'halos' 24 hours post-radiation using a Keyence fluorescence microscope.

SDW080 (peft::CCAR-1::GFP::3xflag) animals were anesthetized with 10mM levamisole and photographed using a Keyence fluorescence microscope.

***Lifespan Assays***

Lifespan assays were conducted on wildtype N2 and CF1903 glp-1 (e2144) III at both 16˚C and 25˚C with forty worms per condition in biological triplicates. Animals were transferred to fresh plates daily for five days to avoid progeny contamination. Adult worms were scored every other day and counted as dead upon no response to poking with a platinum wire. Survivability was plotted using Graphpad Prism v.6 (Graphpad software www.graphpad.com) and statistical analysis done by log rank (Mantel-Cox) test.

***Data Availability***

*Gene expression data are available* in the SRA database (Accession Number SUB8460771).

**Results**

***Germline genes are regulated by CCAR-1***

We used whole transcriptome RNA-sequencing to examine gene regulation by *C. elegans* CCAR-1. The CCAR-1 deletion mutant strain VC1029 ccar-1 (gk433) IV, which contains a deletion of the first three exons of the *ccar-1* gene resulting in a null mutant [62], was outcrossed three times with our laboratory wildtype N2 strain to create strain SDW040. RNA samples were then generated from worms at day 1 of adulthood for wildtype (N2) and CCAR-1Δ (SDW040) strains in biological duplicates and sent for RNA sequencing. (For complete results for regulated genes see Table S1 and Supplementary Data 1. For upregulated genes see Table S2 and Supplementary data 2and for downregulated genes see Table S3 and Supplementary Data 2).

The top two gene categories regulated by *ccar-1* deletion in both upregulated and downregulated genes were germline genes and DAF-2/SIR-2.1-regulated genes (Fig. 1A). The other categories of genes found in the upregulated gene dataset included neuronal genes, zinc-factor proteins, DNA-binding proteins and non-coding RNAs compared to intestinal genes, non-coding RNAs and neuronal genes found in the downregulated dataset.

The DAF-2/SIR-2.1-regulated gene category was expected, as previous work in both *C. elegans* and mammalian cells has linked CCAR family members with SIR-2.1 and SIRT1, respectively [10, 25, 29, 159]. However, a role for CCAR-1 in the regulation of germline genes has not been previously reported. The germline-specific gene categories from our dataset included piRNA/21U-RNA genes, P-granule-associated protein genes, and male-spermatogenic genes (Fig. 1B). For the full gene ontology analysis, see Supplementary Data 3.

To validate our findings, we then used qRT-PCR on a couple of the top-most downregulated and upregulated germline genes (Fig. 1C). We tested the genes R07H5.11 and Y54G2A.36 from the upregulated gene dataset and F14H8.8 and C04C314 from the downregulated gene dataset and found that the qRT-PCR results were consistent with the RNA-sequencing results. We thus conclude that CCAR-1 regulates the expression of germline-enriched genes, amongst other genes, in *C. elegans.*

***CCAR-1 is localized in the germline.***

As CCAR-1 regulates germline gene expression, we hypothesized that the CCAR-1 protein might be highly expressed in the germline. The CCAR-1 protein has been shown to localize throughout the various cell types of the worm [62], but its localization in the germline has not been previously documented. To test for CCAR-1 protein localization, we constructed a peft::CCAR-1::GFP::3XFLAG strain (SDW082) and found that CCAR-1 is expressed in the

germline (Fig. 2). CCAR-1 is expressed in the the oocytes and the mitotic/late pachytene region, supporting its role in germline gene regulation.

***CCAR-1 protects germline cells from DNA damage-induced apoptosis***

Mammalian CCAR1 has been implicated in regulating apoptosis [54, 163-165]. In *C. elegans*, apoptosis occurs during development and in the gonad of adult hermaphrodites [74, 95]. As we found that CCAR-1 is important in regulating germline genes, we wondered whether it may also be important in regulating germline apoptosis.

During apoptosis, cell corpse engulfment is carried out by neighboring cells, which involves substantial rearrangement of the actin cytoskeleton as it surrounds the dying cells [166]. We thus used a transgenic strain expressing the actin isoform ACT-5 fused to YFP (ACT-5::YFP strain WS2170 (unc-119(ed-3)) III; opls110 IV) in the sheath cells to highlight apoptotic cells [96, 167]. We compared worms treated with empty vector (EV) RNAi as compared to CCAR-1 RNAi to visualize any differences in actin "halos" that appear around early apoptotic cells after treatment with UV damage.

Transgenic ACT-5::YFP strain WS2170 (unc-119(ed-3)) III; opls110 IV worms were bleach synchronized and placed on RNAi plates until development into L4. L4 worms were then UV irradiated (400uJ/$m^3$) using a Stratalinker and assessed for DNA damage-induced apoptosis 24 hrs later. For control images (no UV radiation), see Supplementary Data 5.

We found that in the empty vector control RNAi-treated worms (EV) there was an occurrence of several ACT-5::YFP "halos" signifying the expected UV damage-induced apoptosis (Fig. 2A). As expected, knockdown of either CEP-1/p53 or CED-4/Apaf-1, two key regulators of apoptosis [96, 167], inhibited the production of the UV-induced apoptotic "halos". Interestingly, upon knockdown of CCAR-1, we see an increase in UV-induced ACT-

5::YFP “halo”' as compared to control, signifying that CCAR-1 functions in protecting against UV damage-induced apoptosis (Fig. 2A). Data quantification indicates an approximate three-fold increase in actin “halos” with CCAR-1 RNAi as compared to control RNAi (EV) (Fig. 2B). Altogether, these data demonstrate a novel role for CCAR-1 in germline apoptosis.

***CCAR-1 is required for normal levels of progeny production***

To further investigate a functional role for CCAR-1 in the germline, we assessed progeny production. We compared wildtype N2 worms with ourSDW040 in a brood assay and counted the number of progeny until day three of adulthood (Fig. 3). To validate our results, we used three additional CCAR-1 mutant strains available from the CGC: MT7019 ccar-1 (n2667) IV, DM1153 ccar-1 (ra14) IV and DM1154 ccar-1 (ra5) IV,. Our results show that CCAR-1 is required for normal levels of progeny production, as progeny levels in the mutants dropped by 2.5-4-fold in the various CCAR-1Δ strains.

***CCAR-1 regulates longevity in a manner that is dependent on the presence of a germline.***

In a previous study, we showed that CCAR-1 RNAi increased C. elegans longevity [2]. The germline in C. elegans is known to affect longevity [159, 168, 169]. Therefore, to test whether the effect of CCAR-1 on longevity is dependent on the ability of CCAR-1 to regulate progeny number, we performed lifespan assays using a temperature-sensitive strain in which we could turn off progeny production. CF1903 glp-1 (e2144) III worms display a temperature-sensitive loss of the germline at 25˚C, but not at the permissive temperature of 16˚C. We compared the lifespans of wildtype (N2) versus CF1903 glp-1 (e2144) III worms treated with and without CCAR-1 RNAi (Fig. 4). The worms were scored every other day starting at day 1 of adulthood for survival, and dead worms were scored when non-responsive to poking with a platinum wire. At the permissive temperature of 16˚C, both N2 and CF1903 glp-1 (e2144) III

worms showed no change in survival between the control and CCAR-1 RNAi groups, with a mean survival of thirteen days in all cases (Fig. 4A). Thus, CCAR-1 does not affect lifespan at 16˚C in the presence of the germline. At 25 ˚C, wildtype N2 worms fed control RNAi had a mean survival of three days, whereas N2 worms fed CCAR-1 RNAi had a mean survival of five to seven days (Fig. 4B). As expected, based on our previous work [2], CCAR-1 RNAi affects lifespan at 25 ˚C. However, this effect on lifespan extension was lost in the CF1903 glp-1 (e2144) III worms grown at this temperature (Fig. 4B). At the restrictive temperature of 25 ˚C in which the germline is absent, CF1903 glp-1 (e2144) III worms fed control RNAi had a mean survival of eight days, whereas CCAR-1 RNAi fed worms had a mean survival of six days (Fig. 4B). Therefore, these data suggest that decreased CCAR-1 expression enhances longevity through decreasing progeny production (Fig 5).

**Discussion**

Here, we reveal the genome-wide effect of CCAR-1 on gene expression in *C. elegans.* Previous mammalian tissue culture studies have linked the CCAR family of proteins to the regulation of genes involved in metabolism. We used a strain in which the single worm CCAR family member, CCAR-1, is deleted in these studies. We found that in addition to having a prominent role in regulating metabolic genes, CCAR-1 also controls germline-specific gene expression. CCAR-1 expression is required to protect germline cells in the worm against UV-induced apoptosis and is required for normal levels of progeny. Additionally, CCAR-1 regulates longevity in a manner that is dependent on the presence of a germline.

Our RNA-seq transcriptional dataset shows that the two major categories of genes regulated by the deletion of CCAR-1 in the worm are DAF-2/SIR-2.1-regulated genes, and germline-linked genes. The DAF-2/SIR-2.1-regulated gene category was expected, as human

CCAR2 binds to the catalytic domain of SIRT1, forming a stable complex that inhibits SIRT1 deacetylase activity [10, 25]. SIR-2.1 modulates the well-studied DAF-2/DAF-16 signaling cascade [170, 171]. Additionally, our previous studies show that CCAR-1 affects the regulation of the heat shock response in a SIR-2.1-dependent manner [2, 29].

The regulation of germline-related genes by CCAR-1 is a novel finding. The germline-specific gene categories from our dataset included piRNA/21U-RNA genes, P granule-associated protein genes, and male-spermatogenic genes. In the germline of *C. elegans* and other species, piRNAs work together with PIWI argonaute proteins to repress transposable elements from causing detrimental effects in the next generation [172]. In *C. elegans*, piRNAs have also been shown to have a function in spermatogenesis, maintaining germline cells and cell totipotency [173, 174]. Future work can help to determine whether CCAR-1 regulates the transcription of these piRNA genes, and/or if it is involved in regulating piRNA processing.

P granules are protein- and RNA-containing membrane-less organelles which are important in *C. elegans* germline development. Interestingly, P granule-associated protein-coding transcripts are another category of genes regulated by CCAR-1. P granule assembly depends on self-interaction domains that are present in the PGL P granule scaffolding proteins. P granules are found throughout the life cycle of worm germline cells, and proteins associated with P granules have a critical role in germline cell differentiation during post-embryonic development [175]. As P granules sit on the cytoplasmic side of nuclear pores, most mRNAs transcribed in germ cells pass through a P granule on their way to the cytoplasm, consistent with a role of these granules in mRNA surveillance. Our finding that CCAR-1 regulates the expression of both piRNAs, and P granule-associated proteins suggests that CCAR-1 may regulate mRNA surveillance in the *C. elegans* germline.

The last category of germline-related genes identified to be regulated by CCAR-1 are male-spermatogenic genes. Previous studies have reported that there are precise sex-specific expression levels of spermatogenic and oogenic functional genes in the male and hermaphrodite germline cells. [176] With this distinction, several male-spermatogenic genes were shown to be regulated by the deletion of CCAR-1 including spermatid genes. Thus, by using a whole animal model, we have identified a novel role for CCAR-1 in regulating germline genes.

Given that CCAR-1 regulates genes that are important in the germline, our results that extend CCAR-1 protein expression to the germline make sense. While our work is the first to identify CCAR-1 protein expression in the germline, previous high-throughput experiments have shown that the CCAR-1 mRNA is present in the *C. elegans* germline. From a Serial Analysis Gene Expression (SAGE) high-throughput analysis of the hermaphrodite germline, *ccar-1* appeared to be one of 1063 genes enriched in the germline as compared to the soma [177]. This gene was also identified as differentially expressed between wildtype N2 and GLP-4 worms, which are lacking a fully functional germline [178]. Therefore, both *ccar-1* mRNA and CCAR-1 protein are localized to the germline, allowing the possibility for a role in regulating germline gene expression.

Interestingly, P granules and their resident PGL proteins are linked to germline apoptosis. In the *C. elegans* germline, and in the germlines of other organisms, several hundreds of cells die by apoptosis due to physiological signals, genotoxic stress and/or bacterial infection [95]. Here, we show that knockdown of CCAR-1 not only regulates germline-specific genes, but also increases the formation of apoptotic germline cells following DNA damage induction. Previous work has shown that PGL-depleted germ cells are

selectively committed to apoptosis [111]. In future work, it will be interesting to test the hypothesis that CCAR-1 affects germline apoptosis through the regulation of piRNAs and P granule function.

Investigation of genes involved in lifespan extension has been an area of interest in *C. elegans* research. In a previous study, we showed that knockdown of CCAR-1 affects an increase in the lifespan of wildtype N2 *C. elegans* at 23˚C [2]. Here, we confirm this finding in wildtype N2 worms; however, with the use of a germline-defective worm strain, we see that CCAR-1 knockdown loses its lifespan extension capabilities at 25˚C. Thus, the effect of CCAR-1 on lifespan regulation depends on the germline.

In summary, our experiments have established that CCAR-1 has a vital role in regulating the *C. elegans* germline. CCAR-1 regulates germline transcription, reproduction, DNA-damage induced apoptosis, and lifespan. We find it intriguing that CCAR-1 regulates various genes affiliated with P granules, membraneless organelles through which all germline mRNAs must pass through as they exit the nuclear pores. Are many of the germline-specific biological effects of CCAR-1 occurring through mRNA surveillance that occurs in the P granules? In future work, it will be important to unravel the precise mechanism of action of CCAR-1 in the germline.

**Figure legends**

Figure 1: Germline genes are enriched in the gene set regulated by CCAR-1. A. Pie chart showing the genome-wide categories of genes transcriptionally regulated by CCAR-1. RNA sequencing was performed using total RNA samples from N2 (control) and CCAR-1Δ (SDW040) strains. The largest category of regulated genes from the RNASeq analysis are germline genes (24.1%) followed DAF-2/SIR-2.1-regulated genes (20.7%). B. The

subcategories of germline-related genes found in our RNASeq dataset are listed. C. qRT-PCR validation of the RNASeq data for genes that are upregulated (blue) or downregulated (red) in the CCAR-1Δ (SDW040) strain as compared to control N2 worms. Significance was determined using the Student's T test, where * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$. n=3 for all qPCR data

Figure 2: CCAR-1 protects germline cells from UV damage-induced apoptosis. A. ACT-5::YFP strain WS2170 (unc-119(ed-3)) III; opls110 IV worms expressing actin fused to YFP in the gonad sheath cells were treated with control (EV), CED-4/Apaf-1, CEP-1/p53, and CCAR-1 RNAi prior to exposure to a UV dose of 400J/$m^2$. Images were taken 24 hrs after the exposure. YFP, brightfield and merged images are used to visualize germ cell corpses, which are represented as actin "halos" during early-stage apoptosis and indicated by white arrows. B. Quantification of the number of apoptotic actin "halos" from Figure 3A. There is significant change ($p<0.001$) between EV and CCAR-1 apoptotic 'halos'. n= 10 for all imaging samples.

Figure 3: CCAR-1 is required for normal levels of progeny production. The graph shows a brood assay in which the N2 control strain is compared to various CCAR-1 mutant allele strains SDW040 ccar-1 (gk433) IV, MT7019 ccar-1 (n2667) IV, DM1153 ccar-1 (ra14) IV and DM1154 ccar-1 (ra5) IV) to determine the effects of CCAR-1 on the number of progeny. n =50.

Figure 4: CCAR-1 regulates longevity in a manner that is dependent on the presence of a germline. A. Lifespan analysis was performed at 16°C in wild-type (N2) worms and in CF1903 glp-1(e2144) III worms fed control RNAi or ccar-1 RNAi. GLP-1Δ worms grown at this permissive temperature contain a germline. B. Lifespan analysis was performed at 25°C in wild-type (N2) and CF1903 glp-1 (e2144) III worms fed control RNAi or ccar-1 RNAi

throughout lifespan. CF1903 glp-1(e2144) III worms grown at this restrictive temperature lack a germline. For (A-B), worms were scored every other day for survival, and significance was determined using the Log-rank (Mantel-Cox) Test. n=3 (40 worms per n)

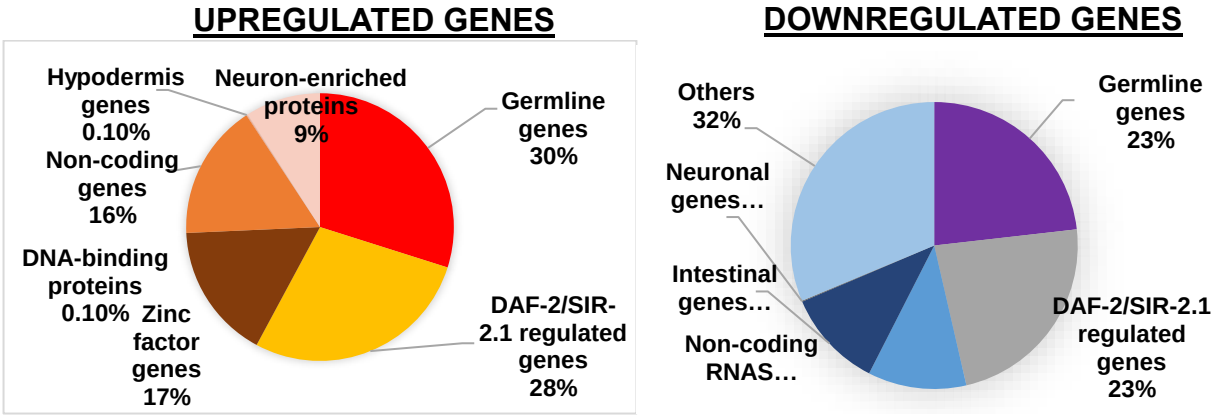
Figure 5: Proposed model for CCAR-1 functions in the germline. We show in this work that CCAR-1 regulates germline gene expression and protects from UV-induced apoptosis in the germline. Reductions in CCAR-1 expression led to decreased progeny numbers and an enhanced lifespan.

**Acknowledgements**

The wildtype N2,) VC1029 ccar-1 (gk433) IV, MT7019 ccar-1 (n2667) IV, DM1153 ccar-1 (ra14) IV, DM1154 ccar-1 (ra5) IV, WM161 prg-1 (tm872) I, ACT-5::YFP strain WS2170 (unc-119(ed-3)) III; opls110 IV and CF1903 glp-1 (e2144) III, strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). This work was funded by NIH grant AG052149.

**Conflict of Interest**

None declared.




| Germline genes | Function |
|---|---|
| **piRNAs/21U-RNAs** | Known to have a function in germline gene regulation in *C.elegans* |
| **P-granule-associated protein genes** | Genes associated with P-granule proteins such as PRG-1, PLG-1 |
| **Male-spermatogenic genes** | Spermatid genes |

**Figure 2.1: Germline genes are enriched in the gene set regulated by CCAR-1.** A. Pie chart showing the genome-wide categories of genes transcriptionally regulated by the deletion CCAR-1. RNA sequencing was performed using total RNA samples from N2 (control) and SDW040 ccar-1 (gk433) IV strains. The largest category of regulated genes from the RNASeq analysis are germline genes followed DAF-2/SIR-2.1-regulated genes. B. The subcategories of germline-related genes found in our RNASeq dataset are listed.

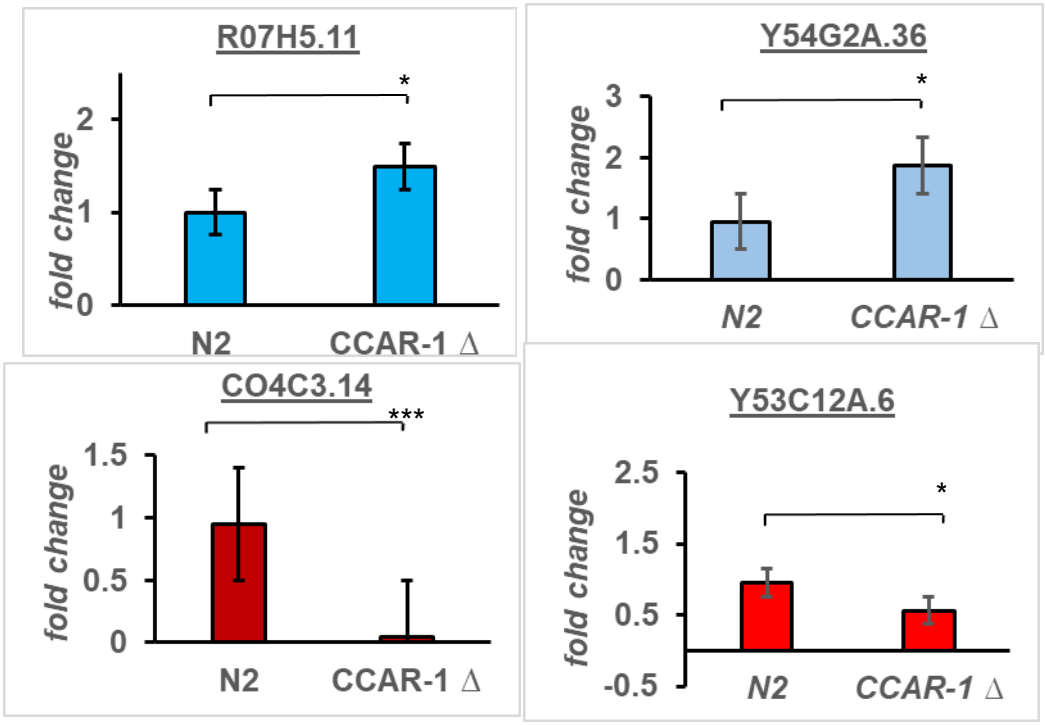



**Figure 2.2: Germline genes are enriched in the gene set regulated by CCAR-1 (continued).** qRT-PCR validation of the RNASeq data for genes that are upregulated (blue) or downregulated (red) in the CCAR-1 mutant (SDW040) strain as compared to control N2 worms. Significance was determined using the Student's T test, where * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$. n=3 for all qPCR data.

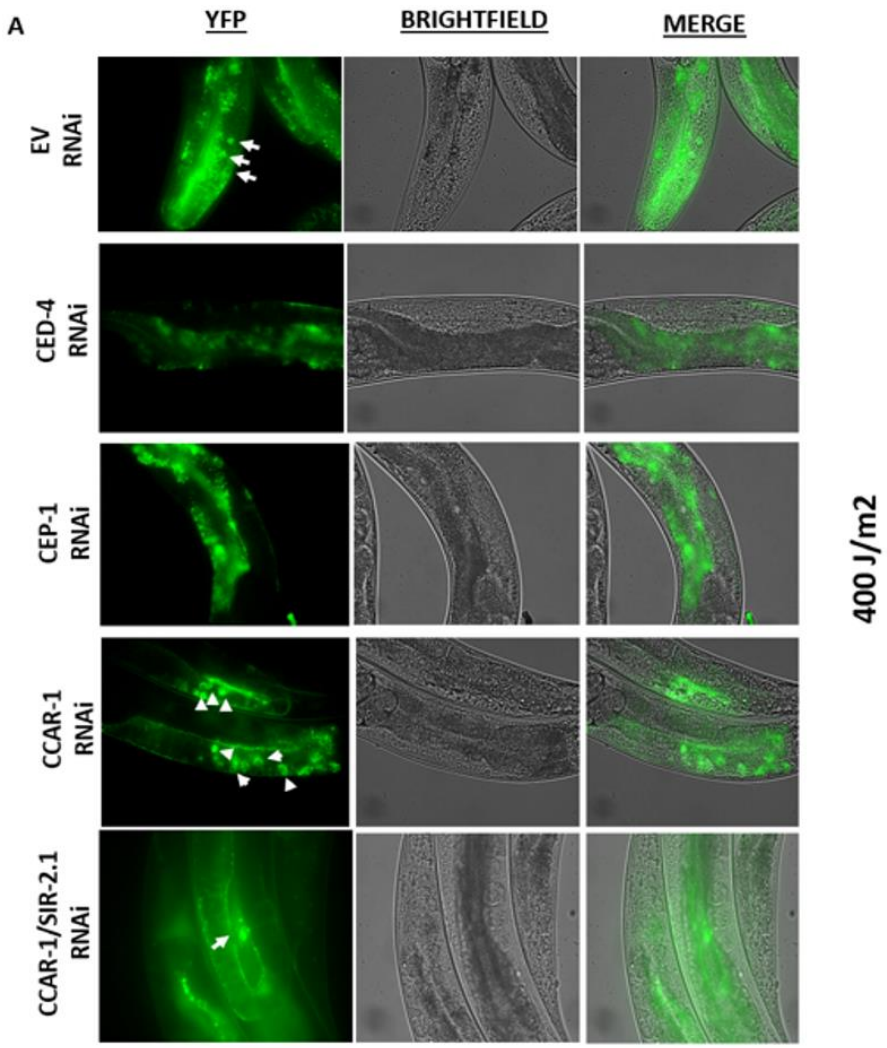



**Figure 2.3: CCAR-1 protects germline cells from UV damage-induced apoptosis** A. Control (EV), genes required for DNA damage-induced apoptosis (CED-4 and CEP-1) and CCAR-1 were knocked down by RNAi to highlight their role in promoting apoptotic responses. Images were taken 24hrs after exposure of worms to 400J/m2. Comparison of strains expressing actin fused to YFP (ACT-5::YFP strain WS2170 (unc-119(ed-3)) III; opls110 IV) in the gonad sheath

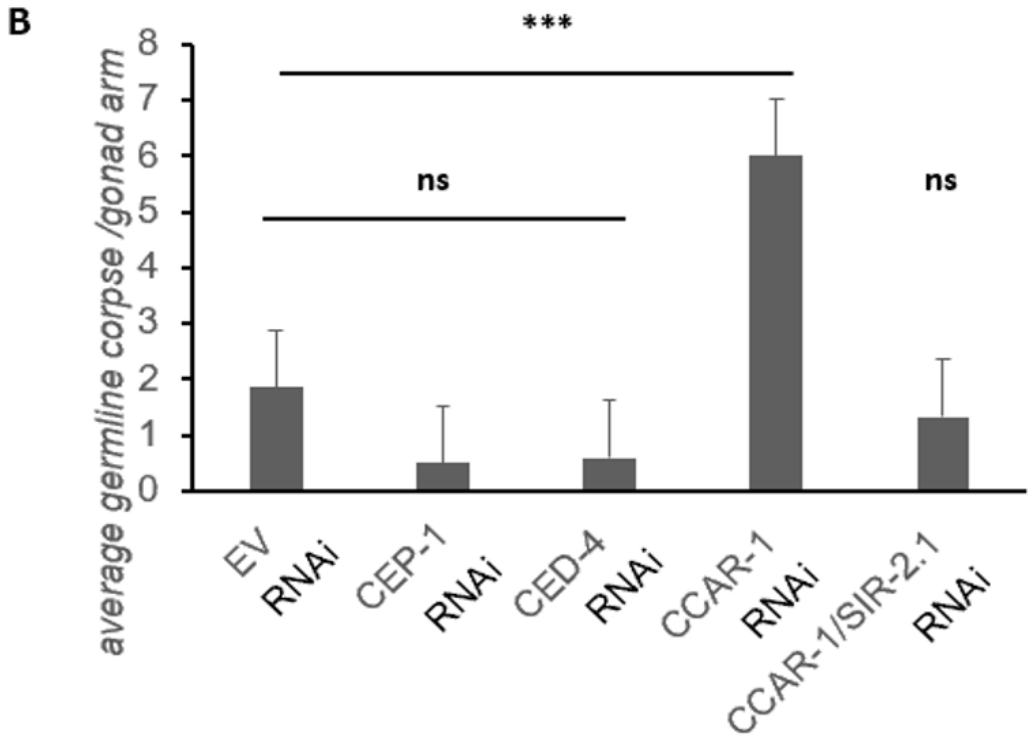



**Figure 2.4: Quantification of the number of apoptotic actin 'halos' from Figure 3A. n= 10 for all imaging samples.**

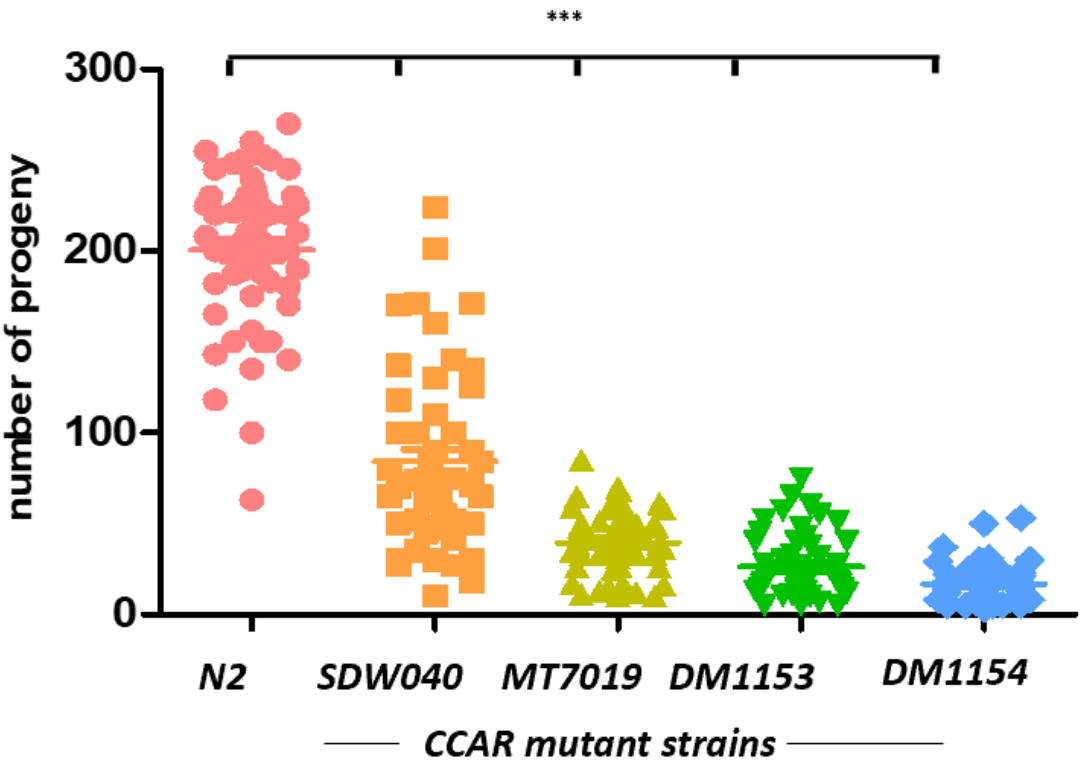



**Figure 2.5: CCAR-1 is required for normal levels of progeny production.** The graph shows a brooding assay of the n2 control strain as compared to various ccar-1 mutant allele strains (sdw040 ccar-1 (gk433) iv, mt7019 ccar-1 (n2667) iv, dm1153 ccar-1 (ra14) iv and dm1154 ccar-1 (ra5) iv) to determine the effects on the number of progeny. n=50.

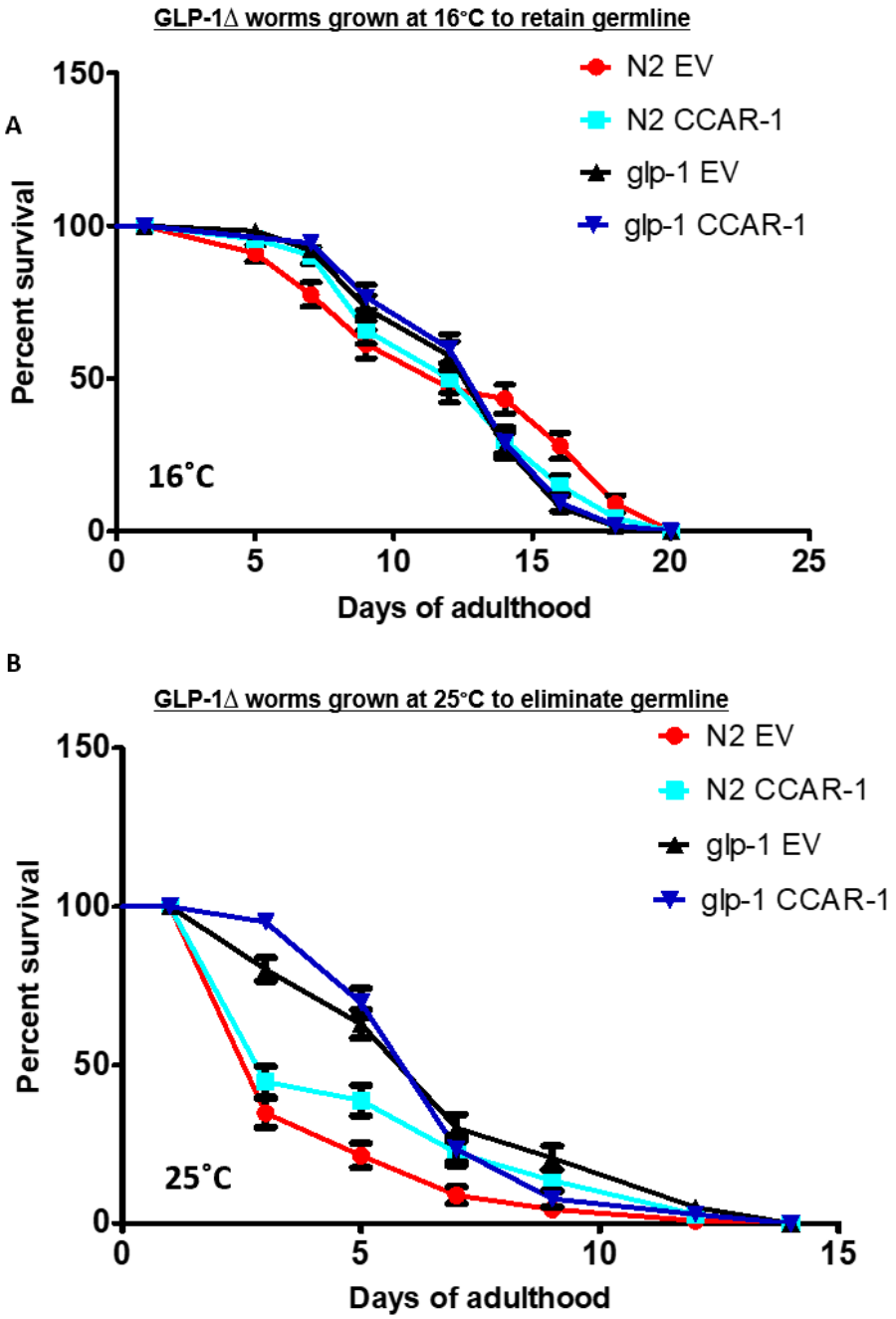



**Figure 2.6: CCAR-1 regulates longevity in a manner that is dependent on the presence of a germline.** A. Lifespan analysis was performed at 16°C in wild-type (N2) worms and CF1903 glp-1 (e2144) III in worms fed control RNAi or ccar-1 RNAi. CF1903 glp-1 (e2144) III worms grown at this permissive temperature contain a germline. B. Lifespan analysis was performed at 25°C in wild-type (N2) and CF1903 glp-1 (e2144) III worms fed control RNAi or ccar-1 RNAi throughout lifespan. CF1903 glp-1 (e2144) III, worms grown at this restrictive temperature lack a germline. For (A-B), worms were scored every other day for survival, and significance was determined using the Log-rank (Mantel-Cox) Test. N=3 (40 worms per n)

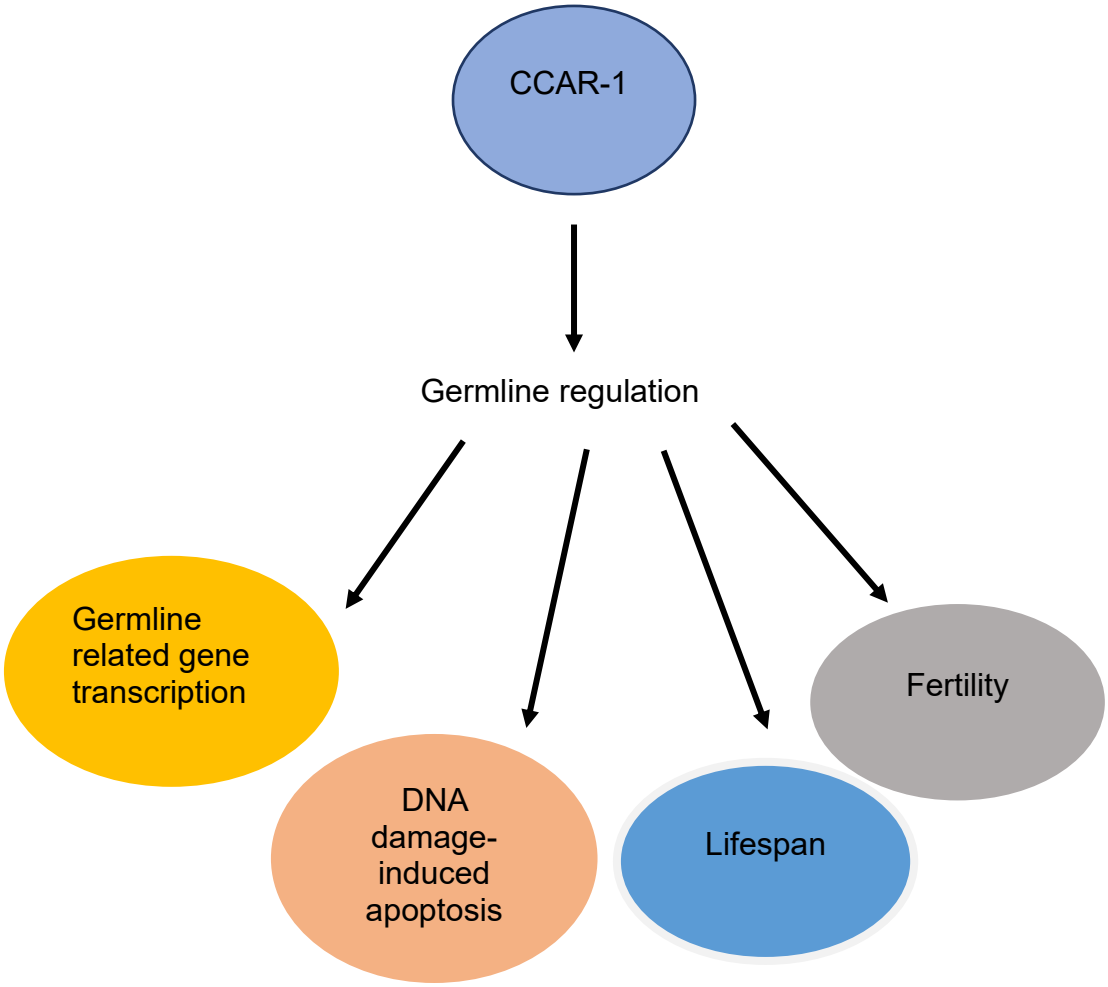



**Figure 2.7: Proposed model for CCAR-1 functions in the germline**. We show in this work that CCAR-1 regulates germline gene expression and protects from UV-induced apoptosis in the germline. Reductions in CCAR-1 expression lead to decreased progeny numbers and an enhanced lifespan.

# CHAPTER 3: CCAR-1 WORKS TOGETHER WITH U2AF LARGE SUBUNIT UAF-1 TO REGULATE ALTERNATIVE SPLICING.


## Abstract

Aberrant forms of alternatively spliced proteins are a common consequence of genotoxic stress and diseases like cancer and multiple sclerosis. Hence, the need to identify *trans*-acting factors involved with these divergent forms of the proteins. The Cell Division Cycle and Apoptosis Regulator (CCAR) protein family regulates alternative splicing and other key physiological functions. For example, mammalian CCAR2/DBC1 forms a complex with the zinc factor protein ZNF326 to integrate alternative splicing with RNA polymerase II elongation in AT-rich regions of the DNA. Additionally, *Caenorhabditis elegans* CCAR-1, a homolog to mammalian CCAR2, facilitates the alternative splicing of the perlecan /*unc-52* gene by excising exon 17 during development. Nonetheless, much about the CCAR family's role in alternative splicing is unknown. We are interested in uncovering the role of the CCAR family in alternative splicing *in vivo* using *Caenorhabditis elegans*. By utilizing this model, we can identify genome-wide alternative splicing changes in a whole organism and utilize available *trans*-acting factor mutants and behavioral assays to understand the mechanism of action. Mammalian and *C. elegans* CCAR family members share a significant similarity in structure and domains, making the latter a good model for the study. Our work identifies new alternative splicing targets of CCAR-1 in *C. elegans* and a novel interaction with spliceosome factors, UAF-1 and UAF-2.

## Introduction

The human genome requires approximately 400,000 proteins but contains only 30,000 genes. To overcome this impediment, alternative splicing of mRNAs allows one gene to produce multiple isoforms of a protein, causing an expansion of the proteome [59, 60, 114, 116, 120, 123, 125]. This process occurs in 70% of human genes and is facilitated by members of the spliceosome, *cis*-acting sequences, and *trans*-acting factors [116, 117, 120, 123]. Furthermore, AS is regulated by genotoxic stress such as heat shock and disease states in the cell; diseases such as multiple sclerosis and cancer have significant AS changes [116, 120]. Hence, there is a need to investigate AS changes during genotoxic stress and diseases and the factors involved.

The human cell division cycle apoptosis regulator family (CCAR family) are multifaceted proteins involved in regulating cancer, metabolism, apoptosis, stress response, and, more recently, alternative splicing [1, 7, 9, 10, 25, 29, 43, 46]. This ability to participate in multiple processes is because of the various domains in their structure [9]. These domains include an S1-like putative RNA binding domain, a nuclear localization domain, and an L.Z. that interacts with transcription factors NFκB family members, RelB, and p52 [5]. In addition, a nudix domain binds metabolites such as NAD+ and acts as an inactive holdase. Furthermore, an EF-hand domain is involved in calcium-dependent regulation, and a coiled-coiled domain is involved in protein-protein interactions.

As previously mentioned, an emerging function of the CCAR family is the regulation of alternative splicing. Mammalian CCAR2 forms a complex with a zinc factor, ZNF326 forming the DBIRD complex, which binds both RNA polymerase II and the ribonucleoprotein particle [61]. This interaction of the DBIRD complex integrates alternative splicing with RNA

polymerase II elongation and is most prevalent in A+T regions of the DNA [61]. Interestingly, this process is independent of SIRT1, a histone deacetylase that is primarily associated with CCAR2. In *C. elegans*, CCAR-1 regulates the alternative splicing of *unc-52*/perlecan, a basement extracellular matrix (ECM) ligand [62]. This alternative splicing allows the excision of *unc-52* exon 17 during development. Additionally, CCAR-1 physically interacts with HRP-2/hnRNP R, a splicing factor previously shown to mediate the alternative splicing of *unc-52* [62].

The role of CCAR-1 in alternative splicing in *C. elegans* is only just emerging and remains largely unstudied. For example, how does CCAR-1 regulate the global alternative splicing of genes in this whole model organism? How does this regulation change during stress? And further, what is the mechanism of action of CCAR-1 in the alternative splicing of these genes?

*C. elegans* is an excellent model to study alternative splicing due to the conservation of the splicing machinery, the ability to manipulate the worm genetically, and the availability of viable mutants of splicing factors [9, 59, 60]. In addition, there is high conservation of the CCAR protein family structure within species, with mammalian CCAR2 having 30% sequence similarity to *Caenorhabditis elegans* CCAR-1 [9].

Here we use RNA sequencing analysis to show genome-wide alternative splicing changes with the deletion of CCAR-1 in *C. elegans*. We identify new alternative splicing targets of CCAR-1 in both heat shock and no heat shock condition. Additionally, by using mass spectrometry, we show that CCAR-1 interacts with splicing factors of the spliceosome, UAF-1, and UAF-2. Furthermore, we demonstrate that CCAR-1 RNAi affects the alternative splicing patterns and motility of UAF-1 mutants. Finally, we show that CCAR-1 RNAi reduces

the amount of polyglutamate aggregates caused by UAF-1 RNAi in a *C.elegans* Huntington's disease model worm.

## Methods

### *C. elegans strains and maintenance*

The following strains were used in the study: Wild-type Bristol N2, CCAR-1 mutant VC1029 *ccar-1 (gk433)* IV, UAF-1 mutant MT16492 *uaf-1*(n4588) III , UAF-2 mutant VC3010 *uaf-2* (gk3159) IV , AM140 rmls132 (unc-54p::Q35::YFP) and SDW080 (p*eft*::CCAR-1::GFP::3xflag). The CCAR-1 mutant VC1029 *ccar-1 (gk433)* IV was outcrossed three times to our N2 wildtype strain to generate the SDW040 strain. All strains were maintained at 20 °C on standard NGM plates seeded with *Escherichia coli* OP50-1. Synchronous populations of nematodes were obtained by bleach synchronization and plating for 19 hrs on NGM plates without food.

### *RNA preparation for RNA sequencing*

RNA samples were prepared using Trizol reagent (Ambion).The samples were sent to the Brigham Young University DNA sequencing center. RNA libraries were prepared using the TruSeq Total RNA kit (Illumina, Inc.) with a Ribo-zero gold kit (Illumina, Inc.) to remove rRNA and mitochondrial RNA transcripts.

### *RNA-sequencing analysis*

HISAT and STRING-Tie were used in the alignment and assembly of raw FASTQ files from the RNA sequencing. The R program, DEXSeq was used to determine exon usage from the dataset.

***Venn Diagram analysis***

Bioinformatics and genomics website http://bioinformatics.psb.ugent.be/cgi-bin/liste/Venn/calculate_venn.htpl and LucidChart were used to construct Venn diagrams. Venn diagrams were generated for significantly altered exon usage for each condition tested (p-value <0.05).

***qRT-PCR***

The worms were collected, and RNA extraction was done using Trizol reagent (Ambion) followed by cDNA synthesis using the SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. cDNA was diluted to 50ng/µl and used as a template for qRT-PCR. qRT-PCR and the normalization were done as previously described [162].

***RT-PCR***

RNA samples were prepared using Trizol reagent (Ambion) followed by cDNA synthesis using SuperScript III reverse transcriptase kit (Invitrogen) on all samples. The samples were amplified through conventional PCR with designed primers, spanning 200-800 base pairs of each gene. The primers were specifically designed to identify splicing changes between alternative exons and introns. The samples were separated in a 5% TBE polyacrylamide gel [179]. The gels were stained with SYBR gold nucleic acid stain (Invitrogen), and the images were taken by Molecular Imager F.X. (Bio-Rad). The densitometries of the bands were calculated through Image J for quantitation, and the ratios of the isoform bands were calculated.

***Thrashing Assay***

The worms were grown in desired OP50-1/RNAi plates until the fourth larval stage. Individual worms were placed in a drop of nematode growth media on the lid of a petri dish. The number of body bends by each worm per minute was recorded. This was repeated for 15 worms per condition.

***Protein aggregation assay***

AM140 (Q35::YFP) worms were grown in desired RNAi plates until the fourth larval stage. Worms were picked to fresh plates until day 3 of adulthood. Protein aggregates were scored for at least 15 worms per condition in independent biological triplicates

***Mass Spectrometry***

Immunoprecipitation eluates were prepared for mass spectrometry-based proteomics using filter-aided sample preparation (FASP). Briefly, proteins were alkylated with iodoacetamide (IAA), buffer exchanged with urea followed by ammonium bicarbonate, and finally digested with Trypsin/Lys-C overnight at 37˚C. Peptides were eluted and subsequently desalted using C18 SPE cartridges (Waters) with a vacuum manifold. Desalted peptides were dried in a vacuum concentrator. Peptides were resuspended in $H_2O$/0.1% formic acid for LC-MS/MS analysis.

Peptides were separated using a 75 µm x 50 cm C18 reversed-phase-HPLC column (Thermo Scientific) on an Ultimate 3000 UHPLC (Thermo Scientific) with a 120 minute gradient (2-32% ACN with 0.1% formic acid) and analyzed on a hybrid quadrupole-Orbitrap instrument (Q Exactive Plus, Thermo Fisher Scientific). Full MS survey scans were acquired at 70,000 resolution. The top 10 most abundant ions were selected for MS/MS analysis.

Raw data files were processed in MaxQuant (www.maxquant.org) and searched against the Uniprot *Caenorhabditis elegans* protein sequence database. Search parameters included constant modification of cysteine by carbamidomethylation and the variable modification, methionine oxidation. Proteins were identified using the filtering criteria of 1% protein and peptide false discovery rate.

**Results**

***CCAR-1 regulates the alternative splicing of genes with and without heat shock in C. elegans.***

We used whole transcriptome RNA-sequencing to examine changes in alternative splicing with the deletion of CCAR-1. Previous work shows that mammalian CCAR-2 regulates the alternative splicing of several genes, and *C. elegans* CCAR-1 regulates the alternative splicing of a perlecan gene, *unc-52*. However, the genome-wide alternative splicing targets of CCAR-1 in a whole model organism and during stress is unknown.

To investigate this, we used RNA sequencing to understand the global alternative splicing changes with the deletion of CCAR-1. The CCAR-1 deletion mutant strain VC1029 *ccar-1 (gk433)* IV, which contains a deletion of the first three exons of the *ccar-1* gene resulting in a null mutant [62], was outcrossed three times with our laboratory wildtype N2 strain to create strain SDW040 *(gk433)* IV. RNA samples were then generated and sequenced from day 1 of adulthood of wildtype (N2) and CCAR-1 mutants (SDW040 *(gk433)* IV) worms in biological duplicates in both heat shock and no heat shock conditions.

We used differential exon usage analysis (DEXseq) to analyze the RNA sequencing data. In wildtype N2, 13 genes are significantly alternatively spliced with heat shock. This result is expected because stresses such as heat shock cause alternative splicing changes in *C.*

*elegans* and other species [180]. In the SDW040 *(gk433)* IV, 17 genes are significantly alternatively spliced during heat shock. Lastly, between N2 and CCAR-1Δ in control conditions, 17 genes are significantly alternatively spliced. Altogether, this data shows that other genes, not just *unc-52*, are alternatively spliced with the deletion of CCAR-1 in *C. elegans*. This data also indicates that CCAR-1 affects the alternative splicing of *C. elegans* genes during heat shock.

Table 1 includes the list of significantly alternative genes spliced in wildtype N2 and the SDW040 *(gk433)* IV during heat shock. We show that other than *unc-52*, CCAR-1 regulates the alternative splicing of different structural proteins including, *apl-1, ketn-1,* and *vab-10*. Furthermore, CCAR-1 regulates the alternative splicing of the target of splicing gene, *tos-1*, a widely used splicing reporter gene in *C. elegans*. In addition, stress response genes, heat shock proteins *hsp-90* (HSP90AA1) and *dnj-20* (DNAJB11), aging gene *idh-1* and rRNA demethylase, *tsfbm-1* are also shown in the dataset. Interestingly, CCAR-1's deletion regulates the alternative splicing of a spliceosome factor *uaf-2* (U2AF35). We also identify *unc-52* as one of the alternative splicing targets of CCAR-1 shown in previous studies [62]. From this analysis, we detect new CCAR-1 alternative splicing targets.

***Deletion of CCAR-1 changes the alternative splicing patterns of target genes.***

We used RT-PCR to validate our RNA sequencing dataset and show the changes in splicing patterns of target genes. We show the RT-PCR validation of two genes, F30F8.10 and *tos-1,* from our CCAR-1's deletion no heat shock dataset and *unc-52* and *uaf-2* from our CCAR-1 mutant heat shock dataset.

To determine these genes' isoform ratios, we used populations of approximately 300 worms of N2 and SDW040 *(gk433)* IV. The box plots represent the distribution of all

populations' isoform ratios, and the RT-PCR images show a visual representation of isoform changes.

Altogether, we demonstrate a similar trend as the RNA sequencing data in alternative splicing changes in the various populations. This confirms our previous conclusion that deletion of CCAR-1 regulates the alternative splicing of previously unknown *C. elegans* genes.

***CCAR-1 interacts with members of the spliceosome, UAF-1, and UAF-2 and affects the alternative splicing pattern of UAF-1 in a tos-1 splicing reporter.***

CCAR-1 and its family members regulate the alternative splicing of genes, but there is no experimental evidence to show their direct binding to RNA; due to the lack of an identified RNA binding domain. Therefore, we hypothesize that CCAR-1 is binding to a splicing factor(s) which enables alternative splicing changes. We constructed a peft: CCAR-1::GFP::3XFLAG strain (SDW082) to test this hypothesis, which we used to pull down CCAR-1 for mass spectrometry. As a result, we identified three splicing factors from the mass spectrometry dataset, RSP-3, the *C. elegans* homolog of SRSF1, a serine and arginine-rich splicing factor, and spliceosome factors, UAF-1 (U2AF65) and UAF-2 (U2AF35).

To determine if CCAR-1 is working with these members of the spliceosome, we used a *tos-1* splicing reporter in UAF-1 mutant MT16492 *uaf-1*(n4588) III and UAF-2 mutant VC3010 *uaf-2* (gk3159) IV. Alternative splicing changes were determined by quantifying the isoform ratios of the *tos-1* gene.

N2, CCAR-1 mutant SDW040 *(gk433)* IV, UAF-1 mutant MT16492 *uaf-1*(n4588) III and UAF-2 mutant VC3010 *uaf-2* (gk3159) IV worms were bleach synchronized and placed on OP50-1 plates until development into day one adults. RNA extraction using Trizol reagent

(Sigma) was followed by cDNA synthesis using the Superscript III reverse transcriptase kit (Invitrogen). Finally, the samples were run through RT-PCR analysis for the *tos-1* gene.

We assessed four different worm populations to detect the changes in isoform ratios in the worm strains. From our analysis, the *tos-1* gene predominantly produced three isoforms, A,B, and C. We measured isoform A/C and isoform B/C changes. Wildtype N2 mostly makes isoform A/C and has a low ratio for B/C. The CCAR-1 mutant SDW040 *(gk433)* IV also produces isoform A/C, with a slightly different ratio than N2. On the other hand, UAF-2 mutant VC3010 *uaf-2* (gk3159) IV does not show significant changes in both isoform ratios A/C or B/C compared to N2 and CCAR-1 mutants. However, UAF-1 mutant MT16492 *uaf-1*(n4588) III remarkably shows a 6-fold change in isoform A/C ratio and up to a 30-fold change in isoform B/C.

To determine if CCAR-1 affects the alternative splicing pattern of UAF-1, we used CCAR-1 RNAi in the UAF-1 mutant MT16492 *uaf-1*(n4588) III and assessed for changes in the *tos-1* splicing reporter. We show that CCAR-1 RNAi in the UAF-1 mutant increases the isoform A and C (not significant) but significantly increases the isoform ratio of B&C by up to 2-fold. This data suggests that CCAR-1 RNAi affects the splicing patterns of UAF-1, in a *tos-1* splicing reporter.

***CCAR-1 RNAi significantly increases the motility of UAF-1*Δ**

To further investigate if CCAR-1 is working with UAF-1, we assessed the motility of the N2 and UAF-1 mutant MT16492 *uaf-1*(n4588) III worms. *C. elegans* U2AF65 genes (*uaf-1*) and U2AF35 (*uaf-2*) cause genome-wide changes in 3' splice site [118, 119, 135], and *uaf-1* mutants have embryonic viability and motility defects [136]. We previously showed that CCAR-1 RNAi significantly increases of motility of N2 worms in a thrashing assay [2].

Therefore, we wanted to determine the effects of CCAR-1 RNAi on the motility of UAF-1 mutants.

To do this, we compared wild-type N2 worms with UAF-1 mutants MT16492 *uaf-1*(n4588) III in a thrashing assay. The worms were bleach synchronized and placed on EV (empty vector) and CCAR-1 RNAi plates at 25 ˚C until development into day one adults. On day 1 of adulthood, we counted the number of body bends per minute for each worm. Like our previous work, we show that CCAR-1 RNAi significantly increases the motility of N2 worms. However, we also observe that CCAR-1 RNAi rescues UAF-1Δ mutants motility, further suggesting that the two proteins could interact, affecting the alternative splicing of genes.

***CCAR-1 RNAi significantly decreases the number of PolyQ aggregates in UAF-1 RNAi***

Our previous work demonstrates that CCAR-1 impacts proteostasis in a *C. elegans* Huntington's disease model. To determine if CCAR-1 is working with UAF-1, we assessed polyglutamine aggregate formation in response to control, *ccar-1*, *uaf-1*, and *ccar-1/uaf-1 RNAi* in a Huntington's disease model strain AM140 rmls132 (unc-54p::Q35::YFP). AM140 strain contains 35 polyglutamine repeats fused to YFP (Q35::YFP) under the control of a muscle-specific promoter, which develops aggregates in the body wall muscle.

AM140 worms were bleach synchronized and grown in respective RNAi plates until day 3 of adulthood. Fluorescent images, as well as quantification of aggregates, are shown. As expected, we see an average of 17 aggregates/worm in empty vector (control) and a significantly lower average of 12 aggregates/worm in CCAR-1 RNAi [2]. In *uaf-1* RNAi, we see an average of 18 aggregates/ worm, slightly more than the N2 control. However, if we knockdown *ccar-1* and *uaf-1* simultaneously, the average is further reduced to 11 aggregates/worm, significantly reducing the number of aggregates per worm. These results

suggest that CCAR-1 restores proteostasis in a Huntington's *C. elegans* disease model with UAF-1 RNAi. It also indicates that CCAR-1 is working with a spliceosome factor UAF-1.

**Discussion**

Mammalian and *C. elegans* CCAR family members regulate the alternative splicing of genes [61, 62]. There is a gap in knowledge in how this regulation occurs in a whole model organism and during stress conditions. To address this, we used RNA sequencing and mass spectrometry to show new targets of CCAR-1 alternative splicing in an entire organism. From the RNA sequencing, identify new targets of CCAR-1's alternative splicing.

*C. elegans* CCAR-1 is a splicing regulator of the structural gene *unc-52* [62]. We confirm that *unc-52* is indeed alternatively spliced by CCAR-1 and further show other structural genes in our dataset. The additional structural genes from our dataset include *apl-1*, *ketn-1,* and *vab-10*. Our findings suggest that CCAR-1 has a more significant role in regulating structural genes through alternative splicing. Various structural genes are alternatively splicing during their biogenesis, showing the importance of understanding this regulation [141, 181]. In addition, CCAR-1 has a crucial role in cancer progression [43, 164, 182-184]. Studies show that cancers associated with CCAR family members are epithelial related and occur in hemidesmosome-related tissues [185]. From our studies, we identify that CCAR-1 regulates various structural genes. These findings further suggest that CCAR affects these structural genes during tumor progression.

Also, from our dataset, we see that CCAR-1 regulates the alternative splicing of stress response genes *dnj-20* and *hsp-90*. Our previous studies show that CCAR-1 regulates the heat shock response in a sirtuin-dependent manner [2, 29]. Therefore, our findings on *dnj-20* and

*hsp-90* suggests that CCAR-1 regulates the heat shock response through the alternative splicing of heat shock genes.

The overall mechanism of action of CCAR-1 alternative splicing in *C. elegans* genes is unknown. However, our mass spectrometry data identifies splicing factors RSP-3, UAF-1, and UAF-2 as interacting partners of CCAR-1. UAF-1 and UAF-2 are splicing factors of the spliceosome. They aid snRNA U2 interaction with the intron binding the polypyrimidine tract and recognizing the AG nucleotide on the 3' end of the intron [59, 118, 119, 135, 136]. *C. elegans* U2AF65 genes (*uaf-1*) and U2AF35 (*uaf-2*) cause genome-wide changes in 3' splice site [118, 119, 135], and *uaf-1* mutants have embryonic viability and motility defects [136].

Using the worm's advantages, which are the availability of splicing factor mutants, we show that CCAR-1 RNAi affects UAF-1 alternative splicing patterns in the *tos-1* splicing reporter. Furthermore, in other behavioral assays, we show that CCAR-1 RNAi rescues the motility of the worms and reduces the number of aggregates in the AM140 UAF-1 RNAi worm. Thus, our work suggests a potential mechanism of action of CCAR-1 in the alternative splicing of genes. This mechanism could be through its regulation of UAF-1, spliceosome factors, alternative splicing patterns. However, further studies, are needed to determine how CCAR-1 works with UAF-1 in various developmental stages and stresses.

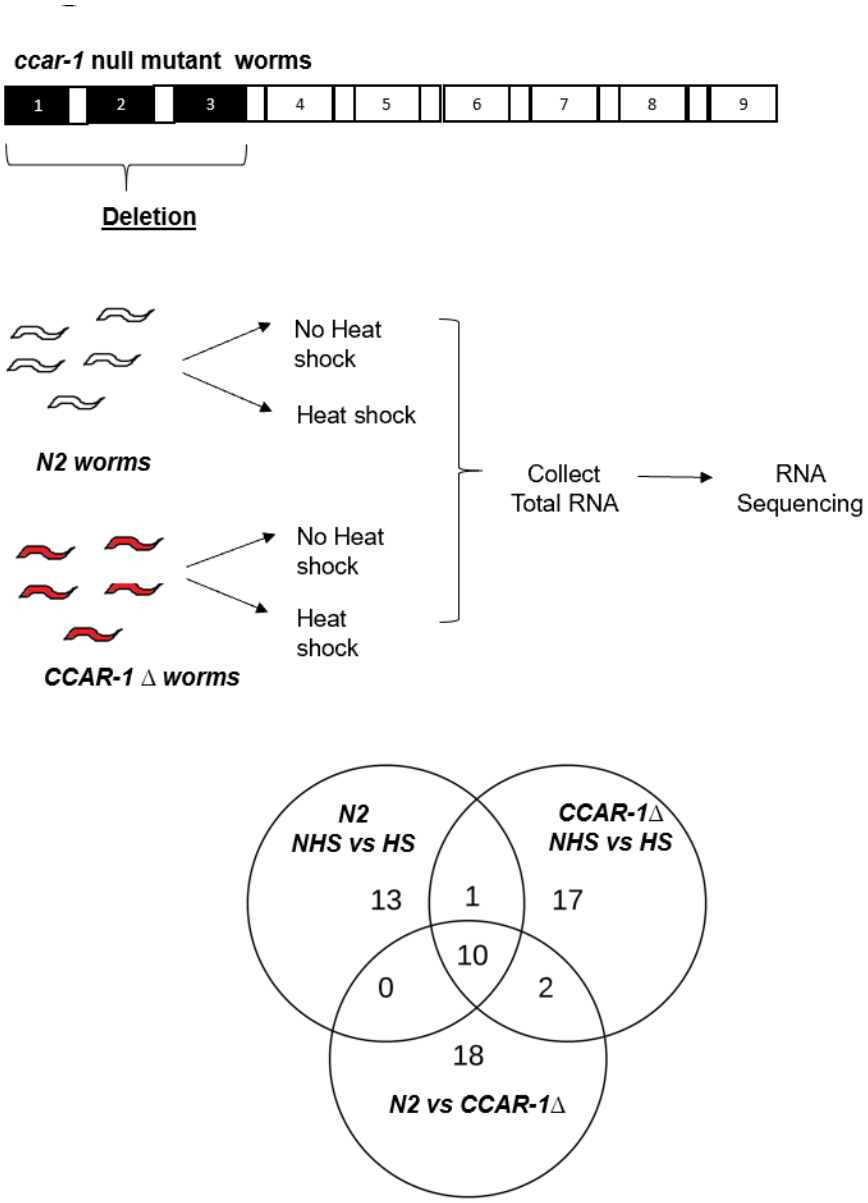



**Figure 3.1: The genome wide regulation of alternative splicing by CCAR-1 with and without heat shock**. A. Schematic of the CCAR-1 (null mutant) worm used in the analysis. This worm contains a deletion of the first three exons of the *ccar-1* gene. B. Methodology of RNA sequencing samples preparation. C. Venn diagram of significantly alternatively spliced gene from the RNA sequencing analysis by DEXSeq.

**Table 1: List of genes alternatively spliced by CCAR-1 both with and without heat shock**.

| *Gene* | *qPCR/RT-PCR validation* | *Function* |
|---|---|---|
| ***apl-1*** | NO | Involved in body morphogenesis; ecdysis, collagen and cuticulin-based cuticle |
| ***gld-3*** | NO | Involved in cell division, multicellular organism development and positive regulation of meiotic nuclear division. |
| ***ccar-1*** | NO | Ortholog of Cell cycle apoptosis regulator, involved in hemidesmosome assembly |
| ***ketn-1*** | NO | Involved in actin filament binding activity |
| ***vab-10*** | NO | Involved in actin filament binding activity and epidermis morphogenesis |
| ***C33A11.2*** | NO | Ortholog of DNA damage regulated autophagy modulator (DRAM2). |
| ***pyp-1*** | NO | Ortholog of human PPA-1. Involved in inorganic diphosphatase activity and magnesium ion binding |
| ***F30F8.10*** | YES | Ortholog of human NAA60, (N-alpha-acetyltransferase 60). Involved in N-acetyltransferase activity |
| ***K09G1.1*** | YES | Ortholog of human CMTM3, transmembrane protein |
| ***srap-1*** | NO | Serine Rich Adhesion Protein -like |
| ***T19A6.1*** | NO | Ortholog of human SLC66A2 (solute carrier family 66 member 2) |
| ***mtl-11*** | NO | Ortholog of human SPINT2. Involved in serine peptidase inhibitor activity. |
| ***symk-1*** | NO | Ortholog of human SYMPK (symplekin) |
| ***F57C9.4*** | NO | Ortholog of Zinc Factor ZNF709 |
| ***H24G06.1*** | NO | Ortholog of human WDR62, function in primary autosomal microcephaly |
| ***tos-1*** | YES | Target of splicing gene |
| ***T23E7.2*** | NO | Ortholog of human FAM186A (family of sequence similarity 186 member A) |

| *Gene* | *qPCR/RT-PCR validation* | *Function* |
|---|---|---|
| ***col-73*** | NO | Structural constituent of the cuticle |
| ***hsp-90*** | YES | Ortholog of human HSP90AA1 and HSP-90 Involved in ATPase activity, glucocorticoid receptor binding activity and protein phosphatase 5 binding activity. |
| ***dnj-20*** | YES | Ortholog of human DNAJB11 (DnaJ heat shock protein family (Hsp40) member B11.) Involved in unfolded protein binding activity. |
| ***lev-11*** | NO | Ortholog of human TPM1 and TPM-3. Involved in actin filament binding activity. |
| ***pqn-44*** | NO | Ortholog of human TENT5A. Involved in RNA adenyl transferase activity |
| ***uaf-2*** | YES | Ortholog of human U2AF1. Involved in pre-mRNA 3' splice site binding activity |
| ***unc-52*** | YES | Ortholog of human HSPG2. Involved in extracellular matrix, cellular component assembly, mitochondrion organization and multicellular organism development |
| ***idh-1*** | YES | Ortholog of human IDH-1. Involved in isocitrate dehydrogenase activity and metal ion binding activity. |
| ***C17F4.7*** | NO | Enriched in cholinergic neurons, head mesodermal cells, intestine, pharyngeal cells, and retro-ventricular ganglion |
| ***F56D3.1*** | NO | Enriched in the germline, germline precursor cells and hypodermis. |
| ***tfbm-1*** | NO | Ortholog of TFBM1M. Involved in RNA binding activity and rRNA dimethyl transferase activity |
| ***nspc-12*** | NO | Nematode specific Peptide family, group C |
| ***F59E12.16*** | NO | Pseudogene. Enriched in the intestine, muscle cell, neurons and retroventricular ganglion |

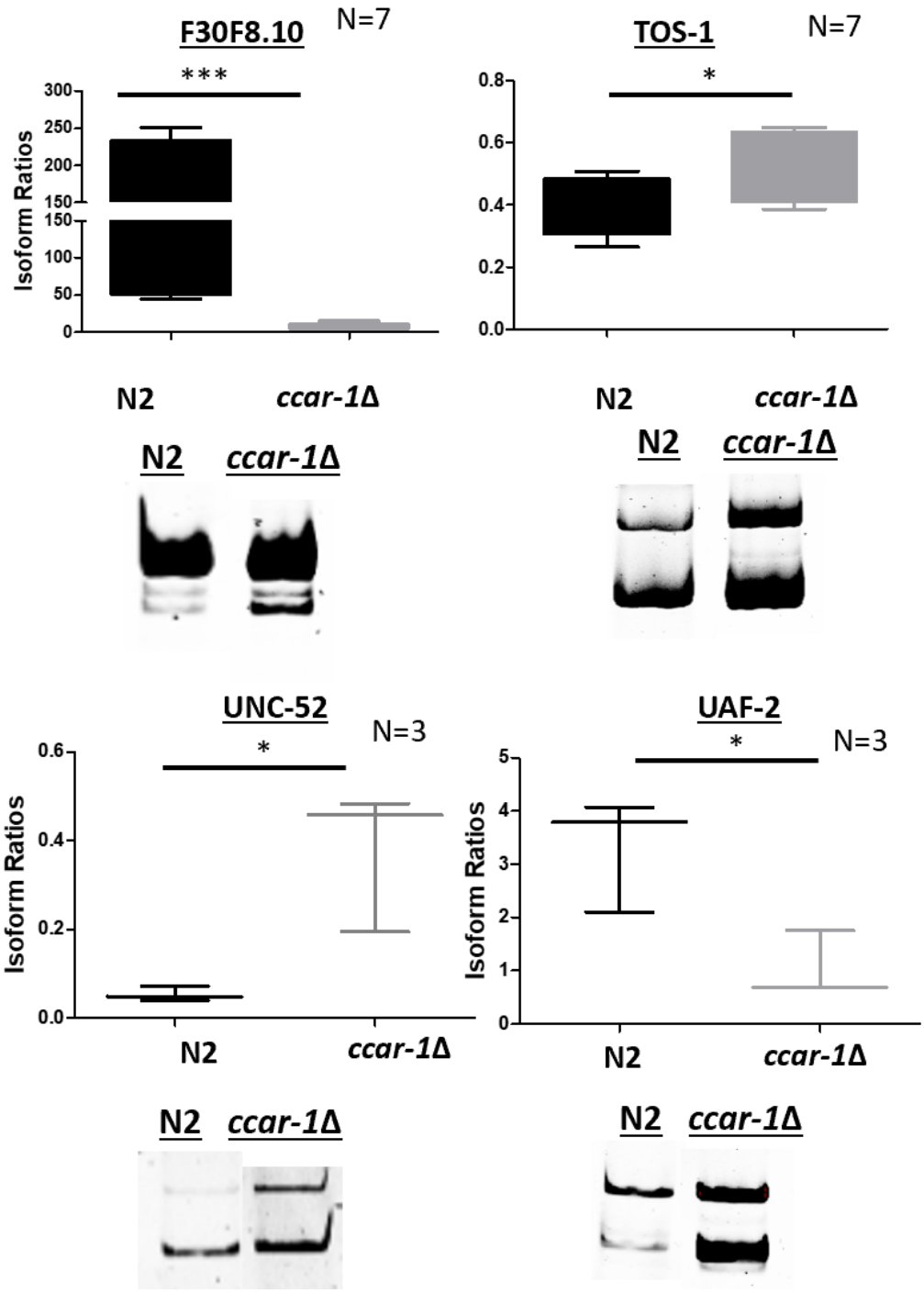



**Figure 3.2: CCAR-1 alternatively splices genes both with and without heat shock.** This figure shows the RT-PCR validation of CCAR-1 alternative splicing from the RNA sequencing dataset. The box plots represent the distribution of the isoform ratios of all populations of N2 and CCAR-1Δ worms. The RT-PCR images show the visual representation of isoform changes in N2 and CCAR-1Δ.

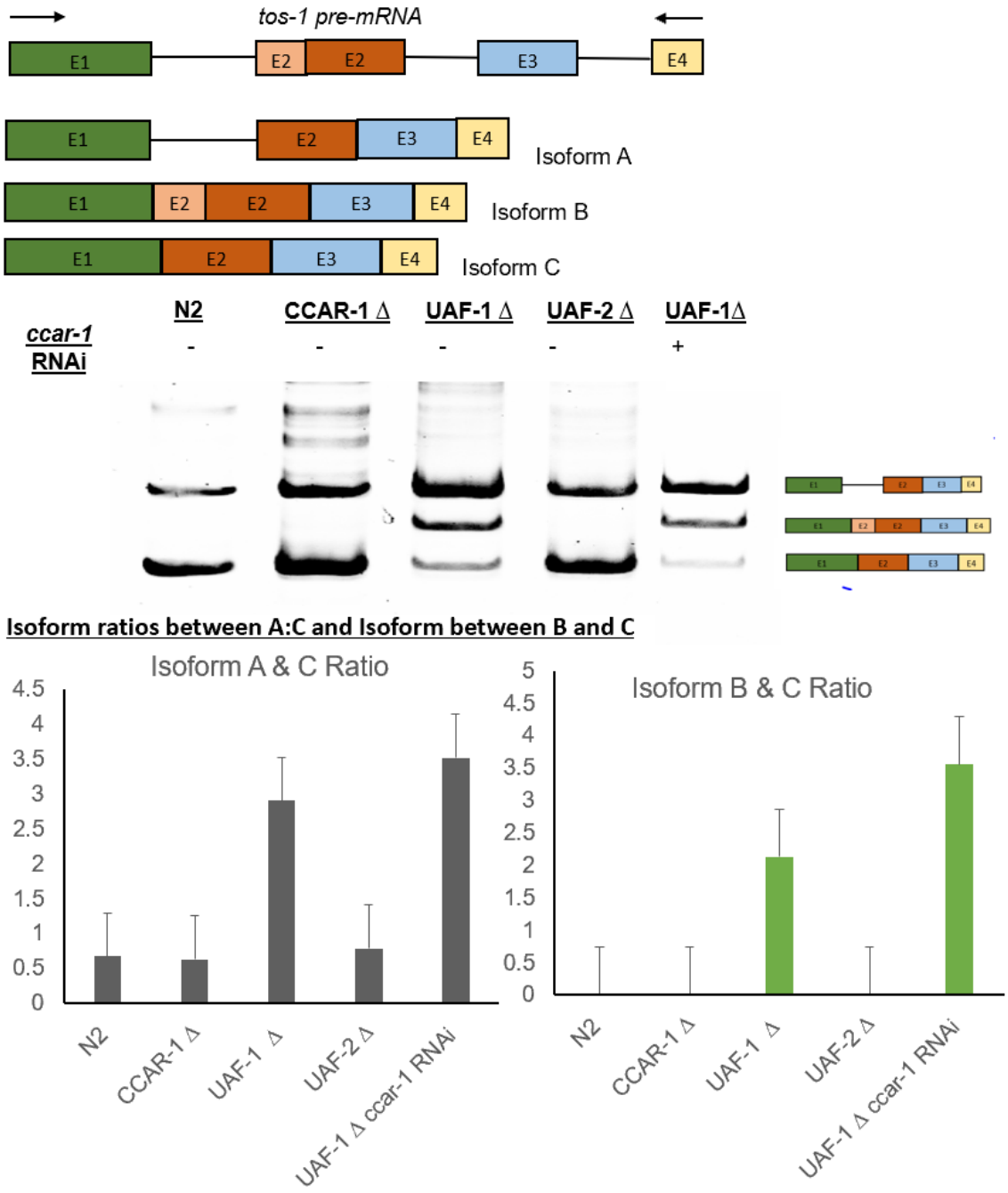



**Figure 3.3: CCAR-1 RNAi affects the alternative splicing of UAF-1Δ.** A. Shows table of splicing factors that interact with CCAR-1 based on mass spectrometry. B. Shows a tos-1 alternative splicing reporter C &D. Shows how CCAR-1 significantly increases the isoform ratios of isoform A/C and B/C of the UAF-1Δ.

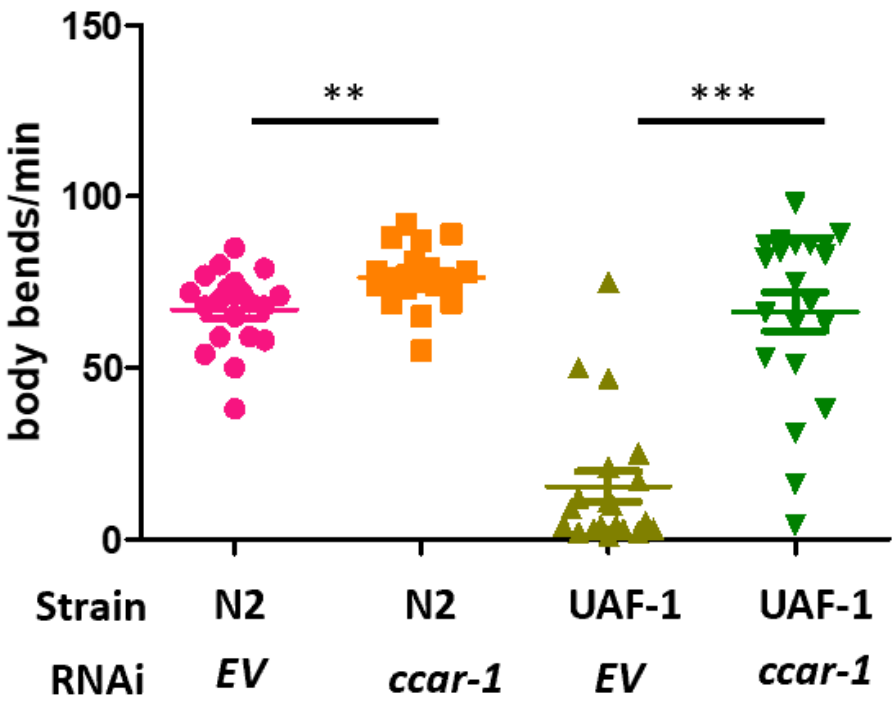



**Figure 3.4: CCAR-1 RNAi rescues the motility of UAF-1Δ.** N2 and UAF-1Δ grown at 25˚C, were assessed for motility in a thrashing assay. Body bends per minute were measured for individual worms. This was done in N=15 for two biological replicates.

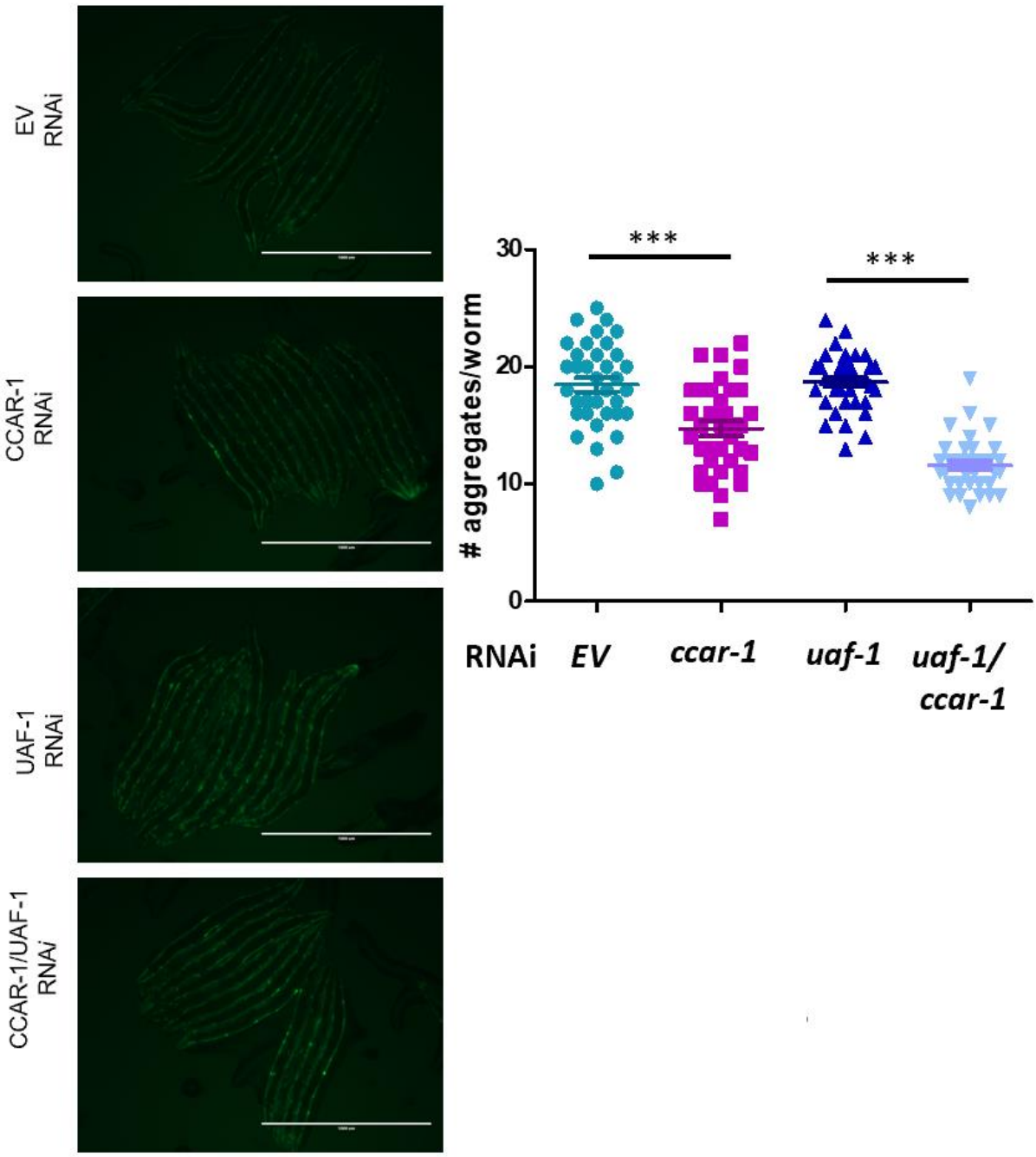



**Figure 3.5: CCAR-1 RNAi significantly decreases the number of PolyQ aggregates in UAF-1 RNAi.** A. Shows the fluorescent images of AM140 (Q35::YFP) fed control, ccar-1, uaf-1 and ccar-1/uaf-1 RNAi from L1 larval stage to day 3 of adulthood. B. Shows quantification of number of aggregates per worm in each condition.

# CHAPTER 4: CONCLUSIONS AND IMPLICATIONS OF THE ROLE OF CCAR-1 IN GERMLINE REGULATION

## Conclusions

In Chapter 5, we showed using RNA sequencing that CCAR-1 regulates the *C. elegans* germline by transcribing many genes. These genes include germline enriched genes, p-granule genes, piRNAs, *cep-1/p53* protein, and spermatogenic genes. With further investigation of the role of CCAR-1 in the germline, we show that it is localized in the germline; CCAR-1 mutants show significant defects in the numbers of offspring, it regulates the DNA-damage induced apoptosis, and its extension of lifespan is dependent in part on its role in the germline.

*C. elegans* is a valuable model for studying gene pathways; however, we want to understand how to translate the discoveries in this model to higher organisms. Here are some implications of the study in future works.

## Implications in the reproduction of higher organisms

Mammalian CCAR2 and *C. elegans* CCAR-1 have similarities in protein structure and molecular functions such as the epigenetic regulation of sirtuin, alternative splicing, and apoptosis.

We show that CCAR-1 deletion significantly reduces the number of offspring and the transcriptional regulation of germline genes. Gene expression during embryogenesis is essential for the development of the offspring. Various studies in human cells are gearing towards understanding the transcriptional landscape during embryogenesis. During early

development, changes in gene expression of human trophoblast cells (TB), a type of cell lineage cell, is linked to abnormal placental functions that could cause pregnancy-related complications such as preeclampsia, intrauterine growth restriction, preterm labor, and low birth weight [186]. Insights into early human development in human and mouse preimplantation embryos have used RNA-sequencing to understand the transcriptional landscape [182] further. With CCAR-1 regulating the transcriptional landscape of the germline in *C. elegans,* it would be intuitive to determine if this function is conserved, which would suggest a role in the reproduction/embryogenesis of higher organisms.

**Implications in piRNAs regulation and fertility in higher organisms**

We show from our RNA-Sequencing dataset that CCAR-1 mutants have differential expression of p granule proteins and piRNAs. P-granules are RNA-rich germ cell granules associated with germline development [72, 94]. They are vital in delivering maternal proteins and RNA to the nascent germline. They contain RNA and nucleus proteins that bind to RNA and others related to translational control [94]. piRNAs are small, conserved RNAs found in the germline, with the role of silencing retrotransposons through binding to argonaute proteins, PIWIs. Previous studies have shown that piRNAs/21U-RNAs and PRG-1, the sole PIWI argonaute protein in *C. elegans,* are required for average progeny production [105, 187].

In humans and mice studies, piRNAs have a role in spermatogenesis [188]. piRNAs are expressed in both male and female testis and ovaries; however, various studies show that piRNAs are essential for the specificity and differentiation of the male germline. This role of piRNAs is a major cause of infertility. According to worldwide statistics, 10-15% of couples are affected by infertility, and half of the cases are male infertility [189].

piRNAs are dependent on PIWI argonautes; which have a significant role in their biogenesis and functions. In addition, human and mouse studies have shown that silencing of PIWI's leads to meiotic arrest and male sterility [98].

In our study, we show that CCAR-1 regulates both piRNA gene expression and PRG-1/PIWI protein regulation. In unpublished data, we also show that CCAR-1 colocalizes with PRG-1. Our work suggests a role of CCAR-1 in regulating the piRNA/PIWI pathway of germline regulation that could be conserved in higher organisms such as humans and mice.

With the conservation of the protein structure and molecular functions of the CCAR family of proteins within species, it would be intuitive to investigate CCAR-1 role in this pathway. Furthermore, these similarities in both structure and function could unravel a transcriptional regulator of the piRNAs pathways that affect male fertility in humans and mice.

# CHAPTER 5: FUTURE STUDIES

Based on the results from Chapter 5, we show that CCAR-1 regulates germline transcription, apoptosis, progeny production, and lifespan. However, we do not show the mechanism of action of this regulation. Therefore, here are a few future studies that could address the mechanism of action of CCAR-1 in the germline.

**Determine the role of SIR-2.1 in the germline regulation by CCAR-1**

In previous studies, we have shown that CCAR-1 regulates the heat shock response in a *sir-2.1*-dependent manner. This regulation affected HSF-1 binding to the promoter region of heat shock proteins, the transcription of heat shock proteins, and the proteostasis of the worms. Therefore, it is key to evaluate if the role of CCAR-1 in the *C. elegans* germline is sirtuin (*sir-2.1* ) dependent.

In preliminary data, we used four CCAR-1 mutant strains available from the CGC: MT7019, DM1153, DM1154, and SDW040 to determine changes in the number of progenies compared to N2 control with and without knockdown by *sir-2.1* RNAi. Our results show that CCAR-1 is required for normal levels of progeny production, as progeny levels in the CCAR-1 mutants dropped by 2.5-4-fold. However, we show that *sir-2.1* RNAi does not influence progeny. Thus, CCAR-1 is required for normal progeny production levels in a mechanism independent of SIR-2.1.

Next, we wanted to test *sir-2.1* involvement in CCAR-1 regulation of germline apoptosis in ACT-5::YFP strain. The ACT-5::YFP strain contains ACT-5 isoform bound to

YFP and expressed in the sheath cells with higher efficacy in corpse labeling for early apoptotic cells than DIC optics [96, 167]. Increased apoptosis is visualized as 'halos' around early apoptotic cells and decreased apoptosis as less ACT-5::YFP ' halos.' We showed in Chapter 5 that knockdown of CCAR-1 showed a significant increase in ACT-5::YFP apoptotic 'halos.'

To determine if the increase of ACT-5:: YFP' halos' with knockdown of CCAR-1 is affected by SIR-2.1 RNAi, we did a CCAR-1/SIR-2.1 RNAi double knockdown. From the results, we show a significant reduction of ACT-5:: YFP' halos' with an additional knockdown of SIR-2.1. Altogether, this result suggests that CCAR-1 plays a role in the DNA damage-induced apoptosis in the *C. elegans* germline, leading to the formation of more early-stage apoptotic corpse/'halos'. Furthermore, we also determine that CCAR-1 function in DNA-damage-induced apoptosis is affected by SIR-2.1 function.

Our preliminary data show involvement of SIR-2.1 in CCAR-1 regulation of DNA-damage induced apoptosis but no significant change in the production of progeny.

In future studies, it would be intuitive to determine the *sir-2.1* role in the lifespan of CCAR-1 in the germline. Sirtuin has functions in the longevity of *C. elegans* [171]. It also interacts with other longevity factors of the DAF-2/DAF-16 pathway, which could be working with CCAR-1 to cause these longevity effects [83, 171, 190-192]. We also show from our RNA-sequencing dataset that CCAR-1 deletion changes the transcription of DAF-16/SIR-2.1 regulated genes, further testing their involvement in CCAR-1 extension of lifespan.

**Determine the role of CCAR-1 in the piRNA/PRG-1 pathway of germline regulation.**

The piRNA/21U-RNA family is a significant category of germline genes that CCAR-1 from our dataset regulates. Previous studies have shown that piRNAs and PRG-1, the sole

PIWI argonaute protein in C. elegans, are required for average progeny production [105, 187]. We show from our dataset that CCAR-1 regulates the transcription of p-granule protein-associated genes such as PRG-1 and piRNAs. Therefore, CCAR-1 could be regulating this key small RNA pathway in the *c. elegans* germline.

We tested whether CCAR-1 affects PRG-1 function in preliminary data, we employed a temperature-sensitive brooding assay using the PRG-1Δ strain (WM161). These temperature-sensitive mutants show a decrease in progeny when grown at 20ºC, an effect that is enhanced at 25˚C [173, 174]. We used RNA interference (RNAi) to knockdown CCAR-1 in N2 worms and the PRG-1Δ strain (WM161) at 25˚C and counted the number of progenies until day three of adulthood. We found that the PRG-1 mutant worms have up to a twenty-fold reduction of normal progeny at 25˚C, as expected. Upon knockdown of CCAR-1, we observed that the progeny is further decreased, though not statistically significant. CCAR-1 RNAi on its reduced progeny by 1.5-fold at 25ºC in the N2 strain. Together, this data shows that CCAR-1 knockdown enhances the impact of the PRG-1 mutation on progeny production. These findings could also suggest that CCAR-1 could be working through the PRG-1/piRNA pathways to regulate reproduction in the germline. This pathway is associated with the occurrence of numerous germline defects.

To further test whether CCAR-1 colocalizes with argonaute of the piRNA pathway, PRG-1, we crossed SDW082 (CCAR-1::GFP::3xflag) to a PRG-1::RFP from Dr. Dustin Updike, MDI Labs. As a result, we show colocalization of CCAR-1::GFP to PRG-1::RFP.

In conclusion, we show that CCAR-1 regulates piRNA and PRG-1 related transcription, knockdown of CCAR-1 by RNAi further reduces PRG-1 mutants progeny production (not statistically significant), and CCAR-1 colocalizes with PRG-1.

In future studies, it would be intuitive to determine the mechanism of the role of CCAR-1 in the piRNA/PRG-1 pathway of regulation. Future questions to ask are: Does CCAR-1 directly interact with PRG-1? Does this binding affect its binding to piRNAs? Does binding affect piRNA biogenesis and functionality?

Interestingly, other studies have shown PRG-1 mutants to have similar functions in the germline as CCAR-1. For example, PRG-1 mutants show reduced fertility, increased stress-induced apoptosis, and changes in the germline transcription [90, 91, 105]. It would, therefore, be essential to test whether the changes caused by CCAR-1 are solely through its interactions with PRG-1 or another pathway.

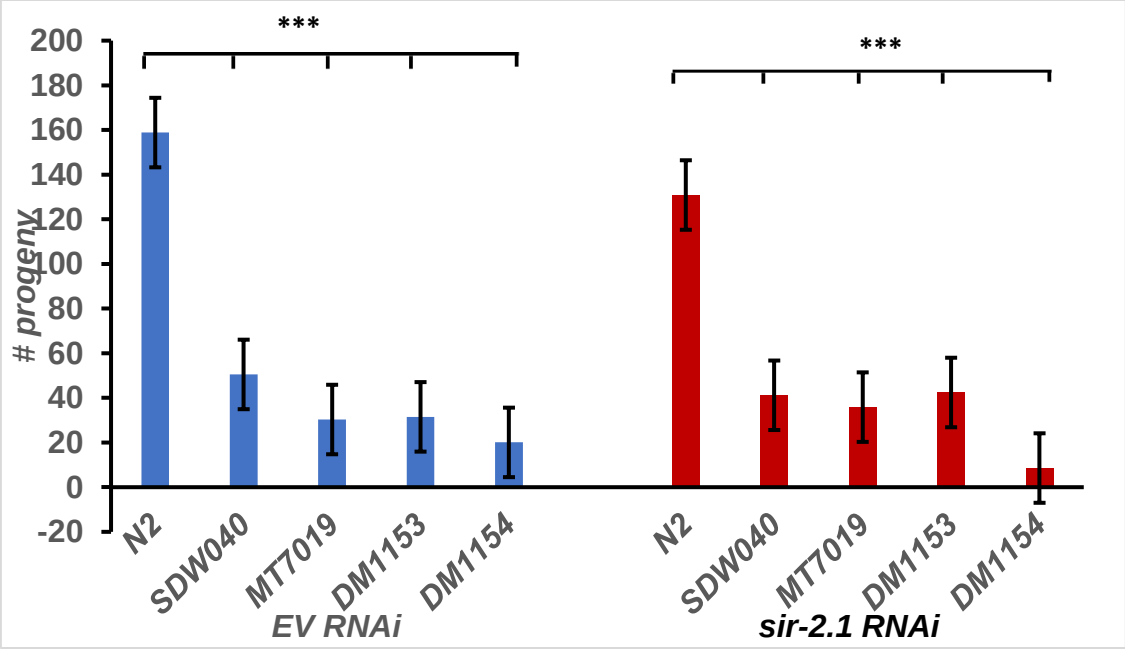



**Figure 5.1: CCAR-1 is required for normal levels of progeny production.** The graph shows a brooding assay in which the N2 control strain is compared to various CCAR-1 mutant allele strains (SDW040, MT7019, DM1153 and DM1154) to determine the effects of CCAR-1 on the number of progeny in Empty vector (control) and SIR-2.1 RNAi conditions.

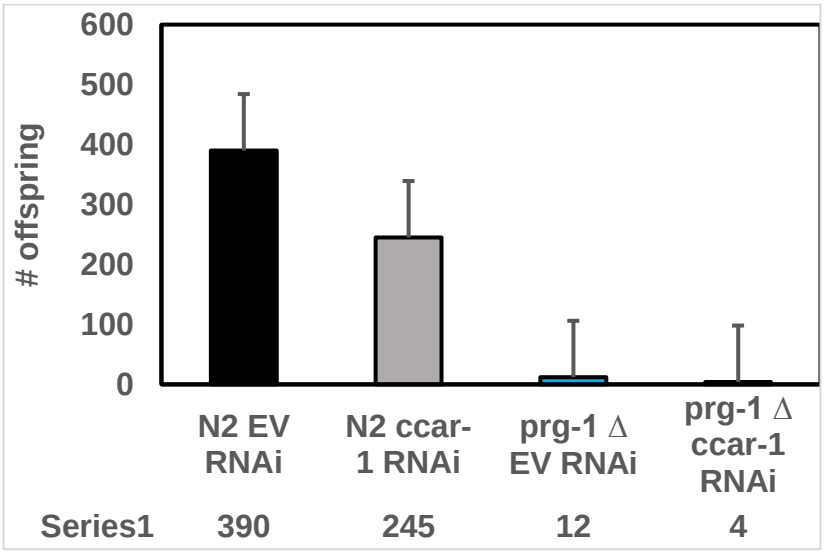



**Figure 5.2: CCAR-1 protects germline cells from UV damage-induced apoptosis.** A. ACT-5::YFP worms expressing actin fused to YFP in the gonad sheath cells were treated with CCAR-1 and CCAR-1/SIR-2.1 RNAi prior to exposure to a UV dose of 400J/m$^2$. Images were taken 24 hrs after the exposure. YFP, brightfield and merged images are used to visualize germ cell corpses, which are represented as actin "halos" during early-stage apoptosis and indicated by white arrows.

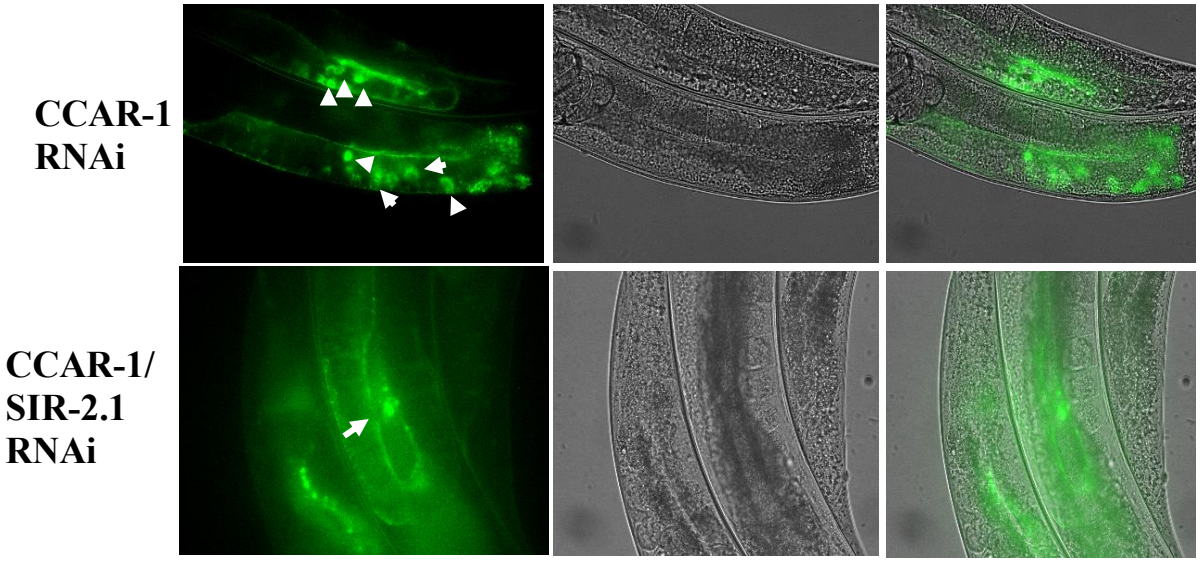



**Figure 5.3: CCAR-1 RNAi reduces number of offspring in both N2 and PRG-1 mutants at 25˚C.** There is a significant decrease in the number offspring in N2 CCAR-1 RNAi when compared to EV RNAi. In the PRG-1 mutants, there is a decrease in the number of offspring with CCAR-1 RNAi, however this is not significantly changing.

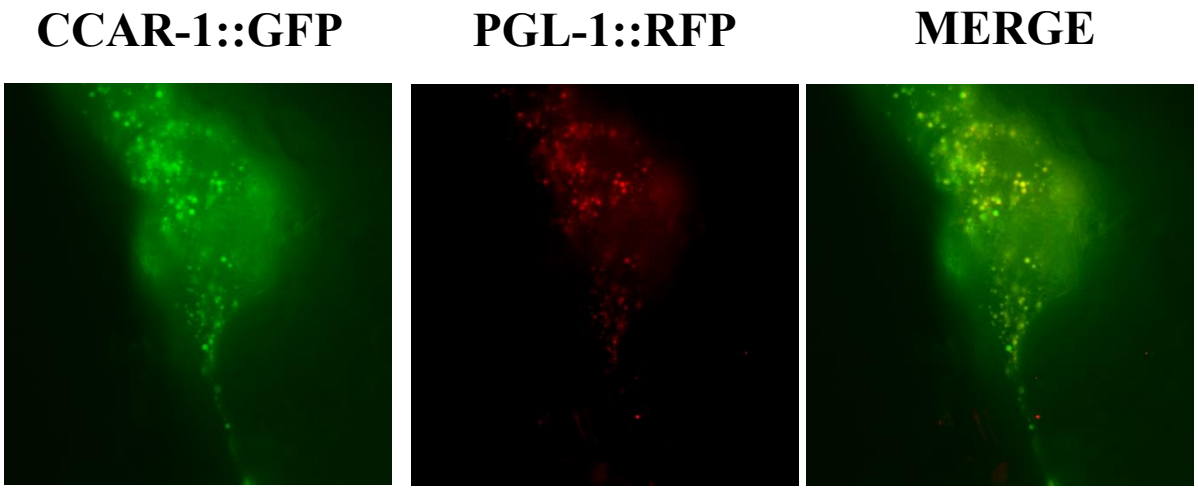



**Figure 5.4: CCAR-1::GFP colocalizes with PGL-1::RFP.** The figure shows the colocalization of CCAR-1::GFP and PGL-1::RFP in a double mutant strain of the two tagged proteins.

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# APPENDICES

**Appendix A: Supporting Figures for Chapter 5: Methodology For RNA-Sequencing and Venn Diagram of Results**

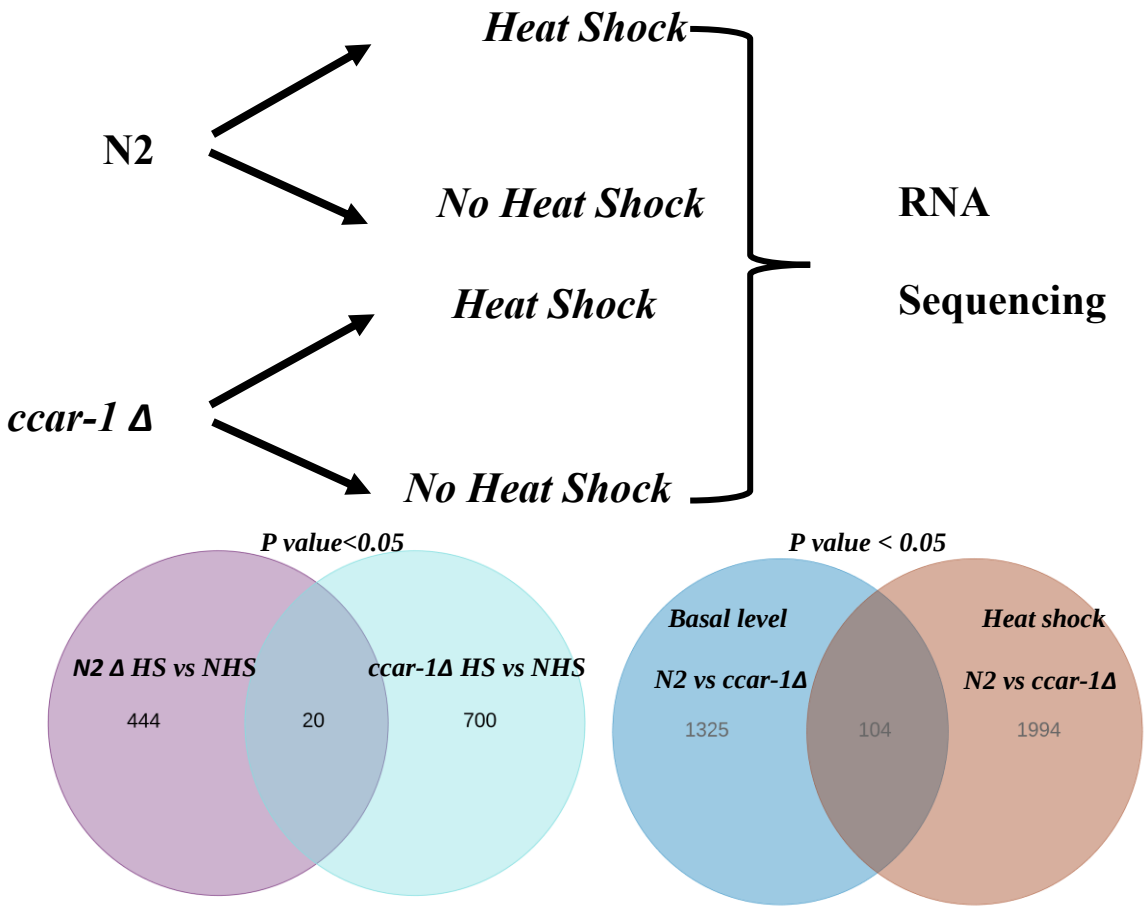


**Figure A1: Methodology of RNA sequencing samples.** A. Shows the results of significantly changing genes between N2 and ccar-1Δ, during heat shock vs no heat shock. The second Venn diagram shows significantly changing genes during no heat shock and during heat shock in N2 vs ccar-1Δ.

**Table A1: Significantly altered genes in RNASeq between N2 and CCAR-1 Δ**

| WormBase ID | pval | WormBase ID | pval | WormBase ID | pval |
|---|---|---|---|---|---|
| **WBGene00001553** | 0.000189 | **WBGene00077490** | 0.008 | **WBGene00022411** | 0.014249 |
| **WBGene00199771** | 0.000264 | **WBGene00008978** | 0.008059 | **WBGene00015720** | 0.014586 |
| **WBGene00021799** | 0.000369 | **WBGene00010705** | 0.008352 | **WBGene00018738** | 0.014632 |
| **WBGene00043066** | 0.000699 | **WBGene00015043** | 0.008558 | **WBGene00020324** | 0.014652 |
| **WBGene00021418** | 0.001117 | **WBGene00013611** | 0.008605 | **WBGene00012952** | 0.015222 |
| **WBGene00195436** | 0.001216 | **WBGene00008127** | 0.008809 | **WBGene00199661** | 0.015344 |
| **WBGene00008668** | 0.001369 | **WBGene00011294** | 0.008812 | **WBGene00200723** | 0.015418 |
| **WBGene00172117** | 0.001393 | **WBGene00017453** | 0.00885 | **WBGene00019729** | 0.015879 |
| **WBGene00010702** | 0.001409 | **WBGene00021818** | 0.009104 | **WBGene00011397** | 0.015929 |
| **WBGene00010447** | 0.001601 | **WBGene00016645** | 0.00927 | **WBGene00021388** | 0.016143 |
| **WBGene00012810** | 0.001623 | **WBGene00015429** | 0.009435 | **WBGene00044687** | 0.016294 |
| **WBGene00003862** | 0.001627 | **WBGene00198528** | 0.009619 | **WBGene00006198** | 0.016326 |
| **WBGene00014999** | 0.001675 | **WBGene00001577** | 0.009637 | **WBGene00235262** | 0.016357 |
| **WBGene00197191** | 0.001703 | **WBGene00022736** | 0.009713 | **WBGene00007176** | 0.016511 |
| **WBGene00011041** | 0.001831 | **WBGene00019022** | 0.009858 | **WBGene00008292** | 0.016563 |
| **WBGene00011263** | 0.00243 | **WBGene00001783** | 0.009998 | **WBGene00012170** | 0.016705 |
| **WBGene00017957** | 0.003169 | **WBGene00020852** | 0.010122 | **WBGene00045400** | 0.016729 |
| **WBGene00008192** | 0.003181 | **WBGene00008892** | 0.010123 | **WBGene00198470** | 0.016903 |
| **WBGene00045115** | 0.003279 | **WBGene00011607** | 0.010376 | **WBGene00012841** | 0.017845 |
| **WBGene00012421** | 0.003518 | **WBGene00197473** | 0.010391 | **WBGene00012225** | 0.01806 |
| **WBGene00000708** | 0.003698 | **WBGene00018461** | 0.010501 | **WBGene00001475** | 0.018066 |
| **WBGene00019216** | 0.003931 | **WBGene00197822** | 0.010535 | **WBGene00044006** | 0.018113 |
| **WBGene00021089** | 0.004039 | **WBGene00008084** | 0.010876 | **WBGene00006237** | 0.018197 |
| **WBGene00009137** | 0.004105 | **WBGene00018186** | 0.010908 | **WBGene00043051** | 0.018699 |
| **WBGene00008200** | 0.004119 | **WBGene00023371** | 0.011076 | **WBGene00044616** | 0.018874 |
| **WBGene00045412** | 0.004139 | **WBGene00015790** | 0.011211 | **WBGene00020086** | 0.018901 |
| **WBGene00174308** | 0.0043 | **WBGene00002224** | 0.011228 | **WBGene00012726** | 0.01898 |
| **WBGene00011630** | 0.004452 | **WBGene00015495** | 0.011352 | **WBGene00008967** | 0.019591 |
| **WBGene00194650** | 0.004716 | **WBGene00014977** | 0.011482 | **WBGene00016239** | 0.019745 |
| **WBGene00022815** | 0.004834 | **WBGene00015559** | 0.011506 | **WBGene00000445** | 0.019855 |
| **WBGene00044491** | 0.005702 | **WBGene00003386** | 0.012011 | **WBGene00009572** | 0.019946 |
| **WBGene00196123** | 0.005734 | **WBGene00172412** | 0.012493 | **WBGene00007725** | 0.020105 |
| **WBGene00019741** | 0.006262 | **WBGene00006364** | 0.012685 | **WBGene00003621** | 0.020122 |
| **WBGene00045363** | 0.006346 | **WBGene00007208** | 0.012836 | **WBGene00017178** | 0.020436 |
| **WBGene00015270** | 0.006424 | **WBGene00009930** | 0.013216 | **WBGene00020144** | 0.020436 |
| **WBGene00020004** | 0.006558 | **WBGene00017117** | 0.013507 | **WBGene00043990** | 0.02062 |
| **WBGene00011822** | 0.006583 | **WBGene00008353** | 0.0137 | **WBGene00018299** | 0.020642 |
| **WBGene00201577** | 0.006584 | **WBGene00220029** | 0.013763 | **WBGene00015784** | 0.020726 |
| **WBGene00009726** | 0.007124 | **WBGene00022246** | 0.013943 | **WBGene00014132** | 0.020797 |
| **WBGene00010773** | 0.007209 | **WBGene00020804** | 0.013991 | **WBGene00008863** | 0.020819 |
| **WBGene00196138** | 0.007962 | **WBGene00016911** | 0.014074 | **WBGene00012537** | 0.02088 |

**Table A1 (continued): Significantly altered genes in RNASeq between N2 and CCAR-1 Δ**

| WormBase ID | pval | WormBase ID | pval | WormBase ID | pval |
|---|---|---|---|---|---|
| **WBGene00199417** | 0.020894 | **WBGene00017233** | 0.025615 | **WBGene00195613** | 0.03112 |
| **WBGene00044074** | 0.020923 | **WBGene00197451** | 0.025658 | **WBGene00050947** | 0.031264 |
| **WBGene00185096** | 0.020956 | **WBGene00197454** | 0.025696 | **WBGene00011438** | 0.031368 |
| **WBGene00019230** | 0.020961 | **WBGene00045210** | 0.026024 | **WBGene00015085** | 0.031504 |
| **WBGene00022207** | 0.021135 | **WBGene00007076** | 0.026149 | **WBGene00010073** | 0.031541 |
| **WBGene00016517** | 0.021453 | **WBGene00023162** | 0.02623 | **WBGene00197434** | 0.031659 |
| **WBGene00168565** | 0.021894 | **WBGene00013778** | 0.026401 | **WBGene00011935** | 0.031683 |
| **WBGene00019025** | 0.021956 | **WBGene00168245** | 0.026494 | **WBGene00201908** | 0.032137 |
| **WBGene00008186** | 0.022059 | **WBGene00015642** | 0.026647 | **WBGene00006696** | 0.03238 |
| **WBGene00015198** | 0.022244 | **WBGene00009301** | 0.026821 | **WBGene00201519** | 0.032444 |
| **WBGene00045833** | 0.022329 | **WBGene00007692** | 0.026847 | **WBGene00019156** | 0.032665 |
| **WBGene00018241** | 0.022498 | **WBGene00019448** | 0.027035 | **WBGene00004369** | 0.03275 |
| **WBGene00219713** | 0.022586 | **WBGene00013914** | 0.027076 | **WBGene00201735** | 0.032763 |
| **WBGene00200906** | 0.022713 | **WBGene00003610** | 0.027274 | **WBGene00018223** | 0.032942 |
| **WBGene00011838** | 0.022822 | **WBGene00012690** | 0.027321 | **WBGene00200836** | 0.032979 |
| **WBGene00013356** | 0.023078 | **WBGene00008105** | 0.027346 | **WBGene00015984** | 0.033044 |
| **WBGene00015183** | 0.023172 | **WBGene00044207** | 0.027351 | **WBGene00044297** | 0.03326 |
| **WBGene00022163** | 0.023223 | **WBGene00201778** | 0.027372 | **WBGene00220203** | 0.033284 |
| **WBGene00018648** | 0.023566 | **WBGene00020150** | 0.02766 | **WBGene00003547** | 0.033504 |
| **WBGene00171442** | 0.023586 | **WBGene00044450** | 0.027768 | **WBGene00219415** | 0.033549 |
| **WBGene00174703** | 0.023586 | **WBGene00020657** | 0.027797 | **WBGene00011673** | 0.033632 |
| **WBGene00017673** | 0.023588 | **WBGene00002051** | 0.027815 | **WBGene00044009** | 0.034134 |
| **WBGene00022978** | 0.024017 | **WBGene00219722** | 0.027908 | **WBGene00016355** | 0.034167 |
| **WBGene00022799** | 0.024374 | **WBGene00044301** | 0.0281 | **WBGene00017873** | 0.034193 |
| **WBGene00201840** | 0.024378 | **WBGene00007247** | 0.028538 | **WBGene00023234** | 0.034199 |
| **WBGene00169693** | 0.024402 | **WBGene00007374** | 0.028699 | **WBGene00012623** | 0.034302 |
| **WBGene00023086** | 0.024437 | **WBGene00015399** | 0.028889 | **WBGene00011014** | 0.034421 |
| **WBGene00007288** | 0.024508 | **WBGene00196002** | 0.028989 | **WBGene00010119** | 0.034785 |
| **WBGene00197453** | 0.024512 | **WBGene00197729** | 0.029002 | **WBGene00200456** | 0.034864 |
| **WBGene00050892** | 0.024607 | **WBGene00022741** | 0.029504 | **WBGene00006067** | 0.035054 |
| **WBGene00014695** | 0.024673 | **WBGene00014107** | 0.029504 | **WBGene00015081** | 0.035061 |
| **WBGene00021371** | 0.024913 | **WBGene00002975** | 0.029684 | **WBGene00012425** | 0.035082 |
| **WBGene00198382** | 0.024971 | **WBGene00005188** | 0.02974 | **WBGene00045058** | 0.035433 |
| **WBGene00166882** | 0.025001 | **WBGene00013607** | 0.029804 | **WBGene00018789** | 0.035474 |
| **WBGene00169184** | 0.025001 | **WBGene00219271** | 0.029961 | **WBGene00000086** | 0.03572 |
| **WBGene00171552** | 0.0252 | **WBGene00014130** | 0.030127 | **WBGene00020325** | 0.035835 |
| **WBGene00198764** | 0.025223 | **WBGene00194651** | 0.030288 | **WBGene00011131** | 0.036263 |
| **WBGene00008950** | 0.025289 | **WBGene00014564** | 0.030374 | **WBGene00011715** | 0.036412 |
| **WBGene00008306** | 0.025581 | **WBGene00201373** | 0.030504 | **WBGene00018195** | 0.036699 |
| **WBGene00198005** | 0.025589 | **WBGene00219604** | 0.030944 | **WBGene00017036** | 0.036711 |

**Table A1 (continued): Significantly altered genes in RNASeq between N2 and CCAR-1 Δ**

| WormBase ID | pval | | WormBase ID | pval |
|---|---|---|---|---|
| **WBGene00077537** | 0.036928 | | **WBGene00007356** | 0.044381 |
| **WBGene00012906** | 0.037279 | | **WBGene00022823** | 0.044428 |
| **WBGene00220160** | 0.03732 | | **WBGene00012626** | 0.044443 |
| **WBGene00201626** | 0.037383 | | **WBGene00220003** | 0.044533 |
| **WBGene00016809** | 0.03763 | | **WBGene00019924** | 0.044647 |
| **WBGene00219304** | 0.037693 | | **WBGene00018595** | 0.044727 |
| **WBGene00013252** | 0.037907 | | **WBGene00201335** | 0.044909 |
| **WBGene00014060** | 0.038235 | | **WBGene00022568** | 0.044927 |
| **WBGene00018734** | 0.038694 | | **WBGene00017462** | 0.045123 |
| **WBGene00011515** | 0.038742 | | **WBGene00219667** | 0.045125 |
| **WBGene00200747** | 0.038937 | | **WBGene00020889** | 0.04514 |
| **WBGene00003165** | 0.039211 | | **WBGene00014021** | 0.04523 |
| **WBGene00016338** | 0.039295 | | **WBGene00169649** | 0.045729 |
| **WBGene00002881** | 0.039528 | | **WBGene00012847** | 0.045902 |
| **WBGene00021965** | 0.0399 | | **WBGene00006765** | 0.045945 |
| **WBGene00196946** | 0.039956 | | **WBGene00010822** | 0.046136 |
| **WBGene00044298** | 0.040371 | | **WBGene00008100** | 0.046295 |
| **WBGene00005652** | 0.040732 | | **WBGene00018020** | 0.046335 |
| **WBGene00013073** | 0.040772 | | **WBGene00018569** | 0.046459 |
| **WBGene00009226** | 0.041021 | | **WBGene00012861** | 0.046978 |
| **WBGene00003677** | 0.041108 | | **WBGene00077536** | 0.047264 |
| **WBGene00014838** | 0.04133 | | **WBGene00199534** | 0.047695 |
| **WBGene00199923** | 0.041545 | | **WBGene00008350** | 0.047911 |
| **WBGene00020575** | 0.041695 | | **WBGene00012624** | 0.047931 |
| **WBGene00018787** | 0.041894 | | **WBGene00006731** | 0.048021 |
| **WBGene00201272** | 0.041898 | | **WBGene00000759** | 0.048062 |
| **WBGene00004233** | 0.041957 | | **WBGene00017393** | 0.048199 |
| **WBGene00199158** | 0.041971 | | **WBGene00022252** | 0.048728 |
| **WBGene00011928** | 0.042101 | | **WBGene00044980** | 0.049059 |
| **WBGene00004229** | 0.042261 | | **WBGene00012642** | 0.04912 |
| **WBGene00000434** | 0.042363 | | **WBGene00194814** | 0.049206 |
| **WBGene00021094** | 0.042513 | | **WBGene00195808** | 0.049256 |
| **WBGene00021928** | 0.042707 | | **WBGene00016984** | 0.049283 |
| **WBGene00020075** | 0.042792 | | **WBGene00007734** | 0.049309 |
| **WBGene00012760** | 0.043677 | | **WBGene00019312** | 0.049318 |
| **WBGene00007723** | 0.043768 | | **WBGene00018918** | 0.049511 |
| **WBGene00018958** | 0.043771 | | **WBGene00013184** | 0.04955 |
| **WBGene00012308** | 0.043917 | | **WBGene00020326** | 0.049553 |
| **WBGene00006983** | 0.043948 | | **WBGene00044734** | 0.04963 |
| **WBGene00199819** | 0.044058 | | **WBGene00003638** | 0.049692 |
| **WBGene00200302** | 0.044334 | | **WBGene00000455** | 0.049804 |
| **WBGene00004193** | 0.049994 | | **WBGene00198171** | 0.049943 |

**Table A2: Top Downregulated Genes in RNASeq between N2 and CCAR-1 Δ.**

| Wormbase ID | N2 p-value | ccar-1 Δ p value | Wormbase ID | N2 p-value | ccar-1 Δ p value |
|---|---|---|---|---|---|
| **WBGene00008822** | -0.89343 | 0.020552 | **WBGene00189953** | 0.212167 | 0.778616 |
| **WBGene00012211** | -0.77266 | 0.098552 | **WBGene00235389** | -0.23472 | 0.331457 |
| **WBGene00219241** | -0.53032 | 0.324263 | **WBGene00219718** | -0.18215 | 0.374271 |
| **WBGene00022237** | -0.7041 | 0.105125 | **WBGene00047500** | -0.21699 | 0.332717 |
| **WBGene00011509** | -0.65842 | 0.138673 | **WBGene00003965** | -0.37224 | 0.176773 |
| **WBGene00019789** | -0.0057 | 0.736159 | **WBGene00220029** | -0.33909 | 0.20939 |
| **WBGene00010981** | -0.49255 | 0.247758 | **WBGene00048305** | -0.26772 | 0.274345 |
| **WBGene00009302** | -0.60285 | 0.133211 | **WBGene00016099** | -0.41061 | 0.131063 |
| **WBGene00015729** | -0.05964 | 0.671027 | **WBGene00004883** | -0.37586 | 0.164497 |
| **WBGene00269434** | -0.04425 | 0.674564 | **WBGene00011131** | -0.0738 | 0.459944 |
| **WBGene00012436** | -0.74212 | -0.02673 | **WBGene00020700** | 0.004901 | 0.538018 |
| **WBGene00199786** | -0.17108 | 0.504924 | **WBGene00000797** | -0.51796 | 0.01154 |
| **WBGene00011435** | -0.00314 | 0.670722 | **WBGene00016823** | -0.48058 | 0.047195 |
| **WBGene00004837** | -0.40299 | 0.263932 | **WBGene00013319** | -0.47694 | 0.048031 |
| **WBGene00235180** | -0.36821 | 0.286679 | **WBGene00195403** | -0.14102 | 0.379961 |
| **WBGene00044975** | -0.48183 | 0.156803 | **WBGene00018195** | -0.07378 | 0.446103 |
| **WBGene00043051** | -0.52507 | 0.102809 | **WBGene00019140** | -0.21072 | 0.308576 |
| **WBGene00196409** | 0.096415 | 0.723678 | **WBGene00003601** | -0.54819 | -0.03185 |
| **WBGene00018363** | -0.22636 | 0.397645 | **WBGene00016017** | -0.07344 | 0.442045 |
| **WBGene00015355** | -0.31283 | 0.301103 | **WBGene00021620** | -0.29236 | 0.222394 |
| **WBGene00017461** | -0.51317 | 0.099225 | **WBGene00008747** | -0.47449 | 0.039572 |
| **WBGene00008027** | 0.030252 | 0.642591 | **WBGene00022669** | -0.54094 | -0.03054 |
| **WBGene00008536** | -0.73137 | -0.12331 | **WBGene00021199** | -0.41548 | 0.092767 |
| **WBGene00012878** | -0.56894 | 0.035045 | **WBGene00004750** | -0.50575 | 0.002476 |
| **WBGene00007509** | -0.42836 | 0.165206 | **WBGene00016023** | -0.26331 | 0.244137 |
| **WBGene00013756** | -0.42127 | 0.170909 | **WBGene00016457** | -0.56925 | -0.06446 |
| **WBGene00016695** | -0.53782 | 0.053916 | **WBGene00023492** | 0.030441 | 0.53389 |
| **WBGene00008537** | -0.78449 | -0.1965 | **WBGene00201953** | -0.11544 | 0.386561 |
| **WBGene00010158** | -0.62969 | -0.04192 | **WBGene00000708** | -0.56013 | -0.05945 |
| **WBGene00194981** | -0.36912 | 0.218512 | **WBGene00013633** | -0.46644 | 0.033747 |
| **WBGene00220003** | 0.020403 | 0.605524 | | | |
| **WBGene00048235** | -0.10396 | 0.477059 | | | |
| **WBGene00008526** | -0.53444 | 0.045821 | | | |
| **WBGene00023427** | -0.47054 | 0.108892 | | | |
| **WBGene00045150** | -0.46037 | 0.118017 | | | |
| **WBGene00219788** | -0.04322 | 0.534947 | | | |
| **WBGene00003977** | -0.59457 | -0.01854 | | | |
| **WBGene00010064** | -0.72205 | -0.14893 | | | |
| **WBGene00021898** | -0.31072 | 0.26075 | | | |
| **WBGene00201211** | -0.0303 | 0.537113 | | | |
| **WBGene00077558** | -0.22471 | 0.342331 | | | |

**Table A2 (continued): Top Downregulated Genes in RNASeq between N2 and CCAR-1 Δ**

| WormBase ID | N2 p-value | ccar-1 Δ p value | WormBase ID | N2 p-value | ccar-1 Δ p value |
|---|---|---|---|---|---|
| **WBGene00220075** | 0.747261 | -0.60724 | **WBGene00009059** | 0.432479 | -0.23985 |
| **WBGene00013819** | 0.837594 | -0.26334 | **WBGene00077582** | 0.151881 | -0.5129 |
| **WBGene00173615** | 0.464926 | -0.53638 | **WBGene00008273** | 0.195576 | -0.46514 |
| **WBGene00235361** | 0.255589 | -0.7311 | **WBGene00219733** | 0.492794 | -0.16791 |
| **WBGene00003351** | 0.520049 | -0.42217 | **WBGene00269383** | 0.223536 | -0.43541 |
| **WBGene00165536** | 0.24549 | -0.65757 | **WBGene00018702** | 0.500763 | -0.15274 |
| **WBGene00168743** | 0.27793 | -0.62425 | **WBGene00045494** | 0.444628 | -0.20576 |
| **WBGene00044895** | 0.17881 | -0.68591 | **WBGene00000759** | 0.315115 | -0.33317 |
| **WBGene00046721** | 0.362823 | -0.48551 | **WBGene00018210** | 0.378617 | -0.25664 |
| **WBGene00013172** | -0.10369 | -0.92402 | **WBGene00018425** | 0.353523 | -0.27919 |
| **WBGene00005657** | 0.611889 | -0.19566 | **WBGene00018485** | 0.382083 | -0.24738 |
| **WBGene00201473** | 0.269799 | -0.52042 | **WBGene00043985** | 0.375031 | -0.24997 |
| **WBGene00195147** | 0.323167 | -0.46644 | **WBGene00016807** | 0.30564 | -0.31921 |
| **WBGene00195448** | 0.45278 | -0.33467 | **WBGene00016714** | 0.513868 | -0.10688 |
| **WBGene00045114** | 0.55656 | -0.22722 | **WBGene00016565** | 0.361258 | -0.25882 |
| **WBGene00219410** | 0.685014 | -0.09603 | **WBGene00199513** | 0.182361 | -0.43599 |
| **WBGene00020455** | 0.600164 | -0.17228 | **WBGene00021630** | 0.280975 | -0.33695 |
| **WBGene00013521** | -0.19973 | -0.96688 | **WBGene00006678** | 0.462784 | -0.15443 |
| **WBGene00013141** | -0.00603 | -0.75644 | **WBGene00017193** | 0.258503 | -0.35645 |
| **WBGene00013154** | -0.19635 | -0.94401 | **WBGene00000191** | 0.260721 | -0.35385 |
| **WBGene00017869** | 0.204821 | -0.54232 | **WBGene00022180** | 0.651409 | 0.037421 |
| **WBGene00007823** | 0.759826 | 0.013653 | **WBGene00002118** | 0.504166 | -0.10604 |
| **WBGene00016684** | 0.115015 | -0.61987 | **WBGene00006983** | 0.365179 | -0.24431 |
| **WBGene00008310** | 0.693353 | -0.03604 | **WBGene00000645** | 0.237581 | -0.37169 |
| **WBGene00021403** | 0.621864 | -0.10683 | **WBGene00001984** | 0.704082 | 0.099434 |
| **WBGene00019022** | 0.510253 | -0.20684 | **WBGene00009239** | 0.287188 | -0.31242 |
| **WBGene00016645** | 0.504297 | -0.21149 | **WBGene00008661** | 0.296093 | -0.30136 |
| **WBGene00195051** | 0.336125 | -0.37048 | **WBGene00012761** | 0.075209 | -0.52123 |
| **WBGene00044574** | 0.232061 | -0.4712 | **WBGene00000845** | 0.492007 | -0.10199 |
| **WBGene00011551** | 0.559738 | -0.1434 | **WBGene00195790** | 0.214774 | -0.37858 |
| **WBGene00196687** | -0.00253 | -0.70295 | **WBGene00021010** | 0.244942 | -0.34801 |
| **WBGene00194785** | 0.35647 | -0.34093 | **WBGene00199006** | 0.288537 | -0.30327 |
| **WBGene00021415** | 0.746864 | 0.049605 | **WBGene00019829** | 0.139088 | -0.45271 |
| **WBGene00015335** | 0.694204 | -0.00132 | **WBGene00011756** | 0.486383 | -0.1052 |
| **WBGene00050939** | 0.900848 | 0.212172 | **WBGene00003660** | 0.482644 | -0.10861 |
| **WBGene00255441** | 0.433333 | -0.2475 | **WBGene00017478** | 0.310467 | -0.28047 |
| **WBGene00196443** | 0.174311 | -0.5065 | **WBGene00019356** | 0.056182 | -0.53012 |
| **WBGene00219304** | 0.290699 | -0.38966 | **WBGene00014156** | 0.170205 | -0.41572 |
| **WBGene00011263** | 0.338744 | -0.34103 | **WBGene00008047** | 0.372116 | -0.21282 |
| **WBGene00138719** | 0.840845 | 0.162945 | **WBGene00009956** | 0.351135 | -0.23058 |
| **WBGene00009059** | 0.432479 | -0.23985 | **WBGene00014870** | 0.207478 | -0.37257 |

**Table A2 (continued): Top Downregulated Genes in RNASeq between N2 and CCAR-1 Δ**

| Wormbase ID | N2 p-value | ccar-1 Δ p value | Wormbase ID | N2 p-value | ccar-1 Δ p value |
|---|---|---|---|---|---|
| **WBGene00202469** | 0.32995 | -0.19774 | **WBGene00007723** | 0.04204 | -0.53751 |
| **WBGene00016212** | 0.30892 | -0.21828 | **WBGene00011838** | 0.61662 | 0.0371 |
| **WBGene00017550** | 0.26365 | -0.26282 | **WBGene00016177** | 0.29041 | -0.28868 |
| **WBGene00000610** | 0.34926 | -0.17134 | **WBGene00003677** | 0.28819 | -0.29041 |
| **WBGene00009060** | 0.44194 | -0.07783 | **WBGene00196852** | 0.19343 | -0.38298 |
| **WBGene00048701** | 0.20096 | -0.31874 | **WBGene00077682** | -0.0792 | -0.65132 |
| **WBGene00004153** | 0.39789 | -0.12169 | **WBGene00008711** | 0.54615 | -0.02123 |
| **WBGene00013175** | 0.16521 | -0.35333 | **WBGene00012850** | 0.44981 | -0.11693 |
| **WBGene00194747** | 0.16595 | -0.35188 | **WBGene00268208** | 0.2371 | -0.32662 |
| **WBGene00010782** | 0.34484 | -0.17215 | **WBGene00167575** | 0.12068 | -0.44277 |
| **WBGene00019277** | 0.03801 | -0.47853 | **WBGene00012912** | 0.33834 | -0.22019 |
| **WBGene00196123** | -0.01826 | -0.53314 | **WBGene00006371** | 0.35745 | -0.19754 |
| **WBGene00010381** | 0.35176 | -0.16175 | **WBGene00003747** | 0.26273 | -0.29057 |
| **WBGene00008651** | 0.19256 | -0.32056 | **WBGene00023408** | 0.21755 | -0.33554 |
| **WBGene00017052** | 0.47065 | -0.04197 | **WBGene00195785** | 0.43005 | -0.11797 |
| **WBGene00010968** | 0.40211 | -0.10768 | **WBGene00197695** | 0.23756 | -0.30885 |
| **WBGene00016946** | 0.17408 | -0.33517 | **WBGene00016130** | 0.46966 | -0.07558 |
| **WBGene00195184** | 0.17098 | -0.33804 | **WBGene00007446** | 0.36566 | -0.17931 |
| **WBGene00011081** | 0.3779 | -0.12955 | **WBGene00016533** | 0.25267 | -0.29147 |
| **WBGene00011949** | 0.45465 | -0.05248 | **WBGene00195276** | 0.27495 | -0.26877 |
| **WBGene00220111** | 0.28779 | -0.21768 | **WBGene00004226** | 0.5734 | 0.03012 |
| **WBGene00007339** | 0.38512 | -0.11856 | **WBGene00019644** | 0.52676 | -0.01613 |
| **WBGene00044463** | 0.64853 | 0.14487 | **WBGene00019116** | 0.35517 | -0.18712 |
| **WBGene00044484** | 0.23873 | -0.26472 | **WBGene00206358** | 0.21057 | -0.33084 |
| **WBGene00020324** | 0.35635 | -0.14704 | **WBGene00022867** | 0.20425 | -0.33658 |
| **WBGene00206384** | 0.18291 | -0.32036 | **WBGene00219271** | 0.4126 | -0.12735 |
| **WBGene00004971** | 0.49087 | -0.01233 | **WBGene00044640** | 0.44989 | -0.08946 |
| **WBGene00010717** | 0.38133 | -0.12026 | **WBGene00020019** | 0.04147 | -0.49758 |
| **WBGene00235343** | 0.13185 | -0.36973 | **WBGene00018706** | 0.63281 | 0.09398 |
| **WBGene00011188** | 0.38637 | -0.11509 | **WBGene00013693** | 0.32743 | -0.21012 |
| **WBGene00195375** | 0.49444 | -0.00676 | **WBGene00167978** | 0.10651 | -0.43009 |
| | | | **WBGene00020646** | 0.45542 | -0.08103 |
| | | | **WBGene00013089** | 0.19689 | -0.33939 |
| | | | **WBGene00017835** | 0.49232 | -0.04345 |

**Table A2 (continued): Top Downregulated Genes in RNASeq between N2 and CCAR-1**

| | | | | | |
|---|---|---|---|---|---|
| | | | **WBGene00013279** | 0.23967 | -0.29547 |
| | | | | | |
| | | | **WBGene00019973** | 0.27109 | -0.26341 |
| | | | **WBGene00007687** | 0.45662 | -0.07656 |
| | | | **WBGene00003680** | 0.34478 | -0.18572 |
| | | | **WBGene00009155** | 0.28277 | -0.24773 |
| | | | **WBGene00015511** | 0.23571 | -0.29391 |
| | | | **WBGene00201188** | 0.32995 | -0.19774 |

**Appendix B: Heat Maps Of Top Upregulated And Downregulated Genes In Rna-Seq Between N2 And Ccar-1 ∆, P-Value Of < 0.05**

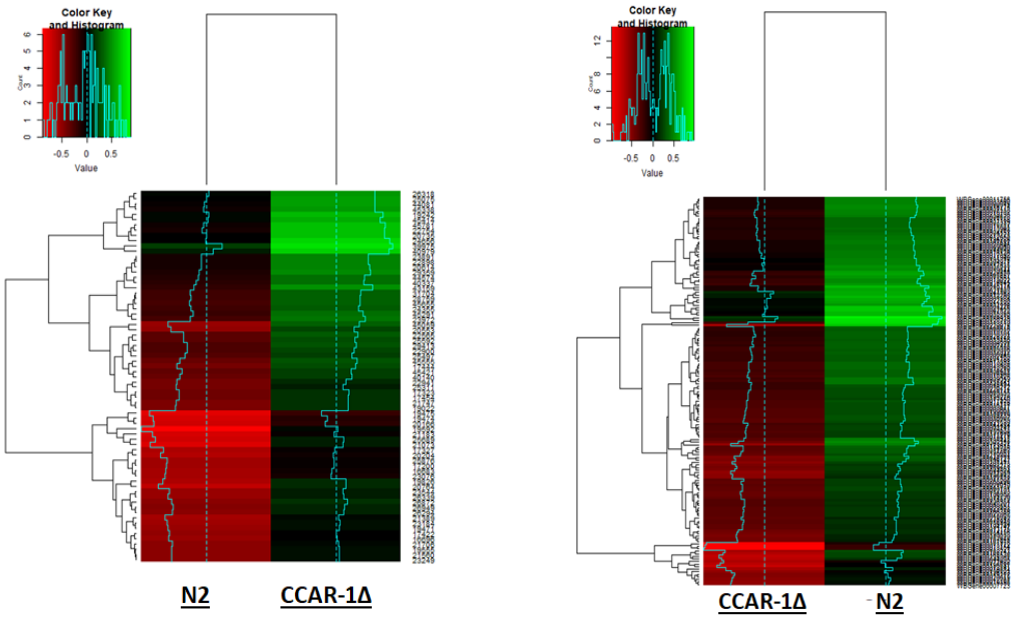


**Figure B1: Identification of genes that are transcriptionally regulated in a CCAR-1Δ compared to wildtype N2 control strain dependent and independent of heat shock.** The heatmap represents the hierarchical clustering of top 500 genes upregulated in a CCAR-1Δ compared to N2 dependent during heat shock as determined by RNA-sequencing. D. The heatmap represents hierarchical clustering of top 500 genes downregulated in a CCAR-1Δ compared to N2 dependent on heat shock as determined by RNA sequencing. The heat maps were organized using heatmap.2 in gplot package of R programming.

**Appendix C: David Gene Ontology Analysis Of Rnaseq Between N2 And Ccar-1 ∆, P-Value Of < 0.05**

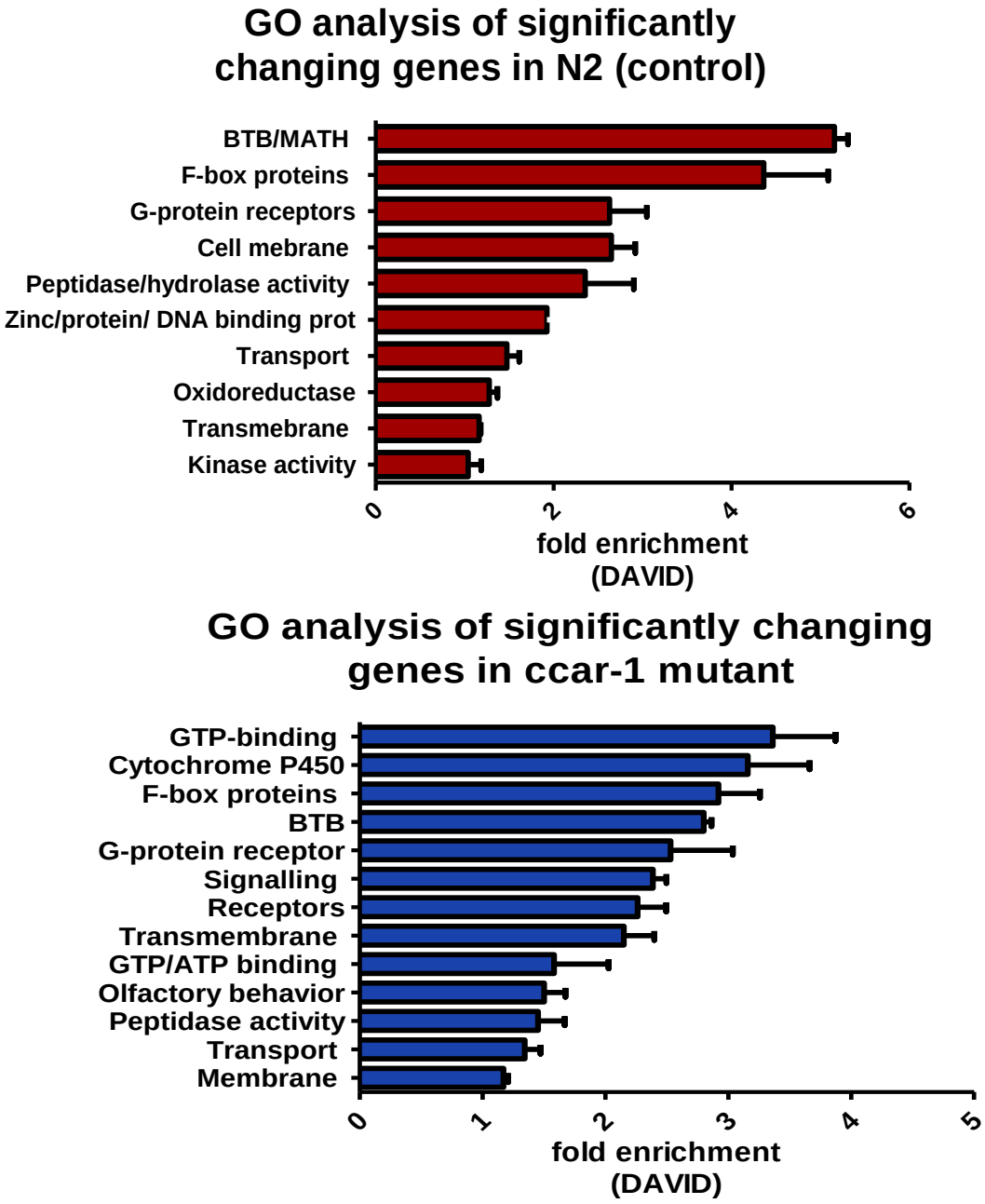


**Figure C1: Gene ontology analysis of significantly changing genes in N2 and CCAR-1Δ using DAVID.** A. Shows genes ontology of significantly changing genes in N2 with and without heat shock. B. Shows genes ontology of significantly changing genes in CCAR-1Δ with and without heat shock.

## Appendix D: Extended Protocols

### *Freezing of C. elegans*

1. Allow OP50 plates to starve, leaving a population of 10,000-20,000 starving L1 and L2 with a few adults. This stage of worms survives freezing better.

N/B: If the plates are contaminated, they are bleached, and the process is repeated.

2. Chunk each strain to a new 0P50/NA22 plate in case the freezing process goes poorly.
3. Wash the worms into 15ml tubes until clear of bacteria.
4. Remove supernatant leaving 2mls in the tube.
5. Add 2ml of worm freezing media and mix thoroughly.
6. Pipet 1ml of the mixture into cryovials and place them in a thawed freezing chamber.
7. Place in the cryovials in an -80 degrees freezer.

### *Thawing of C. elegans*

1. Take a cryovial of worms from the -80 degrees freezer and thaw them in your hands.
2. Make sure there are frozen stocks of the same worm strain remaining in the freezer.
3. Pour the thawed worms into an OP50/NA22 plate.
4. Dry the plate in the fume hood.
5. Allow worms to recover over the week while monitoring them.

N/B

*Some worms recover faster than others depending on the condition of freezing as well as strain in question.*

### *Bleach Synchronization*

1. Examine plates (NA22/OP50-1) for the presence of gravid adults.
2. Take out a clean NGM plate to warm to room temperature.

3. Wash bleaching plate with NGM buffer using a transfer pipette into a 15ml tube.
4. Wash worms two times with NGM buffer in the 15ml tube until the solution is clear and all bacteria have been eliminated.
5. When the solution is clear, remove supernatant up to 3.5mls and discard transfer pipet.
6. Add 1000 µl of bleach and 250 µl of NaOH to worms.
7. Cap the 15 ml tube tightly and shake for a maximum of 5 minutes.
8. Examine the tube for unlysed worms; if none, move on to the next step.
9. Wash the eggs with 10mls of water, centrifuge at 1600 rpm for 30 seconds, and pour off the supernatant.
10. Wash the worms two times with water and transfer them to a warmed NGM plate with a transfer pipette.
11. Observe the plates for the presence of eggs with a dissecting microscope.
12. Dry plate in the fume hood, wrap with parafilm, and place in 20˚C incubator to hatch overnight.

***C.elegans RNAi feeding protocol.***

1.Determine the gene of interest to knockdown using RNAi.

2.Pick gene of interest from the Ahringer library in the fume hood and grow in LB + Carb overnight.

3.The next day, spread 200µl of the RNAi bacteria onto 10 cm or 100 µl for 6 cm plates.

4.Allow the plates to grow a bacteria lawn at room temperature. This growth allows the production of double-stranded RNA, which will knock down the gene of interest.

5.Use RNAi plates within 2-3 weeks.

***Thrashing Assay***

1. Grow worms in desired RNAi/OP50-1/NA22 plates.
2. Add a drop of NGM buffer to the lid of the petri dish.
3. Pick a worm onto the drop of the liquid and count the number of body bends for 30-60 seconds.
4. Repeat for n=12-15, calculate statistics and plot the graph.
5. Determine significance with appropriate statistical analysis.

***Brooding Assay***

1. Grow worms in desired RNAi/OP50-1/NA22 plates to the desired age.
2. Transfer worms (6-12 worms) to a fresh plate /12 well plate well the next day
3. Repeat this transfer for 2-3 days.
4. Count the offspring from the old plate/well each day.
5. Calculate the number of worms laid per day and the total number of offspring per day.
6. Plot the data and perform an appropriate statistical test.

***Lifespan Assay***

1. Grow worms in desired RNAi/OP50-1/NA22 plates to the right age.
2. Transfer worms to a fresh plate for the first three days and every other day after Day 3.
3. Count the number of dead worms by poking until all worms are dead in each condition.
4. Plot the data and perform an appropriate statistical test.

***Visualization of Apoptotic corpses by Sheath Cell Engulfment***

1. Grow worms in desired OP50-1/NA22/RNAi plates
2. Irradiate worms with 400uJ/$m^3$ UV using a Stratalinker.
3. After 24 hours, anesthetize worms with 10mM levamisole

4. Image for the presence/absence of act-5::YFP 'halos' using a Keyence fluorescence

N/B: *Representative images are available in Boulton et al., 2004). Although this method is highly sensitive, it is not suited for use in conditions where high levels of cell death occur, as the CED-1::GFP signal then appears diluted, thus making detection of individual cells difficult. Furthermore, time-lapse analyses indicate that some cells surrounded by CED-1::GFP may not end up forming apoptotic corpses ( Aymeric Bailly and Anton Gartner, unpublished data)*

***Induction of EGL-1/CED-13 Transcription Is an Apoptotic Marker***

1. Grow worms in desired OP50-1/NA22/RNAi plates

2. Irradiate worms with 400uJ/$m^3$ UV using a Stratalinker.

3. After 24 hours, isolated total RNA using the Westerheide lab RNA extraction protocol

3. cDNA synthesis, use the Westerheide lab protocol.

4. Estimate relative amounts of EGL-1 and CED-13 cDNAs by quantitative PCR (qPCR). Primer sequences are taken from Schumacher et al.(2005).

**egl-1primers**:5'-TACTCCTCGTCTCAGGACTT-3', 5'-CATCGAAGTCATCGCACAT-3.'

**ced-13primers**:5'-ACGGTGTTTGAGTTGCAAGC-3', 5'-GTCGTACAAGCGTGATGGAT-3'

**tbg-1primers**:5'-CGTCATCAGCCTGGTAGAACA-3'5'-TGATGACTGTCCACGTTGGA-3'

***RNA extraction***

1. Grow worms in desired OP50-1/NA22/RNAi plates.
2. Wash worms in NGM buffer until clean.
3. Spin down and distribute 50 µl packed worm pellets to a 1.5ml tube for a large RNA prep.

4. For a small RNA prep, pick 30-35 worms to 20µl of NGM in 1.5ml tubes.
5. Place tubes immediately on ice and remove supernatant.
6. In a fume hood, add 250ul of Trizol to each tube of worms.
7. Sonicate for 10 minutes in Bioruptor.
8. Add 250ul of 100% ethanol to samples and vortex to mix.
9. Load the mixture into spin columns and centrifuge at max speed for 1 minute.
10. Transfer spin column to the new collection tube and discard Trizol waste.
11. DNase treats the samples by adding 75ul of DNase reaction buffer and 5ul of DNase I enzyme per sample directly to the spin column and incubating at RT for 15 minutes.
12. Add 400ul of Direct Zol Prewash to the spin column and centrifuge at max speed for 1 minute and discard flow through. Repeat this step.
13. Add 700 ul of RNA wash buffer to the column and centrifuge at max speed for 2 minutes, discard the flow-through
14. Place the spin column into a new DNase/RNAse free 1.5ml tube.
15. Add 20-30 ul of RNAse free water to elute RNA.
16. Proceed to quantification with Nanodrop.

***Competitive RT-PCR assay /Alternative Splicing Assay***

***<u>Growth and Collection of Worms</u>***

*1.* Grow worms on OP50 plates until there is adequate amounts of worms and gravid adults. *Proceed to bleach synchronization. (Refer to Westerheide lab bleach synchronization protocol)*
2. Allow worms to arrest at L1 on NGM plates overnight.

3. Transfer worms onto OP50/RNAi plate and allow growth until desired developmental stage. Then, you can administer desired treatments as well.
4. Collect worms at the desired developmental stage by washing the worms off plates with NGM buffer and transferring them to a 15ml conical tube. Immediately place on ice.

***PLACE WORMS ON ICE FROM THIS STEP FORWARD***

5. Centrifuge for 30seconds at 1600 rpm to pellet worms.
6. Wash worms using sterile water until the liquid is clear/ no longer cloudy from the bacteria on the plates.
7. Centrifuge for 30 seconds at 1600rpm after every wash.
8. Label the 1.5 ml tubes with the desired information.
9. Pipette 20 µl of NGM buffer to each 1.5ml tube and place on ice.
10. Transfer 50 µl of the worm pellet to the labeled 1.5ml tubes containing 20 µl of NGM buffer and immediately place on ice.
11. Proceed to administer heat shock or desired treatment or proceed to RNA extraction.
12. Alternatively, transfer worms to -80 degrees fridge

***RNA Extraction***

13. Westerheide lab RNA extraction procedure using Trizol will be utilized at this point.
14. RNA should then be quantified to determine concentration and purity using the Nanodrop Spectrophotometer.
15. All figures should be recorded for the normalization of samples.

***cDNA Production***

16. RNA should normalize at 250 ng per sample, and the amount of RNA diluted to water and added to the cDNA master mix will be determined.

17. The master mix should be calculated according to the number of samples tested and accounting for negative control (water control/ no RNA sample).
18. Proceed to cDNA synthesis using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit.
19. Run sample in a conventional PCR machine.
20. Determine cDNA concentrations using Nanodrop Spectrophotometer.

***PCR***

21. Normalize cDNA concentrations to 300ng per sample, calculating the amount of DNA diluted with PCR water (Molecular Grade water).
22. Dilute all cDNA samples to 300ng and store cDNA stock solutions. Include the control from the cDNA synthesis.
23. Calculate Taq polymerase master mix according to the number of samples.
24. Make master mix and pipette 24 µl to each sample tube.
25. Add 1 µl of diluted cDNA sample to respective PCR tube.
26. Run PCR using Taq polymerase cycle temperatures and adjust annealing temperatures depending on the primers. Calculate primer annealing temperature using *NEB Tm calculator*

***Making of DNA Polyacrylamide Gels***

1. Make the recipe below in a 50ml conical tube
2. Add 3 µl of 6x loading Dye to PCR product /3 µl per 25ul sample
3. 3. Add 3 µl of 100 bp ladder to the gel
4. 4. In each well, load 10 µl of sample
5. 5. Run the gel at 110V for 1 hour / until the dye is at the bottom of the gel

6. 6. Use 0.5 x TBE buffer to run the gel (950 ml to 50 ml of 10x TBE)

**Table D1: Recipe for DNA Polyacrylamide Gels**

| | 1 Gel | 2 Gels |
|---|---|---|
| 30% Acrylamide | 1.8 ml | 4.0ml |
| 10x TBE | 0.5ml | 1ml |
| ddH20 | 7.7 ml | 15ml |
| TEMED | 20 µl | 50 µl |
| APS | 35 µl | 70 µl |

***Imaging***

1. In a plastic container, add 0.5xTBE (enough to submerge the gel)
2. Add 3 µl of SYBR gold stain and swirl gently.
3. Immerse the gel into the buffer and remove only the top thin glass to allow the stain to access the gel.
4. Place on a shaker and allow for shaking for 5-15 minutes.
5. Image gels on Molecular Imager FX (Bio-Rad).
6. Calculate densitometries using Image J and determine ratios of splice variants.

***C. elegans Immunoprecipitation Protocol***

***Low salt wash buffer:***

**Table D2: Reagents for C.elegans co-immunoprecipitation**

| **Reagents** | **Low Salt Wash Buffer** | **High salt Wash Buffer** | **Lysis Buffer** |
|---|---|---|---|
| 1M T-HCl (7.4) | 0.4mls | 0.4 mls | 0.4 mls |
| 5M NaCl | 1.2 mls | 4mls | 1.2 mls |
| 0.5M EDTA | 40 µl | 40 µl | 40 µl |
| Water | 38.4mls | 35.4 mls | 36.4 mls |
| 10% NP-40 | 0 | 0 | 2 mls |

1. Wash worms from 10-12 NA22 plates into a 15mL tube with NGM buffer. Wash three times with NGM until the solution is no longer cloudy. Transfer worms to a 1.5ml tube.

2. Make Lysis Buffer + Protease Inhibitor in a 15ml tube, depending on the number of samples.

3. Add 250 µL of the lysis buffer supplemented with the protease inhibitor cocktail to the worm pellet and mix well. Centrifuge for 1 minute at 14,000 rpm. Remove the supernatant.

4. Add 400uL of LyP to the worm pellet.

5. To lyse the worms, run them in a Bioruptor for 30 minutes.

6. Centrifuge in the refrigerated centrifuge for 20 min and 14,000 rpm at 4˚C.

7. Transfer the supernatant to a new labeled 1.5 ml tube. The volume of the supernatant should be about 350-415uL. Keep on ice.

N/B: *Do not pipette up the cloudy white layer just above the beads.*

8. Take out 50 µl for the input sample.

9. Make two 40uL dilutions in 1:5 (6:24) and 1:10 (4:36) dilutions. These will be used for the protein concentration determinations.

10. Calculate the protein concentrations.

11. *After protein estimation*: From each lysate, pipette out the amount corresponding to between 800-1000ug protein (Can go up to 1200 ug of total protein). This is usually in approx. 100-350uL.

***Preparation of beads and immunoprecipitation***

12. 40/60 uL of anti-flag beads). Cut the pipette tip dispense the beads easily. Wash the antibody conjugated beads 3X with 600uL of cold Chromotek low salt wash buffer using the magnetic separator.

13. Wash beads with low wash buffer

14. While washing the beads, dilute 1000 ug of protein lysates in a total of 900/1000 uL. using protease inhibitor supplemented low salt wash buffer. (Can use less total protein if the protein concentration is lower)

15. After the three washes, add the lysate mix to the washed beads.

16. Incubate overnight at four ˚C, rotating in the end-over-end invertor. Make sure that the liquid is rolling correctly in the tube.

***Washing steps***

17. Separate the beads from the lysate using the magnet. Discard the supernatant. Wash with 600uL of low salt wash buffer (containing 150mM NaCl) for 10 min using the end-over-end rotator at RT.

18. Separate, discard the wash buffer. Repeat with a high salt wash buffer containing 500mM NaCl.

19. Repeat with low salt wash buffer containing 150mM NaCl.

***SDS Gel to confirm IP***

20.After the last wash, place in a low-speed centrifugation to bring down all the liquids. Add twice 17.5uL of 2X SDS sample buffer + BME directly to the beads. (we elute in 35uL because our SDS_PAGE gels take a max of 50uL). Heat for 10 min at 95C. Quickly spin the tubes to bring down the droplets. Separate by the magnet and transfer the eluate to a new tube.

***Preparing inputs samples.***

21. Take 10ug of total protein in a total of 10uL. Add water to bring the final volume.

22. Add 5uL of 4X SDS sample buffer + BME to the inputs and boil them for 10 min at 95C. "Input" lanes (i.e. lysate before the IP): Use from 0.5 to 10ug (depending from strain) for each in a final volume of 10uL + 5uL of 4X SDS-sample buffer+BME. No need for empty lanes between input lanes since the volume is only 15uL.

23. Load 15uL of 1X SDS sample buffer to each empty lane.

24. Run the gel for 1 hour at 120V.

***Western Blot Protocol***

Western Blots detect the presence of a specific protein in a complex mixture extracted from cells. The procedure relies upon three key elements to accomplish the task:

-separate the protein mixtures by size using gel electrophoresis

-efficient transfer of separated proteins to a solid support

-specific detection of target proteins by appropriately matched antibodies

***Protein Extraction***

1. Grow worms to desired age/condition
2. Label 15ml falcon tubes with the specific strain and date

3. Wash worms from 10-12 NA22 plates into a 15mL tube with NGM buffer. Wash three times with NGM until the solution is no longer cloudy. Transfer worms to a 1.5ml tube.
4. Proceed to Western Blot or store at -80°C
5. Place the samples on ice / if samples are from the -80°C thaw on ice

***Preparation of buffers***

**RIPA buffer** is useful for whole-cell extracts and membrane-bound proteins and may be preferable to NP-40 or Triton-X-100 buffers for extracting nuclear proteins. It disrupts protein-protein interactions and may therefore be problematic for immune precipitations and pull-down assays

**Protease Inhibitor** - as soon as lysis occurs, proteolysis, dephosphorylation, and denaturation begin. These events can be slowed down significantly by having the samples on ice or at four °C at all times, and the inhibitors added to slow the process

**DTT** - Used for the disruption of protein disulfide bonds in SDS polyacrylamide gel electrophoresis

**Table D3: Reagents for Western Blot lysis protocol**

| Components | Amount per samples |
|---|---|
| Buffer C | 435 µl |
| DTT (0.5M) | 0.44µl |
| Protease Inhibitor | 4.4µl |

1. 100 µl of the prepared buffer will be added to each sample, therefore calculate before set up.

***Worms Lysis***

2. Prepare corresponding Eppendorf tubes to the 1.5 ml sample tubes. If using different buffers, separate them at this point. Label all the tubes well.
3. Remove any supernatant using vacuum or fresh tips. Leave a small amount and the pellet
4. Add 100 µl to each tube
5. Place samples on ice immediately

***Bioruptor***

1. Prepare a bucket of ice prepared and cold water from the four °C fridge
2. Set up the bioruptor to appropriate settings
3. Place the sample deck on the bioruptor on ice and load samples
4. Change the water in the Bioruptor if the ice has significantly melted
5. Run samples for 30 minutes

***Centrifugation***

*Turn on the heat-block at this point*

1. Set the centrifuge 10 minutes before sonication ends, run at four °C beforehand to pre-cool
2. Run samples for 8 minutes at full speed
3. Prepare a new set of Eppendorf tubes

   *Chill samples on ice*
4. Transfer supernatant to new labeled tubes (Include date, name, and concentration)

***Preparation of Gels***

6. Use 1mm glass plates, two glass panels- Clean with 70% ethanol and Kim wipes
7. Prepare the gel according to the molecular weight of the protein of interest
8. Spray water at the bottom to seal

9. Only add TEMED and APS when ready to load
10. Acrylamide -4°C fridge

***Bradford Assay***

***Use either Bradford reagent / Coomasie***

1. Perform Bradford solution dilution in 15ml tubes, one in four parts, i.e., *1.5mls of Bradford solution to 6mls water*
2. In a 96 well plate, add 10 µl of water, 20 µl BSA and 200 µl Bradford solution/ coomassie solution in the following format

*Water: A2-A12 and B2-B12*

*BSA: Add to A1 and serially dilute all through to A11*

*A12 and B12 should be undiluted with BSA and should be Bradford /coomassie with water*

3. Switch on Bioteck machine and open Gen 5.1 on the computer to detect protein concentration

***Load Samples on Gel***

1. Sample needs to be 10/20 µg per well; calculate this from the Bradford concentrations 10/concentration= volume to be added.
2. Add equal amounts of laemli buffer from the four °C fridge.
3. Heat solution at 100 °C for 10-15 minutes
4. Spin briefly and cool on ice
5. Add 20 µl each sample into the gel
6. Add page ruler ladder (10 µl of the ladder )

***Running of The Gel***

1. Place the gel in the gel box and a dam on the other side.

2. Put running buffer depending on how many gels are running.
3. Ensure there is no leakage: pour out buffer, create a water pocket, and then place the gel back to the gel box.
4. Fill tank according to the number of gels
5. Prepare sample for loading.
6. Load 20 µl of sample and the ladder
7. Run the gel at 120V for 1.5 hours.

***Membrane Transfer***

8. Prepare four tapper ware; Methanol, transfer buffer (four °C fridge), and water
9. Cut membrane from the box: Make sure NOT TO TOUCH IT
10. Place it in methanol and rock it on slow while preparing the rest
11. Place gel in water and carefully take off the top without tampering with the gel
12. Cut off the stacking gel
13. Place blot paper in both transfer buffers
14. Place gel on the first blot paper and gently push it off the glass. try to center it
11. Pick membrane from methanol and center it on the gel
12. Place other blot paper on top of the membrane and apply pressure
13. Clean transfer machine with water
14. Flip the gel, place lid, and run at 0.3A for 45 min

***Staining***

1. Add porcelain to dish and place membrane
2. Rock on medium
3. Wash with TBST until clear

4. Prepare to block solution 10 ml- 0.5mls non-fat dry milk to 10mls of TBST and shake until there are no lumps
5. Pour onto the blot and rock overnight

***Primary Antibody***

*Add primary antibody to required ratio.*

***Secondary Antibody***

1. Wash blot with TBST for 15minutes and repeat this three times
2. Prepare the blocking solution
3. Add secondary antibody overnight

***Imaging***

1. Prepare ECL solution – reacts with Horseradish. Should be mixed in the dark due to being light-sensitive
2. Probe the ECL on the estimated size of the band

***Stripping of the blot***

1. Add BME solution from 4 degrees fridge – Add 34 µl in 5mls / 68 µl in 10mls
2. Pour on the blot and shake for an hour
3. Pour out and rinse 4-5 times with TBST
4. Repeat antibody procedure

***Tissue Culture***

***Thawing cells***

1. Warm cells in 37˚C
2. Thaw cells from (-80 ˚C and N2 tank) by placing them immediately in 37˚C water bath
3. Pipette 10 ml of media to 15 ml conical flask

4. Pipette all cells from the stock slowly, adding them to the serum one drop at a time.
5. Cell membranes are weakened by storage in DMSO.
6. Spin for 5 minutes at four ˚C
7. Discard supernatant via vacuum aspiration
8. Add 10 ml of media gently to resuspend the pellet. Titrate up and down.
9. Add 8 ml of media to 5 dishes.
10. Add the re-suspended cells to a 100mm plate.
11. Place in 37 ˚C incubator overnight

***Co-Immunoprecipitation***

**Day 1**

1. Split 293T cells. In a 10cm plate per sample.
2. If cells are 90% confluent, split 1:5

**Day 2: Transfections**

1. Cells should be above 50% confluency.
2. Dilute a total of 8 µg CCAR-1- flag plasmid and UAF-2-myc plasmid form maxiprep kit into 1ml serum-free media.
3. Add 24µl of MIRUX Transfection mix reagent and incubate for 30 minutes in the hood.
4. Aspirate media from cells and add 8 ml fresh growth media (without antibiotics)
5. Add 1ml of the growth media with complexes, mix and add the cells.

**Day 3: Harvest cells:**

1. Treat +/- HS at 42 ˚C for 1 hour
2. Aspirate media from cells
3. Wash with cold 1XPBS

4. Scrape cells by adding 1ml of trypsin
5. Spin down to pellet the cells
6. Lyse pellets with 250µl + Protease inhibitors and vortex for 30 minutes in the cold room (shaking)
7. Spin at the four ˚C centrifuges for 10 minutes maximum speed
8. Transfer supernatant to a new tube
9. Quantitate protein
10. Save 5% of total protein as input. If you have 1mg, save 50µg

***Preclear Beads***

1. Add 20ul of Protein A beads to 1mg of protein lysate
2. Make volume to 1ml with 1XPBS
3. Rotate 30 minutes at four ˚C
4. Spin for 1 minute at 1000 rpm, transfer supernatant to a new tube

***Equilibrate beads:***

1. Add 60µl of anti-flag beads per tube
2. Wash three times with Chromotek buffer

***Coimmunoprecipitate proteins***

1. Add equal amounts of cell lysate to equilibrated beads
2. Rotate overnight at four ˚C

***Day 5: Western Blot***

1. Centrifuge 1-minute 1000rpm and aspirate supernatant
2. Wash five times with 500µl RIPA + Protease inhibitor ( each time vortex, nutate 5 min at four ˚C, spin down)

3. Add 40µl of 2x laemmli buffer+ Beta-mecarptenol.
4. Boil at 95 ˚C for 15 minutes
5. Spin at 700 rpm for 5 minutes
6. Load 20µl of the IP and input load 25µg (half of the input saved)
7. Run at 120V for 1:30 min
8. Transfer to membrane
9. Block with 3% milk overnight at 4˚C or 1 hour at 37 ˚C nutating

**Primary antibody** *(anti-myc):*

1. Overnight at four ˚C, nutating
2. Wash 5X in TBST (5 minutes each)

**Secondary antibody**

1. 1 hour at 37 ˚C, nutating
2. Wash 8X (20 minutes in total) in TBST
3. Develop with ECL