# Canagliflozin Shares Common mTOR and MAPK Signaling Mechanisms with other Lifespan Extension Treatments

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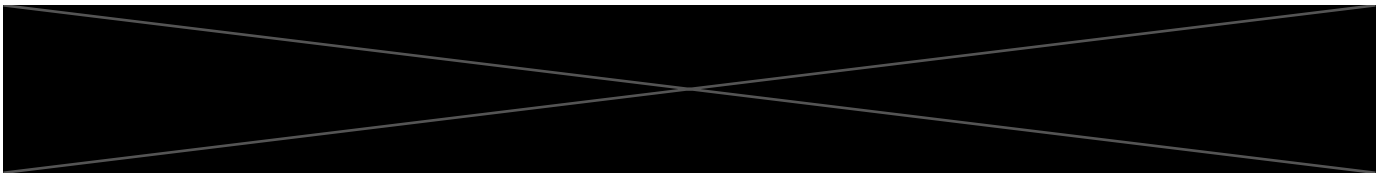

**Running Title**: Canagliflozin effects on mTOR and MAPK signaling.

# Abstract

Long-lived mouse models and treatments that extend lifespan, such as Rapamycin, acarbose and 17α- -estradiol, lead to reduction in mTORC1 activity, declines in cap-dependent translation and increases in cap-independent translation. In addition, these treatments reduce the MEK-ERK-MNK (ERK1-2) signaling cascade, leading to reduction in eIF4E phosphorylation, which also regulates mRNA translation. Here, we report that Canagliflozin, a drug that extends lifespan only in male mice reduces mTORC1 and ERK1-2 signaling in male mice only. The data suggest reduction in mTORC1 and ERK pathways are common mechanisms shared by both genetic and pharmacological models of slowed aging in mice. Our data also reveal a significant sexual dimorphism in the ERK1-2 signaling pathway which might help to explain why some drugs can extend lifespan in males but have no effects in female mice.

## Introduction

Several dietary and pharmacological treatments are known to extend lifespan, including rapamycin [Rapa, (1-3)], acarbose [ACA, (4)] and 17-α-estradiol [17aE2, (4, 5)] as well as canagliflozin [Cana, (6)]. Rapa and ACA can extend lifespan significantly in both male and female mice, although the ACA effect is stronger in males than in females (1, 4), In contrast, 17aE2 and Cana increase lifespan in male mice only, with no significant effects in females (4). The intracellular mechanism(s) underlying the sexual dimorphism in lifespan with these treatments are unknown. Rapa and ACA may share common mechanisms that involve blocking (or reversing) the age-related upregulation of mTORC1 activity (7). In these papers, mTORC1 activity was measured in liver of UM-HET3 mice by changes in the phosphorylation of the ribosomal protein S6 (S6) at Serine 253 (pS6) as well as phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 at threonine 37 (p4EBP1) (7, 8). The canonical pathway implies that p4EBP1 status affects translation by its differential interaction with eIF4E. Our studies also suggested that age can also modify translation by affecting the levels of 4EBP1, as previously found (9). Those effects are independent of the 4EBP1 phosphorylation status, suggesting another level of regulation of eIF4E function independent of mTOR signaling (10, 11). We found that ACA, Rapa and 17a-E2 in liver and kidney blocks the age-related increase in 4EBP1 protein levels, thus reducing the ratio of p4EBP1/4EBP1. In addition, declines in p4EBP1, or increases in 4EBP1 protein levels, have been shown to reduce cap-dependent translation and, indirectly, increase the levels of cap-independent translation or CIT (12). Reduction in translation in lower organisms has shown to modulate aging (13) Although the function and relevance of CIT are not well understood, there is evidence that CIT may lead to enhanced stress resistance, and may modulate metabolic processes and development [i.e (12, 14) ]. Proteins whose levels are influenced by CIT include O-6-methylguanidine-DNA methyltransferase (MGMT), N-myc downstream regulated gene-1 (NDRG1), mitochondrial transcriptional factor-A (TFAM) and heat shock protein 70 (Hsp70) (8, 12, 15-17). Since drugs and mutations that increase mouse lifespan leads to increases in CIT (7, 15), we speculate that CIT may itself promote longevity, but the specific mechanism remains elusive.

17aE2 also leads to declines in mTORC1, reversals of the age-related changes in 4EBP1 protein and increases in CIT, but does so only in male mice (7). The sexual dimorphism mechanism is unknown but may involve changes in the phosphorylation state of eIF4E. eIF4E regulates translation, and in lower organisms a reduction in activity of specific eIF4E isoform in C. elegans has been shown to increase lifespan (18, 19). Function of eIF4E is also regulated by the MAPK1-ERK1/2-MNK1/2-eIF4E signaling pathway (ERK1-2; for details of this pathway see Figure 1 B). The ERK1-2 pathway is involved in the phosphorylation of eIF4E at Ser 209 (peIF4E) via MNK1-2 kinases. MNK1 and MNK2 are the only enzymes known to phosphorylate eIF4E (20). This phospho-site regulates eIF4E translation and thus plays an important role in many biological process (21). Lowering peIF4E by pharmacological or genetic manipulation leads to significant improvements in tissue health (22), lipid metabolism (23), oxidative stress (22), reduction in proinflammation responses (24), as well as reduction in cancer incidence (25). Reduction in eIF4E function can also extend lifespan in fruit flies (26-28), but it is unknown if eIF4E can regulate mammalian aging. Several treatments that extend lifespan in mice do show a significant reduction in the ERK1/2 - eIF4E signaling pathway, including ACA, Rapa and

17aE2, and this pathway is also reduced in several long-lived mouse models, including Snell Dwarf (DW) and GHR-/- mice (GHRKO). The data suggest that the ERK1-2 – eIF4E axis may be a common pathway in regulating aging (7, 29, 30) and we hypothesize that this pathway could be complementary to the effects of changes in mTORC1 signaling.

Canagliflozin (Cana) extended median lifespan of UM-HET3 male mice by 14% but had no effect on female lifespan (6). Cana is an inhibitor of sodium-glucose co-transporter 2 (SGLT2). Cana acts in the kidney to lower blood glucose by increasing glucose loss in the urine. In humans, Cana can reduce the risk of stroke, heart attack, kidney disease or death in people who have type 2 diabetes (31, 32). Although Cana does not extend lifespan in female UM-HET3 mice, both male and female mice have lower body weight after a 7-month exposure to Cana, and also have lower fasting glucose and improved glucose tolerance (6, 33). This suggests that Cana is effective in lowering glucose in both sexes. In addition, UM-HET3 male mice treated with Cana for 16 months did not have altered total body mass, lean body mass or fat mass, and there were no changes in HbA1c in either sex. In contrast, Cana does, however, lead to significantly diminished total body mass and fat mass in female mice (6). Cana-treated males and females show an improvement in the glucose tolerance test (6), insulin sensitivity test and locomotor activity and overall motion behavior (33). Furthermore, there is significant reduction in the age-associated proinflammatory response in both sexes (33), similar to what has been found in other interventions that extend lifespan, such as 17aE2 and Rapamycin (30), suggesting a dissociation between lifespan extension and proinflammatory phenotype in the UM-HET3 mice that may be the result of a sexual dimorphism in downstream signaling affected by Cana. Interestingly, the levels of Cana in plasma and other tissues are 3-5-fold higher in female mice (6). The higher levels of Cana in female mice suggest the absence of a Cana effect on lifespan is not the result of diminished Cana absorption or concentration. Cana can reduce mTORC1 activity in the brain of male mice but not females (33), but it is not known whether responses to Cana in other tissues show a sexual dimorphism similar to what previously shown for 17aE2 treatments (7, 30). Although Cana and 17aE2 have very different primary targets, we hypothesize that both have common downstream mechanism(s) that affect sex-specific aspects of lifespan control. Here we show data suggesting that Cana leads to reduction in mTORC1 and ERK1/2, and to regulation of translation via eIF4E function, specifically in male mice. In addition, we noted a significant sexual dimorphism in ERK1-2 pathways, with higher activity in males than in females, which could help to explain why these drugs, and possible others that affect the ERK1-2 pathway, can extend lifespan in males with little or no effect in female mice.

## Results

### Effects of Cana on mTORC1 signaling pathways

To determine if Cana has effects on mTORC1 similar to those produced by other anti-aging drugs (7), we analyzed liver, kidney, and muscle samples from untreated and Cana treated mice. Tissues were collected from mice fed ad libitum, a change from the 18 hr fasting protocol used in previously studies. (7). The effects of Cana treatments on mTORC1 activity were assessed by immunoblotting, measuring the level and phosphorylation status of two mTORC1 substrates (Figure 1A): S6 protein levels and phosphorylation of S6 at serine 235/236 (pS6), and 4EBP1 protein levels and phosphorylation at threonine 37/46 (p4EBP1). The mRNA levels for each mTORC1 substrate protein were measured by qRT-PCR using the same set of mice. Figure 2 shows representative immunoblots for S6, pS6, 4EBP1, and p4EBP1 from liver, along with mRNA data and statistical analysis of each protein, phosphoprotein, and phosphorylation ratio. The statistical tables in the figure show results of two separate two-way ANOVA calculations, one evaluating age effects (Cana mice omitted), and one involving Cana effects (Young mice omitted). In each case the calculation provided estimates for effects of Sex, Age or Drug, and the corresponding interaction term as shown. When the two-factor ANOVA revealed significant age, drug, or interaction terms, we then performed a one-way ANOVA (with Tukey post-hoc test) to determine which groups differed, and we illustrate this result by asterisks in the bar plot.

Analysis of liver samples shows no significant effects of Cana on S6 protein levels, and no sex or interaction effects (Figure 2A top left panel). This corresponds well with the lack of changes in the S6 mRNA levels (Figure 2A scatterplots). In contrast, we noted significant effects of both age and Cana both on pS6 and on the pS6/S6 ratio. The [Sex x Age] interaction term is significant for both pS6 and the ratio, indicating that the age-related increase in pS6 is stronger in males than in females, males having approximately 30% higher levels of both pS6 and the pS6/S6 ratio than females. Phosphorylation ratios of pS6 have been suggested to be a good indicator of mTORC1 activity, suggesting that under ab lib conditions older male mice may have higher mTORC1 activity than females. However, other pathways known to regulate pS6, such as RSKs, may also contribute to this sexual dimorphism in pS6 (34).

Cana (Figure 2A right panels) significantly reduces pS6 and the pS6 ratio, but in males only. The interaction term (Sex x Drug) is significant ($p = 0.04$) for the ratio and reaches $p = 0.06$ for pS6. These results are consistent with the sex-specificity in pS6 previously seen in mice treated with 17aE2 (7), whose lifespan benefit is also seen in males only. In contrast to S6, 4EBP1 protein levels do show sex-specific effects of both age and Cana in ad lib fed mice (Figure 2B); $p < 0.0001$ for the [Sex x Age] and [Sex x Drug] interaction terms. Young males have a significantly higher level (20-30%) of 4EBP1 than females (Figure 2B), and old males have significantly less 4EBP1 than old females. We do not know the reason for these sexual dimorphisms, which were not seen in fasted mice (7). Nevertheless, with age there is a significant decline in the levels of 4EBP1 in males and females (see box for ANOVA analysis); these results correspond well with our previous reports in fasted mice (7). The sexual dimorphism and the aging effects correlate well with the changes in the mRNA levels shown in the 4EBP1 scatter plot (see * bars in 4EBP1 expression levels and mRNA levels of Figure 2B) suggesting that the age and Cana effects may

reflect a transcriptional mechanism. As expected, Cana blocks or reverse this age effect in 4EBP1 male mice but has no effect in females (see * bars graphs of Figure 2B). Again, this pattern resembles what was found with 17aE2 treatments (7). Figure 2B also shows data on p4EBP1 and the phosphorylation ratio, which show parallel patterns, i.e. an age associated increase, blocked in male mice only. Interestingly, analysis of the p4EBP1 levels (in contrast to the 4EPB1 and ratio data) do not show a sexual dimorphism. The reason for this discrepancy with the data pS6 is unclear but may be due to other upstream pathways are capable of will have effects on pS6, such is the case of MAPK-RSK-pS6 signaling.
Kidney data for S6 and 4EPB1 are shown in Figure 3A. The results are quite similar to those seen in liver, with S6 protein and mRNA levels showing no significant differences by sex, age, or Cana. The levels of pS6 and its ratios (Figure 3A right panels) suggest an age-related increase for both sexes, similar to liver. The statistical analysis suggested significant sexual dimorphism with around 40% higher levels in male males regardless of age (see Figure 3A right panel and box). As in the case of liver, Cana blocks or reduces these effects in kidneys of male mice but has no significant effects in female mice. The pattern of results for 4EBP1 in kidney also resemble those seen in liver (Figure 3A lower panels), with significant age-related reductions of 4EBP1 protein levels in both sexes, opposed by Cana in male mice only. These effects are well correlated with the levels of 4EBP1 mRNA (Figure 3A scatter plots) suggesting that they may be the result of transcriptional regulation. The p4EBP1 and phosphorylation ratio of p4EBP1 (also Figure 3A right panels) follow the same pattern as liver, with a significant age-related upregulation in both sexes. In addition, there was a small but significant sexual dimorphism, with older males showing around 10-20% higher level of p4EBP1 and phosphorylation ratio. As expected, Cana treatments reduce or block the age effects in both parameters (see Figure 3A, * cross bars) but only in male mice, with no effects in the female mice.

S6 expression (protein or mRNA levels) in skeletal muscle shows the same pattern seen in liver and kidney, with no changes in S6 expression by sex, age, or Cana treatment (Figure 3B). As in the case of liver and kidney, pS6 and the ratio of pS6/S6 show an age-related increase with a trend of sexual dimorphism that does not reach significance. Cana treatments can block or reduce both parameters in male mice but not females (Figure 3B, * cross bar). Regulation of 4EBP1 in muscle, however, appears to differ from that seen in liver and kidney. Aging increases 4EBP1 protein and mRNA in muscle in both sexes, with a significant difference between the sexes in the protein data ($p = 0.002$ for Age x Sex interaction). This is the opposite of what was found in liver and kidney and suggests that control of the 4EBP1 protein levels and mRNA involves age-sensitive pathways that are also tissue specific. Nevertheless, levels of p4EBP1 and ratio of p4EBP1 show the same pattern seen in liver and kidney, with an age-related increase that Cana can block or reverse in males only.

Overall, the data suggest that with age an increase in mTORC1 activity is responsible for the phosphorylation of S6 and 4EBP1 that is blocked or reduced by Cana treatment in males. Independently, there is tissue specific regulation of 4EBP1 levels (protein and mRNA) affected by age in both sexes and regulated by Cana in male mice only. The underlying mechanisms that modulate levels, and phosphorylation status, of 4EBP1 are unknown but may reflect sex-specific changes in hormones, including sex hormones (35).

**Effects of Cana on the MEK1-ERK-MNK-eIF4E signaling pathway**

We have previously noted effects of aging and of anti-aging drugs (Rapa, ACA, and 17aE2) on the ERK1-2 signaling pathway diagrammed in Figure 1B (30), We therefore evaluated this MAPK cascade in tissues of ad lib fed Cana-treated mice, with the same analytic approach illustrated in Figures 2 and 3 for the mTORC1 substrates.

The first element in this pathway, MEK1, showed small but significant sexual dimorphism with males having around 10-20 % higher MEK1 protein levels than female mice at both ages and regardless of Cana treatment (Figure 4A). These effects seem to be the result of higher MEK1 mRNA levels in male mice (Figure 4A mRNA panel). Neither Age nor Cana affected MEK1 protein or mRNA levels.

Analysis of pMEK1 reveals a significant sexual dimorphism, with male mice having higher levels of pMEK1 and higher pMEK1 phosphorylation ratio than female mice (Figure 4A right panels). These sexual differences may be more pronounced in older samples, although the [Sex x Age] interaction effect is not significant for pMEK1 or the ratio. Age increases pMEK1 and the pMEK1 ratio in both sexes, while Cana significantly reduces the pMEK1 and the ratio in male mice only, with [Cana x Sex] interaction at $p = 0.02$ in both cases.

ERK1/2 are the downstream substrates of MEK1 action. Neither sex, age, nor Cana has any effect on ERK1 or ERK2 protein or mRNA (Figure 4B right panels). In contrast, pERK1, pERK2 and the respective ratios follow the same pattern shown by pMEK1. Aging leads to increases in pERK1 and pERK2 that are stronger in male mice than in females, and Cana treatment diminishes this increase in pERK1 and pERK2 in males only (Figure 4B).

The pattern of age, sex, and Cana effects in kidney and in skeletal muscle (Figure 5) is very similar to those seen in liver. MEK1 levels are higher in males than in females in both tissues; for kidney the sex effect is stronger in young mice than in old. MEK1 mRNA shows the same sex effect, and a small but significant increase with age. Cana does not alter MEK1 in either tissue. pMEK1 increases with age in both tissues, a change that is blocked in males by Cana, but this Cana effect reaches significance in muscle only. The pMEK1 phosphorylation ratio is diminished by Cana in a sex-specific fashion, i.e. principally in males, in both tissues. Second, the ERK1-2 protein levels and mRNA in kidney and muscle show no significant changes with age, sex, or treatment, similar to the situation for liver (Figure 4). pERK1 and pERK2 increase with age in both tissues (males more than females for pERK1), with the phosphorylation ratios showing the same age effects. Cana reduces this age-related effect in male mice only, significantly in all cases except that the [Sex x Drug] interaction term is marginal ($p = 0.07$) for the pERK1 ratio in kidney. These sex-specific effects shown by Cana are similar to those previously reported for treatment with 17aE2, whose effect on lifespan is also limited to males (30).

The key downstream target of ERK1-2 is the MNK family of kinases (this pathway is summarized in Figure 1B). MNK1 and MNK2 protein levels and mRNA in liver (Figure 6A right panels) show sexual dimorphism that is not affected by age or Cana treatment. Male mice have 20 to 50% higher levels of protein and mRNA, in agreement with the sexual dimorphism we previously noted in fasted mice (30). In the other hand, phosphorylation of MNK at

Threonine 197/202 (pMNK) in liver samples produces a single band (Figure 6A). Because phosphorylation sites of MNK1 and MNK2 predicted by amino acid sequence should be indistinguishable by western blots, it is not possible to distinguish pMNK1 and pMNK2 or to estimate the corresponding ratios for each isoform independently. Therefore, statistical analysis was done on pMNK without respect to the levels of MNK1 or MNK2 protein for each sex. Analysis of pMNK in liver (Figure 6A right panel) reveals a pronounced sexual dimorphism, with males higher than females independently of age and Cana treatment (see Figure 6A ANOVA box for pMNK). This was expected, in that the data on MEK1/ERK also suggested higher activity in male mice. In addition, there is a significant age-related increase in pMNK in both sexes that Cana can block or reduce in male mice only (see Figure 6A * cross bar).

One of the key downstream substrates of MNK is eIF4E. MNK is the only kinase responsible for the phosphorylation of eIF4E at Ser 209 (peIF4E), a critical step in cap-dependent translation (20). Our data (Fig 6B) show no effects of sex, age, or Cana treatment on eIF4E protein or mRNA levels, in good agreement with a previous publication (30). In contrast, analysis of peIF4E and ratios of peIF4E reveal highly significant effects of both sex and age. Male mice have approximately 50% higher levels of peIF4E, and age increases this phosphoprotein in both sexes, with the ratio values showing the same effects. The sexual dimorphism and age effects are consistent with the data on activity of MNK1/2 (Figure 6A). Cana treatment reduces peIF4E and the phosphorylation ratio in male mice only, consistent with the male-specific lifespan effect and with the other biochemical endpoints tested in this MAPK cascade.

The MNK and eIF4E results in kidney (shown in figure 7A) and skeletal muscle (shown in figure 7B) closely resemble the patterns seen in liver samples. MNK1 and MNK2 show a significant sexual dimorphism in both tissues at the level of protein and mRNA, with males having 20-30% higher levels of both enzymes. Age or Cana treatments do not seem to affect these sexual differences in either kidney or muscle. pMNK is higher in males than in females for kidney, independent of age, but this sex effect is not significant in muscle. Age increases pMNK in both tissues, and Cana treatments reduce it in males only. Consistent with the liver data, eIF4E shows no effect of sex, age, or treatment (Fig 7). In contrast, peIF4E and ratios follow the pattern seen for pMNK, with male mice having higher phosphoprotein levels independently of age. Age increases peIF4E and ratio status in both sexes, while Cana treatment blocks this upregulation in male mice only.

Overall, the results suggest that male mice have a higher activity in the ERK1-2 signaling pathway, in particular higher MNK function that would lead to increase in the eIF4E function and changes in the translation of specific set of mRNAs (36). Age seems to increase eIF4E function in both sexes while Cana blocks this in male mice only. This pathway correlates well with the effects of drugs and treatments in lifespan extension, suggesting that higher ERK1-2 at older ages may have negative effects, similar to age-related effect of mTORC1 (37). In addition, the sexual dimorphism could explain why some drugs and treatments, if they affect ERK1-2 signaling directly or indirectly, have more dramatic effects on lifespan in male mice.

## Discussion

Several genetic models of slow aging, such as Snell dwarf, Ghr-/-, and Pappa-KO mice, show increases in proteins that can be synthesized by cap-independent translation (CIT) (8, 15). CIT proteins include those involved in mitochondrial function (i.e. TFAM) and cellular stress resistance, such as MGMT and NDRG (15, 38). The relative increase in multiple CIT proteins could result from several factors in combination, potentially including (a) a decline in mTOR or (b) increases in the pathways involved reading or writing 6-methyladenosine tags in the 5'UTR of mRNA (8, 15, 38). In addition, some of these varieties of long-lived mutant mice show a significant reduction in the ERK1-2 signaling pathway (29, 39, 40) leading to reduction in the ratio of peIF4E, a key regulator of the translation (25). These slow-aging mutants also show a reduction in pro-inflammatory/stress signaling responses mediated by a separate MAPK cascade, one involving p38 MAPK (41, 42). The association of lower mTORC1 function with extended lifespan suggests the possible involvement of diminished translation in aging, health maintenance, and CIT activation, but details are sparse, and in particular it is not known if regulation of the ERK-peIF4E axis is involved in these phenomena. Because there is a deep interconnection network of signaling between mTOR, ERK, and inflammation, it has been difficult to discern what role each of these pathways has in aging.

A parallel approach to addressing these mechanistic questions is to evaluate mice that have been exposed to drugs and diets that extend lifespan in genetically normal mice. In this context, rapamycin and acarbose (ACA), both of which extend lifespan in males and females, have shown a common mechanism with the genetic models of slow aging, including reduction in mTORC1 (7), and ERK1-2 signaling and p38-dependent inflammatory control pathways (30). In contrast, 17aE2 extends lifespan in males only, and the ability of 17aE2 to reduce mTORC1 and ERK signaling is also restricted to males only. Interestingly, 17aE2 treatment lowers p38-dependent inflammatory signals equally in both sexes (30), suggesting a dissociation between inflammation and lifespan extension, at least within the set of mouse models tested. Furthermore, Rapa, ACA, and 17aE2 (male only) treatments also block or reverse the age-related declines in 4EBP1 protein levels, suggesting that these treatments can block age-related increases in cap-dependent translation, leading, indirectly, to increases in CIT (7).

Cana, like 17aE2, leads to an increase in lifespan in male mice, but has no significant effect on lifespan of female mice (6). The mechanism(s) leading to this sexual dimorphism in lifespan are unknown. However, UM-HET3 mice treated with Cana for 16 months show an improvement in glucose homeostasis and motor function in both males and female mice. Female mice have higher levels of Cana in plasma, kidneys, and brain as well as a reduction in fat mass (6, 33). These data suggest that the lack of significant Cana effects on female lifespan are not the result of lower drug concentration, but rather an inherent sexual dimorphism that could be related to higher peak glucose level in male mice as well as differential effects in sex hormones or/and signaling pathways contributing to lifespan extension. It is also possible that the higher levels of Cana in females may lead to harmful effects that are not seen in males, and which limit lifespan benefits in females only. In term of intracellular mechanism, we hypothesize that Cana treatments might have similar signaling changes to those in mice exposed to 17aE2 (7, 30), including male-specific declines in mTORC1 and ERK1-peIF4E signaling. This hypothesis was

tested by treating mice with Cana as shown in (Figure 1C) and measuring the changes in mTORC1 and ERK1-2 signaling pathway from three different tissues: liver, kidney, and skeletal muscle. We observed an age-related upregulation of mTORC1 activity in liver, kidney, and skeletal muscle (Figures 2/3), and found that treatment with Cana led to a significant decrease mTORC1 activity in males but not females. This conclusion is based on the declines of the phosphorylation status and ratios of pS6 and p4EBP1 (Figures 2/3 right), suggesting a sex specific reduction in mTORC1 activity. The results are in good agreement to changes seen in the hypothalamus of Cana treated mice (33) and mimic what was found for 17aE2 treatments (7). We do not know the mechanism for these sexual dimorphisms in lifespan with Cana and 17aE2 treatments, but we did note that male mice show significantly higher phosphorylation ratios of pS6, and phosphoprotein from the ERK signaling pathway in the liver, kidney, and muscle (Figure 2 to 7), suggesting that both mTORC1 and ERK activity are higher in male mice

Another way to regulate translation is by modulating levels of 4EBP1. The unphosphorylated form of 4EBP1 interacts with eIF4E, blocking translation, while its phosphorylation leads to release of eIF4E to initiate translation. Increases in 4EBP1 lead to reductions in translation and, indirectly, increases in CIT. Declines in 4EBP1 may lead to increases in protein translation dependent on its phosphorylation status by mTOR (10, 14). Interestingly, there is an age-related decline in 4EBP1 in liver and kidneys (Figures 2/3A) suggesting an increase in overall protein translation. The skeletal muscle, however, showed an age-related increase in 4EBP1 (Figure 3B) suggesting a possible decline in translation. Age-related increases in 4EBP1 could contribute to declines in muscle mass and thus to age-associated sarcopenia (43). Independent of these tissue specific 4EBP1 changes, Cana treatments seem to reverse or block the age effects on muscle function in male mice only (44, 45). The changes in 4EBP1 protein levels are accompanied by, and presumably caused by, parallel changes in 4EBP1 mRNA (Figures 2/3 mRNA levels), suggesting that this regulation may be independent of mTOR or ERK signaling. In this regard, there are data suggesting that sex hormones play a role in the improvement of lifespan extension and muscular function by these treatments (45). What regulates constitutive 4EBP1 transcription and protein levels is unknown, but it would be important to test the idea that hormones, in particular sex hormones such as testosterone, play a key role in 4EBP1 regulation and the aging effects.

The MNK pathway, modulating phosphorylation of eIF4, provides another path that could regulate the balance between cap-dependent and CIT protein translation (20, 21, 42). MNK activity is under the control of the MEK1-ERK signaling pathway (ERK1-2 pathway, see Figure 1B). Slow-aging mutant mice (46) and mice treated with Rapa, 17aE2 (males only), ACA (30) and now Cana (males only) all show reduction of the age-related increases of this ERK1-2 signaling pathway. We hypothesize that this pathway, either in parallel to or perhaps independently of mTORC1, can regulate mouse health and lifespan extension. The hypothesis that the ERK pathway can regulate aging independently of mTOR has been tested in lower organisms, in both worms and flies (26, 47). Interestingly, flies exhibit sexual dimorphism in responses to nutrients that affect lifespan (48), suggesting that effects of Cana could also sexually dimorphic in nature. However, the underlying mechanism is not understood. Interestingly, we found that male mice show higher MEK1 and MNK1/2 protein levels as well as higher levels of pMNK and peIF4E (and ratios) in all three tissues: liver, kidney, and muscle.

This sexual dimorphism is typically independent of age, and is at least in part regulated by differences in transcription (e.g. mRNA data for MEK1 and MNK1/2 in figures 4 to 7), with higher activity of the ERK1-2 signaling pathway in males. It is possible, therefore, that treatments that reduce ERK1-2 activity would show stronger effects in male mice than in females. [NO PARAGRAPH BREAK HERE]Future work could test this hypothesis by using drugs or treatments that can specifically reduce ERK1-2 or MNK1/2 activity, such as Trametinib or CGP-57380 (26) that can extend lifespan in worm and flies independently of mTOR, which have been suggested to have positive effects on tissue and mouse health.

## Material and Methods

### Mice and diets.

Genetically heterogeneous UM-HET3 mice were produced by a four-way cross between CByB6F1 mothers and C3D2F1 fathers and housed as previously described (3, 35). Mice in breeding cages received Purina 5008 mouse chow, and weaned animals were fed Purina 5LG6. At 6-7 months of age, animals in different sibling groups were randomly allocated to control and Cana treatments. Cana was purchased from Steraloids Inc. (New Port, RI, USA) and given at a dose of 14.4 mg/Kg diet (14.4 ppm) as described in Figure 1C.

### Tissues, western blots, and qRT-PCR analysis

Tissues were harvested at the age indicated in Figure 1C; tissues were harvested during the morning from ab lib fed mice. Tissues were frozen with liquid nitrogen and stored at -80°C. Cell lysates were prepared, after which equal amounts of protein were loaded for Western blot analysis of actin. Then samples from each mouse were normalized to the same actin concentration present in young untreated female controls, and the specific proteins or phospho-sites measured by western blots as described previously (8). Each Western Blot contained at least 2 young control females and 2 young control males in addition to at least one sample for each group (old, Cana treatments for males and females). Data on band intensity for each western blots was normalized with respect to the average of the two young female controls to allow combination of results from multiple western blots. The specific antibodies used in the analysis are listed in Supplemental Table 1. qRT-PCR from the same set of samples was performed as previously described (8). qRT-PCR probe sequences are listed in Supplemental Table 2.

### Statistics

All statistics were carried out in GraphPad Prism version 7.0. To normalize between different western blots, we use young females as a reference as we previously described (8). This involves adjusting by serial dilution to identical levels of β-actin for each batch of western blots. After normalization, data for each parameter was measured by western blots and the data analyzed by two-way ANOVA, using a full factorial model, which included estimates of the effect of age, sex, and interaction between samples from young and old untreated mice. A separate two-way ANOVA then evaluated samples from treated and untreated older mice, to evaluate effects of sex, Cana, and their interaction. To determine significance between specific groups, such as effects of sex or of Cana treatments, we used a one-way ANOVA test followed by Tukey's test for multiple comparisons. Significance was evaluated using a criterion of $p<0.05$ and shown in the graphics as * for $p < 0.05$. ** $p<0.01$, *** $p<0.001$ and **** $p<0.00001$.

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**Abbreviations:** ACA, Acarbose; Rapa, Rapamycin; Cana, Canagliflozin, mTOR, mechanistic target of rapamycin; mTORC1, mechanistic target of rapamycin complex 1; S6, ribosomal protein S6; 4EBP1, eukaryotic translation initiation factor 4E binding protein 1; MEK1, Dual specificity mitogen-activated protein kinase kinase 1; ERK1, Mitogen-activated protein kinase 3; ERK2, Mitogen-activated protein kinase 1; MNK1, MAP kinase-interacting serine/threonine-protein kinase 1; MNK2, MAP kinase-interacting serine/threonine-protein kinase 2; eIF4E, elongation and initiation factor 4 E.

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## Figure Legends

**Figure 1. A**) Diagrams of the mTORC1 signaling pathway evaluated in this study. **B**) Diagram of the ERK1-2 MAPK signaling pathway. **C**) Diagram of the experimental design for the Cana treatments.

**Figure 2. Effect of Cana on hepatic mTORC1 signaling. A)** Representative western blots of S6 and pS6(pS235) in livers of males (M) and females (F) from young (Y), old untreated samples (O), or old Cana treated (Cana) as described in Figure 1C. Bars represent the mean ± SEM of each S6 protein level, S6 mRNA scatter plot, pS6 or pS6 ratios from a minimum of 8 mice per group. Each graph is accompanied by a box showing the statistics by age (Age ANOVA) or Cana treatment (Cana ANOVA). The * represents a significant level for the specific group comparation show in the cross bar as described in Material and Methods. **B)** Representative western blots of 4EBP1 and p4EBP1 levels as described in section above.

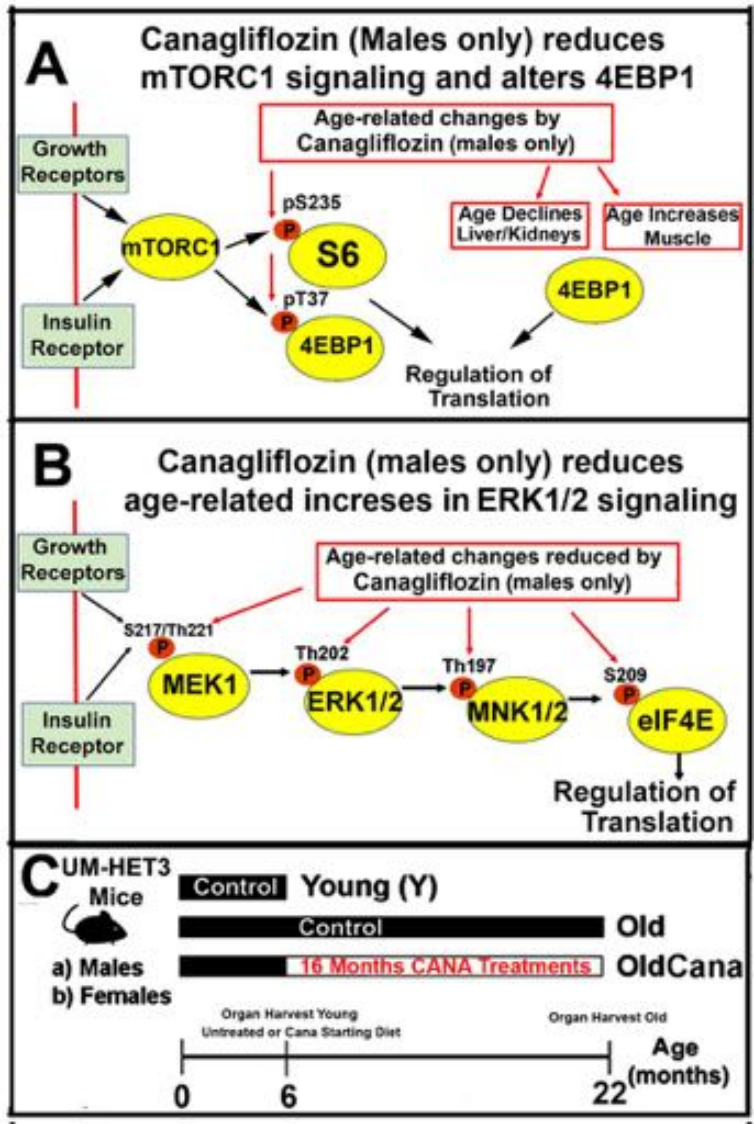
**Figure 3. Effect of Cana on kidney and muscle mTORC1 signaling. A**) **Kidneys**: Representative Western blot follow by bar graphs representing the mean ±SEM of S6, 4EBP1 protein level, S6 and 4EBP1 mRNA scatter plot, plus pS6 and pS6 ratios as well as p4EPB1 and ratios of p4EBP1 from a minimum of 8 individual kidneys per group as described in figure 2. Each graph is accompanied by a box showing the statistics by age (Age ANOVA) or Cana treatment (Cana ANOVA). The * represents a significant level for the specific group comparation show in the cross bar as described in Material and Methods. **B**) **Muscle**: Representative Western blot followed by bar graphs representing the mean ±SEM as described above but samples from skeletal muscle of a minimum of 8 individual per group.

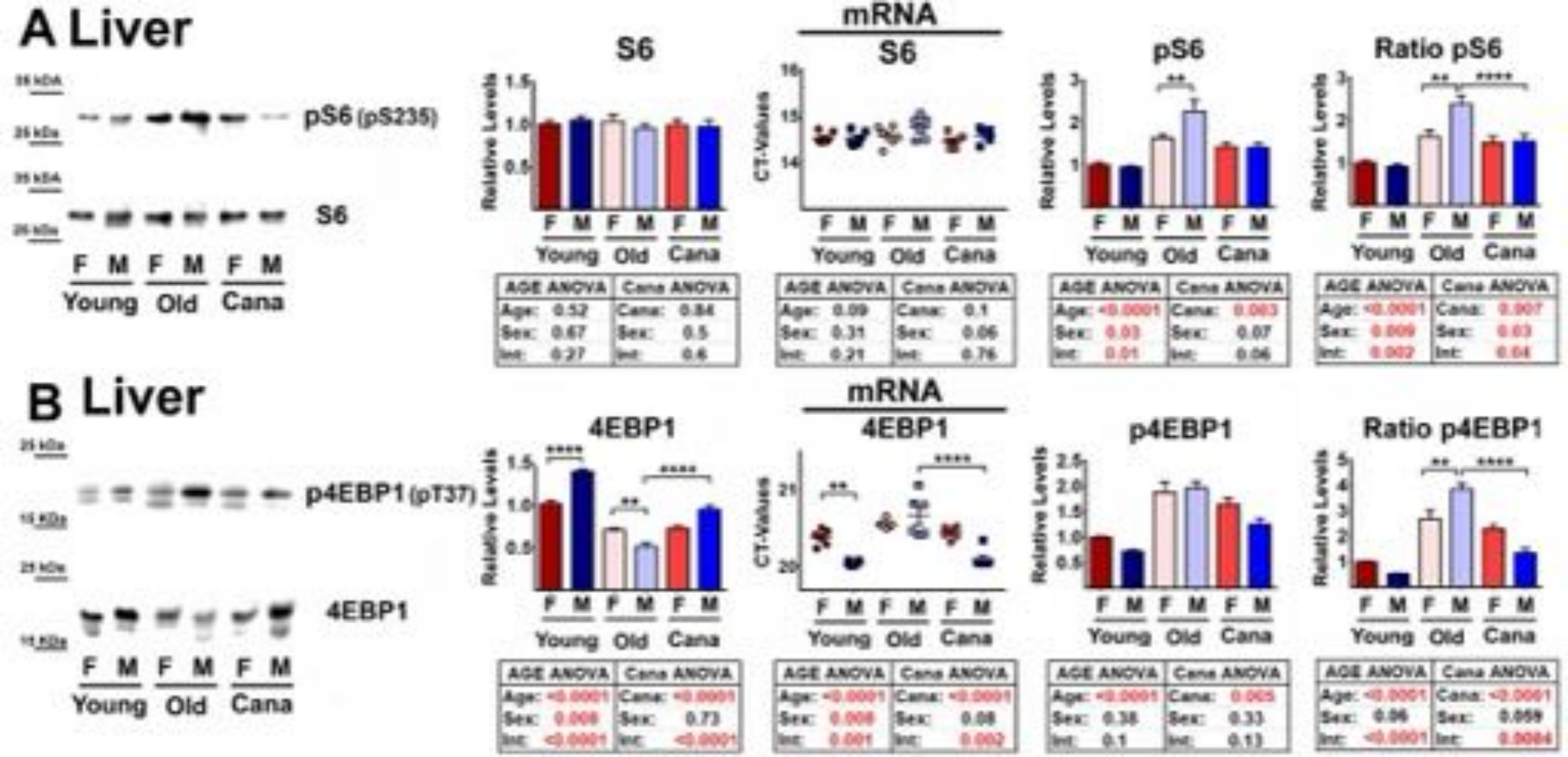
**Figure 4. Effect of Cana on hepatic MEK1 and ERK1-2 signaling. A**) Representative western blots for MEK1 and pMEK1 (pSer217) liver samples. Bar graphs show the mean ± SEM of MEK1 protein, MEK1 mRNA scatter plots, pMEK1, and pMEK1 ratios from at least 8 mice per group. Each graph is accompanied by a box showing the ANOVA analysis as described in figure 2. **B**) Representative western blots for ERK1 (pT202) and ERK2 (pT185) and total levels of ERK1 and ERK2 protein in liver samples. Bar graphs represent the mean ± SEM of ERK1 and ERK2 protein levels, ERK1 and ERK2 mRNA scatter plots, plus pERK1/2 and pERK1/2 ratios from at least 8 mice per group as described in Figure 2.

**Figure 5. Effect of Cana on kidney and muscle MEK1 and ERK1-2 signaling. A**) **Kidneys**: Representative Western blot follow by bar graphs representing the mean ± SEM of MEK1, ERK1 and ERK2 protein levels and mRNA scatter plots, plus pMEK1, pERK1, pERK2 and respectively ratios in kidney samples. All values are analyzed from 8 mice per group, and the statistical analysis by ANOVA is included for each graph described in Figure 2. **B**) **Muscle**: Representative Western blot follow by bar graphs representing the mean ±SEM of each MEK1, ERK1 and ERK2 protein levels, mRNA scatter plots, pMEK1, pERK1, pERK2 and respectively ratios from a minimum of 8 mice per group as described above and in Figure 2.

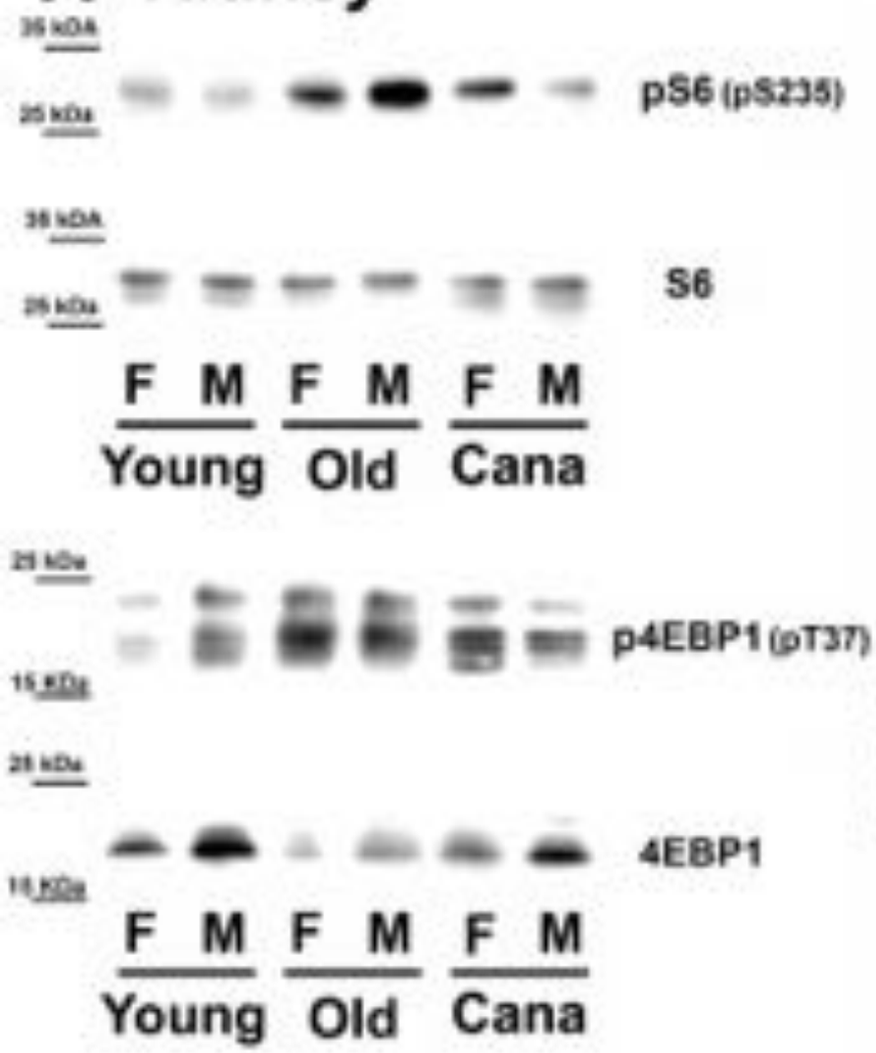
**Figure 6. Effect of Cana on hepatic MNK1/2 and eIF4E signaling**. **A**) Representative western blots of MNK1 and MNK2 protein as well as pMNK at Thr197 (pMNK1) from liver samples. Bar graphs represent the mean ± SEM of MNK1, MNK2 protein levels, mRNA scatter plots, and pMNK from 8 mice per group as described in Figure 2. Each graph is accompanied by statistical analysis using ANOVA methods described in the Material and Methods section and described in figure 2. **B**) Representative western blots for eIF4E and peIF4E at serine S208 (peIF4E) liver samples. Bar graphs represent the mean ± SEM of eIF4E protein levels, mRNA scatter plots, peIF4E and peIF4E ratios from 8 mice per group as described above and figure 2.

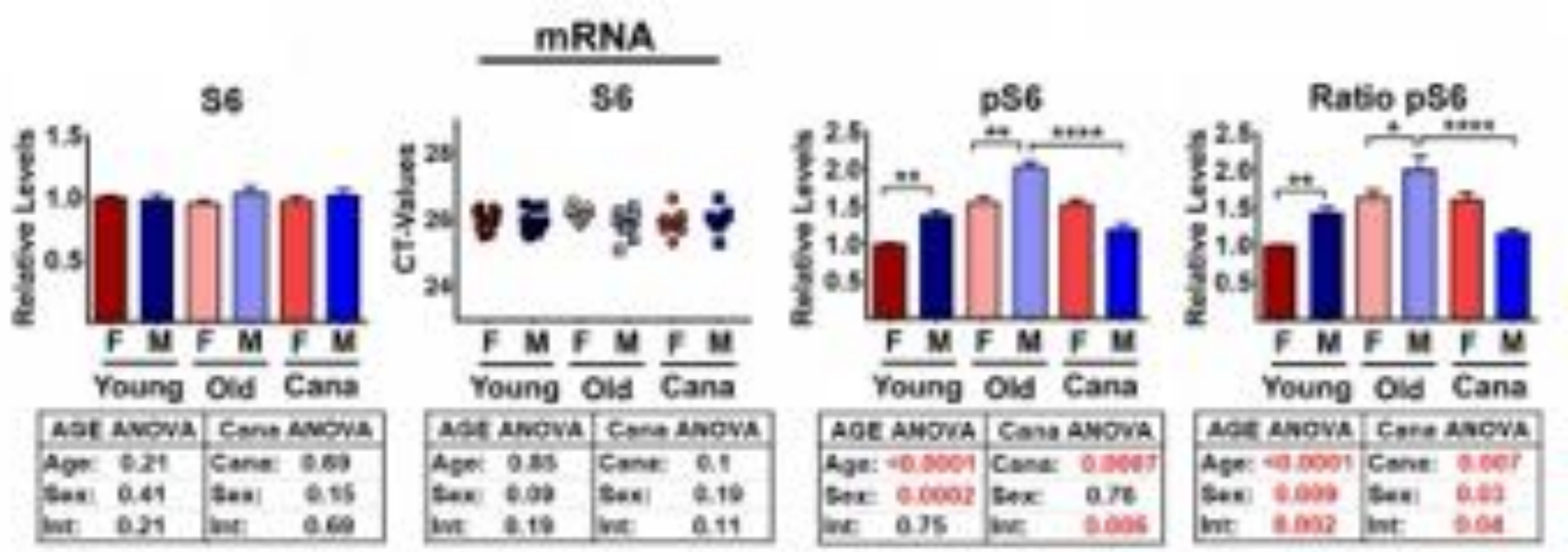
**Figure 7. Effect of Cana on kidney and muscle MNK1/2 and eIF4E signaling. A) Kidneys**: Representative Western blot followed by bar graphs representing the mean ±SEM of each MNK1, MNK2, mRNA scatter plots, and pMNK levels. In addition to eIF4E, mRNA scatter plots follow by peIF4E and ratios levels from a minimum of 8 mice per group as described in Figure 2. Each graph is accompanied by statistical analysis using ANOVA methods described in Material and Methods and in figure 2. **B) Muscle**: same parameters but in skeletal muscle from a minimum of 8 mice per group as described in Figure 2.


A
Canagliflozin (Males only) reduces mTORC1 signaling and alters 4EBP1
Age-related changes by Canagliflozin (males only)
Growth Receptors
Insulin Receptor
mTORC1
pS235
S6
pT37
4EBP1
Age Declines Liver/Kidneys
Age Increases Muscle
4EBP1
Regulation of Translation
B
Canagliflozin (males only) reduces age-related increses in ERK1/2 signaling
Growth Receptors
Insulin Receptor
Age-related changes reduced by Canagliflozin (males only)
S217/Th221
MEK1
Th202
ERK1/2
Th197
MNK1/2
S209
eIF4E
Regulation of Translation
C
UM-HET3 Mice
a) Males
b) Females
Control
Young (Y)
Control
Old
16 Months CANA Treatments
OldCana
Organ Harvest Young
Untreated or Cana Starting Diet
Organ Harvest Old
Age (months)
0
6
22


A Liver
pS6 (pS235)
S6
F M F M F M
Young Old Cana
mRNA
S6
pS6
Ratio pS6
Relative Levels
CT-Values
AGE ANOVA
Cana ANOVA
Age: 0.52
Cana: 0.84
Sex: 0.67
Sex: 0.5
Int: 0.27
Int: 0.6
Age: 0.09
Cana: 0.1
Sex: 0.31
Sex: 0.06
Int: 0.21
Int: 0.76
Age: <0.0001
Cana: 0.083
Sex: 0.03
Sex: 0.07
Int: 0.01
Int: 0.06
Age: <0.0001
Cana: 0.907
Sex: 0.009
Sex: 0.03
Int: 0.002
Int: 0.04
B Liver
p4EBP1 (pT37)
4EBP1
mRNA
4EBP1
p4EBP1
Ratio p4EBP1
Age: <0.0001
Cana: <0.0001
Sex: 0.008
Sex: 0.73
Int: <0.0001
Int: <0.0001
Age: <0.0001
Cana: <0.0001
Sex: 0.008
Sex: 0.08
Int: 0.001
Int: 0.002
Age: <0.0001
Cana: 0.065
Sex: 0.38
Sex: 0.33
Int: 0.1
Int: 0.13
Age: <0.0001
Cana: <0.0001
Sex: 0.06
Sex: 0.059
Int: <0.0001
Int: 0.0084

# A Kidney




| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.21 | Cana: | 0.69 |
| Sex: | 0.41 | Sex: | 0.15 |
| Int: | 0.21 | Int: | 0.69 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.85 | Cana: | 0.1 |
| Sex: | 0.09 | Sex: | 0.19 |
| Int: | 0.19 | Int: | 0.11 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.0087 |
| Sex: | 0.0002 | Sex: | 0.76 |
| Int: | 0.75 | Int: | 0.006 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.007 |
| Sex: | 0.009 | Sex: | 0.03 |
| Int: | 0.002 | Int: | 0.04 |

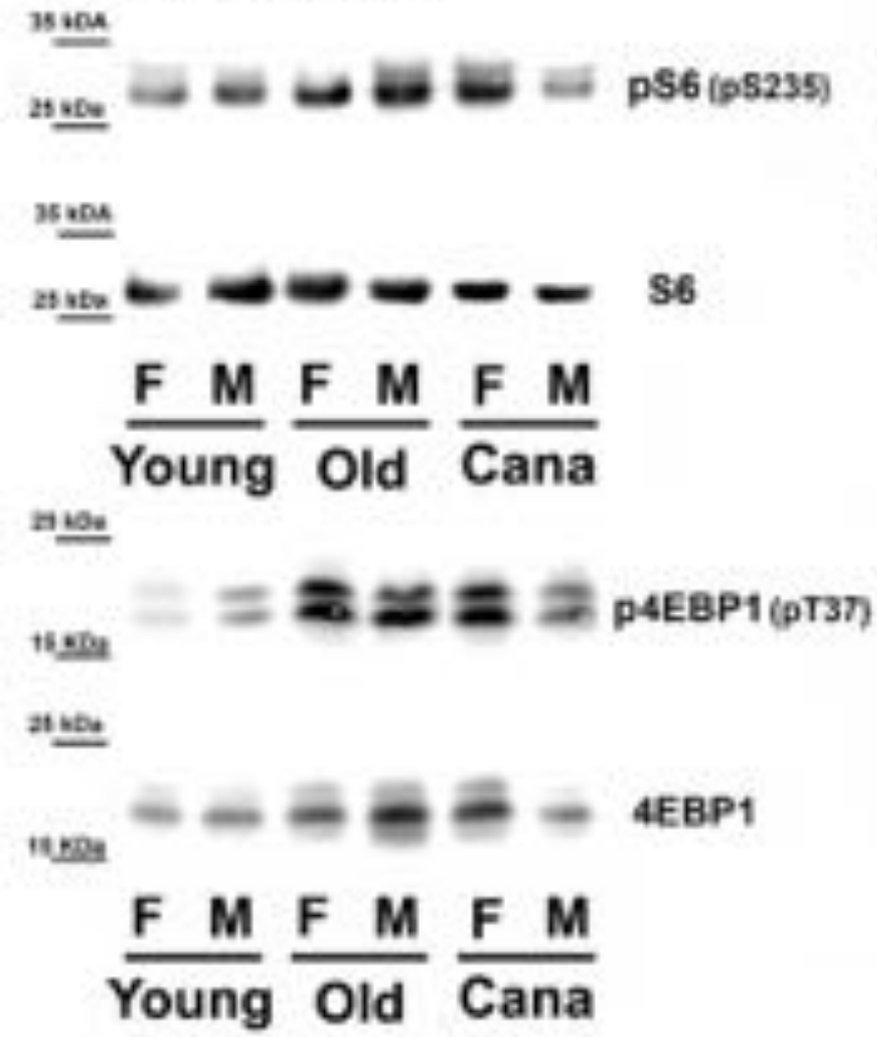
| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | <0.0001 |
| Sex: | 0.001 | Sex: | 0.0007 |
| Int: | <0.0001 | Int: | <0.0001 |

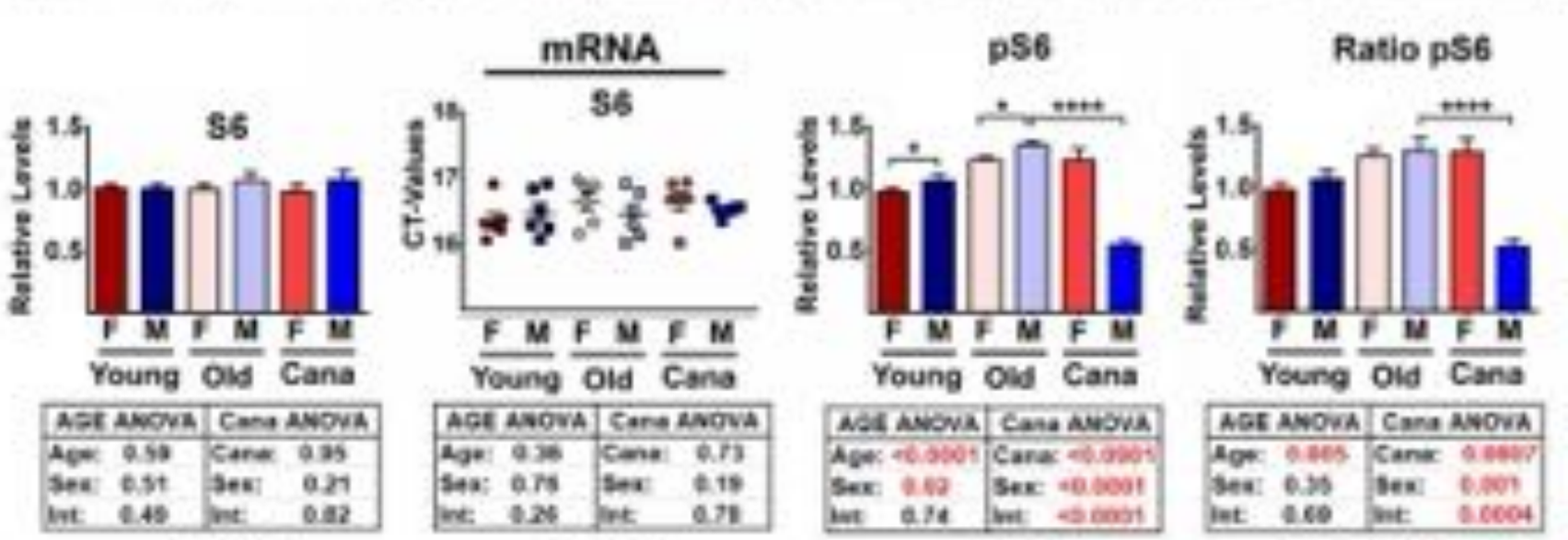
| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.0001 | Cana: | 0.005 |
| Sex: | 0.14 | Sex: | 0.28 |
| Int: | 0.0001 | Int: | 0.007 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.03 |
| Sex: | 0.005 | Sex: | 0.92 |
| Int: | 0.33 | Int: | 0.13 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.9901 |
| Sex: | 0.01 | Sex: | 0.52 |
| Int: | 0.057 | Int: | 0.0002 |

# B Muscle




| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.59 | Cana: | 0.95 |
| Sex: | 0.51 | Sex: | 0.21 |
| Int: | 0.49 | Int: | 0.82 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.36 | Cana: | 0.73 |
| Sex: | 0.75 | Sex: | 0.19 |
| Int: | 0.26 | Int: | 0.78 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | <0.0001 |
| Sex: | 0.62 | Sex: | <0.0001 |
| Int: | 0.74 | Int: | <0.0001 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.885 | Cana: | 0.8897 |
| Sex: | 0.35 | Sex: | 0.001 |
| Int: | 0.69 | Int: | 0.0004 |

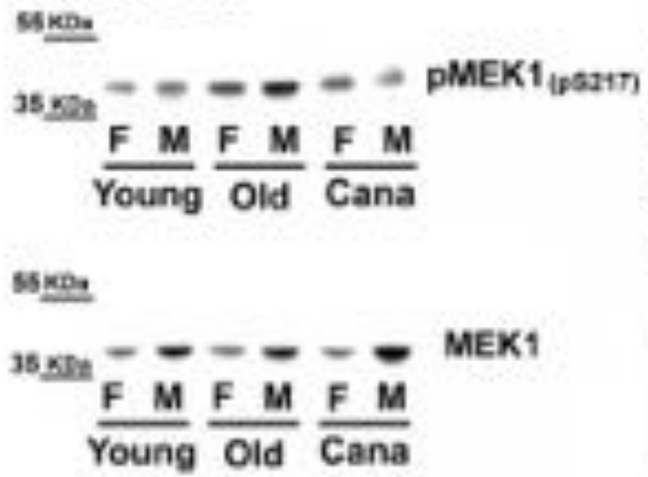
| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.001 |
| Sex: | 0.0006 | Sex: | 0.1 |
| Int: | 0.002 | Int: | 0.002 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.005 | Cana: | 0.04 |
| Sex: | 0.15 | Sex: | 0.69 |
| Int: | 0.52 | Int: | 0.007 |

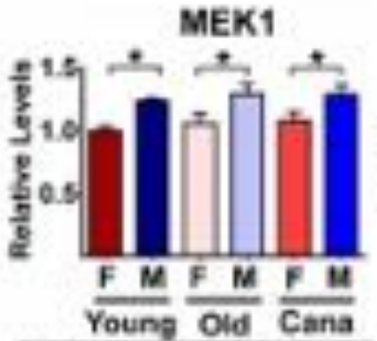
| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.0005 | Cana: | 0.07 |
| Sex: | 0.24 | Sex: | 0.63 |
| Int: | 0.6 | Int: | 0.04 |

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.05 | Cana: | 0.008 |
| Sex: | 0.14 | Sex: | 0.020 |
| Int: | 0.21 | Int: | 0.019 |

# A Liver




## mRNA




| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
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| Sex: | 0.0002 | Sex: | 0.009 |
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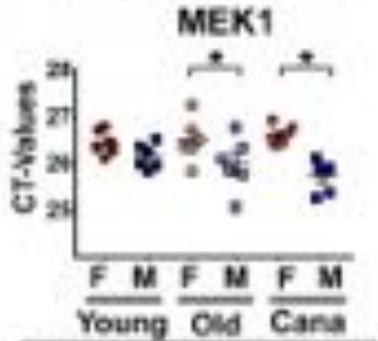
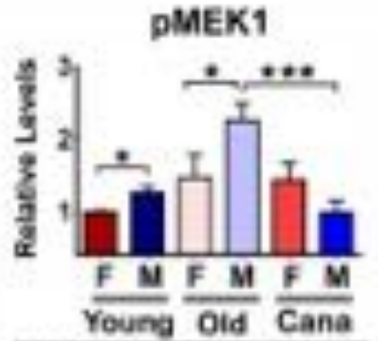
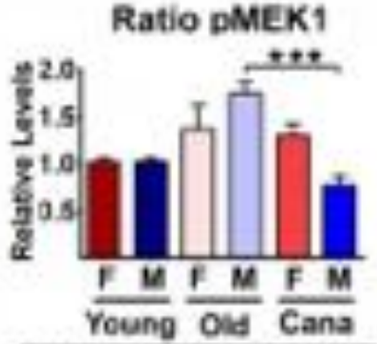



| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.54 | Cana: | 0.71 |
| Sex: | 0.03 | Sex: | 0.0006 |
| Int: | 0.54 | Int: | 0.31 |

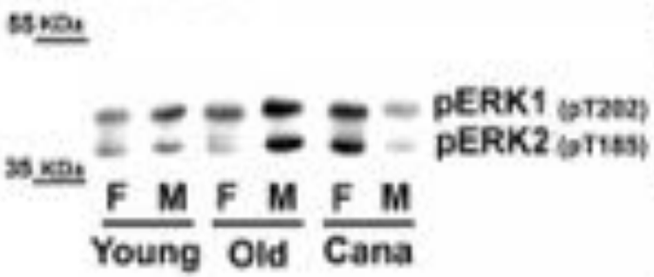



| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.0063 | Cana: | 0.013 |
| Sex: | 0.807 | Sex: | 0.51 |
| Int: | 0.16 | Int: | 0.02 |

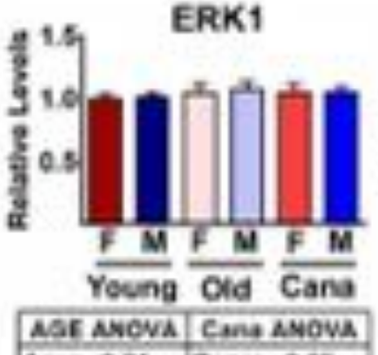



| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.0005 | Cana: | 0.005 |
| Sex: | 0.18 | Sex: | 0.64 |
| Int: | 0.17 | Int: | 0.02 |

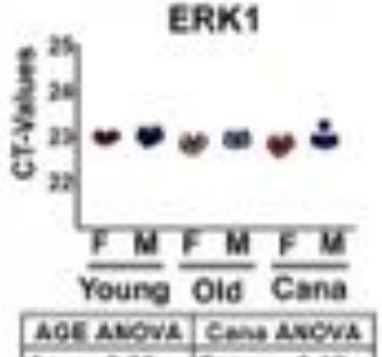
# B Liver



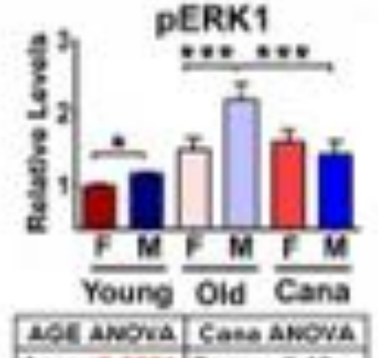

## mRNA



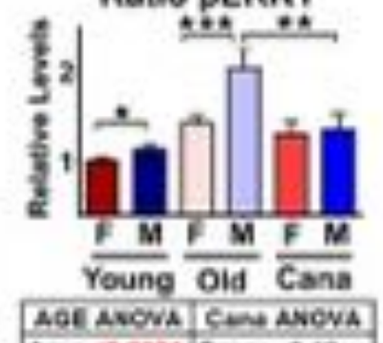

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.31 | Cana: | 0.65 |
| Sex: | 0.71 | Sex: | 0.52 |
| Int: | 0.92 | Int: | 0.84 |



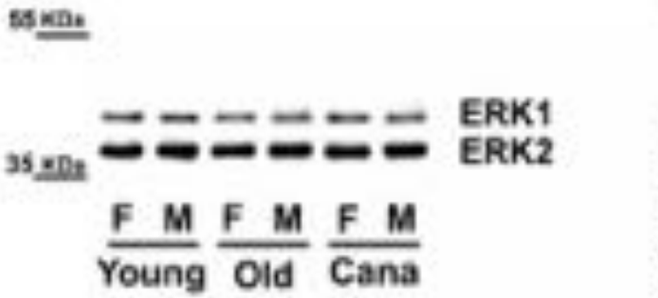

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.06 | Cana: | 0.12 |
| Sex: | 0.09 | Sex: | 0.06 |
| Int: | 0.29 | Int: | 0.6 |



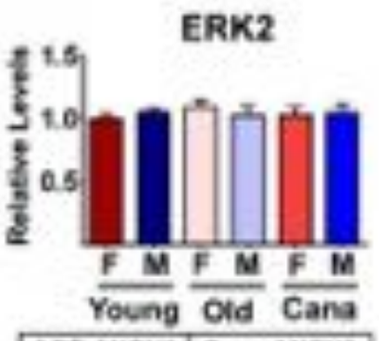

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.12 |
| Sex: | 0.802 | Sex: | 0.2 |
| Int: | 0.05 | Int: | 0.05 |



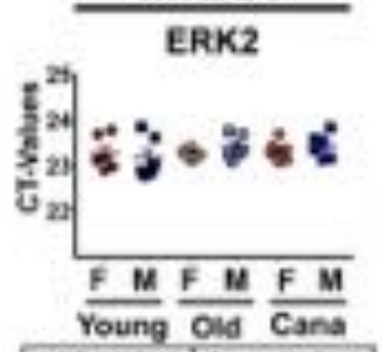

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.19 |
| Sex: | 0.004 | Sex: | 0.33 |
| Int: | 0.08 | Int: | 0.03 |



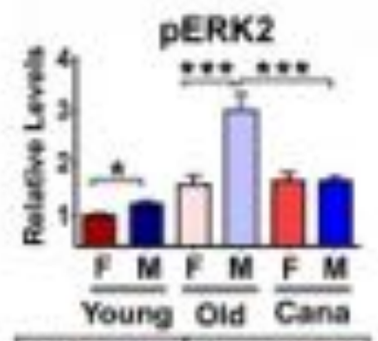

## mRNA



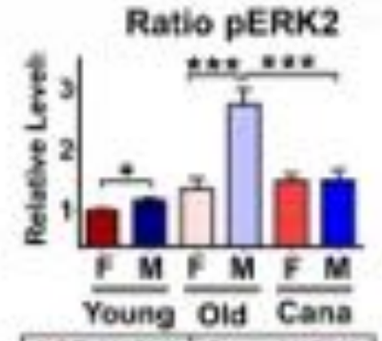

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.18 | Cana: | 0.41 |
| Sex: | 0.28 | Sex: | 0.33 |
| Int: | 0.11 | Int: | 0.25 |




| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.46 | Cana: | 0.54 |
| Sex: | 0.85 | Sex: | 0.17 |
| Int: | 0.34 | Int: | 0.8 |

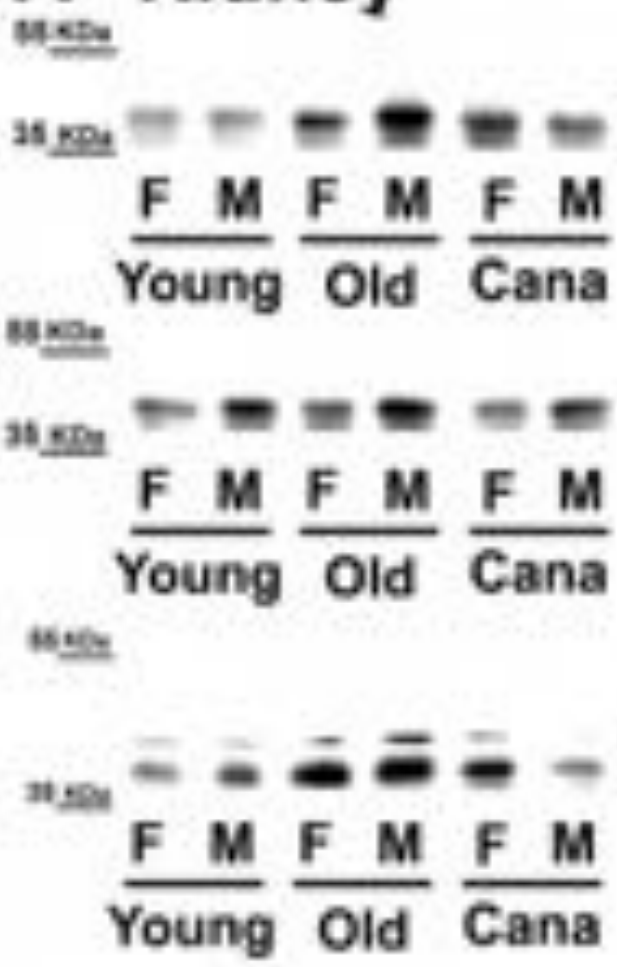


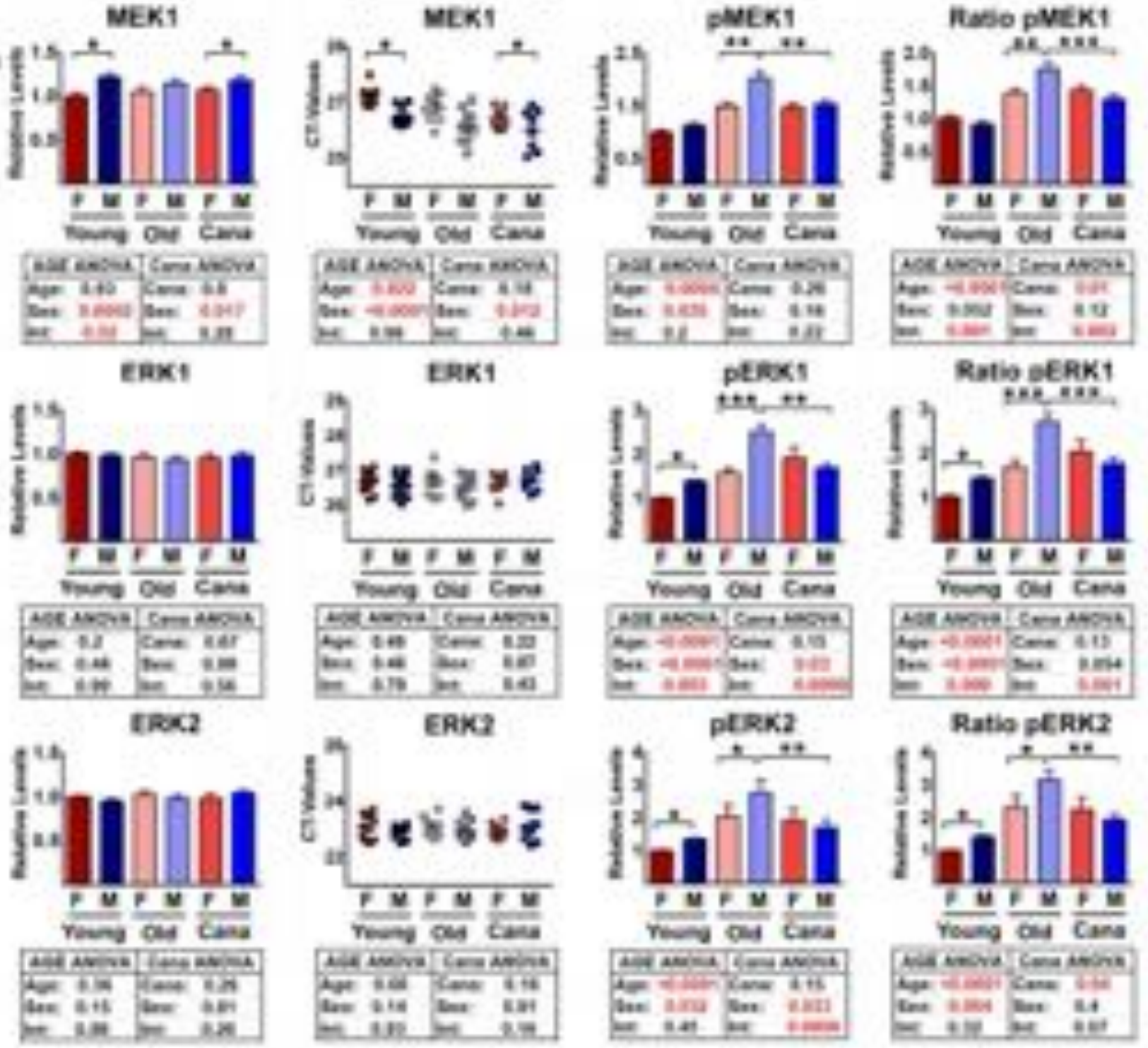

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.021 |
| Sex: | <0.0001 | Sex: | 0.0013 |
| Int: | 0.0006 | Int: | 0.0009 |



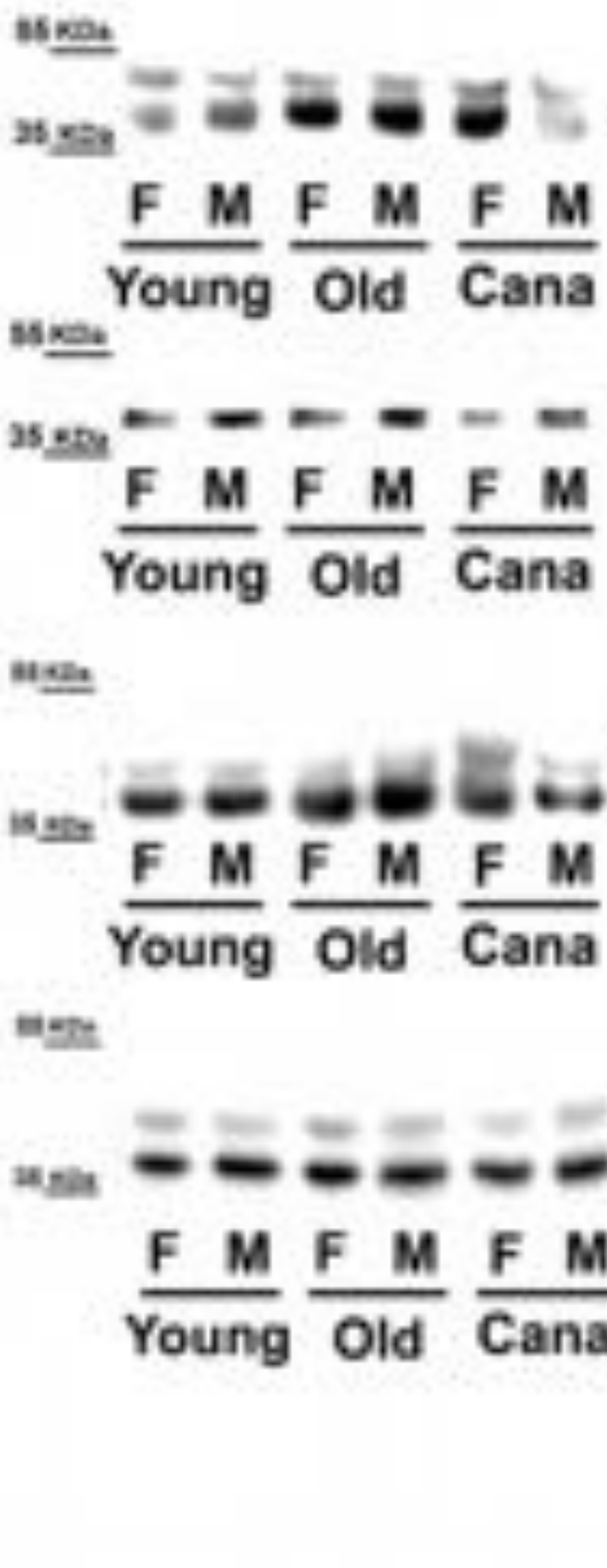

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.005 |
| Sex: | <0.0001 | Sex: | 0.0014 |
| Int: | 0.0002 | Int: | 0.002 |

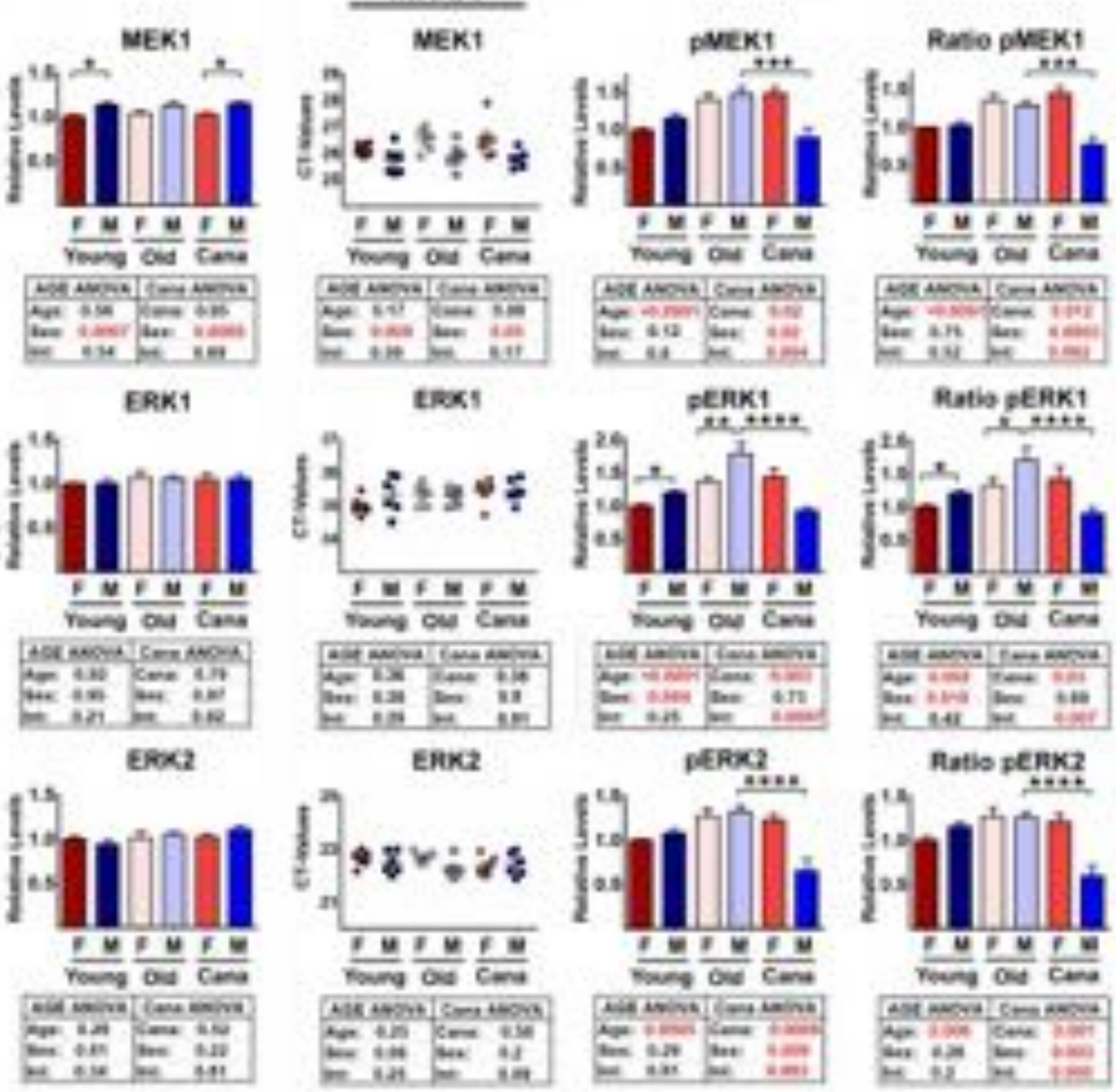
# A Kidney



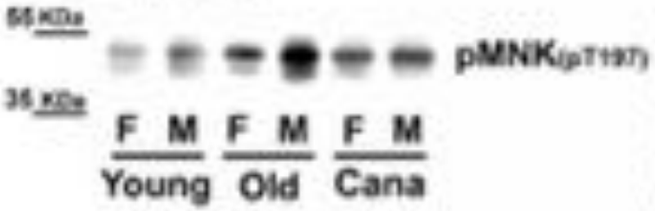



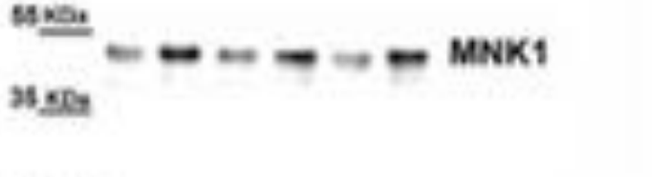

# B Muscle



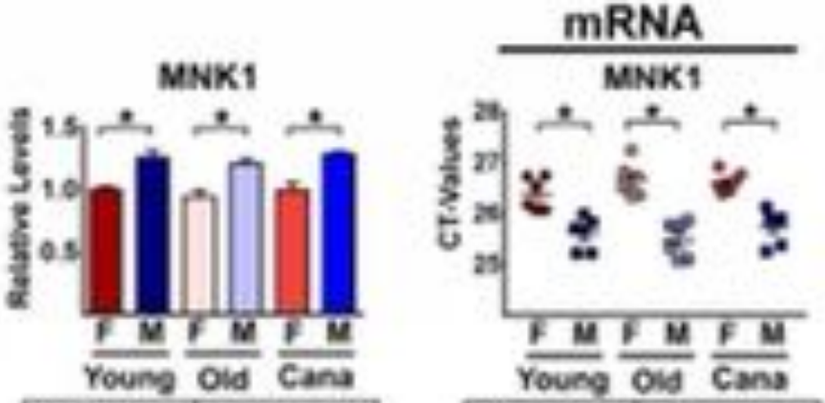



# A Liver



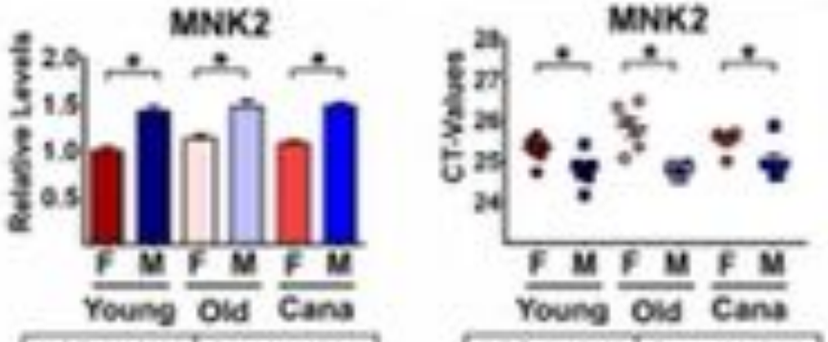





| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.23 | Cana: | 0.19 |
| Sex: | <0.0001 | Sex: | <0.0001 |
| Int: | 0.93 | Int: | 0.76 |



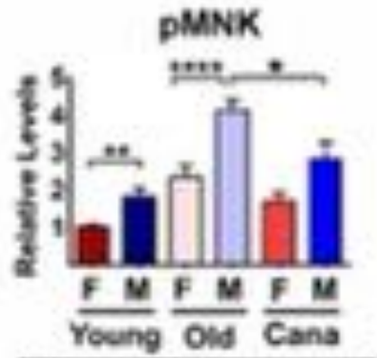

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.57 | Cana: | 0.45 |
| Sex: | <0.0001 | Sex: | <0.0001 |
| Int: | 0.17 | Int: | 0.25 |




| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.11 | Cana: | 0.68 |
| Sex: | <0.0001 | Sex: | <0.0001 |
| Int: | 0.49 | Int: | 0.55 |

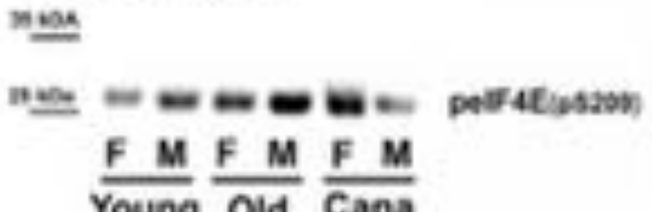


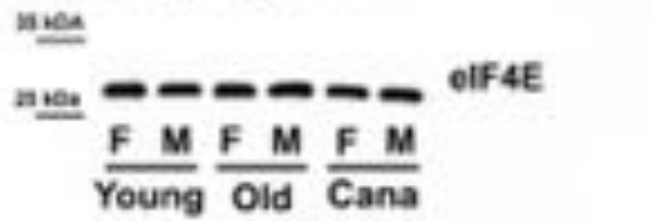

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.09 | Cana: | 0.72 |
| Sex: | <0.0001 | Sex: | <0.0001 |
| Int: | 0.064 | Int: | 0.1 |

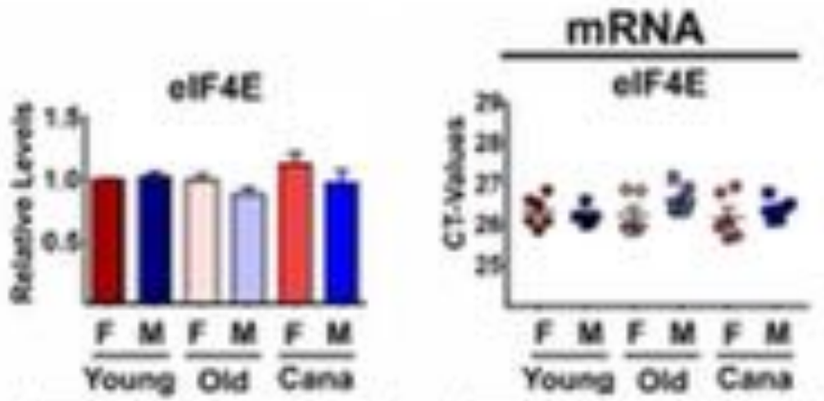



| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.009 |
| Sex: | <0.0001 | Sex: | 0.0003 |
| Int: | 0.051 | Int: | 0.35 |

# B Liver



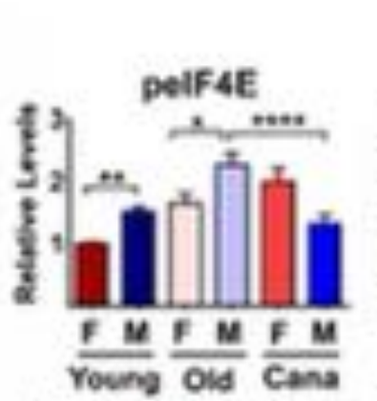



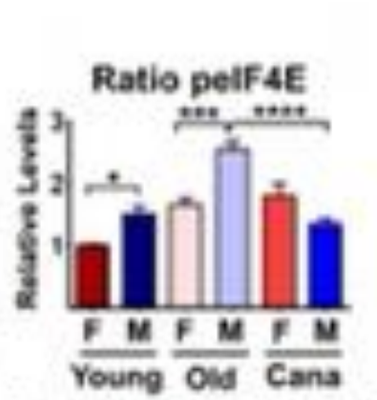

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.67 | Cana: | 0.16 |
| Sex: | 0.28 | Sex: | 0.1 |
| Int: | 0.09 | Int: | 0.79 |




| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | 0.33 | Cana: | 0.41 |
| Sex: | 0.4 | Sex: | 0.11 |
| Int: | 0.09 | Int: | 0.58 |

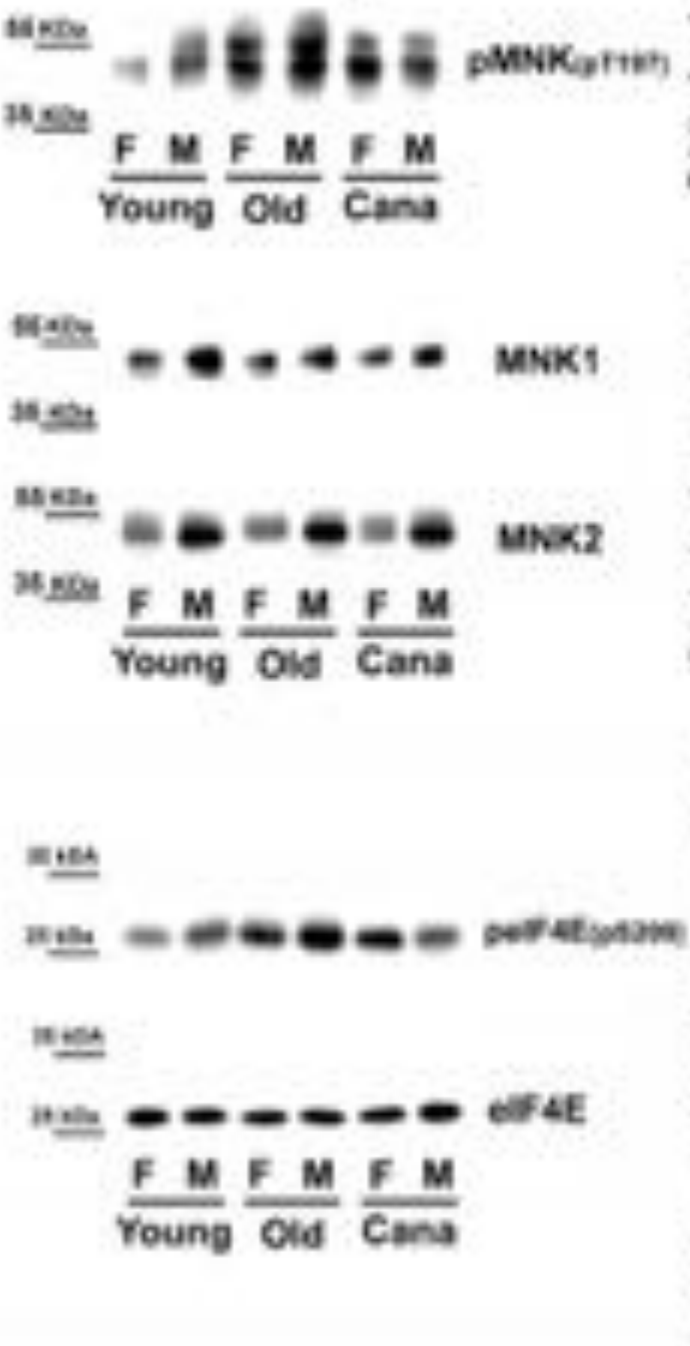


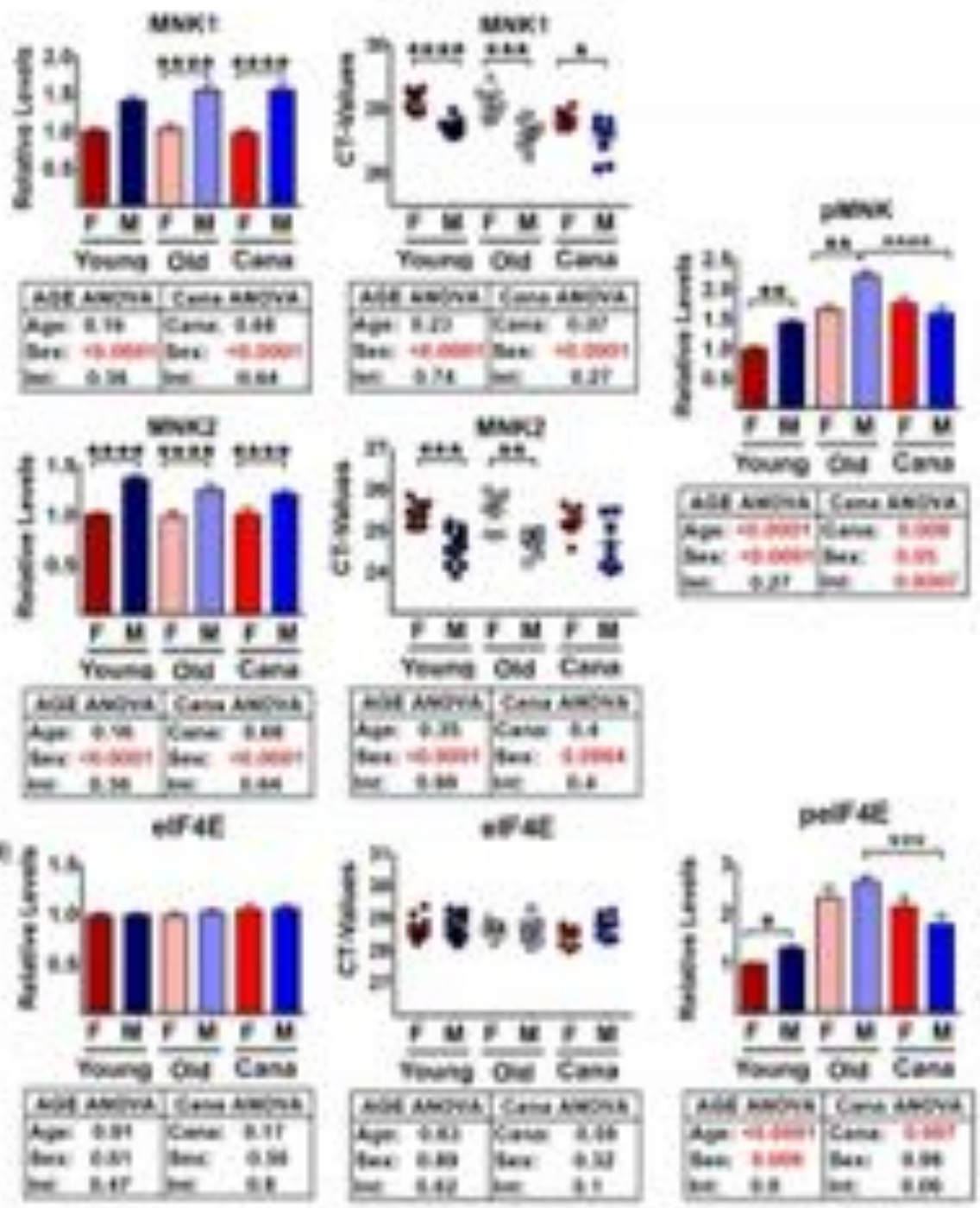

| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.13 |
| Sex: | <0.0001 | Sex: | 0.85 |
| Int: | 0.72 | Int: | 0.003 |




| AGE ANOVA | | Cana ANOVA | |
|---|---|---|---|
| Age: | <0.0001 | Cana: | 0.002 |
| Sex: | <0.0001 | Sex: | 0.15 |
| Int: | 0.09 | Int: | 0.0002 |

# A Kidney




# B Muscle