A Protein-trap allele reveals roles for *Drosophila* ATF4 in photoreceptor degeneration, oogenesis and wing development

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Summary Statement

A protein-trap line for *Drosophila* ATF4 (*cryptocephal*) reveals how this gene responds to physiological stress, and this gene’s role in *Drosophila* development and retinal degeneration.

Abstract

Metazoans have evolved various quality control mechanisms to cope with cellular stress inflicted by external and physiological conditions. ATF4 is a major effector of the Integrated Stress Response (ISR), an evolutionarily conserved pathway that mediates adaptation to various cellular stressors. Loss of function of *Drosophila* ATF4, encoded by the gene *cryptocephal* (*crc*), results in lethality during pupal development. The roles of *crc* in *Drosophila* disease models and in adult tissue homeostasis thus remain poorly understood. Here, we report that a protein-trap MiMIC insertion in the *crc* locus
generates a crc-GFP fusion protein that allows visualization of crc activity in vivo. This allele also acts as a hypomorphic mutant that uncovers previously unknown roles for crc. Specifically, the crc protein-trap line shows crc-GFP induction in a Drosophila model for Retinitis Pigmentosa (RP). This crc allele renders flies more vulnerable to amino acid deprivation and age-dependent retinal degeneration. These mutants also show defects in wing veins and oocyte maturation. Together, our data reveal previously unknown roles for crc in development, cellular homeostasis and photoreceptor survival.

Introduction

Virtually all organisms have evolved stress response mechanisms to mitigate the impact of homeostatic imbalance. The Integrated Stress Response (ISR) pathway, conserved from yeast to humans, is initiated by a collection of stress-responsive kinases. The ISR pathway has been linked to the etiology of a number of human diseases including neurodegenerative disorders, diabetes, and atherosclerosis, amongst others (Back et al., 2012; Chan et al., 2016; Ivanova and Orekhov, 2016; Ma et al., 2013). Thus, there is immense interest in identifying specific ISR signaling factors and their roles in these pathologies.

Each ISR kinase responds to a different type of stress: PERK, an ER-resident kinase, responds to disruption in endoplasmic reticulum (ER) homeostasis (e.g. misfolding proteins, calcium flux); GCN2, a cytoplasmic kinase, responds to amino acid deprivation; PKR, a cytoplasmic kinase, responds to double stranded RNA, and; HRI, a cytoplasmic kinase, responds to oxidative stress (Donnelly et al., 2013). More recently, MARK2 has been identified as an additional eIF2α kinase which responds to proteotoxic stress (Lu et al., 2021). When activated by the corresponding cellular stress, the ISR kinases phosphorylate the same downstream target: the α-subunit of the initiator methionyl-tRNA (Met-tRNA$^{\text{Met}}$) carrying complex, eIF2. Such phosphorylation of eIF2α kinases leads to decreased availability of Met-tRNA$^{\text{Met}}$, resulting in lowered cellular translation (Sonenberg and Hinnebusch, 2009). However, the translation of some mRNAs with unusual 5′ leader arrangements, such as the one encoding the ISR
transcription factor ATF4, is induced even under such inhibitory conditions (Hinnebusch et al., 2016). ATF4 is a bZIP (basic Leucine Zipper) transcription factor that induces the expression of stress response genes, including those involved in protein folding chaperones, amino acid transporters, and antioxidant genes (Back et al., 2009; Fusakio et al., 2016; Han et al., 2013; Shan et al., 2016).

The number of ISR kinases varies depending on organismal complexity, e.g. GCN2 in *Saccharomyces cerevisiae* (yeast), GCN2 and PERK in *Caenorhabditis elegans* (worms) and *Drosophila melanogaster* (flies), and additional ISR kinases in *Danio rerio* (zebrafish) and other vertebrates (Mitra and Ryoo, 2019; Ryoo, 2015). While these kinases induce a few downstream transcription factors (Andreev et al., 2015; Brown et al., 2021; Palam et al., 2011; You et al., 2021), ATF4 remains the best-characterized (Donnelly et al., 2013). *Drosophila* has a functionally conserved ortholog referred to as *cryptocephal* (*crc*) (Fristrom, 1965; Hewes et al., 2000). In addition to its well-characterized roles during cellular stress, a plethora of studies have demonstrated roles for ISR signaling components during organismal development (Mitra and Ryoo, 2019; Pakos-Zebrucka et al., 2016). In *Drosophila*, loss of Gcn2 results in decreased lifespan and increased susceptibility to amino acid deprivation and bacterial infection (Kang et al., 2017; Vasudevan et al., 2017). *Drosophila Perk* is highly expressed in the endodermal cells of the gut during embryogenesis, and has also been demonstrated to regulate intestinal stem cells in adults (Wang et al., 2015). Although both Gcn2 and Perk *Drosophila* mutants survive to adulthood (Kang et al., 2017; Vasudevan et al., 2020), mutations in *crc* result in significant lethality during larval stages. The *crc* hypomorphic point mutant, *crc^t^*, which causes a single amino acid change, results in delayed larval development and subsequent pupal lethality (Fristrom, 1965; Hewes et al., 2000; Vasudevan et al., 2020). The most striking phenotype of the *crc^t^* mutants is the failure to evert the adult head during pupariation, along with failure to elongate their wings and legs (Fristrom, 1965; Gauthier et al., 2012; Hewes et al., 2000; Vasudevan et al., 2020).
The larval and pupal lethality of known \( \text{crc} \) alleles have limited our understanding of its role in adult tissues. Additionally, study of the role of \( \text{crc} \) using mitotic clones has been impeded by the cytogenetic proximity of \( \text{crc} \) to the widely used FRT40 element. Here, we report that a GFP protein-trap reporter allele in the \( \text{crc} \) locus acts as a hypomorphic mutant that survives to adulthood. We use this allele to discover that loss of \( \text{crc} \) results in higher rates of retinal degeneration in a \emph{Drosophila} model of autosomal dominant retinitis pigmentosa (adRP), a human disease whose etiology is linked to ER stress. Adult \( \text{crc} \) mutants show increased susceptibility to amino acid deprivation, consistent with what was previously known for GCN2. Additionally, we observe several developmental defects in adult tissues, including reduced female fertility due to a block in oogenesis. We also observe wing vein defects and overall reduced wing size in both male and female \( \text{crc} \) mutants.

\section*{Results}
\emph{crc}\textsuperscript{GFSTF} \textit{is a faithful reporter for endogenous crc levels}

In seeking endogenous reporters of \( \text{crc} \) activity, we examined a “protein trap” line for \( \text{crc} \), generated as part of the Gene Disruption Project (Nagarkar-Jaiswal et al., 2015a; Nagarkar-Jaiswal et al., 2015b; Venken et al., 2011). The Gene Disruption project is based on a MiMIC (Minos Mediated Integration Cassette) element inserted randomly into various regions in the \emph{Drosophila} genome. The cassette can be subsequently replaced with an EGFP-FIAsH-StrepII-TEV-3xFlag (GFSTF) multi-tag cassette using recombination mediated cassette exchange. One such insertion recovered through this project is in the intronic region of the \emph{Drosophila} \( \text{crc} \) locus, which has been subsequently replaced with an EGFP-FIAsH-StrepII-TEV-3xFlag (GFSTF) multi-tag cassette (Fig. 1). The splice donor and acceptor sequences flanking the cassette ensure that the GFSTF multi-tag is incorporated in the coding sequence of most \( \text{crc} \) splice isoforms to generate a \( \text{crc} \) fusion protein (Fig. 1). This \( \text{crc} \) reporter allele is henceforth referred to as \( \text{crc} \textsuperscript{GFSTF} \), with the encoded fusion protein referred to as to \( \text{crc}-\text{GFP} \).
Our lab and others have utilized acute misexpression of Rh1\textsuperscript{G69D}, an ER stress-imposing mutant protein, in third instar larval eye disc tissues, using a \textit{GMR-Gal4} driver (\textit{GMR}>Rh1\textsuperscript{G69D}), as a facile method to activate the \textit{Perk-crc} pathway (Kang et al., 2015; Kang et al., 2017; Ryoo et al., 2007). We tested the utility of \textit{crc\textsuperscript{GFSTF}} allele as an endogenous reporter for \textit{crc} levels, and found robust induction of \textit{crc-GFP} in third instar larval eye discs in response to misexpression of Rh1\textsuperscript{G69D} protein, but not in response to control lacZ protein in the \textit{crc\textsuperscript{GFSTF/+}} background (Fig. 2a, b). To validate that such induction was downstream of PERK activation, caused by the misexpression of Rh1\textsuperscript{G69D}, we generated \textit{Perk} mutant FRT clones negatively marked by DsRed expression in the GMR compartment using ey-FLP. While control clones showed no change in induction of \textit{crc-GFP} (Fig. 2c), \textit{Perk\textsuperscript{e01744}} mutant clones showed a complete loss of \textit{crc-GFP} in \textit{GMR>Rh1\textsuperscript{G69D}} eye imaginal discs (Fig. 2d). We also validated these observations in whole animal \textit{Perk\textsuperscript{e01744}} mutants, where we observed a complete loss of \textit{crc-GFP} in \textit{GMR>Rh1\textsuperscript{G69D}} eye imaginal discs (Fig. S1).

To examine if \textit{crc-GFP} could also be utilized as a readout for GCN2 activation, we dissected fat bodies from wandering third instar larva, where we have previously reported GCN2-dependent \textit{crc} activation (Kang et al., 2015; Kang et al., 2017). We observed \textit{crc-GFP} signal localized to the nucleus in the larval fat bodies (Fig. S2). Such signal was substantially depleted using fat body-specific RNAi knockdown of ATF4 or GCN2 (Fig. S2).

Since the induction of \textit{crc} in response to stress is regulated at the level of mRNA translation, we wanted to ensure that the induction of \textit{crc-GFP} we observed in Fig. 2a-d is reflective of translation regulation via the \textit{crc 5’ leader} sequence. The \textit{crc 5’ leader} is structured such that the main ORF of \textit{crc} is favorably translated when eIF2 availability is reduced, such as during phospho-inactivation of eIF2\textsubscript{\alpha} by ISR kinases (Hinnebusch, 1984; Kang et al., 2015). It has also been previously demonstrated that phosphorylation of a subset of cellular eIF2\textsubscript{\alpha} is sufficient to diminish initiator methionine availability, thus mimicking ISR activation (Ramaiah et al., 1994). To imitate reduction of eIF2 availability by ISR kinases, we generated a phospho-mimetic transgenic line where the Ser51 in eIF2\textsubscript{\alpha} is mutated to Asp51 (\textit{UAS-eIF2\textsubscript{\alpha}\textsuperscript{S51D}}). We also generated a corresponding control transgenic line containing wild type eIF2\textsubscript{\alpha} (\textit{UAS-eIF2\textsubscript{\alpha}\textsuperscript{WT}}). We used this genetic
mimetic of ISR activation to test if $\text{crc}^{\text{GFSTF}}$ reported crc translation induction under reduced elf2 availability conditions. While $\text{GMR}>\text{elf2}^{\text{WT}}$ discs showed no detectable levels of crc-GFP, we found that $\text{GMR}>\text{elf2}^{\text{S51D}}$ led to robust induction of crc-GFP in eye discs, as detected by immunostaining with anti-GFP (Fig. 2e, f). These data demonstrate the applicability of $\text{crc}^{\text{GFSTF}}$ as a reliable reporter of endogenous crc expression downstream of ISR activation.

$\text{crc}^{\text{GFSTF}}$ is a hypomorphic crc mutant allele

Similar to the previously characterized crc hypomorphic mutant allele, $\text{crc}^{1}$ (Fristrom, 1965; Hewes et al., 2000), we observed that flies homozygous for $\text{crc}^{\text{GFSTF}}$ exhibited a delay in head eversion and showed anterior spiracle defects. However, unlike the $\text{crc}^{1}$ mutants, none of the $\text{crc}^{\text{GFSTF}}$ homozygotic pupae exhibited complete loss of head eversion, indicating that $\text{crc}^{\text{GFSTF}}$ is likely a weaker hypomorphic allele than $\text{crc}^{1}$. To further assess the effects of the $\text{crc}^{\text{GFSTF}}$ allele, we performed lethal phase analysis of development, starting at the first instar larva. We found that a little over 50% of $\text{crc}^{\text{GFSTF}}$ homozygotes were larval lethal (Fig. 3a), which is remarkably similar to the larval lethality we previously reported for $\text{crc}^{1}$ (Vasudevan et al., 2020). However, unlike $\text{crc}^{1}$ homozygotes, only a small percentage of $\text{crc}^{\text{GFSTF}}$ homozygotes showed prepupal and pupal lethality, with ~30% of animals eclosing as adults (Fig. 3a). To ensure that these developmental defects cannot be attributed to background mutations in $\text{crc}^{\text{GFSTF}}$, we performed lethal phase analysis on $\text{crc}^{\text{GFSTF}}$ in transheterozygotic combinations with the hypomorphic $\text{crc}^{1}$ allele. We found that $\text{crc}^{\text{GFSTF}}/\text{crc}^{1}$ transheterozygotes showed similar levels of larval and pupal lethality to $\text{crc}^{\text{GFSTF}}$ homozygotes, with ~25% of animals surviving to adulthood (Fig. 3a). These data together suggested that the $\text{crc}^{\text{GFSTF}}$ allele may function as a crc loss-of-function allele.

To examine if crc transcript levels are affected in $\text{crc}^{\text{GFSTF}}$ mutants, we performed qPCR in the wandering 3rd instar larval stage, when crc activity is known to be high in fat tissues (Kang et al., 2015; Kang et al., 2017). We found that $\text{crc}^{\text{GFSTF}}$ homozygotes showed a ~65% decrease in crc transcript levels in comparison to control animals (Fig. 3b). We also tested crc activity by measuring mRNA levels of the well-characterized crc transcriptional target, $4E$-BP ($\text{Drosophila Thor}$). We observed ~40% lower levels of $\text{Thor}$
in \textit{crc}^{GFSTF} in comparison to control animals (Fig. 3b). This reduction in transcript levels of crc targets was also reproducible in \textit{crc}^{GFSTF}/\textit{crc}^{1} transheterozygotes (Fig. 3b). Taken together, these data indicate that \textit{crc}^{GFSTF} acts as a mild hypomorphic mutant allele of \textit{crc}.

\textit{crc has a protective role in age-related retinal degeneration and amino acid deprivation}

Nearly 30\% of all adRP mutations are found in the Rhodopsin gene (Illing et al., 2002; Kaushal and Khorana, 1994). Several of these Rhodopsin mutations result in misfolding proteins, which inflict ER stress (Kroeger et al., 2019). However, the role of ATF4 in adRP has remained unclear. We sought to resolve this using the \textit{crc}^{GFSTF} allele in a \textit{Drosophila} model of adRP.

Clinically, adRP is characterized by age-related loss of peripheral vision, resulting in ‘tunnel vision’ and night blindness, due to degeneration of rod photoreceptors (Kaushal and Khorana, 1994). The \textit{Drosophila} genome encodes several Rhodopsin genes, including \textit{ninaE}, which encodes the Rhodopsin-1 (Rh1) protein. The \textit{ninaE}^{G69D} mutation captures essential features of adRP etiology: flies bearing one copy of the dominant \textit{ninaE}^{G69D} allele exhibit age-related retinal degeneration as seen by photoreceptor cell death (Colley et al., 1995; Kurada and O'Tousa, 1995). This mutant encodes a protein with a negatively charged Asp residue in the transmembrane domain, therefore, is predicted to disrupt Rh1’s folding properties. Consistently, the \textit{ninaE}^{G69D} mutant activates ER stress markers in photoreceptors (Ryoo et al., 2007). More recent gene expression profiling experiments found that \textit{ninaE}^{G69D}/+ photoreceptors induce many ISR-associated genes, including \textit{crc} itself (Huang and Ryoo, 2021).

We found that \textit{crc}^{GFSTF}/\textit{crc}^{1}; \textit{ninaE}^{G69D}/+ animals exhibited rapid retinal degeneration in comparison to \textit{crc}^{GFSTF}/++; \textit{ninaE}^{G69D}/+ control animals, as monitored by pseudopupil structures in live flies over a time course of 30 days (Fig. 4a). While the earliest time point when control animals exhibit retinal degeneration is typically around 13-15 days, \textit{crc} homozygous mutant animals exhibited onset of retinal degeneration as early as 4 days, with all animals displaying complete loss of pseudopupil structures by day 14 (Fig. 4a). Further analysis of photoreceptor integrity by actin immunostaining
following dissection showed that even young (2 days old) \( \text{crc}^{\text{GFSTF}}/\text{crc}^{1} ; \text{ninaE}^{\text{G69D}}/+ \) flies showed evident disruption of ommatidial organization in comparison to \( \text{ninaE}^{\text{G69D}}/+ \) animals, where ommatidial organization was relatively unperturbed (Fig. 4b-d). At day 7, the majority of \( \text{ninaE}^{\text{G69D}}/+ \) animals showed intact pseudopupils, and identifiably regular ommatidial arrangements (Fig. 4a, e). In contrast, we observed considerable disruption of ommatidial structures in both \( \text{crc}^{1} \) and \( \text{crc}^{\text{GFSTF}}/\text{crc}^{1} \) mutants bearing the \( \text{ninaE}^{\text{G69D}} \) allele. (Fig. 4a, f, g). Interestingly, we also found that \( \text{crc}^{\text{GFSTF}}/\text{crc}^{1} \) animals exhibited age-dependent retinal degeneration even in the absence of \( \text{ninaE}^{\text{G69D}} \), indicating that age-related physiological-decline requires a protective role for \( \text{crc} \) in photoreceptors (Fig. 4a).

To measure the expression of \( \text{crc} \) in aging photoreceptors, we performed western blotting of adult fly heads from young (2 days old) and old (14 days old) flies to detect \( \text{crc-GFP} \). While young control flies (\( \text{crc}^{\text{GFSTF}}/+ \)) showed very low levels of \( \text{crc-GFP} \), flies bearing one copy of \( \text{ninaE}^{\text{G69D}} \) showed a substantial induction of \( \text{crc-GFP} \) (Fig. 4h, i). We observed that \( \text{crc-GFP} \) increases with age in both 14 day old control flies (\( \text{crc}^{\text{GFSTF}}/+ \)), and a concomitant increase in \( \text{crc-GFP} \) in \( \text{ninaE}^{\text{G69D}}/+ \) flies as well (Fig. 4h, i). These data substantiate the role of \( \text{crc} \) in photoreceptors suffering from ER stress induced by misfolding-prone Rh1\(^{\text{G69D}} \), thus providing a basis for the protective roles of \( \text{Perk} \) in retinal degeneration.

In addition to rendering a protective effect during ER stress inflicted by Rh1\(^{\text{G69D}} \), we also tested if \( \text{crc} \) had an effect during amino acid deprivation in adult animals. We tested this by subjecting \( \text{crc}^{\text{GFSTF}}/\text{crc}^{1} \) animals to amino acid deprivation by rearing animals on 5% sucrose-agar. While a majority of control animals survived up to 8 days, \( \text{crc}^{\text{GFSTF}}/\text{crc}^{1} \) animals steadily succumbed to amino acid deprivation starting at day 2 with no survivors by day 6 (Fig. 4j). This is consistent with the idea that \( \text{crc} \) mediates the GCN2 response to amino acid deprivation in adult \text{Drosophila}.

**\text{crc} \text{mutants show wing size and vein defects}**

\( \text{crc}^{\text{GFSTF}} \) provided an opportunity to examine previously unreported roles for \( \text{crc} \) in adult flies. We first observed that wings from both \( \text{crc}^{\text{GFSTF}} \) homozygotes and \( \text{crc}^{\text{GFSTF}}/\text{crc}^{1} \) transheterozygotes showed a range of venation defects (Fig. 5a-c). The \text{Drosophila}
wing has five longitudinal veins (annotated L1-L5) and two cross veins, anterior and posterior, labelled ACV and PCV, respectively (Fig. 5a). Severe wing defects in \textit{crc}^{GFSTF} homozygous flies were characterized by ectopic venation on L2, between L3 and L4, on L5, and ectopic cross veins adjacent to the PCV (Fig. 5b, b’). \textit{crc}^{GFSTF}/\textit{crc}^{1} transheterozygotes largely showed milder wing defects, characterized by ectopic venation on the PCV and on L5 (Fig. 5c, c’). We quantified these wing phenotypes in over forty animals of each sex, and found that the penetrance and severity of the phenotype was much stronger in females than in males (Fig. 5e). To ensure that the phenotypes were not due to background mutations, we performed a genomic rescue experiment using a BAC-clone based chromosomal duplication covering the \textit{crc} locus, \textit{Dp (90599)}. We found venation phenotypes in \textit{crc}^{GFSTF} homozygotic mutants to be substantially, albeit incompletely, rescued by \textit{Dp (90599)} (Fig. 5d, d’, e).

We also observed that \textit{crc} mutant wings were smaller than in control animals (Fig. 5a-c). Quantification of the wing area revealed a statistically significant decrease in wing blade size in \textit{crc}^{GFSTF} and \textit{crc}^{GFSTF}/\textit{crc}^{1} (Fig. 5f). To exclude the possibility of dominant negative effects of \textit{crc}^{GFSTF}, we also tested wings from \textit{crc}^{GFSTF}/+ heterozygotes but found no wing defects in these animals (Fig. S3). We were unable to detect \textit{crc}-GFP expression in the developing wing discs. This may be due to insufficient sensitivity of the reporter, or possibly indicate a non-autonomous role for \textit{crc} in wing development. It is notable that \textit{Gcn2} depletion in the wing reportedly causes venation (Malzer et al., 2018). Thus, our results suggest that \textit{Gcn2}-mediated \textit{crc} activation contributes to proper wing vein development.

**\textit{crc} mutants exhibit decreased fertility due to defects in oogenesis**

In trying to establish a stock of \textit{crc}^{GFSTF}, we observed that when mated to each other, \textit{crc}^{GFSTF} homozygotic males and females produced no viable progeny, with very few of the eggs laid hatching to first instar larvae. To determine if this loss of fertility in \textit{crc}^{GFSTF} is due to loss of fertility in males, females, or both, we separately mated \textit{crc} mutant females to healthy control (genotype; \textit{yw}) males and vice versa. We observed that while \textit{crc}^{GFSTF} and \textit{crc}^{GFSTF}/\textit{crc}^{1} males produced viable progeny at similar rates to control \textit{yw} males (data not shown), \textit{crc} mutant females showed \textasciitilde50\% reduction in egg
laying in comparison to control females (Fig. 6a), again with very few of the eggs laid hatching to first instar larvae. Upon closer observation we saw defects in the dorsal appendages of eggs laid by *crc* mutant females, from mild phenotypes such as the shortening of the appendages, to complete absence of one, or both appendages (Fig. 6b). The proportion of eggs showing such dorsal appendage defects were significantly higher in *crc* mutants than in control *yw* animals. Both the overall fertility defect and dorsal appendage defects in *crc* mutants were significantly rescued with the introduction of *Dp(90599)* (Fig. 6a).

Dorsal appendages are specified and develop in the final stage of oogenesis. Each *Drosophila* ovary is comprised of 14-16 developing follicles called ovarioles, with germline stem cells, residing at the anterior apex, undergoing differentiation along the ovariole in individual egg chambers (Lobell et al., 2017). Each egg chamber represents a distinct stage in ovulation, with stage 14 representing a mature egg (see schematic in Fig. 6c). To examine if the dorsal appendage defects resulted in decreased fertilization of eggs, we measured fertilization rates of laid eggs by mating control and *crc* mutant females with *don juan-GFP* (*dj-GFP*) males (Santel et al., 1997). *Dj-GFP* marks individual spermatids, which can be observed in fertilized eggs under fluorescent microscope. Our analysis showed no significant change in rates of fertilization between eggs laid by *yw*, *crc<sup>GFSTF</sup>* and *crc<sup>1</sup>/crc<sup>GFSTF</sup>* females (Fig. 6d). These data indicated that the fertility defects in *crc* mutants were due to loss of *crc* function in female flies.

To further dissect the fertility defects, we examined ovaries from *crc* mutant animals. We observed that ovaries from *crc<sup>GFSTF</sup>* and *crc<sup>GFSTF/crc<sup>1</sup></sup>* were considerably swollen in comparison to control ovaries (Fig. S4a). Several ovarioles within *crc* mutant ovaries showed enlarged stage 10 egg chambers, indicative of a stall in oogenesis (yellow arrowheads in Fig. S4a). Indeed, examination of individual ovarioles from *crc* mutant ovaries counterstained for actin showed that loss of *crc* results in an abnormal arrangement of early stage egg chambers (Fig. 6e-f). While ovarioles from control animals showed sequentially staged and spaced egg chambers culminating in mature stage 14 eggs (Fig. 6c, e), ovarioles from *crc<sup>GFSTF</sup>* and *crc<sup>GFSTF/crc<sup>1</sup></sup>* appeared to be arrested in stage 10, with improper spacing between egg chambers in earlier stages (white arrowheads, Fig. 6f, g). We quantified the number of ovarioles that displayed
such arrest, and found that more than half of $crc$ mutant ovarioles (~9) in each ovary showed stage 10 arrest in comparison to an average of 2-3 ovarioles arrested in ovaries from corresponding control animals (Fig. 6h).

To determine if the arrested egg chambers underwent subsequent cell death, we immunostained ovaries with an antibody that detects proteolytically activated (cleaved) caspase, Dcp-1 (Vasudevan and Ryoo, 2016). We observed that stage 7/8 egg chambers from several $crc^{GFSTF}$ and $crc^{GFSTF/crc^I}$ ovarioles showed strong cleaved Dcp-1 staining (Fig. 7a-c). Analysis from over 10 young animals (2 day old) indicated that at least one ovariole in each $crc$ mutant ovary showed strong cleaved Dcp-1 staining in stage 7/8 egg chambers, in stark contrast to none in control ovaries (Fig. 7d). These data suggest that the decrease in fertility in $crc^{GFSTF}$ and $crc^{GFSTF/crc^I}$ females is correlated with cell death in stage 7 and 8 egg chambers during oogenesis.

Reduced fertility has also been demonstrated to be a consequence of dysregulation of stalk cells, which connect the egg chambers of an ovariole (Fig. 6c) (Borensztejn et al., 2018). Specifically, either a failure to reduce stalk cell numbers by apoptosis during development, or excessive culling of stalk cells was shown to decrease fertility (Borensztejn et al., 2018). To examine if the fertility defects in $crc$ mutants could be attributed to dysregulation in stalk cell numbers, we stained control and mutant ovaries with a stalk cell marker, castor (Chang et al., 2013). We observed that while ovarioles from control egg chambers showed ~7 stalk cells between their stage 5/6 and 7/8 egg chambers, $crc$ mutants showed significantly fewer stalk cells (Fig. 7e-h). These data suggest that dysregulation of stalk cell apoptosis may contribute to the fertility defects seen in $crc$ mutants.

To examine which cell types express $crc$ in the ovary, we immunostained ovaries with GFP antibody to detect $crc$-GFP. However, we were unable to detect $crc$-GFP with anti-GFP (Fig. S4b, c), suggesting that $crc$ may regulate ovulation non-autonomously. We also attempted western blotting of ovary extracts to detect $crc$-GFP, but did not observe any detectable signal (data not shown). A previous study had suggested a non-autonomous role for fat body $Gcn2$ in the regulation of oogenesis (Armstrong et al., 2014). Consistent with these observations, we were able to detect high levels of $crc$-GFP fusion protein in adult abdominal fat tissues from $crc^{GFSTF}$ animals (Fig. S5a, b).
Further, using a fat body-specific driver to deplete \textit{crc} in adult fat tissues led to decreased egg laying (Fig. S5c), similar to the phenotypes observed in \textit{crc} mutants (Fig 6a). These data raise the possibility that \textit{crc} mediates Gcn2-signaling in fat tissues to non-autonomously regulate oogenesis.

**Discussion**

ISR signaling is associated with various pathological conditions, but the role of \textit{Drosophila crc} in adult tissues had remained unclear. This may be partly because the cytogenetic location of \textit{crc} is very close to FRT40, and therefore, attempts to study \textit{crc} function using conventional genetic mosaics have been unsuccessful. Our understanding of the role of \textit{crc} in adult \textit{Drosophila} tissues thus far has entirely relied on RNAi experiments. Loss-of-function mutants, however, allow for unbiased discovery of developmental phenotypes, as is exemplified in our present study, where we examined the role of \textit{crc} in later developmental stages, adult tissues, and during aging.

Generally, misfolding-prone membrane proteins such as Rh1\textsuperscript{G69D} are thought to activate the PERK-mediated ISR response, amongst other ER stress responses (Donnelly et al., 2013). It is worth noting here that while both \textit{Drosophila} and mouse models of adRP describe a protective role for \textit{Perk} in retinal degeneration (Athanasiou et al., 2017; Chiang et al., 2012; Vasudevan et al., 2020), there has been conflicting evidence on the role of ATF4 in the mouse adRP model (Bhootada et al., 2016). In this study, we show that loss of \textit{crc} accelerates the age-related retinal degeneration in a \textit{Drosophila} model of adRP. As we have previously shown that \textit{Perk} mutants similarly accelerate retinal degeneration in this model (Vasudevan et al., 2020), we interpret that \textit{crc} mediates the effect of \textit{Perk} in this model. Our data finds that loss of \textit{crc} renders photoreceptors susceptible to age-related retinal degeneration in \textit{ninaE} animals (solid red line, Fig. 4a). Along with our observation showing an increase in \textit{crc} protein levels in older flies (Fig. 4b, c), these data indicate that photoreceptors suffer from physiological stress which requires \textit{crc} for their proper function and survival during aging.

One of the visible phenotypes in adult \textit{crc} mutants is ectopic wing venation (Fig. 5). It has previously been demonstrated that \textit{Gcn2} depletion in the posterior compartment of imaginal discs results in ectopic wing vein formation, though RNAi
experiments from this study indicated this phenotype to be \textit{crc}-independent (Malzer et al., 2018). This raises the possibility of insufficient \textit{crc} suppression in the prior study. The study proposed that GCN2 regulates BMP signaling by modulating mRNA translation in wing discs via eIF2α phosphorylation and \textit{Thor} induction. Our results are consistent with this proposal, since \textit{Thor} is a transcription target of \textit{crc}. In addition, we report here that \textit{crc} loss affects wing size, a finding that has not been reported previously. Given that BMP signaling has also been extensively implicated in determining wing size (Gibson and Perrimon, 2005; Shen and Dahmann, 2005), it is possible that GCN2-crc signaling regulates wing size via BMP signaling. It is equally possible that GCN2-crc signaling affects tissue size through regulating amino acid transport and metabolism through autonomous and non-autonomous means.

While wing development is not known to be sexually dimorphic, fat tissues are known to have sex-specific effects, with particularly profound effects on female fertility in flies and other sexually dimorphic organisms (Valencak et al., 2017). It has been previously demonstrated that loss of \textit{crc} in \textit{Drosophila} larvae leads to reduced fat content and increased starvation susceptibility (Seo et al., 2009). Hence it is possible that the block in oogenesis in \textit{crc} mutants (Fig. 6, 7) is due to metabolic changes in the female fat body, though this remains to be directly tested. This hypothesis integrates well with our data showing high \textit{crc} activity in adult fat tissues (Fig. S5a, b) and observations from a previous study that amino acid sensing by GCN2 in \textit{Drosophila} adult adipocytes regulates germ stem cells in the ovary (Armstrong et al., 2014). Indeed, our preliminary analysis with fat body specific depletion of \textit{crc} using the 3.1Lsp2-Gal4 driver leads to reduced fertility and increased dorsal appendage defects, similar to those seen in \textit{crc} mutants (Fig. S4c). Nonetheless, it remains possible that \textit{crc} acts autonomously in the ovary but is undetectable using our current methods (Fig. S3b, c).

In summary, our study has found utilities for the \textit{crc}^{GFSTF} allele in discovering a new role for ISR signaling in disease models and during development, and also as an endogenous reporter for ISR activation.
Materials and Methods

Flies were reared on cornmeal-molasses media at 25°C under standard conditions except for retinal degeneration experiments when they were reared under constant light. All fly genotypes and sources used in the study are listed in Table S1.

Generation of UAS-eIF2α transgenic lines

Full length Drosophila eIF2α cDNA was amplified from DGRC plasmid (clone LD21861) with EcoRI and XbaI restriction sites using the following primers

Fwd: GGAATTCATGGCCCTGACGTCGCGCTTCTAC
Rev: GCTCTAGACTAATCCTCTTCCTCCTCCTCATCCTC

The resulting DNA fragment was cloned into the EcoRI and XbaI sites of pUAST-attB to generate pUAST-attB-eIF2αWT. For the eIF2α phosphorylation mutants, unique cut sites across the phosphorylation site were identified (AatII, AgeI) and gene fragments corresponding to S51A and S51D mutations were ordered from IDT (sequences below with mutant residues underlined). The pUAST-attB-eIF2αWT plasmid described above was then restriction digested with AatII and AgeI and the WT fragment was replaced with the synthetic mutant fragment (mutated nucleotides underlined) to generate pUAST-attB-eIF2αS51A and pUAST-attB-eIF2αS51D. The plasmids were then targeted to the VK13 attP-9A landing site (BDSC #9732) by BestGene inc to generate transgenic lines that were placed in the same 76A2 genomic locus.

eIF2αS51A fragment: atg gcc ctg acg tcg cgc ttc tac aac gag cgg tat ccg gag atc gag gat gtc gtt atg tgt aac gtt ctg tcc atc gcc gag atg ggc gcc tac gtt cat ctg ctt gag tac aac aac atc gag ggc atg atc ctg ctg tcg gag ctg GCC cgc cgg cgc atc cgc tcc atc aac aag ctg att cgt gtc ggc aag acc gaa ccg gctgt gtt eIF2αS51D fragment: atg gcc ctg acg tcg cgc ttc tac aac gag cgg tat ccg gag atc gag gat gtc gtt atg tgt aac gtt ctg tcc atc gcc gag atg ggc gcc tac gtt cat ctg ctt gag tac aac aac atc gag ggc atg atc ctg ctg tcg gag ctg GAc cgc cgg cgc atc cgc tcc atc aac aag ctg att cgt gtc ggc aag acc gaa ccg gctgt gtt

Phenotype analysis

Lethal phase analysis was performed as described previously (Vasudevan et al. 2020). Right wings were severed from 1-4 day old flies and imaged using a Nikon SMZ1500
microscope outfitted with a Nikon 8MP camera with NIS-Elements software. Wing size was measured using the regions of interest (ROI) feature in ImageJ software. Female fertility was quantified by placing five 1-4 day old virgin females with five yw males (or Dj-GFP males for fertilization assays) in a vial containing standard media enhanced with yeast to encourage egg laying. After allowing a day for acclimatization, the flies were moved to a new vial and the number of eggs laid in a 24-hour period were counted and quantified. Eggs were imaged for Fig. 5b by placing them on an apple juice plate and captured with the Nikon SMZ1500 microscope outfitted with 8MP Nikon camera controlled by NIS elements software. Ovaries from female flies in this experiment were dissected in cold PBS and similarly imaged on apple juice plates for Fig. S3a.

qPCR analysis
Total RNA was prepared using TriZol (Invitrogen) from five wandering third instar larva, and cDNA was generated using random hexamers (Fisher Scientific) and Maxima H minus reverse transcriptase (Thermo Fisher) according to manufacturer’s protocol. qPCR was performed using PowerSYBR Green Mastermix (Thermo Fisher) using the following primers

crc- Fwd: GGAGTGGGCTGTATGACGATAAC  
Rev: CATCACTAAGCAACTGGAGAGAA
Thor- Fwd: TAAGATGTCCGCTTCACCCA  
Rev: CGTAGATAAGTTTGGTGCCTCC
Rpl15-Fwd: AGGATGCACTTATGGCAAGC  
Rev: CCGCAATCCATACGAGTTC

Immunostaining
Ovaries and fat bodies were dissected in cold PBS from female flies reared for 2-3 days along with yw males on standard media enhanced with dry yeast. Tissues were fixed in 4% PFA in PBT (0.2% Triton-X 100, 1X PBS) for 30 minutes, washed 3x with PBT, and blocked in 1% BSA, PBT for 3 hours, all at room temperature. Tissues were stained overnight at 4°C with the primary antibodies diluted in PBT, following which they were
washed 3X with PBT and incubated with AlexaFluor-conjugated secondary antibodies (Invitrogen) in PBT for 3 hours at room temperature. Tissues were mounted in 50% glycerol containing DAPI.

Eye imaginal discs were dissected from wandering 3rd instar larva in cold PBS and fixed in 4% PFA in PBS for 20 minutes, washed 2x with PBS, and permeabilized in 1X PBT for 20 minutes, all at room temperature. Discs were incubated in primary antibodies diluted in PBT for 2 hours, washed 3x in PBT, incubated in AlexaFluor-conjugated secondary antibodies (Invitrogen) in PBT for 1 hour, and washed 3x in PBT, prior to mounting in 50% glycerol containing DAPI.

Adult retina were dissected and visualized with phalloidin as described previously (Huang et al., 2018).

Antibodies: Phalloidin-Alexa647 (1:1000, Invitrogen cat # A22287), chicken anti-GFP (1:500, Aves Labs cat# GFP-1020), rabbit anti-GFP (1:500, Invitrogen cat # A6455), rabbit anti-cleaved Dcp-1 (1:100, Cell Signaling cat# 9578S), mouse anti-4C5 for Rh1 (1:500, DSHB), rabbit anti-eIF2α (1:500, AbCam cat# ab5369), rabbit anti-S51 peIF2α (1:500, AbCam cat# ab32157), rabbit anti-castor (1:50, gift from Dr. Erika Bach).

All images were obtained on a Zeiss LSM 700 confocal microscope with ZEN elements software and a 20X air or 40X water lens.

Retinal degeneration

All experiments were performed in a white mutant background since crcGFSTF, crc1, and ninaE69D, do not have eye color. 0-3 day old male flies were placed (20 animals/vial) under 1000-lumen light intensity, and their pseudopupil structures (reflecting photoreceptor integrity) monitored with a blue fluorescent lamp at 3-day intervals for a 30-day period. Media was replaced every 3 days, and flies with disrupted pseudopupils in one or both eyes were marked as having retinal degeneration.

Western blotting

Fly head extracts were prepared from 6 severed male fly heads in 30 μl lysis buffer containing 10mM Tris HCl (pH 7.5), 150mM NaCl, protease inhibitor cocktail (Roche), 1mM EDTA, 1% SDS. Following SDS-PAGE and western blotting, proteins were
detected using primary antibodies and IRDye-conjugated secondary antibodies (LI-COR) on the Odyssey system. Primary rabbit anti-GFP (1:500, Invitrogen) and mouse anti-beta Tubulin (1:1000, DSHB).

**Amino acid deprivation**

0-3 day old female flies were placed (10 animals/vial) in standard media or in vials containing 5% sucrose, 2% agarose prepared in dH$_2$O. The number of survivors was counted every 24 hours and survivors were moved to new media.

**Acknowledgements**

We thank Hugo Bellen’s laboratory for making available the *crc* MIMIC RMCE line, and Drs. Lacy Barton and Lydia Grmai for discussions on the ovary phenotypes, and Drs. Erika Bach and Jessica Treisman and their laboratories for helpful discussions that improved this work. We thank the Bloomington Drosophila Stock Center (NIH P40OD018537) for supplying many of the fly stocks, DGRC (NIH 2P40OD010949) and FlyBase (U41 HG000739) for curating plasmids and sequence data used in this study.

**Contributions**

D.V. and H.D.R. conceptualized the project, analyzed the data, and wrote the manuscript. H.T. performed all the wing phenotype analyses, G.T. executed all western blotting experiments, and D.V. performed all other experiments.

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Bibliography


**Fig. 1. Schematic of the *crc* cytogenetic locus**

The *crc* gene (blue bar) is known to encode at least four splice variants *crc*-RA, -RB, -RE, and -RF. These splice isoforms vary in their 5' leader sequences (gray bars) and their coding exons (beige bars). MiMIC-mediated insertion of the GFSTF cassette in the genomic locus (green triangle) with a splice acceptor (SA) and splice donor (SD) sequences predicts the inclusion of a multi-tag exon (green box) in all the *crc* isoforms except *crc*-RB.
Fig. 2. \( \text{crc}^{\text{GFSTF}} \) is a reporter for crc activity in vivo

a-b. Confocal images from eye imaginal discs from wandering third instar larva where \( \text{GMR-Gal4} \) drives the expression of either a control protein (\( \text{GMR>lacZ} \)) or mutant Rh1 (\( \text{GMR>Rh1}^{\text{G69D}} \)), in the \( \text{crc}^{\text{GFSTF}/+} \) background. Here and in following images, the \( \text{crc-GFP} \) fusion protein was detected with anti-GFP (green), Rh1 was detected with 4C5 antibody (magenta), DAPI (blue) stains the nucleus; anterior is the left, posterior to the right; scale bars represent 25 \( \mu \text{M} \).

c-d. Confocal images of eye imaginal discs misexpressing Rh1\( ^{\text{G69D}} \) (\( \text{GMR>Rh1}^{\text{G69D}} \)) showing control clones (c, FRT82) and \text{Perk} mutant clones (d, FRT82.\text{perk}\text{e01744}) generated by eyeless-flippase (ey-FLP) in the \text{crc}^{\text{GFSTF}/+} \) background. Clones are negatively marked with DsRed (red) also driven by \text{GMR>} (white arrowheads) demonstrate the effect of loss of \text{Perk} on \text{crc-GFP} induction in response to Rh1\( ^{\text{G69D}} \).

e-f. Confocal images of eye imaginal discs showing \text{crc-GFP} expression in response to wildtype eIF2\( ^{\alpha} \) (eIF2\( ^{\alpha}^{\text{WT}} \)) or phospho-mimetic eIF2\( ^{\alpha} \) (eIF2\( ^{\alpha}^{\text{S51D}} \)) ectopically expressed with \text{GMR-Gal4} (\text{GMR}>). Ectopic expression was confirmed by staining with anti-eIF2\( ^{\alpha} \) (red).
Fig. 3. \textit{crc}^{GFSTF} is a \textit{crc} hypomorphic allele

a. (Top) Schematic showing transitions and stages during \textit{Drosophila} development. (Bottom) Lethal phase analysis for control (yw), \textit{crc}^{GFSTF} homozygotes, and transheterozygotes (\textit{crc}^{GFSTF}/\textit{crc}^{1}), color-coded per the schematic. n=100 for each genotype. Results are significant (p<0.0001) as calculated by a Chi-squared test.

b. qPCR analysis of \textit{crc} and its transcriptional target, \textit{Thor}, normalized to \textit{Rpl15} from wandering 3\textsuperscript{rd} instar larval stages when \textit{crc} expression is known to be elevated. Data represent the mean of 3 independent experiments, error bars represent standard error. *** = p<0.0001 calculated by a paired two-tailed $t$-test.
Fig. 4. *crc* mediates *Perk* and *Gcn2* phenotypes in adult animals

a. Time course of pseudopupil degeneration in control and *ninaE*<sup>G69D</sup>/+ flies in *crc* heterozygote (*crc<sup>GFSTF/+</sup>*) and transheterozygous mutants (*crc<sup>GFSTF/crc<sup>1</sup></sup>). The difference in the course of retinal degeneration between the following pairs is statistically significant as assessed by the Log-rank (Mantel-Cox) test (*p* < 0.001): *crc<sup>GFSTF/+</sup>* and *ninaE<sup>G69D</sup>/+, *crc<sup>GFSTF/crc<sup>1</sup></sup>* and *ninaE<sup>G69D</sup>/+, and, *crc<sup>GFSTF/crc<sup>1</sup></sup>*. (*n* = 100).

b-g. Confocal images of retina dissected from young (2 day old) and older (7 day old) adult flies of indicated genotypes, stained with phalloidin (red) to mark actin in photoreceptors. Each individual ommatidium comprises of 8 photoreceptors (R1-R8), with only 7 visible in this projection (marked by white stars).

h. Western blot analysis of fly head extracts from young (1-2 day) and aged (14-16 day) old flies of the control and *ninaE<sup>G69D</sup>*/+ animals also heterozygous for *crc<sup>GFSTF</sup>*. Upper panel shows the blot probed with anti-GFP to detect the crc-GFP fusion protein (distinguished by the black arrowhead) and lower panel shows Tubulin (anti-Tub) as a loading control.
i. Quantification of western blotting data in (b) showing crc-GFP normalized to Tubulin. Data represent the mean from three independent experiments, error bars represent standard error. **= p<0.001, *=p<0.01 calculated by a paired two-tailed t-test.

j. Time course of survival rate of adult females of indicated genotypes when fed with standard media (SM, solid lines) or amino acid deprived media (AA\(^{-}\), broken lines). Note that the curves for the flies fed SM for yw (solid black) and crc\(^{GFSTF}/crc^{1}\) (solid red) overlap entirely. The difference in the survival rates between the following pairs is statistically significant as assessed by the Log-rank (Mantel-Cox) test (p < 0.001): yw (SM) and yw (AA\(^{-}\)), crc\(^{GFSTF}/crc^{1}\) (SM) and crc\(^{GFSTF}/crc^{1}\) (AA\(^{-}\)), yw (AA\(^{-}\)) and crc\(^{GFSTF}/crc^{1}\) (AA\(^{-}\)). (n = 100).
Fig. 5. Adult *crc* mutants display developmental defects in the wing

a-d. Grayscale images of the right wing from female (a-d) or male (a'-d') flies from the indicated genotypes. (a) shows the arrangement of wing veins in control (*w^{1118}* ) flies with L1-L5 marking longitudinal veins, and arrows marking the anterior cross vein (ACV) and posterior cross vein (ACV). Ectopic longitudinal veins in *crc^{GFSTF}* homozygotes and *crc^{GFSTF}/crc^{1}* transheterozygotes (b, b’, c, c’) are marked by arrows and arrow heads point to ectopic cross veins. (d, d’) show rescue of ectopic venation by the introduction of a chromosomal duplication, *Dp (90599)*, spanning *crc*. Scale bar= 1000μm.

d. The severity and penetrance of the ectopic vein phenotype in (a-d) quantified from 40 animals of each sex of the indicated genotypes. Animals showing venation such as in (b) were classified severe and as in (c) were classified mild. Results were statistically significant (p<0.001) as calculated by a Chi-square test.

e. Area of the right wing from male and female flies of the indicated genotypes as measured in ImageJ. n≥27 for each genotype. Data represent the mean and error bars represent standard error. **= p<0.001, ****= p<0.00001 calculated by an unpaired two-tailed t-test.
Fig. 6. *crc* mutant females show reduced fertility

a. Total number of eggs laid per female in a 24-hour period for control (*yw*), *crc* mutants, and *crc*<sup>GFSFF</sup> rescued with Dp(90599), with proportion of eggs showing dorsal appendage (DA) defects in gray. The data are the mean from 4 independent experiments with five females per experiment, error bars represent standard error. *** = p<0.0001, calculated by a paired two-tailed *t*-test.

b. Grayscale images of 0-24 hour eggs from females of the indicated genotypes. White arrowheads indicate dorsal appendage defects in eggs laid by *crc* mutant females, in comparison to well-formed and elongated dorsal appendages in eggs laid by control females (*yw*).

c. Schematic of ovariole from a partially visible ovary. The stages of the egg chambers can be approximately identified by the arrangement of cells within each ovariole as
indicated in gray, with the oocyte becoming readily visible starting at stage 7.

d. Fertilization rate as indicated by percentage of dj-GFP positive eggs laid by females of the indicated genotype and *Dj-GFP* males. Data represent the mean from three independent experiments, error bars represent standard error. n.s = not significant as calculated by an unpaired two-tailed *t*-test.

e-g. Confocal images of individual ovarioles from indicated genotypes, counterstained with phalloidin (actin). Control ovarioles (*yw*) show clearly delineated individual egg chambers (white arrowheads, c) that are appropriately sized for each stage. *crc* mutant ovarioles (*crc*^GFSTF^, *crc*^GFSTF^/*crc*^1^) show enlarged stage 10 egg chambers, with no clear delineation between individual egg chambers (white arrowheads) indicating a defect in oogenesis. Approximate stages of egg chambers are indicated.

h. Percentage of ovarioles per ovary showing enlarged stage 10 egg chambers, which are indicative of a mid-oogenesis arrest. Data represent the mean from individual ovaries of 11 animals, error bars represent standard error. ***= p<0.0001, calculated by an unpaired two-tailed *t*-test.
Fig. 7. *crc* mutant ovaries show increased egg chamber death and stalk cell dysregulation

a-c. Confocal images of ovaries from the indicated genotypes stained with the cell death marker cleaved Dcp-1 (red), nuclei counterstained with DAPI (blue) and phalloidin marking actin (yellow). White arrowheads point to egg chambers in *crc* mutant ovarioles (*crc^GFSTF^, *crc^GFSTF^/crc^1^) that show elevated Dcp-1 staining. The lower panels show Dcp-1 only channel.

d. Quantification of the percentage of ovarioles per egg showing cleaved Dcp-1 positive egg chamber (a-c). Data represent the mean from individual ovaries at least 9 animals, error bars represent standard error. *** = p<0.0001, **=p<0.001, calculated by an unpaired two-tailed t-test.

e-g. Confocal images of ovaries from the indicated genotypes stained with the stalk cell marker castor (red), nuclei counterstained with DAPI (blue) and phalloidin marking actin (green). White arrowheads point to stalk cells between stage 5/6 and 7/8 egg chambers.
h. Quantification of stalk cell numbers counted by castor-positive cells between stage 5/6 and 7/8 egg chambers (arrowheads from images in e-g). Data represent the mean from over 19 ovarioles from at least 10 different animals, error bars represent standard error. **= p<0.001, *=p<0.01, calculated by an unpaired two-tailed t-test.
**Fig. S1.** crc-GFP induction in response to Rh1$^{G69D}$ is PERK-dependent

a-c. Confocal images of third instar larval eye discs misexpressing mutant Rh1 ($GMR>\text{Rh1}^{G69D}$) in Perk $+/+$ control (a), Perk$^{01744/+}$ heterozygous (b) or Perk$^{01744}$ homozygous animals (c) bearing one copy of crc$^{GFPSTF}$. The left-most panels show merged images of crc-GFP (green) Rh1$^{G69D}$ (red) and DAPI (blue). Rh1 and crc-GFP individual channels are also shown in black and white. d, e. Phospho-eIF2α staining (P-eIF2α, magenta) in $GMR>\text{Rh1}^{G69D}$ eye discs containing Perk$^{01744}$ homozygous mosaic clones (e), or with control clones (d). Perk$^{01744}$ homozygous clones are marked by the absence of DsRed (red). DAPI (blue) counterstains the nucleus. Grayscale images of Rh1, DsRed and P-eIF2α only channels are shown in separate panels. Scale bars represent 25 µM.
Fig. S2. crc-GFP expression in larval fat body is GCN2-dependent
(a-d). Fat body tissues dissected from posterior regions of male third instar larva shows strong nuclear expression of crc-GFP as seen by a heatmap of GFP signal (a). A representative nucleus is marked with a dotted circle. RNAi depletion of crc with fat body-specific driver *dcg-Gal4* shows loss of this signal (b), corroborating the specificity of immunostaining. Depletion of GCN2 with two independent RNAi lines shows loss of nuclear crc-GFP signal (c, d).
Fig. S3. \textit{crc}^{GFSTF/+} does not have a dominant effect

Grayscale images of the right wing from female or male \textit{crc}^{GFSTF/+} heterozygotes do not show the wing defects seen in \textit{crc} mutants (Fig. 2b-c).
**Fig. S4. crc reporters are not expressed in the ovary**

a. Brightfield grayscale image of whole ovaries from indicated genotypes, with the germarium on the left (anterior) and oviduct to the right (posterior). Yellow arrowheads point to easily discernible enlarged stage 10 egg chambers in intact ovarioles from *crc* mutants (*crc*GFS*TF* and *crc*GFS*TF*/crc*).

b-c. Confocal images of ovarioles dissected from control (*yw*, b), and *crc*GFS*TF*/+ animals (c) stained with anti-GFP to detect *crc*-GFP (green) and nuclei counterstained with DAPI (blue). Since DAPI staining is not readily visible in the oocyte, ovaries were also stained with lamin to visualize the nuclear envelope (red). Note that no significant *crc*-GFP signal was detected in *crc*GFS*TF* in comparison to *yw*.
Fig. S5. crc-GFP is expressed in the fat body and can regulate fertility

a, b. Confocal images of adult fat bodies from animals of indicated genotypes stained with anti-GFP (green) and nuclei counterstained with DAPI (blue), showing robust expression of crc-GFP in adipocyte cells.

c. Total number of eggs laid per female in a 24-hour period for control (lacZRNAi) and crcRNAi driven by fat body specific 3.1Lsp2-Gal4, with proportion of eggs showing dorsal appendage (DA) defects in gray. The data are the mean from 3 independent experiments with four females per experiment, error bars represent standard error. **= p<0.001, calculated by a paired two-tailed t-test.
Table S1. Stocks used in the study and their source.

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Table S2.

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