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ROS-induced ribosome impairment underlies ZAKα-mediated metabolic decline in obesity and aging

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Abstract

The ribotoxic stress response (RSR) denotes a signaling pathway in which the p38 and JNK-activating MAP3 kinase ZAKα senses stalling and/or collision of ribosomes. Here, we show that reactive oxygen species (ROS)-generating agents trigger ribosomal impairment and ZAKα activation. Conversely, zebrafish larvae deficient for ZAKα are protected from ROS-induced pathology. Livers of mice fed a ROS-generating diet exhibit ZAKα-activating changes in ribosomal elongation dynamics. Highlighting a role for the RSR in metabolic regulation, ZAK knockout (KO) mice are protected from developing high-fat high-sugar (HFHS) diet-induced blood glucose intolerance and liver steatosis. Finally, ZAK ablation slows animals from developing hallmarks of metabolic aging. In sum, our work highlights ROS-induced ribosomal impairment as a physiological activation signal for ZAKα that underlies metabolic adaptation in obesity and aging.
**Introduction**

A high degree of metabolic flexibility allows for optimal release and utilization of energy when resources are scarce and efficient storage of energy when resources are abundant (1, 2). In contemporary societies, continuous access to calorie-rich foods has caused a global obesity epidemic (3). In this setting, otherwise beneficial mechanisms for dynamic metabolic regulation negatively affect homeostasis. A deeper understanding of the underlying signaling pathways is needed to direct the development of new treatment principles for obesity and associated metabolic maladies (4). These include type 2 diabetes, non-alcoholic steatohepatitis (NASH), hypertension, and dyslipidemia. Hallmarks of early obesity-associated metabolic dysfunction include insulin resistance, pancreatic β-cell insufficiency, hepatic accumulation of lipids (steatosis), and adipose tissue hypertrophy (5, 6). Similar changes occur during the process of aging (7), suggesting that the underlying mechanisms of metabolic alteration are related. One potential driver of metabolic dysregulation is reactive oxygen species (ROS), the production of which is increased in both obesity and aging (8, 9). Elevated ROS has the potential to disturb the redox balance of cells and damage macromolecules such as proteins, DNA, and RNA. At the same time, ROS within a physiological range are prerequisite signaling molecules for homeostasis with beneficial functions (10, 11). It is not clear how elevated ROS perturb metabolic functions on an organismal scale, and the underlying mechanism(s) may include indiscriminate oxidative damage to macromolecules and regulation of metabolic signaling pathways.

The stress-activated MAP kinases p38 and JNK are activated by multiple cell stress agents such as ROS, UV light, heat stress, and mechanical perturbation. Signaling from these kinases determines diverse cellular outcomes, including cell cycle arrest, cell death, cell differentiation, stress adaptation, and inflammation (12). Less appreciated are the powerful roles of p38 and JNK in
metabolic regulation, which have been demonstrated in a wide range of conditional knockout (KO) mouse models (13, 14). These studies implicate JNK kinases in the regulation of tissue-specific and systemic insulin sensitivity, hepatic lipid deposition, and production of adipokines, among others (14). Among its many roles in metabolic regulation, p38 regulates β-cell survival (15) and key processes in adipose tissues such as thermogenesis and lipolysis (16). Activation of p38 and JNK is associated with obesity and metabolic syndrome, and targeting of these kinases or non-essential components of their signaling pathways has been proposed as therapeutic approaches for the treatment or prevention of the above-mentioned metabolic diseases (13, 17). In particular, the protection of JNK-deleted mice against high-fat diet-induced insulin resistance and hepatic steatosis (14, 18, 19) points to MAP kinase signaling pathways as key regulators of metabolic flexibility and drivers of metabolic decline.

MAP kinases are activated through signal transduction cascades involving upstream MAP kinase kinases (MAP2Ks) and MAP kinase kinase kinases (MAP3Ks) (12). The most upstream of these components, the MAP3Ks, are a group of 21 human kinases, of which we only know the activation mechanisms and signals for a few. One MAP3K which has recently attracted interest is ZAKα, which interacts with the ribosome and is a sensor of translational impairment (20). ZAKα binds to ribosomes by virtue of two C-terminal ribosome binding domains (21) and is activated by perturbations such as stalling and/or collision of ribosomes (22, 23). This pathway for monitoring ribosomal function and converting ribosomal aberrations into p38 and JNK activation is known as the ribotoxic stress response (RSR) (24). Our knowledge about the function of the RSR has been gleaned in mammalian cell lines with treatments such as ribotoxic enzymes (including ricin, Shiga toxin and α-sarcin which all have pathological relevance as human toxins), antibiotics (including anisomycin, cycloheximide), or UV-irradiation (25, 26), which damage the ribosome, chemically
inhibit the ribosome, or damage mRNA templates, respectively. Except for UV-B-irradiation of keratinocytes in the skin (27), these treatments neither offer much insight into the physiological sources of ribosome stalling, ribosome collision, and RSR activation nor provide any clues as to the physiological roles of this signaling pathway in the context of a whole organism. Here we show that ROS is a powerful activator of ZAKα and downstream RSR signaling, and that a ROS-generating obesogenic diet is associated with altered ribosomal elongation dynamics in the mouse liver. In the context of obesity and aging, the RSR pathway mediates well-known but unwanted metabolic transitions such as deregulated glucose tolerance and liver steatosis. Our work offers mechanistic insights into metabolic regulation by MAP kinase signaling and points to the ribosome as a hitherto unappreciated sensor of metabolic stress.

Results

Reactive oxygen species inhibit protein synthesis and activate the ribotoxic stress response

Oxidative stress and ROS are both inhibitory to translation (28) and lead to activation of p38 and JNK (29). In search of a potential link between these effects, we treated U2OS cells with a p38-activating dose of menadione, which causes intracellular superoxide radical generation through a futile reduction-oxidation cycle (Fig. 1A). Strikingly, p38 activation was completely abrogated both by an inhibitor of ZAK kinase activity and by CRISPR-mediated inactivation of the Zak gene (Fig. 1B). Menadione appeared to activate the RSR, as p38 activation required the ribosome-binding α-isoform but not the unrelated β-isoform of ZAK (Fig. 1C,D). In further support of this notion, we found menadione to strongly inhibit bulk translation in a puromycin-incorporation assay (Fig. 1E) and require functional ribosome-binding domains in ZAKα for p38 activation (Fig. 1F). These effects were reversible, as cells both resumed translation and silenced p38 activity, albeit with some
delay, when menadione was washed out in the presence of the ROS scavenger N-acetylcysteine (NAC) (Fig. 1G). Menadione also activated the Integrated Stress Response (ISR) as evidenced by eIF2α phosphorylation, and all of the above effects could be circumvented by pre-incubation of cells with NAC (Fig. 1E; Fig. S1A). These effects of ROS were not restricted to menadione, as we found the redox cycling agent β-lapachone and the thioredoxin reductase inhibitor auranofin to similarly reduce ribosomal output and activate both p38 and JNK in a ZAK-dependent and NAC-reversible manner (Fig. 1H-J; Fig. S1B-D). Based on these results, we conclude that ROS exposure acutely triggers RSR signaling in cells.

**ROS induce ribosome stalling in vivo and impair translation in vitro**

ZAKα responds to both stalling and collision of ribosomes (22, 23). To understand the mechanistic basis of ROS-induced RSR activation, we analyzed ribosome collisions in cells by digesting polysomes with MNase and resolving the collided ribosomes on a sucrose gradient (30). Upon treatment of cells with menadione for 30 minutes or 1 hour, this approach did not reveal an increase in MNase-resistant polysome peaks, which are indicative of stacked ribosomes (Fig. 2A; Fig. S1E – left). However, treatment of U2OS cells with β-lapachone for 30 minutes produced a small increase in collided ribosomes (Fig. S1F – left). These results were in clear contrast to anisomycin treatment, which gave rise to substantial amounts of ribosome collisions (Fig. S1G) as previously reported (22, 30). We and others have previously reported that amino acid deprivation results in stalling of elongating ribosomes, but these stalled ribosomes are only converted to collisions upon inhibition of the ISR (30, 31). Since ROS also activate the ISR (Fig. 1), we wondered if this response also limits ribosome collisions in response to menadione and β-lapachone. Treatment of cells with the pan-ISR inhibitor ISRIB did stimulate low levels of ribosome collision at 30 minutes after both menadione and β-lapachone addition (Fig. 2A; Fig. S1F – right; Fig. S1H), but these effects were
transient and largely undetectable at the 1 and 2 hour timepoints (Fig. S1E). These data suggest that cells react acutely to ROS-generating agents with both stalling and collision of ribosomes, and that high levels of ROS themselves interfere with translation initiation at later timepoints. Indeed, both menadione and β-lapachone treatments were accompanied by a time-dependent loss of polysomes that could not be reversed by ISRIB (Fig. S1I,J). Targeting of initiating 80S ribosomes with harringtonine resulted in the fast run-off of elongating ribosomes (Fig. 2B - left) as previously shown (32). This run-off was impeded for the remaining translating ribosomes in menadione-treated cells (Fig. 2B - right), further supporting that ROS slow down and/or stall elongating ribosomes. Consistent with the above, and in contrast to anisomycin treatment, ribosome-enriched pellets from menadione-treated cells were negative for all biochemical markers of collided elongating (EDF1 (33, 34), ubiquitylated RPS10 and ZNF598 (35, 36)) and initiating (ubiquitylated RPS2 (37, 38)) ribosomes (Fig. S2A,B). In addition, the endoplasmic reticulum (ER)-stress-responsive kinase PERK, and not ribosome-associated GCN2, appeared to be the more relevant eIF2α kinase upon treatment of cells with menadione and auranofin (Fig. 2C; Fig. S2C). These and previous (23) data indicate that activation of ZAKα upon acute ROS exposure can occur independently of widespread ribosome collision and may thus largely depend on stalling and slowing of individual ribosomes (Fig. S2D). Our results also highlight that the triggers of ER stress and ribotoxic stress are overlapping. Indeed, the ER stress-inducing agent thapsigargin strongly repressed bulk ribosomal translation and induced ZAKα activation (Fig. S2E-G).

ROS have the potential to damage nucleotides in DNA as well as RNA, and oxidative modification of both rRNA and mRNA bases has previously been associated with translational impairment (39). To understand which components of the translation process are sensitive to ROS, we developed a tri-partite in vitro translation system from lysates of HeLa cells (Fig. 2D. Of note, also this cell line
displayed menadione-induced and NAC-reversible translational inhibition and p38 activation (Fig. S2H,I). Our approach consisted of isolating ribosomes from one culture of HeLa cells and a ribosome-free cytoplasmic fraction containing tRNAs, initiation, and elongation factors from another, which only upon combination supported translation of an in vitro-transcribed luciferase-encoding mRNA (Fig. 2D; Fig. S2J). To introduce oxidative damage to the in vitro translation system, we treated only one of the three components at a time with hydrogen peroxide for 10 minutes. To prevent carry-over effects, we subsequently neutralized the hydrogen peroxide with catalase before combining the fractions and determining the extent of luciferase protein production. To our surprise, the ability of ribosomes or luciferase mRNA to support translation was not impaired by hydrogen peroxide treatment, rather it was the cytoplasmic fraction that was exquisitely sensitive to oxidative damage (Fig. 2E). This result was in stark contrast to collision-causing UV-B irradiation, which negatively impacted exclusively the mRNA component (Fig. 2F), as previously described (22). We also prepared the ribosomal and cytoplasmic fractions directly from menadione and NAC-treated HeLa cells (Fig. 2G). Also, in these experiments, our results indicated that the cytoplasmic fraction, but not the ribosomal fraction, contained one or more soluble ROS-sensitive component(s) (Fig. 2H,I). Employing a biotin switch assay (40), we did not find biochemical evidence for cysteine oxidation in a number of relevant translation initiation and elongation factors after menadione treatment (Fig. S3A). Global inspection of tRNA integrity also did not indicate large-scale degradation of the tRNA pool in the cytoplasmic fraction (Fig. 2J). However, northern blotting for the tRNA Arg-TCT revealed marked cleavage and fragmentation, which was induced by menadione and reversed by NAC (Fig. 2K). Similar but much weaker effects were observed for the tRNAs Leu-CAA, Gly-GCC, and iMet-CAT (Fig. S3B-D), without an apparent effect on tRNA charging (Fig. S3E). Angiogenin is a tRNA-cleaving RNase that is activated upon a multitude of cellular stress insults to produce translation-inhibiting tRNA fragments (41, 42). Of note, activation
of angiogenin by menadione and ensuing tRNA cleavage was recently demonstrated (43). While these effects may not be the only relevant ones, we conclude that ROS-inducing agents interfere with translation in vivo and in vitro and activate ZAKα and the RSR.

Additive contributions from ZAKα and ASK1 underlie ROS-induced p38 and JNK activation

When challenged with menadione, p38 activation occurred in two distinct waves, one starting at 5 minutes after exposure and peaking at 10-15 minutes, and another starting at 45 minutes and peaking at 1-2 hours (Fig. 3A). Only the second of these peaks coincided with ISR activation, translation shutdown, and appearance of oxidized proteins (Fig. 3A; Fig. S3F). When analyzing U2OS cells deleted for the ZAK and ASK1 genes, either individually or in combination, the ROS-activated MAP3K ASK1 (44) appeared to be involved only in the first of these responses, while ZAKα was exclusively required for the second wave (Fig. 3B). These data imply that ASK1 is a faster responder to oxidative stress, and that damage to macromolecules and ribosomal impairment precedes activation of the RSR. We also challenged our U2OS cells with hydrogen peroxide and observed a partial dependency for both ZAK and ASK1 kinase activity for stress-associated MAPK activation (Fig. S3G). Our results add to the existing knowledge about ASK1 as a ROS-activated MAP3K and highlight ZAKα as a nexus in a parallel pathway that relays ROS-induced translational impairment towards MAPK signaling (Fig. 3C).

ZAKα mediates ROS-induced death in zebrafish larvae

To probe the organismal consequences of ROS-induced RSR signaling, we turned to zebrafish as a model organism. Zebrafish is amenable to CRISPR-mediated genetic manipulation and is an attractive organism for developmental studies due to its fast extrauterine development (45). Of note, while mammals contain a single Zak gene that encodes two distinct splice variants (Fig. 1C),
zebrafish express clear orthologues of ZAKα and ZAKβ from two independent genes (Fig. S3H). We thus proceeded to interrupt these two genes in zebrafish (Fig. 3D) and generated single and double-knockout (KO) animals (Fig. 3E). The genetically introduced deletions to interrupt the open reading frames of the two paralogues also reduced their mRNA abundances, with zakβ knockout additionally affecting zakα expression (50% reduction) (Fig. S3I).

Exposure of fertilized WT zebrafish eggs to menadione resulted in death (scored as cardiac arrest) of most of the larvae after 3-4 days (Fig. 3F,G). This was preceded by the appearance of a range of developmental phenotypes such as edema of the yolk sac and heart, curved spine/tail, and decreased length (Fig. 3H). All of these dramatic effects could be attributed to pathological ROS generation, as they were prevented by the co-administration of NAC in the culture water (Fig. 3G,H). Strikingly, while zakβ−/− fish were similarly sensitive to the effects of menadione, zakα−/−, and double-KO larvae, were largely resistant to death as well as to the appearance of pathological phenotypes (Fig. 3G,H). Menadione-induced pathology was associated with apoptotic cell death, as visualized by TUNEL-positive cells in the tails of WT larvae, and this effect could also be prevented by co-administration of NAC or zakα deletion (Fig. 3I). We conclude that ZAKα, but not ZAKβ, responds to ROS in an organismal setting and that zakα−/− zebrafish larvae are remarkably protected, at least in the short term, against the harmful effects of a pathological burst of ROS.

**ZAK−/− mice are protected against metabolic dysfunction when fed a calorie-rich diet**

Examining the relative expression levels of ZAKα and ASK1 transcripts across human tissues, we noticed that the liver contains relatively little ASK1 mRNA, while at the same time being one of the very few tissues where expression of ZAKα exceeds that of ZAKβ (Fig. S3J – data from gtexportal.org). Re-analysis of a recent tissue-resolved draft of the mouse proteome (46) also
indicated that the ZAKα protein is expressed at higher levels than ASK1 in several metabolic organs, including the liver (Fig. S3K). We proceeded by subjecting male WT and ZAK<sup>−/−</sup> mice to a diet rich in lipids and sugar (high-fat, high-sugar - HFHS) for 25 weeks, only interrupted by intraperitoneal glucose tolerance tests (ipGTT) and magnetic resonance (MR) scans to determine body composition (Fig. 4A). This diet is known to result in increased adiposity, insulin resistance, and liver steatosis (47), with increased ROS generation serving, at least in part, as an underlying pathological driver (48). As we previously reported (49), the starting weights of the male ZAK<sup>−/−</sup> mice were slightly lower than those of their WT littermates (Fig. S4A). Initial weight gain was also somewhat delayed in ZAK<sup>−/−</sup> mice when shifted from chow to HFHS diet, but mice then proceeded to gain weight in a largely genotype-independent manner (Fig. 4B) and ingested similar amounts of food throughout the experiment (Fig. S4B). MR scanning revealed that fat mass increased as expected in WT mice (Fig. S4C) but with some retardation in ZAK<sup>−/−</sup> mice (Fig. S4C), in both cases with a relatively constant lean mass (Fig. S4D). In WT mice, HFHS feeding also resulted in the expected loss of blood glucose control as determined by ipGTT, being evident at 8 weeks and exacerbated at 19 weeks (Fig. 4C). HFHS-fed ZAK<sup>−/−</sup> mice, on the other hand, were remarkably protected against such functional metabolic decline, and performed similarly to chow-fed mice in ipGTT at the 8-week timepoint (Fig. 4C – top vs. middle). At the 19-week timepoint, ZAK<sup>−/−</sup> mice appeared to have developed a certain degree of glucose intolerance, albeit less pronounced than WT (Fig. 4C - bottom). Consistent with this, ZAK<sup>−/−</sup> mice exhibited less insulin resistance at the 19-week timepoint, as evidenced by 50% reduced HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) values (Fig. 4D; Fig. S4E,F). In contrast to glucose intolerance, frank insulin resistance, assessed by HOMA-IR or an insulin challenge test, develops over a longer timespan (50). In accordance, and in a distinct mouse cohort, WT mice appeared insulin sensitive and thus not significantly different from ZAK<sup>−/−</sup> mice after 8 weeks of HFHS feeding (Fig. 4E), hence not
fully recapitulating the reported phenotype of JNK-deficient mice (51). Liver weights measured after euthanasia were similar between WT and ZAK<sup>−/−</sup> mice (Fig. S4G), yet the hepatic triglyceride content was roughly 50% lower in HFHS-fed ZAK<sup>−/−</sup> mice despite indistinguishable serum triglyceride levels (Fig. 4F Fig. S4H). This difference also manifested as lower steatosis grades in ZAK<sup>−/−</sup> livers (Fig. 4G,H).

The observed early protection against diet-induced glucose intolerance and the long-term protection against hepatic steatosis in ZAK<sup>−/−</sup> over WT mice despite a similar whole-body insulin sensitivity (at 8 weeks of feeding) prompted us to search for alternative explanations for metabolic protection offered by Zak deletion. First, we subjected WT and ZAK<sup>−/−</sup> mice on an HFHS diet for 10 weeks to a glucose tracing experiment using tritiated 2-Deoxy-D-glucose (2DG). Mice were euthanized 1 hour after injection, and uptake of radioactive glucose was determined in several central and peripheral tissues (Fig. S4I). In line with our glucose challenge experiments (Fig. 4C), radioactivity was cleared faster from the bloodstream of ZAK<sup>−/−</sup> mice (Fig. S4J - upper left panel), although uptake of 2DG as well as its conversion to 2DG-6-phosphate (2DG6P) at the endpoint was similar between genotypes (Fig. S4J - remaining panels). These results indicate an equal total capacity for glucose uptake in WT and ZAK<sup>−/−</sup> tissues after 60 minutes with potential kinetic effects at earlier timepoints.

Second, we placed mice of both genotypes, fed chow or HFHS for 6 weeks, in metabolic chambers for 1 week and monitored their movement, consumption of O<sub>2</sub>, and production of CO<sub>2</sub> (Fig. S5A). Locomotor activity and energy expenditure (EE) were largely independent of genotype (Fig. S5B-D, within-genotype variance = 0.0022 (kcal/h)<sup>2</sup> and between-genotype variance = 0.00027 (kcal/h)<sup>2</sup> in Fig. S5C). However, we observed a striking increase in the respiratory exchange ratio (RER) in ZAK<sup>−/−</sup> mice over WT when fed the regular chow diet (Fig. S5E-G). This result indicates a preferred utilization of carbohydrates over lipids as energy sources in ZAK<sup>−/−</sup> mice, likely as a consequence of
the reduced adiposity of these animals (23). Notably, this readout was only observable in chow-fed mice, as the pronounced HFHS-induced suppression of RER exceeded that of the two genotypes, thus impeding further interrogation of obese mice.

To evaluate the contribution of ROS to metabolic phenotypes associated with ZAK KO, we combined HFHS feeding with 10 g/l NAC supplementation in the drinking water. IpGTT assays were performed at 5 and 10 weeks, and mice were euthanized after 12 weeks of treatment (Fig. S6A). In this experiment, weight gain was also initially delayed in ZAK−/− mice and was further reduced by the high dose of NAC (Fig. S6B). IpGTT assays confirmed the progressive development of glucose intolerance and HOMA-IR elevation in WT mice, while ZAK−/− mice were fully protected against such metabolic decline both after 5 and 10 weeks of HFHS-feeding (Fig. S6C-G). Remarkably, NAC supplementation completely nullified these differences between genotypes both in terms of ipGTT (Fig. S6C,D), fasting insulin levels (Fig. S6F), and HOMA-IR (Fig. S6G). On the other hand, NAC supplementation only partly improved the liver steatosis grade in WT mice and did not appear to offer the same protection as ZAK KO against this outcome (Fig. S6H,I). Our results indicate that RSR signaling activated by ROS and other sources of ribosomal impairment mediates at least some aspects of metabolic adaptation to an obesogenic diet.

**Phospho-proteomic analysis reveals deregulation of MAPK signaling in livers of ZAK−/−**

**HFHS-fed mice**

We used phospho-proteomics to interrogate signaling towards p38 and JNK kinases in livers of mice fed HFHS for 5 weeks and euthanized during the dark phase, where hepatic ROS levels are maximal (52) (Fig. 5A). We identified ~5,500 unique proteins and ~12,500 unique phosphorylation sites (Table S1; Table S2). Principal Component Analysis (PCA) revealed little separation of
samples at the protein level (Fig. S7A), but a clear separation and clustering according to both genotype and diet at the level of phosphorylation sites (Fig. 5B). We used this dataset to interrogate phosphorylation changes annotated as regulatory on all recorded MAPK, MAP2K, and MAP3K components (Fig. S7B). This analysis revealed HFHS- and Zak-dependent regulation of activation-associated phosphorylation sites on several components of p38 and JNK signaling cascades. A finer analysis of the relevant phospho-peptides across all conditions revealed a decrease in activation-associated phosphorylation of JNK2 (MAPK9-Y185) upon HFHS-feeding of ZAK−/− but not WT mice (Fig. 5C; Fig. S7C, Table S2). However, western blot analysis on the same liver samples did not indicate decreased HFHS-induced JNK phosphorylation in ZAK−/− mice (Fig. 5D). A portion of the HFHS-induced RSR signaling in WT mice likely occurs in steatotic hepatocytes, in which we managed to detect weak p-JNK positivity by immunohistochemistry (IHC) (Fig. S7D). We also conducted a mouse experiment where we fed WT and ZAK−/− mice either chow or HFHS for 16 weeks. These mice were starved overnight prior to euthanasia (53), and the livers were analyzed for p-JNK by western blotting (Fig. S7E). This approach also failed to highlight deregulation of HFHS-associated JNK activity, likely explained by our small sample size (Fig. S7F).

Liver ribosome profiling reveals effects on global translation and distinct stalling/collision sites on highly expressed transcripts upon HFHS treatment

Using ribosome profiling (ribo-seq), we next investigated to what extent the HFHS diet-induced phenotype was accompanied by translational alterations in liver (Fig. 5E). We prepared ribo-seq libraries from two footprint species: standard ~30 nt monosome-protected footprints and ~60 nt disome footprints, which are indicative of ribosomal stacking/collisions (54-56). Abundant disome footprints can be observed in mouse liver and at specific positions even under physiological conditions (54), likely diagnostic of sites of temporary translation slowdown that will not elicit a
collision response such as the RSR. In our analyses we aimed at identifying exacerbated disome coverage/novel sites dependent on HFHS. First, PCA on footprint signal from the top-500 expressed genes indicated separation by library type (monosome/disome) followed by diet (chow/HFHS) (Fig. 5F). Monosome footprint analyses suggested that HFHS diet was not associated with strong systematic, transcriptome-wide effects on elongation kinetics, as indicated by footprint alignment to the 5' and 3' ends of coding sequences (Fig. S8A) and an analysis of codon dwell times (57) (Fig. S8B). Next, we extended our analyses to specific sites on the translatome. We calculated position-specific ribosome occupancies transcriptome-wide using a recently reported pause score metric (58, 59), yielding 150 sites with increased and 160 sites with decreased relative ribosome occupancy in HFHS- vs. chow-fed animals (Fig. S8C, left panel). Many of these sites corresponded to positions on the translatome with high ribosomal occupancy (Fig. S8C, right panel), and we hence explored the possibility that translational alterations affecting a relatively small number of sites on transcripts with very high translational volume could be associated with HFHS treatment. Thus, we quantified how individual high-expression genes contributed to overall translational activity in liver, which revealed a strong bias towards a few dominating transcripts, in particular mRNAs that encode bloodstream-secreted proteins such as albumin (Alb, 8.9% of all hepatic translation), apolipoprotein E (Apoe, 4.2%), and other mRNAs (Fig. 5G, dark gray curve; 119 genes make up 50% of all hepatic translation). A globally similar distribution was found for disome footprints (Fig. 5G, light gray curve). The group of differential codon sites showed an even stronger bias towards highly dominant transcripts, with three mRNAs – Alb, Apoe, Tfr – accounting for half of the detected sites (Fig. 5G, orange curve; Table S3). To follow up on this observation, we inspected monosome and disome coverage and differential codon sites across the top-ranked transcripts. These analyses established that overall footprint distributions were highly similar and reproducible between HFHS- and chow-fed animals, as exemplified by the monosome and disome
coverage patterns on *Alb* mRNA (Fig. S8D). However, for some highly abundant mRNAs – see *Apoe* (Fig. 5H) and retinol binding protein 4 (*Rbp4*; Fig. 5I) – we identified strongly enriched disome sites specifically in HFHS livers (Fig. 5H,I; marked by orange shading and arrows). Closer inspection of these transcript regions (Fig. 5J-L) revealed that these disome positions were just upstream, in close proximity of increased differential sites from the monosome footprint odds ratio analysis - a constellation compatible with increased collisions at these sites.

**ZAK<sup>−/−</sup> mice are protected against metabolic decline in aging**

Male mice develop insulin resistance during aging (60), which at very old age can be compensated for by pancreatic islet hypertrophy (61). Given the striking protection of ZAK<sup>−/−</sup> mice against metabolic dysfunction in obesity, we allowed chow-fed mice of both sexes to reach between 14 and 16 months of age, before subjecting them to ipGTT (Fig. 6A). At this age, and similar to young mice, WT males were slightly heavier than their Zak-deleted counterparts (Fig. S8E), while the weight of female mice was independent of genotype (Fig. S8F). Aged male WT mice presented with a strikingly impaired blood glucose control in ipGTT assay as expected (Fig. 6B - top), whereas the performance of aged ZAK<sup>−/−</sup> male mice was undistinguishable from young male mice in this assay (Fig. 4C - top; Fig. 6B - top). As previously reported (60), female mice did not present with pronounced aging-induced metabolic decline (Fig. 6B - bottom). The protection against aging-associated decline in glycemic control in ZAK<sup>−/−</sup> male mice was further underscored by reduced fasting blood glucose and insulin as well as lower HOMA-IR values (Fig. 6C-E). Similar to early (8 weeks) HFHS-induced obesity (Fig. 4C-E), these changes in males occurred in the absence of a measurable difference in whole-body insulin sensitivity assessed by an insulin tolerance test (Fig. S8G). Inspection of the livers from these mice revealed different degrees of hepatic steatosis in three out of seven WT mice, while only one of six ZAK<sup>−/−</sup> mice displayed the mildest degree of this
hallmark of metabolic disease (Fig. 6F,G), thus mirroring the genotype-specific characteristics of diet-induced obesity reported in Figure 4.

These results suggest an overlap in the underlying metabolic stress signals in obesity and aging, albeit with a different magnitude and duration. To compare the extent of the oxidative burden of livers in the two conditions, we performed IHC to evaluate the content of 4-HNE in livers from young vs. old and chow vs. HFHS-fed WT mice. 4-HNE is a commonly used marker of lipid peroxidation that has prognostic value in the diagnosis of NASH and other diseases associated with oxidative stress (62). While IHC analysis of 4-HNE in young, chow-fed livers resulted only in homogenous background staining (Fig. S9A), the same analysis of HFHS-fed mice from Fig. 4A revealed a markedly increased overall staining, as well as the typical enrichment in steatotic areas around portal veins (63) (Fig. S9B). These patterns were not observed in livers from aged mice (Fig. S9C). This analysis indicates that oxidative stress load is increased in aged mouse livers but to a lesser extent than in HFHS-fed mice.

Deterioration of brown adipose tissue structure and function is attenuated in ZAK−/− mice

Adipose tissues undergo aging-induced deterioration in male mice as well as humans (64), including “whitening” of brown adipose tissue (BAT) (65). In comparison with BAT sections from aged WT mice, the staining intensity of aged ZAK−/− mice more closely resembled that of BAT from young mice (Fig. S10A - top and middle panels; Fig. S11A). These differences were also evident at the microscopic level, where the transition of multilocular BAT to a more unilocular WAT-like tissue was much more evident in old vs. young WT samples compared to those from ZAK−/− mice (Fig. 6H,I). Although the effects were in general smaller, we observed a similar protection against BAT whitening when re-examining our 25-week HFHS cohort (Fig. 4A; Fig. S10 – lower panel;
These structural differences prompted us to evaluate functional BAT thermogenesis by infrared imaging of interscapular BAT on shaved mice. While HFHS-feeding was associated with the expected reduction of BAT temperature and thus thermogenesis in WT mice, ZAK⁻/⁻ mice maintained a high temperature in this tissue (Fig. S11C,D).

**Discussion**

Here we show that ROS potently interfere with ribosomal function in vitro, in human cell lines, and mice. These aberrations activate the RSR pathway, culminating in oxidative stress-induced activation of the p38 and JNK kinases. This response has clear relevance for both pathological organismal responses to oxidative stress (zebrafish experiments) and physiological adaptations to increased ROS production. In mice, ROS-induced RSR signaling underlies at least some of the metabolic adaptations to high-fat diet-induced obesity and aging, such as the loss of glycemic control and development of hepatic steatosis. This is underscored by the protection of ZAK⁻/⁻ mice against metabolic dysfunction when fed an HFHS diet, as well as the healthy metabolic aging observed in aged ZAK⁻/⁻ male mice (Fig. 6J). The above insights highlight ribosomal impairment as an important metabolic stress signal, which is used for the regulation of metabolism at an organismal level. Ribosomes are perfectly placed to perform the role of scaffolds for mild stress signals in a broad context. Because they are present in high amounts in every cell and constantly performing their function, compromised processivity of just a few ribosomes can provide for a highly sensitive and rapid readout of cellular perturbations (39). Previous work has highlighted oxidative damage to mRNA as a source of translational impairment (39), but our in vitro translation experiments highlight soluble cytoplasmic factors as the more sensitive components. These may include tRNAs, initiation and elongation factors. While we did not obtain evidence of oxidative damage to the latter, we corroborated recently published evidence of ROS-induced tRNA cleavage.
and fragmentation (43). Besides representing a depletion of key translation components, these fragments, likely generated by the RNAse angiogenin, are generally considered to be inhibitory to ribosomal function (41, 42).

JNK signaling promotes both insulin resistance and liver steatosis in obese mice (14). Specifically, mice conditionally deleted for JNK1 and JNK2 in the liver are protected against these outcomes when fed a high-fat diet (19). Among the relevant JNK targets is RXRα, the phosphorylation of which inhibits the PPARα complex and transcriptional activation of the Fgf21 gene (51). FGF21 is a metabolic stress-induced hormone that has beneficial effects on both insulin sensitivity and liver adiposity (66), and these effects are exacerbated in jnk-deleted mice (51). Conversely, mice defective for p38 activation in the myeloid compartment are defective for hepatic FGF21 production and sensitized to metabolic dysfunction when fed a high-fat diet (67). A picture is emerging in which p38 and JNK signaling may compete for opposing outcomes depending on the tissue, cell type, and metabolic context. These intricacies likely allow for a fine-tuned mode of metabolic regulation that collectively manifests in an extremely well-buffered system. Surprisingly, ZAK−/− mice, are protected against key aspects of metabolic dysfunction in the apparent absence of increased insulin sensitivity at intermediate timepoints (8-10 weeks). Alternative explanations may include stimulation of insulin-independent glucose uptake mechanisms and/or altered utilization of energy sources.

MAP3K ASK1 is a major ROS-induced activator of p38 and JNK in cell lines as well as mouse models (44), and has also been linked to metabolic regulation (68-70). Other ROS-activated MAP3Ks that have been implicated in metabolic control are MLK2/3 and MAP3K4 (71, 72). In this work, we describe a clear protection of ZAK KO mice against HFHS-induced glucose intolerance
and liver steatosis, and at least some of these phenotypes were related to ROS-mediated activation of ZAKα and the RSR. In one plausible model for the interplay of ZAKα and ASK1 in ROS-induced activation of p38 and JNK, ASK1 is the more relevant kinase upon sudden bursts of pathological levels of ROS, while ZAKα, through its ability to sense translational impairment, is more sensitive to the effects of slight elevations in intracellular ROS. Furthermore, the division of labor between these two kinases (as well as MLK2/3, MAP3K4, and potentially others) is likely tissue-specific, as especially ZAKα levels are highly variable among organs. It should be noted that the experimental systems used in the present paper differ widely in the source, duration, and magnitude of ROS exposure. In aggregate, we find that the role of ZAKα as the primary inducer of p38/JNK activation and organismal protection was most evident in our experiments featuring excessive ROS levels in cell lines and zebrafish. In settings of prolonged exposure to lower levels of ROS (mouse obesity and aging), alternative mechanisms, as alluded to above, may play equally important roles. Future work will be required to map the full inventory of ROS-activated MAP3 kinases and their relevance for ROS-driven physiological and pathological responses.

Next to glucose intolerance and stochastic liver steatosis, ZAK−/− male mice are also, at least partially, protected against BAT whitening and disruption of BAT integrity upon obesity and aging. These findings suggest that metabolic control exerted by the RSR pathway is not restricted to the liver and that several tissues may contribute to the overall metabolic phenotypes of ZAK−/− mice. Indeed, a recent report described an independent role for the Zak gene in promoting stem cell plasticity in the small intestine of leucine-restricted mice (73). Further adding to the complexity of our current mouse model, the ZAKβ isoform is activated by volumetric cell compression and protects against muscle pathology in mice and humans (49, 74), and this splice variant is also interrupted in the whole-body ZAK KO animals. For the present study, generation, and studies of
mouse models for conditional KO of the Zakα isoform was not achieved. Future efforts in this direction will be crucial for uncovering the tissue-specific contributions of the RSR to the metabolic phenotypes that we describe. We also did not manage to detect Zak-dependent activation of p38 and JNK kinase isoforms in liver, adipose tissues, and other metabolically relevant organs. We suspect that the underlying technical explanation is a prolonged but weak overactivation of these stress kinases during HFHS-feeding, which are hard to detect with conventional methods in the light of high mouse-to-mouse variation. While p38 and JNK are well-established effectors in the RSR, the lack of a direct signaling link from ZAKα to these kinases in vivo limits our conclusions regarding the mechanisms underlying metabolic protection of ZAK KO mice. Finally, we do not know to which extent our findings in mice can be extrapolated to humans. Patients with nonsense mutations in the common kinase domain of ZAKα and β present with severe early-onset myopathy but with no reports of phenotypes of a metabolic nature (74). However, the feeding paradigm for mice used in this study is generally considered a good model of human obesity and associated metabolic complications. Also in humans, Zakα mRNA is robustly identified in all major metabolic tissues (gtexportal.org), and we thus consider it likely that the RSR also mediates metabolic adaptation in our own species.

In summary, our work uncovers a role of the ribosome as a signaling platform for metabolic regulation and highlights the RSR pathway as an important inducer of metabolic changes associated with obesity and aging. These findings may present opportunities for the development of therapeutic strategies to combat metabolic diseases.

Materials and methods summary
**Polysome profiling**

After cells were exposed to various treatments, cytosolic lysates were prepared using 20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 100 µg/ml digitonin, 100 µg/ml cycloheximide, 1X protease inhibitor cocktail (Sigma, #P8340) and 200 U NxGen RNase inhibitor (Lucigen, #30281). Extracts were incubated on ice for 5 min prior to centrifugation at 17,000 g for 5 min at 4 °C. After adding calcium chloride to a final concentration of 1 mM, lysates were optionally digested with 500 U micrococcal nuclease (MNase) (New England Biolabs, #M0247) for 30 min at 22 °C. Digestion was terminated by adding 2 mM EGTA. Equivalent amounts of lysate (250 mg of undigested RNA or 350-400 mg of MNase-digested RNA) were resolved on 15-50% sucrose gradients. Gradients were centrifuged at 38,000 rpm in a Sorvall TH64.1 rotor for two hours at 4 °C. The gradients were passed through an ISCO density gradient fractionation system with continuous monitoring of the absorbance at 254 nm.

**Reconstituted in vitro translation**

Translation reactions were reconstituted by mixing one part isolated ribosomes, five parts cytoplasmic lysates, one part in vitro transcribed luciferase mRNA (final concentration 1-10 ng/µl) and three parts translation buffer (final concentrations of 1.6 mM HEPES, 10 mM creatine phosphate (Sigma Aldrich, #27920), 50 ng/µl creatine kinase (Sigma Aldrich, #10127566001), 10 µM spermidine (Sigma Aldrich, # S0266), 10 µM amino acids (Promega, # L4461), 65 mM KAc, 0.75 mM MgAc₂ and murine RNase inhibitor). Luciferase mRNA with an EMCV IRES was transcribed from a plasmid using HiScribe T7 ARCA mRNA Kit with tailing (New England Biolabs, #E2060) according to manufacturer’s specifications. Translation reactions were incubated at 37 °C for 30 min and translational output was measured as luciferase activity detected with Dual-Glo Luciferase Assay System (Promega, # E2940) according to manufacturer’s specifications. For
H₂O₂-treatment, fractions were treated with 5 mM H₂O₂ (Sigma-Aldrich, #H1009) for 10 min at 25 °C followed by treatment with either 1 µg/µl catalase (Sigma-Aldrich, C1345-1G) or vehicle (1 mM Potassium phosphate). For UV irradiation, fractions were irradiated with 500 J/m² UV-B and immediately added to the in vitro translation reaction.

**Menadione treatment of zebrafish larvae**

Freshly collected zebrafish eggs were incubated in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 0.00001 % Methylene Blue). Unfertilized and dead eggs were removed, and embryos around the early gastrula stage, approx. 6 hpf (hours post fertilization) were randomly distributed into 12-well plates with 10 embryos/well. Menadione powder was dissolved as a 25 mM stock solution in E3 medium and diluted to a working concentration of 9 µM in E3 with or without 60 µM NAC. This medium was refreshed daily, and dead eggs were removed. To enforce full hatching of all genotypes, 1 dpf eggs were incubated overnight with 6 µg/ml Pronase (Sigma-Aldrich, #10165921001). Embryos were scored as dead in the absence of a beating heart. Darkened yolk, cardiac edema, or shortened tails were scored as teratogenic (developmental) effects. All larvae were euthanized at maximally 5 dpf.

**Mouse experiments:**

ZAK KO mice were on a mixed C57BL/6NJ background. Experimental cohorts of WT and ZAK KO mice were obtained by in-house breeding of heterozygotes. For feeding experiments, young male littermate mice with an age span of 10-15 weeks of age were used. Body weight and food intake were measured weekly. Fresh HFHS food pellets were provided to the mice once per week. For N-acetylcysteine supplementation, the compound was added directly to the drinking water at 10 g/l and the solution was refreshed three times per week. Body composition was determined by
quantitative magnetic resonance (MR) using the 4in1 Body Composition Analyzer (EchoMRI). In the aging cohort, male and female mice were group caged according to their sex with ad libitum access to chow diet and water, subjected to ipGTT analysis at 13-15 months of age and euthanized and dissected 1 month later.

**Ribosome profiling (monosome/disome) of mouse livers**

Livers were harvested and flash-frozen in liquid nitrogen. Starting with 200 mg liver pieces from individual animals, lysates and ribosome footprints were generated by RNase I digestion and purified. Briefly, 5 μg of RNase I-digested RNA were separated on 15% urea-polyacrylamide gels to excise monosome (~30nt) and disome (~60nt) footprints. From the gel slices, RNA was extracted overnight at 4 °C on a rotating wheel before precipitation in isopropanol during 3h at -20 °C. RNA 3’-end repair was carried out with 2 U/μl of T4 PNK (Lucigen) prior to a 2 h adaptor ligation at 25 °C using T4 RNA Ligase 1 (NEB) and T4 RNA Ligase 2 Deletion Mutant (Lucigen) and 1 μl of a 20 μM 5’-adenylated DNA adaptor. Adaptor removal was carried out by treating individual libraries with 5’deadenylase (NEB) and RecJf exonuclease (NEB) for 1 h at 30 °C and 1h at 37 °C. Using Zymo Clean & Concentrator columns, samples were purified and pooled. Ribosomal RNA was depleted according to siTools Biotech rRNA depletion kit specifications with a custom-made riboPOOL. The clean-up step was carried out using Zymo Clean & Concentrator columns. Further library preparation steps were performed as described. The amplification of libraries was carried out using i5 and i7 (NexteraD502 or NexteraD503 (monosomes) and NexteraD504 or NexteraD505 (disomes)) primers. Libraries were sequenced on a NovaSeq6000 (Illumina).

**References and notes**
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Author contributions


Competing interests

The authors declare that they have no competing interests.
Data and materials availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (75) partner repository with the dataset identifier PXD043071. Sequencing data files have been deposited in NCBI Gene Expression Omnibus (GEO) with the accession code GSE235700. The scripts for data analysis have been deposited on Zenodo (76). All other data are available in the main text or the supplementary materials. Plasmids and cell lines generated in this study are available upon request and without the need for a material transfer agreement.

Supplementary materials

Materials and methods

Figs. S1 to S11

Tables S1 to S3

References (77-89)

Figure legends

Figure 1.

Reactive Oxygen Species inhibit translation and activate the ribotoxic stress response

(A) U2OS cells were treated with increasing concentrations of menadione (25, 50, 150, 250, 500 µM – 1 h). Lysates were analyzed by immunoblotting with the indicated antibodies. (B) WT U2OS cells or U2OS cells deleted for ZAK (ΔZAK) were treated with a ZAK inhibitor (ZAKi, 2 µM) and menadione (Mena, 250 µM – 1 h). Lysates were analyzed as in (A). (C) Schematic of ZAK protein
isoforms. LZ, Leucine Zipper; SAM, Sterile Alpha-Motif; S, Sensor Domain; CTD, C-Terminal Domain; SFBD, Stress Fiber Binding Domain. (D) U2OS cells were transfected with control (mock) siRNA or siRNAs targeting the α or β isoforms of ZAK. Cells were treated with menadione (250 μM – 1 h) and lysates were analyzed as in (A). (E) U2OS cells were pre-treated with N-Acetyl Cysteine (NAC, 10 mM – 1 h) followed by addition of menadione (250 μM – 1 h) or anisomycin (Ani, 1 μg/ml – 1 h) as indicated. Puromycin (10 μg/ml) was added to the culture 10 min prior to harvest and lysates were analyzed by immunoblotting with the indicated antibodies. (F) U2OS, ΔZAK and ΔZAK cells rescued with WT and mutated forms of ZAKα and WT ZAKβ were treated as in (D) (αΔΔ is a deletion of the “S” and “CTD” domain in (C)). Lysates were analyzed as in (A). (G) U2OS cells were treated with menadione (250 μM – 1 h) followed by washout (WO) in the presence of NAC (10 mM) for the indicated times. Cells were pulse-treated with puromycin (10 μg/ml, 10 min) before harvest as in (E). Lysates were analyzed as in (A). (H) Cells from (B) were treated with ROS-generating β-lapachone (β-lapa, 20 μM - 1 h) and lysates were analyzed as in (A). (I) U2OS cells were pre-treated with NAC (10 mM – 1h) followed by addition of auranofin (Aura, 5 μM – 1 h) as indicated. Lysates were analyzed as in (A). (J) Cells from (B) were treated with auranofin (5 μM - 1 h) and lysates were analyzed as in (A).

Figure 2.

ROS-induced ZAKα activation is associated with ribosome stalling and collision

(A) U2OS cells were treated with menadione (Mena, 250 μM – 30 min) and ISRIB (200 nM).

Lysates were digested with micrococcal nuclease (MNase) and separated on a linear sucrose gradient. Arrows highlight UV-peaks that are indicative of increased ribosome collision. (B) U2OS cells were pre-treated with menadione (250 μM – 1 h) as indicated and harringtonine (HTN, 2
µg/ml) for the indicated times to induce ribosome run-off. Puromycin (10 µg/ml) was added to the culture for 5 min prior to harvest (but after HTN incubation time) and lysates were analyzed by immunoblotting with the indicated antibodies. (C) U2OS cells were treated with menadione (250 µM – 1 h) and inhibitors (i, 1 µM) against PERK and GCN2 as indicated. Lysates were analyzed as in (B). (D) Schematic of tri-partite in vitro translation (IVT) approach. Ribosomes, ribosome-free cytoplasm, and mRNA can be individually treated before combination. (E) The three fractions from (D) were individually treated with H₂O₂ (10 min) and neutralized by addition of catalase as indicated. Translation efficiency in the combined reaction was determined by luciferase assay. (F) As in (E), except that the fractions were individually irradiated with UV-B (500 J/m²) prior to in vitro translation. (G) Schematic of modified tri-partite IVT assay. Compared to (D), HeLa cells were pre-treated with N-Acetyl Cysteine (NAC, 10 mM – 1 h) followed by addition of menadione (250 µM – 1 h) prior to purification of fractions. (H) Combined IVT reactions with treated ribosome-free cytoplasm from (G). (I) As in (H), except that treated ribosomes from (G) were used. All values indicate luciferase activity normalized to the control. (E, F, H, I). Data are plotted as mean and all error bars represent the standard error of the mean (SEM) (n=3 biological replicates). ns., non-significant; *, p≤0.05; **, p≤0.01; ***, p≤0.001; ****, p≤0.0001 in Student’s t-test for two groups and one-way ANOVA with Tukey post hoc test for > 2 groups. (J) Whole cell RNA isolated from HeLa cell fractions from (G) was separated on urea agarose gel and stained for RNA. nt, nucleotides. (K) Northern blot for tRNA-Arg-TCT on RNA samples from (J). Schematic of tRNA intermediates corresponding to distinct bands are shown on the left side of the blot.

**Figure 3.**

*ZAKα mediates menadione-induced apoptosis and death in zebrafish*
(A) U2OS cells were treated with menadione (250 µM) for the indicated times. Lysates were analyzed by immunoblotting with the indicated antibodies. (B) U2OS cells individually deleted for ZAK (ΔZAK), ASK1 (ΔASK1), and both (ΔZAK/ΔASK1) were treated with menadione (250 µM) for the indicated times. Lysates were analyzed as in (A). (C) Model of ROS-induced activation of p38 and JNK kinases by parallel sensing mechanisms. ZAKα responds to ROS-induced impairment of ribosomal translation (left), while ASK1 activity is directly controlled by intracellular ROS levels (right). (D) Genomic location of guide-RNA sequences (blue) and derived knockout alleles in exon 2 of zebrafish zakα (top) and zakβ (bottom) genes. A 33 bp insertion in mutated zakβ is highlighted in red, with the position of an in-frame STOP codon underlined and in bold. PAM, Protospacer Adjacent Motif. (E) Genotyping of WT and CRISPR-modified alleles of zakα and zakβ genes from (D). A single zebrafish larva per genotype was lysed and submitted to genomic PCR with the indicated primer pairs. Amplified bands were resolved by agarose gel electrophoresis. (F) Schematic of experiments with menadione treatment of zebrafish larvae. Zebrafish eggs were incubated in the presence of menadione (9 µM) and/or NAC (60 µM) from 6 hours post fertilization and up to 5 days. Larvae were scored as dead or alive once per day, and the number of fish with developmental phenotypes was counted. For detection of apoptosis, larvae treated for 3 days were fixed and subjected to TUNEL staining. (G) 30 zebrafish larvae with each of the indicated genotypes were treated and scored as in (F). Only larvae with cardiac arrest were scored as dead. The data shown are from one representative experiment out of the three performed. dpf, days post fertilization. (H) Representative images of larvae from (G) – time point day 3. WT fish treated with menadione (Mena) present with darkened yolk sac, cardiac edema, and short kinked tails (red arrows). The addition of NAC or deletion of zakα largely obviates these pathologies. (I) WT and zakα−/− larvae were treated as in (F) for 3 days, fixed, and subjected to TUNEL staining. Pictures show representative images of the tail part of the larvae. All scale bars = 500 µm.
**Figure 4.**

**ZAK**⁻/⁻** mice are protected against obesity-associated metabolic dysfunction**

(A) Schematic of mouse feeding experiment. 10- to 12-week-old male WT (n=13) and ZAK⁻/⁻ (n=12) mice were maintained on chow and subjected to Magnetic Resonance (MR) scanning and intraperitoneal Glucose Tolerance Test (ipGTT) two weeks prior to shifting to a High Fat-High Sugar (HFHS) diet. Mice were subjected to MR scanning and ipGTT after a further 8 and 19 weeks of HFHS feeding. Mice were euthanized and tissues were collected 25 weeks after the diet shift. (B) Percent body weight gain of mice from (A). Arrows mark the time of IpGTT assay and resulting transient weight loss. (C) Blood glucose concentrations of mice from (A) subjected to ipGTT assay. (D) HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) of mice from (A). (E) Blood glucose concentration of mice from Fig. S4I subjected to Intraperitoneal Insulin Tolerance Test (IpITT) at the 8-week timepoint (WT, n=12; ZAK⁻/⁻, n=7). (F) Liver triglyceride content of mice in (A). (G) Scoring of liver steatosis grade (scale 0 – 4) of mice from (A). (H) Images of representative hematoxylin and eosin (H/E)-stained WT and ZAK⁻/⁻ liver sections from mice in (A). Arrows indicate areas of steatosis. (B, C, D, E, F). All data are plotted as mean and all error bars represent the standard error of the mean (SEM). (C, E). Statistical analysis is based on the area under the curve (AUC) for each experimental group ns., non-significant; *, p≤0.05; **, p≤0.01 in Welch’s t-test in (D) and (F) and Mann-Whitney U-test in (C, E). All scale bars = 50 µm.

**Figure 5.**

Ribosome profiling of monosome and disome footprints reveals discreet changes to the translation landscape upon High-Fat High-Sugar feeding
(A) Experimental design and workflow for proteomic and phospho-proteomic profiling of mouse livers. 10- to 12-week-old mice of the indicated genotypes were fed chow or HFHS for 5 weeks (n=4-6). (B) Principal Component Analysis (PCA) of the phospho-proteomic profiles of mouse livers from (A). (C) Barplots of intensities derived from MS2 data for activation-associated phosphorylation sites on MAPK9/JNK2. Height of the bars represents the average of the measurements and error bars the standard error. Missing values in the MS data are plotted as zero values. (D) Lysates of livers from (A) were analyzed by immunoblotting with the indicated antibodies (left) and p-JNK divided by total JNK signals were quantified (right). Data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-significant; *, p≤0.05; **, p≤0.01 in two-way ANOVA with Tukey post hoc test. (E) Schematic of in vivo monosome and disome profiling experiment. 12-week-old male WT mice were fed normal chow or High-Fat High-Sugar (HFHS) diet for 8 weeks (n=3). (F) Principal component analysis (PCA) on monosome (closed circles) and disome (open circles) footprint data from individual mice, using the top-500 expressed genes. (G) Contribution of the most prominent genes to cumulative total signal for monosome footprints (dark gray), disome footprints (light gray), and differential codon sites from Fig. S8C (orange). The locations of Alb, Apoe, and Rbp4 in the cumulative datasets are highlighted. (H) Position-specific A-site signal for monosome and disome footprints across the Apoe mRNA for chow-fed (gray) and HFHS-fed conditions (pink). Below monosome/disome tracks, positions of differential codon sites are depicted, with height corresponding to odds ratio values, and the same color coding as in Fig. S8C. (I) As in panel (H), but for Rbp4. (J) Zoom into the left highlighted region of Apoe from panel (H) with high disome signal specifically in HFHS, and neighboring differential codon sites, in line with increased collisions in this area. (K) As in (J), but for the right highlighted region. (L) As in panels J-K, but for the high-disome site from the Rbp4 transcript in panel (I).
Figure 6.

Healthy metabolic aging in male ZAK<sup>−/−</sup> mice

(A) Schematic of mouse aging experiment. WT (male, n=7; female, n=16) and ZAK<sup>−/−</sup> (male, n=7; female, n=12) mice were maintained on a normal chow diet until 14-16 months of age (“old group”). Mice were subjected to intraperitoneal Glucose Tolerance Test (ipGTT) and euthanized two weeks later for collection of tissues. Tissues from 6-month-old WT and ZAK<sup>−/−</sup> male mice (“young group”) were used for comparison. (B) Blood glucose concentrations of “old” male (top) and female (bottom) mice from (A) subjected to ipGTT assay. Statistical analysis is based on the area under the curve (AUC) for each experimental group. (C) Basal glucose levels, (D) basal insulin levels and (E) HOMA-IR of fasted “old” male mice from (A). (F) Images of representative hematoxylin and eosin (H/E)-stained liver section from male mice in (A). Arrows indicate areas of steatosis. (G) Scoring of liver steatosis grade (scale 0 – 4) of male mice from (A). (H) Images of representative H/E-stained sections of Brown Adipose Tissue (BAT) from male mice in (A). Arrows indicate areas of notable lipid droplet condensation and BAT whitening. (I) Whole mount BAT scans from (Fig. S10A) were segmented according to lipid droplets. Individual droplet sizes for all aged WT and ZAK<sup>−/−</sup> samples were computed and represented in the form of histograms (n=6). (J) Model of metabolic regulation by the Ribotoxic Stress Response (RSR) in obesity and aging in male mice. Reactive Oxygen Species (ROS) produced as a result of High-Fat High-Sugar (HFHS)-feeding or during the course of aging impair ribosomal translation and activate the RSR sensor ZAKα. Downstream MAP kinase (p38, JNK) signaling mediates features of metabolic decline such as glucose intolerance, liver steatosis, and BAT whitening. (B, C, D, E). All data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-
significant; *, p ≤ 0.05; **, p ≤ 0.01 in Welch’s t-test in (C) and Mann-Whitney U-test in (B, D, E).

All scale bars = 50 μm.
Snieckute et al., Figure 3

**A** U2OS

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- p-p38
- p38
- p-eIF2α
- eIF2α
- p150

**B**

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<tr>
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**C**

- ROS
- MAP3Ks
- ZAKα
- ASK1
- MAPKs
- p38
- JNK

**D**

- ZAKα
- Exon 2
- WT
- 5'..GCTGAGATTTTGAGCGTTCTCAGTCATCGGAAN..-3'
- zakα^+/−
- 5'..GCTGAGATTTTGAGCGTTCTCAGTCATCGGAAN..-3'

- ZAKβ
- Exon 2
- WT
- 5'..GCTGAGATTTTGAGCGTTCTCAGTCATCGGAAN..-3'
- zakβ^+/−
- 5'..GCTGAGATTTTGAGCGTTCTCAGTCATCGGAAN..-3'

**E**

- Mw marker
- WT
- α^+/−, β^+/−
- α^+/−, β^−/−
- ZAKα primers
- ZAKβ primers

**F**

- Fertilized egg
- 6 hours
- 9 µM menadione
- +/- NAC
- 3 days
- 1-5 days
- TUNEL staining
- Phenotype scoring

**G**

- Fraction of larvae

**H**

- WT
- zakα^+/−; zakβ^+/−
- zakα^−/−; zakβ^+/−
- zakα^+/−; zakβ^−/−
- zakα^+/−; zakβ^−/−
- Survival
- Survival (+ NAC)
- Teratogenesis
- Teratogenesis (+ NAC)

**I**

- Menadione + TUNEL staining
- WT
- + NAC
- zakα^−/−
- + NAC
Snieckute et al, Figure 5

**A**

Protein extraction
PCA protein digestion
Full proteome analysis
ZrlMAC-HP phospho-enrichment
Full proteome analysis

**B**

phospho-proteomics data

**C**

WT chow  WT HFHS  ZAK-/- chow  ZAK-/-HFHS

**D**

Chow diet  HFHS diet

WT  ZAK-/-  WT  ZAK-/-

p-JNK  JNK  α-tubulin

**E**

Rodent chow  8 weeks  WT

High Fat  High Sugar Diet
Liver ribosome profiling (monosome / disome footprints)

**F**

Liver ribosome profiling (monosome / disome footprints)

**G**

gene count for 50% of total

**H**

ApoE
Monosome  Disome
odds ratio (HFHS vs. Chow)

**I**

Rbp4
Monosome  Disome
odds ratio (HFHS vs. Chow)
Snieckute et al, Figure 6

**Basal glucose**

*Old* males

**Basal insulin**

*Old* males

**HOMA-IR**

*Old* males

**Blood glucose (mmol/L)**

- WT ZAK−/−
- WT ZAK−/−
- WT ZAK−/−

**Liver steatosis score**

- WT ZAK−/−
- WT ZAK−/−
- WT ZAK−/−

**Aging BAT Quantification**

- Lipid droplet size (µm²)

- Density

**J**

Obesity → Aging → ROS

**Metabolic Insufficiency**

Loss of glycemic control
Liver steatosis
BAT whitening

**Ad libitum**

Rodent chow
Supplementary Materials for

ROS-induced ribosome impairment underlies ZAKα-mediated metabolic decline in obesity and aging


Corresponding author: sbj@sund.ku.dk

The PDF file includes:

Materials and Methods
Figs. S1 to S11
Tables S1 to S3
References
Materials and Methods

Cell culture and reagents
All cell lines were obtained from ATCC. Human osteosarcoma cells (U2OS), human near haploid cells (HAP1), and human malignant cervical epithelial cells (HeLa) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Biowest) supplemented with 10% fetal bovine serum (FBS, Biowest), L-glutamine, 1% penicillin and streptomycin. All cells were cultured at 37 °C in a humidified 5-8% CO₂ cell incubator. Stable cell lines expressing WT or truncated versions of ZAKα and ZAKβ were generated previously (21). The chemicals and inhibitors used in this paper were: Menadione (Sigma-Aldrich, #M5750, 250 μM), β-lapachone (Sigma-Aldrich, #L2037, 20 μM), doxycycline (Sigma-Aldrich, D3347, 0.13 μg/ml), anisomycin (Sigma-Aldrich, A9789, 1 μg/ml), puromycin (Cayman Chemicals, CC-13884, 10 μg/mL), N-Acetyl-L-cysteine (Sigma-Aldrich, #C7352, 10 mM), auranoﬁn (Sigma-Aldrich, #A6733, 5 μM), harringtonine (Santa Cruz, #sc-204771, 2 μg/ml), ZAK inhibitor (2 μM, gift from Xiaoyun Lu (Jinan University, China) (77)), ISRIB (Sigma-Aldrich, #SML0843, 200 nM), GCN2 inhibitor A-92 (Axon medchem, #2720, 1 μM), PERK inhibitor GSK2606414 (SelleckChem, #S7307, 1 μM), ASK1 inhibitor Selonsertib, (Selleckchem, #S8292, 2 μM) and thapsigargin (Sigma-Aldrich, #T9033, 1 μM).

Western blotting and antibodies
Cells were lysed in EBC buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease and phosphatase inhibitors). Samples were mixed with Laemmli sample buffer and boiled before they were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in PBS-T + 5% milk and incubated with primary antibody overnight at 4 °C. Subsequently membranes were washed in PBS-T 5 times and incubated with Goat Anti-Rabbit or Goat Anti-Mouse IgG Antibody (H+L) Peroxidase for 1 h at room temperature. Membranes were washed again in PBS-T and visualized by chemiluminescence (Clarity Western ECL substrate, Bio-Rad) using the Bio-Rad Chemidoc imaging system. Antibodies used in this study were: anti-phospho-p38 (Cell Signaling, #9216 and #4511S), anti-p38 (Cell Signaling, #9212), anti-phospho-SAPK/JNK (Cell Signaling, #9255), anti-SAPK/JNK (Cell Signaling, #9258), anti-ZAK (Proteintech, #14945-1-AP), anti-ZAKα (Bethyl #A301-993A), anti-p150 (BD biosciences, #610473), anti-phospho-eIF2α (Cell Signaling, #3398), anti-puromycin (Millipore, #MABE343), anti-EDF1 (Abcam, #ab174651), anti-RPS2 (Bethyl, #A303-794A), anti-Ribosomal RPS10 (Abcam, #ab151550), anti-phospho-PERK (Cell Signaling, #3179), ZNF598 (Abcam, #ab111698 OR Sigma Aldrich, HPA041760), RPL19 (Novus, #H00006143-M01), ASK1 (Thermo Fisher Scientific, #702278), α-tubulin (Sigma, #T9026), anti-eEF2 (Cell Signaling, #2332S), anti-biotin, HRP-linked (Cell Signaling, #7075), anti-eIF3η (Santa Cruz, #sc16377), anti-eIF4E (Cell Signaling, #9742) and anti-peroxiredoxin SO2/3 (crb cambridge, #crb2005004e/T).
siRNAs
siRNA transfections were carried out using RNAiMAX (Life Technologies) following the manufacturer’s instructions. siRNA sequences used in this study were: ZAKα: 5’-ggugcccauuaaguaucaa(dTdT) and ZAKβ: 5’-caugcaagccaagcagaau(dTdT).

Polysome profiling
After cells were exposed to various treatments, cytosolic lysates were prepared using 20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 100 µg/ml digitonin, 100 µg/ml cycloheximide, 1X protease inhibitor cocktail (Sigma, #P8340) and 200 U NxGen RNase inhibitor (Lucigen, #30281). Extracts were incubated on ice for 5 min prior to centrifugation at 17,000 g for 5 min at 4 °C. After adding calcium chloride to a final concentration of 1 mM, lysates were optionally digested with 500 U micrococcal nuclease (MNase) (New England Biolabs, #M0247) for 30 min at 22 °C. Digestion was terminated by adding 2 mM EGTA. Equivalent amounts of lysate (250 mg of undigested RNA or 350-400 mg of MNase-digested RNA) were resolved on 15-50% sucrose gradients. Gradients were centrifuged at 38,000 rpm in a Sorvall TH64.1 rotor for two hours at 4 °C. The gradients were passed through an ISCO density gradient fractionation system with continuous monitoring of the absorbance at 254 nm.

Isolation of ribosomes through sucrose cushions
Crude cellular ribosome fractions were purified by sedimentation through a 30% sucrose cushion. Briefly, cells were lysed (15 mM Tris, pH 7.5, 0.5% NP40, 6 mM MgCl₂, 300 mM NaCl, RiboLock RNase inhibitor) and centrifuged at 12,000 g, 4 °C, 10 min. The supernatant was carefully layered onto a sucrose cushion (30% sucrose in 20 mM Tris, pH 7.5, 2 mM MgCl₂, 150 mM KCl) and ultra-centrifuged at 38,800 rpm for 16 h using Sorvall wX+ Ultrafuge and FIBERlite F50L-8x39 rotor. Pellets were washed thrice in PBS and suspended in 100 mM KCl, 5 mM MgCl₂, 20 mM HEPES, pH 7.6, 1 mM DTT and 10 mM NH₄Cl. Purified ribosome fractions were analyzed by SDS-PAGE and western blotting.

Puromycin incorporation assays
Cells were treated as shown in the figures followed by the addition of puromycin (10 µg/ml) to the medium for 5-10 min. Cells were washed with ice-cold PBS and immediately lysed. Western blotting with anti-puromycin antibodies was performed to visualize puromycin incorporation into nascent polypeptide chains.

Tri-partite in vitro translation assay
Isolation of ribosomes: For isolation of ribosomes, HeLa cells were cultured to 80% confluency and scraped off in ice-cold PBS. Cells were lysed in polysome lysis buffer (20 mM Tris-HCl, 150 mM KCl, 5 mM MgCl₂, 0.5% NP40, 2 mM DTT, Roche EDTA-free Protease Inhibitor and murine RNase inhibitor (New England Biolabs, #M0314)) and incubated while rotating for 10 min at 4 °C. Lysates were cleared by centrifugation at 14,000 g for 10 min and supernatants were recovered and supplemented with 25 µM Hemin (Sigma-Aldrich, #51280). Cleared lysates were loaded on top of a 500 µl 34% sucrose cushion in hypotonic buffer (10 mM HEPES, 10 mM KAc, 0.5 mM MgAc₂) and centrifuged for 240,000 g, 2 h 15 min, 4 °C. Pellets containing
ribosomes were washed thoroughly and resuspended in 0.2 X of loaded lysate volume in hypotonic ribosome buffer (20 mM HEPES, 10 mM NaCl, 25 mM KAc, 1.1 mM MgAc₂). Isolation of ribosome-free cytoplasm: For isolation of cytoplasm, lysates were prepared as described (78) with some modifications: HeLa cells were cultured to 80% confluency and detached by trypsinization followed by trypsin inactivation. Cells were pelleted by centrifugation at 500 g for 4 min, washed in PBS and resuspended in equal volume of hypotonic lysis buffer (10 mM HEPES, 10 mM KAc, 0.5 mM MgAc₂, 5 mM DTT, Roche EDTA-free Protease Inhibitor) and incubated rotating 1 h, 4 °C. After incubation, lysates were pulled through a 27 G, 3/4-inch needle 8-10 times to lyse the cells and debris were washed by centrifugation at 14,000 g for 2 min. Cleared lysates were supplemented with 25 µM Hemin and centrifuged for 240,000 g, 2 h 15 min, 4 °C. Pellets containing ribosomes were discarded and supernatants were recovered as ribosome-free cytoplasmic lysate.

Reconstituted in vitro translation: Translation reactions were reconstituted by mixing one part isolated ribosomes, five parts cytoplasmic lysates, one part in vitro transcribed luciferase mRNA (final concentration 1-10 ng/µl) and three parts translation buffer (final concentrations of 1.6 mM HEPES, 10 mM creatine phosphate (Sigma Aldrich, #27920), 50 ng/µl creatine kinase (Sigma Aldrich, #10127566001), 10 µM spermidine (Sigma Aldrich, # S0266), 10 µM amino acids (Promega, # L4461), 65 mM KAc, 0.75 mM MgAc₂ and murine RNase inhibitor). Luciferase mRNA with an EMCV IRES was transcribed from a plasmid using HiScribe T7 ARCA mRNA Kit with tailing (New England Biolabs, #E2060) according to manufacturer’s specifications. Translation reactions were incubated at 37 °C for 30 min and translational output was measured as luciferase activity detected with Dual-Glo Luciferase Assay System (Promega, # E2940) according to manufacturer’s specifications. For H₂O₂-treatment, reactions were treated with 5 mM H₂O₂ (Sigma-Aldrich, #H1009, 5 mM) for 10 min at 25 °C followed by treatment with either 1 µg/µl catalase (Sigma-Aldrich, C1345-1G) or vehicle (1 mM Potassium phosphate). For UV irradiation, fractions were irradiated with 500 J/m² UV-B and immediately added to the in vitro translation reaction.

tRNA northern blotting
Total RNA was isolated from HeLa cells with TRI reagent (Thermo Fisher Scientific, #AM9738) according to the manufacturer’s instructions. For tRNA charging gels, RNA was prepared in 10 mM sodium acetate (pH 4.5), whilst for TBE-urea gels RNA was prepared in nuclease-free water. Deacylated tRNA controls were generated by incubating total RNA in 1 mM EDTA and 100 mM Tris HCl (pH 9.0) for 1 hour at 42 °C. RNA was then precipitated in 95% ethanol with 300 mM sodium acetate (pH 5.2), RNA pellet was washed twice in 70% ethanol and then resuspended in 10mM sodium acetate (pH 4.5).
To look at tRNA fragments, 5 µg total RNA was resolved on 10% TBE-Urea gels (Thermo Fisher Scientific #EC6872BOX) in 1X loading buffer II (Ambion #AM8546G). Gels were stained with SYBR gold (Thermo Fisher Scientific #S11494), imaged on a FLA-3000 imager (Fujifilm), and then transferred onto positively charged nylon membranes (GE healthcare #RPN119B) in 0.5X TBE buffer using a semi-dry blotter. Membranes were then UV crosslinked and stored until use.
For tRNA charging gels, 5 µg total RNA was resolved at 4 °C on 12% acid-urea (8M) gels prepared in 100 mM sodium acetate (pH 5.2), in 1X loading buffer (8 M urea, 100 mM sodium acetate (pH 5.2), with 0.025% xylene cyanol and 0.025% bromophenol blue), with 100 mM sodium acetate (pH 5.2) running buffer. Gels were stained with SYBR gold (Thermo Fisher
Scientific #S11494), imaged on a FLA-3000 imager (Fujifilm), and then transferred onto positively charged nylon membranes (GE healthcare #RPN119B) in 0.5X TBE buffer using a semi-dry blotter. Membranes were then UV crosslinked and stored until use.

10 pmol of each northern probe was radiolabelled with [γ-32P]ATP (Perkin Elmer #BLU002Z250UC) using T4 PNK (Thermo Fisher Scientific #EK0031) and purified using G25-sephadex columns (GE Healthcare #27-5325-01) according to manufacturer’s instructions. Membranes were prehybridized in hybridization buffer (6X SSC buffer, 4X Denhardt’s solution, 0.1% SDS) at 10 °C below the T_m of the probe of interest for at least 1 hour. Membranes were incubated overnight with the probes in fresh hybridization buffer at 10 °C below the T_m of the probe of interest. Membranes were washed twice in 3X SSC buffer, 0.1% SDS at room temperature and then twice in 3X SSC buffer, 0.1% SDS and hybridization temperature. The membranes were then exposed to phosphoimager screens. Phosphoimager screens were imaged on a FLA-3000 imager (Fujifilm). Probe sequences used: tRNA-Arg-TCT: 5’-GCTATCCATTGCACACAGCC-3’, tRNA-Leu-CAA: 5’-TTAGACCACTCGACATCCTGAC-3’, tRNA-Met-CAT: 5’-CTTCCGCTGCGCCACTCTGAC-3’, tRNA-Gly-GCC: 5’-CTACCACTGAACCACCCATGC-3’, 5S rRNA: 5’-CCGACCCTGCTTAGCTTCC-3’.

Biotin switch assay
Cells were lysed in EBC buffer without DTT and with PMSF. The lysates were incubated for 30 min shaking at 50 °C with 25 mM S-methyl methanethiosulfonate (MMTS, Sigma-Aldrich #208795) in blocking buffer (250 mM HEPES, 1 mM EDTA, 0.1 mM Neocuproine (Sigma-Aldrich #N1501), 2.5% SDS, pH 8.0) to block non-oxidized thiols. Excess of blocking reagents was removed by acetone precipitation. The pellets were left to dry before being resuspended in 1% SDS in PBS. Samples were biotin-labeled by incubating for 30 min, shaking at 25 °C in the dark with 0.0625 mg/mL Biotin-HPDP (Santa Cruz #sc-207359) either in the presence of 10 mM DTT (reducing the oxidized thiols) or as a negative control without DTT. Excess Biotin-HPDP was removed by acetone precipitation. Pellets were resuspended in HENS/10 buffer (25 mM HEPES, 0.1 EDTA, 0.01 mM Neocuproine, 1% SDS, pH 8). Three volumes of neutralization buffer (25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 7.5) were added to each sample. 1% of the samples were kept for input, while the rest were incubated on pre-washed Strep-Tactin Sepharose Resin-beads (IBA Lifesciences #12846436) rotating for 1 h at 4 °C. Samples were centrifuged at 8000 rpm for 30 sec, the supernatant was discarded. The beads were then washed 4 times with 1 mL of washing buffer (neutralization buffer + 600 mM NaCl), followed by 3 washes in 10 mM Tris-HCl (pH 7.5). Beads were eluted with 1x elution buffer E (IBA Lifesciences #2-1000-025) on ice for 1 h. Non-reducing Laemmli sample buffer was added to all samples, including the input samples, and boiled before they were resolved by SDS-PAGE.

Zebrafish
Zebrafish husbandry and egg collection: Zebrafish were maintained under standard conditions in compliance with the European Convention for the Protection of Vertebrate Animals and Other Scientific Purposes. Adult zebrafish (Danio rerio) were maintained in a semi-closed water system with a water temperature of 28 ± 1 °C and pH 7.3-7.5, and a 14:10 hour light:dark cycle. For the breeding and collection of eggs, multiple females and males for each genotype were separately placed into a 1.7-liter breeding tank with a divider one day before egg production.
Eggs were collected the following morning, within 2 h of pulling out the divider, and used as 0 h post-fertilization (hpf) eggs.

**Generation of sgRNAs for genetic knockout:** Guide strand RNAs targeting exon 2 of both ZAK genes in zebrafish were designed using ‘CHOPCHOP’ (https://chopchop.cbu.uib.no/). For each gene, two gene-specific and 20-22 nucleotide long sequences were selected (bold), each including a protospacer adjacent motif (PAM) (bold and underlined). In addition to the target sequence, a T7 promoter region was incorporated into these Forward primers. Templates were generated by PCR using the gene specific and Common Reverse primers with high fidelity Phusion polymerase (Thermo Fisher Scientific) and guide strand RNA was synthesized using MEGA-shortscript T7 transcription kit (Ambion). Common Reverse primer: 5’-AAAAGCACCAGACTCGGTGCCACTTTTTCAAGTTGATAACCGACTAGCCTTTTAACCTTGCTATTTCTAGCTCTAAAAC. Gene Specific Forward primers: zakα (1): 5’-GGAGCTTCTGGAAGCGCCCAATTATGTTTAGAGCTAGAAATA. zakα (2): 5’-GCAGCTAATACGACTCACTATAAGTTTCTGGAAGCGCCCAATTATGTTTAGAGCTAGAAATA. zakβ (1): 5’-GCAGCTAATACGACTCACTATAAGTTTCTGGAAGCGCCCAATTATGTTTAGAGCTAGAAATA. zakβ (2): 5’-GCAGCTAATACGACTCACTATAAGTTTCTGGAAGCGCCCAATTATGTTTAGAGCTAGAAATA.

**Gene targeting:** Each gene was targeted separately by injecting 300 pg of the two gene specific sgRNA together with 1 ng Cas9 protein in a 1 nl volume into zebrafish embryos (AB wildtype) at the 1 - 4 cell stage. F0 zebrafish were raised to sexual maturity and outcrossed with wild-type AB zebrafish. F1s were genotyped by PCR of the relevant genomic DNA region, heterozygotes were identified by heteroduplex analysis (79), and the specific disruption was identified by sequencing after cloning the PCR product into a plasmid. These F1s were back-crossed to AB before selecting F2 heterozygotes, which were outcrossed again before raising a line of heterozygotes with a known mutation. Homozygous zakα and zakβ KO zebrafish lines were generated by in-crossing heterozygotes and genotyping the offspring by fin-clipping at maturity. These were in-crossed to generate a line of double heterozygous knockouts that provided a stock for all genotypes.

**Characterization of null mutations:** Genomic DNA was extracted from F1 embryos, and PCR primers around the target site were used to amplify a ~500bp region which was T-cloned into pGEM T-easy (Promega) and sequenced to identify INDELs. A mutation was identified in zakα with a 71 bp deletion in exon 2, leading to a frameshift and a predicted early stop codon. An INDEL was identified in zakβ resulting in a 33 bp insert in exon 2 with an in-frame stop codon resulting in a predicted truncated protein. To ensure that the mutations detected in the genome persisted in the transcripts of adult zebrafish, brain and skeletal muscle tissue from homozygous zakα and zakβ KO zebrafish lines were generated by in-crossing heterozygotes and genotyping the offspring by fin-clipping at maturity. These were in-crossed to generate a line of double heterozygous knockouts that provided a stock for all genotypes.

**Genotyping of zebrafish line:** Adult zebrafish were anesthetized with MS222 (0.0168% w/v, #E10521, Sigma-Aldrich). A small part of the zebrafish caudal fin was cut and transferred into a 1.5 ml Eppendorf tube with 50 µl of DNA lysis Buffer (10 mM Tris-HCl pH 8, 50 mM KCl, 0.3% Tween 20, 0.3% NP40, 4 mM EDTA pH 8). Samples were incubated for 10 min at 98 °C.
5 µl of 20 mg/ml Proteinase K (Thermo Fisher Scientific, #EO0491) was added upon cooling and incubated overnight at 55 °C and denatured for 10 min at 98 °C. DNA was collected from the supernatant after centrifugation at 4000 rpm for 3 min. DNA samples were diluted at 1:20, of which 2.5 µl were used for PCR reaction (total volume 25 µl). PCR amplifications were performed according to the manufacturer’s instructions (Taq DNA Polymerase, #E00007-1000, GenScript) with the following primers: drZAKα-Fw: 5’-GCTTCATATTATTGGTGCTCTGTC, drZAKα-Rv: 5’-ACCGTCTGCTGTTCACTTTT, drZAKβ-Fw: 5’-TCATGGCCTCAGACGAAA and drZAKβ Rv: 5’-TGCAGCTTTTGGGTGACGTA. PCR products were analyzed by electrophoresis on a 3% agarose gel (drZAKα WT, 561 bp; drZAKα KO, 490 bp; drZAKβ WT, 501 bp; drZAKβ KO, 534 bp).

RT-qPCR analysis
Total RNA was purified using TRI reagent (Sigma Aldrich) according to the manufacturer’s instructions and DNase treated/purified using ZYMO RNA Clean and Concentrator-5 (ZYMO research). For reverse transcription 500 ng of purified RNA was used with 3.6 µM random hexamer primers (Thermo Fisher Scientific), 0.7 µM dNTP’s (Thermo Fisher Scientific) and Superscript IV RT Transcription Kit (Invitrogen) according to manufacturer’s protocol. For qPCR reactions 3 µl of 3-fold diluted cDNA was used together with Fast SYBR green mastermix (Thermo Fisher Scientific) according to the manufacturer’s protocol. RNA abundances were deduced from ΔCt values, normalized to ef1α mRNA abundance, and compared to the corresponding Wildtype sample replicate. Primer sequences used: Ef1α (F 5’-TTGAGAAGAAATCGGTGGTGCTG-3’ and R 5’-GGAACGGTGTGATTGAGGGAAATTC-3’), ZAKα (F 5’-GGCACAGGACAAAAGGACTG-3’ and R 5’-ATGTACGCTATCGCCACCTC-3’), ZAKβ (F: 5’- CCGATCCTGCACCTCCTCTGT-3’ and R: 5’-CCTCAGAGGCTGTGCTCTCC-3’).

Menadione treatment of zebrafish larvae
Freshly collected zebrafish eggs were incubated in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.00001 % Methylene Blue). Unfertilized and dead eggs were removed, and embryos around the early gastrula stage, approx. 6 hpf (hours post fertilization) were randomly distributed into 12-well plates with 10 embryos/well. Menadione powder was dissolved as a 25 mM stock solution in E3 medium and diluted to a working concentration of 9 µM in E3 with or without 60 µM NAC. This medium was refreshed daily, and dead eggs were removed. To enforce full hatching of all genotypes, 1 dpf eggs were incubated overnight with 6 µg/ml Pronase (Sigma-Aldrich, #10165921001). Embryos were scored as dead in the absence of a beating heart. Darkened yolk, cardiac edema, or shortened tails were scored as teratogenic (developmental) effects. All larvae were euthanized at maximally 5 dpf.

Whole-mount TUNEL assay
TUNEL assay to visualize apoptotic cells in zebrafish larvae was performed (80). 0.004% of 1-phenyl 2-thiourea (PTU) was added into the E3 medium from 1 dpf to inhibit pigment formation (81). At 72 hpf, 20 dechorionated larvae were placed into 2 ml Eppendorf tubes and fixed in fresh 4% cold paraformaldehyde in PBS overnight at 4 °C. The following day larvae were washed twice for 5 min in PBST at room temperature. Larvae were transferred into fresh methanol at -20 °C for at least 2 hours and rehydrated in serial methanol dilutions (75%, 50%, 25%) for 5 min each. Samples were washed in PBST and permeabilized by incubation for 10
in 10 µg/ml proteinase K in PBST at room temperature. Digestion was stopped by re-fixing in 4% paraformaldehyde for 30 min before incubation in ApopTag Equilibration buffer for 1 h at room temperature. Larvae were incubated in ApopTag TdT Enzyme solution (In Situ Cell Death Detection Kit, Roche Applied Science, #S7101, Sigma-Aldrich) overnight, consisting of 70% ApopTag Reaction Buffer and 30% ApopTag TdT Enzyme. Subsequently, larvae were washed in ApopTag Stop/Wash Buffer for 3 hours at room temperature and subsequently incubated with anti-digoxigenin-AP Fab fragment (Roche, #11093274910) overnight at 4 °C with mild agitation. On the next day, larvae were rinsed thoroughly with PBST 6 times for 20 min and 3 times for 5 min, each time in fresh NTMT buffer (100 mM NaCl, 50 mM MgCl\(_2\), 100 mM Tris-HCl pH 7.5, 1% Tween 20), before staining in fresh NBT/BCIP (10 µl of NBT/BCIP in 1 ml of NTMT buffer) for 10 min. To prevent over-staining, samples were washed in PBST immediately after incubation for a suitable length of time. Images were captured immediately after, alternatively slides were re-fixed in 4% paraformaldehyde and stored at 4 °C.

**Mice**

**Husbandry:** Mice were housed at the animal facility of the Department of Experimental Medicine at the University of Copenhagen. The research was monitored by the Institutional Animal Care and Use Committee. Permissions for mouse breeding and experimentation was obtained from the Danish Animal Experiments Inspectorate (permission numbers 2022-15-0202-00162 and 2022-15-0201-01126). ZAK KO mice in the mixed C57BL/6NJ background were previously described\(^{(49)}\). WT and ZAK KO mice were obtained by in-house breeding of hetereozygotes. All mice were maintained on a 12:12 hour light:dark cycle in ventilated cages at room temperature and were allowed ad libitum access to water and commercial rodent chow or high-fat high-sugar (HFHS) diet (Research Diets, #D12331) and water at all times. At the time of the experiment, littermate animals were randomly assigned to experimental groups.

**Genotyping:** Genomic DNA was extracted from ear clippings using the QuickExtract DNA Extraction Solution (Lucigen #QE09050). A 700 bp PCR product was amplified (Fw: 5'-gcaaggggtgaattagggag-3'; Rv: 5'-gtgagtgctttcatttcgacttg-3') and digested with EcoRV. KO mutation disrupts a single EcoRV restriction site in the WT product (WT bands, 430 bp and 270 bp; KO band, 700 bp).

**Mouse experiments:** For feeding experiments, young male littermate mice with an age span of 10-15 weeks of age were used. Body weight and food intake were measured weekly. Fresh HFHS food pellets were provided to the mice once per week. For N-acetylcysteine supplementation, the compound was added directly to the drinking water at 10 g/l and the solution was refreshed three times per week. Body composition was determined by quantitative magnetic resonance (MR) using the 4in1 Body Composition Analyzer (EchoMRI). In the aging cohort, male and female mice were group caged according to their sex with ad libitum access to chow diet and water, subjected to ipGTT analysis at 13-15 months of age and euthanized and dissected 1 month later.

**Glucose and insulin tolerance test, insulin measurements and HOMA-IR**

For glucose and insulin tolerance tests, mice fasted for 5 h following intraperitoneal injection of glucose at 2 g/kg or insulin 0.75 mU insulin/g body weight. A small drop of tail blood was taken at 0, 15, 30, 60, 90, and 120 minutes after injections and measured for glucose content using a Contour XT glucometer (Bayer). Insulin levels in blood serum were measured after 5 h of fasting using a commercially available kit (Crystal Chem, #90082) according to the manufacturer’s
instructions. Homeostasis model assessment–insulin resistance (HOMA-IR) index was calculated according to the formula: [fasting glucose levels (mmol/L)] × [fasting serum insulin (μU/mL)]/22.5.

Metabolic cages
For the indirect calorimetry study, male mice were fed either a chow diet or switched to an HFHS diet 6 weeks prior to the study. Physical activity and indirect calorimetry were assessed by Promethion Core Metabolic System. Briefly, mice were acclimatized to single cages for 1 week prior to measurements and data were collected in 5 minutes intervals for 7 days. Data presented in Figure S5b is an average of 7 days and in S5c-g represents data collected from day 3. Data collection was integrated into Promethion Core Metabolic System.

Glucose uptake assay
Prior to the experiment, male mice were fed an HFHS diet for 10 weeks. Mice were fasted for 5h and intraperitoneally injected with 0.2 g/kg glucose (Sigma, G8270) and 0.6 mCi/g of bodyweight of 3H-2DG (Perkin Elmer, #NET549005MC). Blood glucose concentration was measured every 20 minutes (T0, T20, T40, and T60), and blood samples were collected 20 and 60 minutes post glucose tracer injections. At T60 mice were euthanized and tissues were snap frozen in liquid nitrogen. To measure 2-Deoxy glucose uptake frozen tissues were crushed and weighed (20mg of liver, heart, and quadricep and 40mg of the adipose tissues). Tissues were lysed using a Qiagen TissueLyser II (2 x 90 s, 30 Hz). To measure unphosphorylated 2-DG 150 μl of lysate was mixed with 300 μl of 0.3N Ba(OH)₂ and 300 μl of ZnSO₄ and to measure phosphorylated 2-DG 150 μl of lysate was mixed with 600 μl 4.5% PCA (HClO₄). Mixtures were centrifuged at RT for 5 minutes at 10000 rpm and 500 μl of the supernatant was mixed with 4 ml of the Ultima Gold scintillation fluid in a 7 ml scintillation vial. Samples were analyzed in a SCINTILLATION 300SL machine.

BAT thermogenesis
Mouse backs were shaved a day before and imaged using a FLIR ONE PRO camera at room temperature. The average temperature was measured using FLIR software by drawing a 60x60 pixel region on the mouse back where BAT is located.

Triglyceride extraction and quantification
Lipids were extracted from 100-200 mg of liver tissue by overnight incubation at 55 °C in ethanolic KOH (66.6% of 96% ethanol with 33.3% KOH). Samples were brought to a volume of 1.2 ml with 50% ethanol followed by centrifugation (5 min, 16,000 g). 100 μl of supernatant was mixed with 100 μl of 0.5 M MgCl₂, and samples were kept on ice for 10 min before centrifugation (5 min, 16,000 g). Supernatants were transferred to a new tube and triglycerides were measured with Triglycerides Reagent (Thermo Fisher Scientific, #TR22421) according to the manufacturer’s protocol. Alternatively, undiluted mouse serum was measured with the same reagent.
Tissue processing
Snap frozen mouse livers were crushed with a tissue pulverizer device and lysed in ice-cold RIPA buffer (65 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, pH 7.4) for protein extraction, using steel beads and a TissueLyzer II (QIAGEN). Homogenates were centrifuged at 16,000 g for 20 min and lysates were collected and stored at -80 °C for later western blot analyses.

Histology
Mouse liver and BAT tissues were fixed in 10% formalin solution, (Sigma Aldrich, #HT501128) for 48 h at 4 °C. Fixed tissues were embedded in parafilm and sections were stained with hematoxylin and eosin (H/E). Images were acquired through a 40X objective. Lipid deposits were identified as perfectly round unstained regions. Liver steatosis grades were scored from the area of such regions according to the following scheme: <2%, score 0; 2-5%, score 1; 6-33%, score 2; 34-66%, score 3; >67%, score 4.

Immunohistochemistry
The paraffin-embedded sections were rehydrated in the following steps: 2x xylene washes (10 min), 3x 99% ethanol (1-2 min), 2x 96% (1 min) and 1x70% ethanol (3 min). Subsequently, slides were transferred to Easy-Dip staining jar (VWR, # 720-0791) and rinsed in deionized (DI) water. For antigen retrieval, slides were boiled for 3x 6 min in sodium citrate buffer (100mM of citric acid monohydrate, pH 6.0, Sigma-Aldrich, #1002440500). After the sections had cooled down to RT, they were rinsed in DI water and treated for 10 min with a 3% hydrogen peroxide solution diluted in methanol (Sigma-Aldrich, #H1009). Slides were then rinsed with TBS-T (Tris-Buffered Saline, 0.1% Tween 20) and stained using the Epredia UltraVision Quanto Detection System HRP Kit (Thermo Fisher Scientific, # Epedia TL-060-QHD). Antibodies anti-phospho-SAPK/JNK (Cell Signaling Technology, #4668, dilution 1:50) and anti-4 Hydroxynonenal (Abcam, # ab48506, dilution 1:500) were diluted in 2% BSA in either TBS or PBS and applied onto the slides for O/N incubation in a humidified chamber (Thermo Fisher Scientific, #15518996). The next day, slides were washed 3x 5 min in PBS-T or TBS-T before staining with secondary antibody according to the manufacturer’s protocol. Afterward, slides were washed twice in TBS or PBS and exposed to 3′-Diaminobenzidine (DAB, Thermo Fisher Scientific, # Epredia TA-060-QHDX) solution for 20 min (p-JNK) or 1.5 min (4-HNE). Slides were then rinsed in DI water and placed in hematoxylin (Cell Signaling Technology, #CST-14166S) for 30 sec and again rinsed in DI water. The dehydration process was performed in the following steps: 2x 96% ethanol (1-2 min), 2x 99% ethanol (1 min), and 3x Xylene (4 min). Slides were mounted in Pertex (Histolab, # 00840-05).

BAT scanning and quantification
BAT tissue slides were imaged by ZEISS Axioscan 7 (Zeiss Microscopy), with the Plan Apochromat objective, 20 X magnification, and 0.8 of the numerical aperture. The analysis was performed in QuPath (82) (https://qupath.github.io/). The lipid droplets detection was performed using the extension of Cellpose (83) to QuPath (https://github.com/BIOIP/qupath-extension-cellpose).

Ribosome profiling (monosome/disome) of mouse livers
Livers were harvested and flash-frozen in liquid nitrogen. Starting with 200 mg liver pieces from individual animals, lysates and ribosome footprints were generated by RNase I digestion and purified according to previously published protocols (84) with minor modifications. Briefly, 5 μg of RNase I-digested RNA were separated on 15% urea-polyacrylamide gels to excise monosome (~30nt) and disome (~60nt) footprints. From the gel slices, RNA was extracted overnight at 4 °C on a rotating wheel before precipitation in isopropanol during 3h at -20 °C. As described and adapted from (85), RNA 3'-end repair was carried out with 2 U/μl of T4 PNK (Lucigen) prior to a 2 h adaptor ligation at 25 °C using 1.5 μl of ligase mix (final concentration: 0.33 U/μl of T4 RNA Ligase 1 (NEB), 6.66 U/μl of T4 RNA Ligase 2 Deletion Mutant (Lucigen), 1.33 U/μl of riboguard (Lucigen)) and 1 μl of a 20 μM 5'-adenylated DNA adaptor (containing sample barcode and 8N unique molecular identifier (UMI)). Adaptor removal was carried out by treating individual libraries with 2.7 U/μl of 5’deadenylase (NEB) and 1.8 U/μl of RecJf exonuclease (NEB) for 1 h at 30 °C and 1h at 37 °C. Using Zymo Clean & Concentrator columns, samples were purified and pooled. Ribosomal RNA was depleted according to siTools Biotech rRNA depletion kit specifications with a custom-made riboPOOL. The clean-up step was carried out using Zymo Clean & Concentrator columns. Further library preparation steps were performed as described (85). The amplification of libraries was carried out using i5 (AL2 (5’-AATGATACGGCCACACGAGATCTACACATAGAGGCacacTCTTTCCCTACACGACGCTC-3’) or AL3 (5’-AATGATACGGCCACACGAGATCTACACCTATCCTacacTCTTTCCCTACACGACGCTC-3’) (monosomes) and AL4 (5’-AATGATACGGCCACACGAGATCTACACCGGTCTCTGAacacTCTTTCCCTACACGACGCTC-3’) or AL5 (5’-AATGATACGGCCACACGAGATCTACACAGGCGAAGacacTCTTTCCCTACACGACGCTC-3’) (disomes)) and i7 (NexteraD502 or NexteraD503 (monosomes) and NexteraD504 or NexteraD505 (disomes)) primers. Libraries were sequenced on a NovaSeq6000 (Illumina).

Ribosome profiling data analysis
Read mapping was performed following our published protocol (54). Briefly, reads were trimmed from adapter sequence using cutadapt (version: 3.5; options: --match-read-wildcards --overlap 8 --discard-untrimmed --minimum-length 30) and quality filtered using fastx_toolkit (version: 0.0.14; options: -Q33 -q 30 -p 90). UMIs were extracted from each read with UMItools (version: 1.0.0git; options: extract --extract-method string --bc-pattern NNNNNNNNCCCCC --3prime --filter-cell-barcode --error-correct-cell). Then, reads were size-selected for single ribosome (monosomes; size 26 to 37). Subsequently, to estimate rRNA and tRNA contaminations, reads were mapped to human and mouse rRNA and mouse tRNA databases using bowtie2 (version: 2.3.5; options: -p 2 -L 15 -k 20 --trim5 2). Reads that failed to map to these, were then mapped to the mouse transcript database (Ensembl database v. 100). Barcode demultiplexing was carried out with UMItools (options: group --method=directional --per-cell --read-length) and deduplication with an in-house script. Further analyses were restricted to one transcript per gene. Selection was based on APPRIS database (86) primary isoform classification. If selection was not conclusive, the longer transcript with primary annotation was selected. PCA was performed using total RPF counts normalized by library depth of the top-500 most expressed genes (Fig. 5F). The metagene analysis of monosome footprints (Fig. S8A) was done using ChIP-seq tools (version: 1.5.5 (87)). The global codon dwell times analysis (Fig. S8B) was done using the Ribo-DT pipeline (57) on monosome footprint data under chow and
HFHS feeding conditions. Single codon odds ratios (Fig. S8C) were calculated as described (58). Statistical significance was evaluated using a two-sided Fisher’s exact test with Benjamini-Hochberg correction (adjusted p-value < 0.05). Position-specific A-site signals for monosome footprints were generated from the predicted A-sites counts (15 nt from read 5’ end) and normalized by library depth. Sample replicates were aggregated by merging input bam files. For disome data, a similar approach was applied (restricted to footprint lengths 56-63 nt), with A-site defined by an offset of 45 nt from read 5’ end. For visualization purposes and to avoid biases from different sequencing depths, the libraries were subsampled to a common depth between chow and HFHS.

Mouse liver proteomics
Sample Preparation: Mouse livers were immediately snap-frozen after dissection in liquid nitrogen into tissue extraction bags (tissueTUBE TT1, Covaris Inc) and stored at -80 °C. Samples were placed into liquid nitrogen immediately before extraction. Then, the extraction bag was placed in a Covaris CP02 Automated Pulverizer set to impact level 2 to pulverize the frozen sample. This operation was repeated three times. The pulverized samples were poured into a 5 ml Eppendorf tube and solubilized with 3000 μL of boiling lysis buffer (5% SDS, 100 mM Tris, 5 mM TCEP, and 10 mM CAA). The samples were mixed at 1000 rpm for 10 minutes at 95°C. Samples were cleared by centrifugation for 1 minute at 800 g. Afterwards, protein extracts were sonicated on ice for 1 min (1 sec ON, 1 sec OFF, 50 % amplitude). This was followed by centrifugation at 10,000 g for 5 min. Clarified extracts were quantified by BCA, and 2 mg of each sample was digested using PAC method in the KingFisher robot. Digested peptides were desalted with SepPaks and quantified with Nanodrop (A280). 200 μg of resulting peptide was used for subsequent phospho-proteomics enrichment with Zr-IMAC HP beads in the KingFisher robot. Full proteome and phospho-proteomes were analyzed on the Evosep One system using EV-1137 column (PepSep, 15 cm × 150 μm, beads 1.5 um) and EV-1086 emitter (30 μm). The column temperature was maintained at 40 °C using a butterfly heater (PST-ES-BPH-20, Phoenix S&T) and interfaced online using an EASY-Spray source with the Orbitrap Exploris 480 MS (Thermo Fisher Scientific). Data were acquired in Data Independent Acquisition (DIA) mode. Full MS resolution was set to 120,000 at m/z 200 and full MS AGC target was 300% with an IT of 45 ms. Mass range was set to 350–1400. AGC target value for DIA scans was set at 1000%. MS2 scans were acquired using 15,000 resolution and 2 seconds cycle time (49 windows of 13.7 Da), scanning from 361 to 1033 m/z in full proteome and 472 to 1142 m/z in phosphoproteome.

Data processing: MS files were searched using Spectronaut (v18) with a library-free approach (directDIA+) using a mouse database (Uniprot reference proteome 2022 release, 21,984 entries) complemented with common contaminants (246 entries). Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine, acetylation of protein N-termini and, in the case of phosphoproteomics, phosphorylation of serine, threonine and tyrosine were set as possible variable modifications. Maximum number of modifications per peptide was limited to 3, and cross-run normalization was unchecked. Protein quantification was performed using Quant2.0 and Top 3 peptides. PTM identification was turned one and localization probability was set to 0.75. Phosphorylation site collapse was performed using the Perseus Peptide Collapse (v 1.4.4) (88) in Perseus (1.6.5.0) using liner model for aggregation and localization probability cut-off of 0.75. Proteomics and phosphoproteomics data (phospho-site level) were further processed in R (v1.4.3). Data were log2 transformed and filtered out to remove values not measured in at least
three replicates in one experimental condition. Data were normalized by “normalizeCyclicLoess” from limma package (v3.50.3). Data was imported as MSnset format using DAPAR package (v1.26.1) where two-step imputation was performed (89). First, partially-observed values (i.e. values missing in some of the technical replicates within a biological condition) were imputed using “wrapper.impute.slsa” function. Secondly, values missing for an entire condition were imputed using the “wrapper.impute.detQuant” using qval=0.025. Phosphorylation levels were further corrected to account for protein changes. To do so, protein intensities were scaled to the me and the corresponding scaling factor per protein in the proteomic sample was applied to each phosphorylation site of the protein. Phosphorylation sites from proteins not quantified in the proteomics data were not corrected. Differentially regulated protein and phospho-sites were calculated using limma robust analysis, requiring at least three valid values in one condition for each pairwise comparison and p-values were corrected for FDR using Benjamini-Hochberg correction.

Data representation and statistical analysis
All data are plotted as mean and all error bars represent the standard error of the mean (SEM). Statistical testing was performed using t-tests or Mann-Whitney U-test for comparisons between two groups and ANOVA with Tukey post hoc test or Dunnett post hoc test for comparisons with more than two groups. An interaction term was used when test for interaction was significant. For the statistical analysis of ipGTT experiments, the area under the curve (AUC) was calculated and tested using Mann-Whitney U-test for two groups and two-way ANOVA for > 2 groups. ns., non-significant; *, p≤0.05; **, p≤0.01, *** p≤0.001 and **** p ≤ 0.0001.
### Figure S1

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**E**

Menadione

- 1h + MNase
- 2h + MNase

- Mock
- Mena
- Mena + ISRIB

**F**

β-lapachone

- 30 min + MNase
- 1h + MNase
- 2h + MNase

- Mock
- β-lapa
- β-lapa + ISRIB

**G**

Anisomycin (1h) + MNase

- Mock
- Ani (1h)

**H**

ISRIB + MNase

- Mock
- ISRIB

**I**

Menadione

- 30 min no MNase
- 1h no MNase
- 2h no MNase

- Mock
- Mena
- Mena + ISRIB

**J**

β-lapachone

- 30 min no MNase
- 1h no MNase
- 2h no MNase

- Mock
- β-lapa
- β-lapa + ISRIB
**Fig. S1. ROS-induced translation arrest is reversible and associated with stalling and collision of ribosomes.** (A) U2OS cells were pre-treated with N-Acetyl Cysteine (NAC, 10 mM – 1 h) followed by addition of menadione (Mena, 250 µM – 1 h) as indicated. Lysates were analyzed by immunoblotting with the indicated antibodies. (B) U2OS cells were treated with increasing concentrations of auranofin (0.5, 1, 2.5, 5, 7.5, 10 µM – 1 h). Lysates were analyzed as in (A). (C) U2OS cells were pre-treated with NAC (10 mM – 1 h) followed by addition of auranofin (Aura, 5 µM – 1 h) or anisomycin (Ani, 1 µg/ml – 1 h) as indicated. Puromycin (10 µg/ml) was added to the culture 10 min prior to harvest and lysates were analyzed as in (A). (D) U2OS cells were treated with auranofin (5 µM – 1 h) followed by washout (WO) in the presence of NAC (10 mM) for the indicated times. Cells were pulse-treated with puromycin (10 µg/ml, 10 min) before harvest as in (C). Lysates were analyzed as in (A). (E) U2OS cells were treated with menadione (250 µM) for the indicated times in the presence or absence of the Integrated Stress Response inhibitor ISRIB (200 nM). Lysates were digested with micrococcal nuclease (MNase), separated on a linear sucrose gradient and ribosomes were detected by UV spectrophotometry. (F) As in (E), except that cells were treated with β-lapachone (20 µM) for the indicated times in the presence or absence of ISRIB (200 nM). Arrows highlight peaks that are indicative of increased ribosome collision. (G) As in (E), except that cells were treated with ribosome collision-inducing anisomycin (1 µg/ml – 1 h) in absence of ISRIB. (H) As in (E), except that cells were treated with ISRIB alone (200 nM – 1 h). (I) U2OS cells were treated with menadione (250 µM) for the indicated times in the presence or absence of ISRIB (200 nM). Lysates were separated without prior MNase digestion on a linear sucrose gradient and ribosomes were detected by UV spectrophotometry. (J) As in (I), except that cells were treated with β-lapachone (20 µM) and ISRIB (200 nM – 1 h).
Fig. S2. ROS does not induce widespread ribosome collision and predominantly activates the Integrated Stress Response via PERK. (A) U2OS cells were treated with menadione (250 µM – 1 h) or anisomycin (1 µg/ml – 1 h) as indicated. Lysates were ultra-centrifuged through a sucrose cushion. Input lysates and pelleted material were analyzed by immunoblotting with the indicated antibodies. (B) as in (A), except that HAP1 cells were used. (C) U2OS cells were treated with auranofin (Aura, 5 µM – 1 h) and inhibitors (i, 1 µM) against PERK and GCN2 as indicated. Lysates were analyzed as in (A). (D) Activation of ZAKα and downstream kinases p38 and JNK occurs in response to stalled as well as collided ribosomes. (E) WT U2OS cells or U2OS cells deleted for ZAK (ΔZAK) were treated with thapsigargin (thaps, 1 µM – 1 h). Lysates were analyzed as in (A). (F) U2OS cells were treated with thapsigargin (1 µM – 1 h) or anisomycin (1 µg/ml – 1 h) as indicated. Puromycin (10 µg/ml) was added to the culture 10 min prior to harvest and lysates were analyzed as in (A). (G) U2OS cells were pre-treated with N-Acetyl Cysteine (NAC, 10 mM – 1 h) followed by addition of thapsigargin (1 µM – 1 h) as indicated. Lysates were analyzed as in (A). (H) HeLa cells were pre-treated with NAC (10 mM – 1 h) and followed by addition of menadione (250 µM – 1 h) or anisomycin (1 µg/ml – 1 h) as indicated. Cells were pulse-labeled with puromycin (10 µg/ml, 10 min) as in (F) and lysates were analyzed as in (A). (I) HeLa cells were pre-treated with puromycin (10 µg/ml, 10 min) and puromycin (10 µg/ml, 10 min) as in (F) and lysates were analyzed as in (A). (J) Drop-out control reactions for tri-partite in vitro translation (IVT) assay. Ribosomes and ribosome-free cytoplasm were isolated from cultured HeLa cells and combined with in vitro transcribed luciferase mRNA to perform IVT. Luciferase activity is obtained only when all three fractions are combined (n=3 biological replicates).
Fig. S3. Menadione induces tRNA cleavage in cells. (A) U2OS cells were pre-treated with NAC (10 mM – 1 h) and treated with menadione (250 µM – 1 h) as indicated. Oxidized thiols were labelled with biotin (Biotin-HPDP) by blocking free, reduced thiols prior to reduction of oxidized thiols via DTT. Biotin-labeled proteins were pulled down (PD) using streptavidin-beads. Input lysates and PD material were analyzed by immunoblotting with the indicated antibodies. (B) Northern blot for tRNA-Leu-CAA, (C) tRNA-Gly-GCC and (D) tRNA-iMet-CAT on whole cell RNA isolated from HeLa cell fractions from Figure 2G separated on a urea agarose gel. A northern probe against 5S rRNA was used as a loading control. Schematic of tRNA intermediates corresponding to distinct bands are shown on the left side of the blots. (E) As in (B)-(D), except that a probe against tRNA-Arg-TCT was used and a deacylated RNA sample was added. (F) U2OS cells were treated with menadione (250 µM – 1 h) for the indicated times. Puromycin (10 µg/ml) was added to the culture 5 min prior to harvest and lysates were analyzed as in (A). Per SO2/3, an oxidized form of peroxiredoxin. (G). U2OS cells were pre-treated with NAC (10 mM – 1 h) or kinase inhibitors (ZAKi, ASK1i – 2 µM) and treated with H2O2 (5 mM – 15 min) as indicated. Lysates were analyzed as in (A). (H) Top: Genomic structure of the human MAP3K20 (ZAK) gene, giving rise to ZAKα and ZAKβ transcripts through alternative splicing. Bottom: Genomic structure of the distinct zebrafish (Danio rerio) zakα and zakβ genes. (I) qPCR analysis of zakα and zakβ expression in WT and mutated zebrafish lines at 3 days post fertilization (dpf). Data are plotted as mean and error bars represent the standard error of the mean (SEM) (n=6 biological replicates). ns., non-significant; *, p≤0.05; **, p≤0.01, ***, p<0.001, **** p<0.0001 in two-way ANOVA with Dunnett post hoc test. (J) Reported expression levels of ZAKα and ZAKβ transcripts (Map3k20) and ASK1 (Map3k5) gene in human liver. TPM, normalized Transcript Per kilobase Million. Values extracted from gtxexportal.org. (K) Log10-transformed iBAQ values in different tissues for MAP3K5 (ASK1) (green) and MAP3K20 (ZAK) (blue) proteins obtained from a recently curated mouse proteome dataset. Liver, intestines, and adipose tissues are highlighted in yellow. iBAQ, intensity-based absolute quantification.
Fig. S4. Body composition and glucose uptake of WT and ZAK<sup>−/−</sup> mice fed a High-Fat High-Sugar diet. (A) Total body weight of mice from feeding experiment in Figure 4A. (B) Accumulative food intake of mice in Figure 4A. (C) Total fat mass from mice in Figure 4A as determined by MR scanning. (D) Total lean mass in mice from Figure 4A as determined by MR scanning. (E) Basal blood glucose levels at times of Intraperitoneal Glucose Tolerance Test (ipGTT) in mice from Figure 4A. X-axis indicates the time after switching to High-Fat High-Sugar (HFHS) diet. (F) Basal insulin levels of mice from Figure 4A at the 19-week timepoint. (G) Liver weight from mice in Figure 4A. Values indicate tissue weight as a percentage of whole-body weight. (H) Serum triglyceride concentrations of mice in Figure 4A. (I) Schematic of experiment for Intraperitoneal Insulin Tolerance Test (ipITT) and glucose uptake in mice. 10-12-week-old male WT (n=12) and ZAK<sup>−/−</sup> (n=7) mice were fed HFHS diet for 10 weeks before being subjected to a glucose uptake assay with radioactive 2DG. Mice were additionally subjected to IpITT assay after 8 weeks of feeding. (J) Glucose uptake measurements in mice in various tissues measured by scintillation counting of tritium. 2DG is an unphosphorylated and 2DG6P is a phosphorylated form of glucose, respectively. All data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-significant; *, p≤0.05; **, p≤0.01; ***, p<0.001, **** p≤0.0001 in Welch’s t-test in (F, G, H, J) or Mann-Whitney U-test (J-ipGTT) for two groups and two-way ANOVA with Tukey post hoc test for > 2 groups in (C, D). For ipGTT in (J), statistical analysis is based on the area under the curve (AUC) for each experimental group.
A

Rodent chow
High Fat – High Sugar Diet

Group housing

HFHS start or kept on chow

B

Locomotor activity
Average per day

ns

Day

Night

All Meters

W T Chow
ZAK−/− Chow
ZAK−/− HFHS

C

Energy expenditure vs body weight

Average EE (kcal/hour)

Body weight (g)

W T Chow
ZAK−/− Chow

D

Energy expenditure vs body weight

Average EE (kcal/hour)

Body weight (g)

W T HFHS
ZAK−/− HFHS

E

Oxygen consumption (VO₂)

VO₂ (ml/min)

24h

W T Chow
W T HFHS
ZAK−/− Chow
ZAK−/− HFHS

F

Carbon dioxide production (VCO₂)

VCO₂ (ml/min)

24h

W T Chow
W T HFHS
ZAK−/− Chow
ZAK−/− HFHS

G

Respiratory exchange ratio

Respiratory exchange ratio

24h

W T Chow
W T HFHS
ZAK−/− Chow
ZAK−/− HFHS
Fig. S5. Metabolic cage studies indicate enhanced respiratory exchange ratio in chow-fed ZAK<sup>−/−</sup> mice. (A) Schematic drawing of mice subjected to indirect calorimetry in metabolic cages. 10-12-week-old male WT and ZAK<sup>−/−</sup> mice were fed either chow (n=6,6) or HFHS diet (n=8,9) for 6 weeks before being moved to metabolic cages. (B) Average distance traveled over the period of 7 days determined by beam breaks. Data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-significant; *, p≤0.05 in two-way ANOVA with Tukey post hoc test. (C) Average energy expenditure (EE) versus body weight (g) of mice fed chow diet. (D) As in (C), except for mice fed HFHS. (E) Oxygen consumption rates (VO<sub>2</sub>), (F) carbon dioxide release (VCO<sub>2</sub>) rates and (G) Respiratory Exchange Ratio (RER) for mice in (A). (E, F, G), data from first three days were excluded. Gray and light bars represent dark and light periods, respectively.
Fig. S6. NAC supplementation phenocopies ZAK knockout with respect to glucose tolerance but not hepatic steatosis. (A) Schematic of mouse experiment combining HFHS-feeding and supplementation of N-Acetyl Cysteine (NAC, 10 g/l) in the drinking water. 10-12-week-old male WT (n=8,6) and ZAK−/− (n=7,7) mice were used. Timing of ipGTT assay and euthanasia is indicated (weeks). (B) Percent body weight gain of mice from (A). Arrows mark the time of ipGTT assay and resulting transient weight loss. (C) Blood glucose concentrations of mice from (A) subjected to ipGTT assay at the 5-week timepoint. Statistical analysis is based on the area under the curve (AUC) for each experimental group. (D) As in (E), except that results from the 10-week time point are shown. (E) Basal glucose levels, (F) basal insulin levels and (G) HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) of mice from (A) - 10-week time point is shown. (H) Images of representative hematoxylin and eosin (H/E)-stained liver section from male mice in (A). Arrows indicate areas of steatosis. (I) Scoring of liver steatosis grade (scale 0 – 4) of male mice from (A), (B, C, D, E, F, G). All data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-significant; *, p≤0.05; **, p≤0.01 in two-way ANOVA with Tukey post hoc test for > 2 groups. All scale bars = 50 µm.
**Fig. S7. Proteomic analysis of mouse livers.** (A) Principal Component Analysis (PCA) of the proteomic profiles for total proteins of mouse livers from Figure 5A. (B) Changes in phosphorylation sites annotated as “regulating activity” in several MAPK, MAP2K and MAP3Ks from analysis in Figure 5A. Color of data points indicate relative change, and area of the point the statistical significance of the ratio (log2-transformed fold change, limma robust analysis, Benjamini-Hochberg FDR correction, 3 valid values required in one condition) between ZAK−/− and WT mice. (C) Barplots of intensities derived from MS2 data for activation-associated phosphorylation sites on MAP2K4, MAP2K6 and MAPK14 (p38α). Height of the bars represent the average of the measurements and error bars the standard error. Missing values in the MS data are plotted as zero values. (D) Immunohistochemical detection of activated JNK (p-JNK) in steatotic areas in a liver from a HFHS-fed WT mice from cohort in Figure 4A. Scale bar = 50 µm. (E) Schematic of mouse experiment combining 16 weeks of feeding HFHS or chow diet to 10-12-week-old male WT (n=6,6) and ZAK−/− (n=5,4) mice followed by overnight fasting prior to euthanasia. The experiment included infrared imaging for BAT (brown adipose tissue) temperature at 13 weeks. (F) Left: Lysates of livers from (E) were analyzed by immunoblotting with the indicated antibodies. Right: Quantification of p-JNK divided by total JNK signals in immunoblotting analysis from (left). Values indicate the average background corrected signal. Data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-significant in two-way ANOVA and Tukey post hoc test for > 2 groups. HFHS, High-Fat High-Sugar.
Fig. S8. Ribosome profiling of monosome and disome footprints from mouse livers. (A) Metagene analysis of ribosome footprint data, showing the predicted A-site distribution around start and stop codons for chow (gray) and HFHS (pink) conditions. Counts from replicates were aggregated; data normalized by total library depth. (B) Analysis of global codon dwell times inferred from monosome footprint data under chow (top) and HFHS (middle) feeding conditions, using the Ribo-DT pipeline. The comparison of HFHS vs. chow ratios (lower) indicates that transcriptome-wide there are no prominent changes in dwell time associated with specific codons or amino acids in the E/P/A-sites. (C) Specific sites with changes in relative ribosome occupancy (monosomes) in livers of HFHS-fed vs. chow-fed animals. Codon positions with significantly increased and decreased pause scores (adjusted p < 0.05) are indicated in orange (n=150) and ochre (n=160), respectively; all other translatome positions in gray. Statistical analysis was performed using a two-sided Fisher’s exact test with Benjamini-Hochberg correction. Left side shows odds ratio vs. adjusted p-value; right side: odds ratio vs. mean.
footprint signal at the positions, allowing for an evaluation of whether sites are highly/lowly translated. (D) Position-specific A-site signal for monosome (+15 nt position; all footprints from 26-37 nt length analyzed) and disome footprints (+45 nt position of 56-63 nt footprints) across the Albumin (Alb) mRNA for chow-fed (gray) and HFHS-fed conditions (pink). Signal height is presented on the same linear scale for chow and HFHS, with boundaries indicated to the right of tracks. Sample replicates were aggregated to depict a single track per feeding condition. Different library depths were compensated by subsampling to a common depth between chow and HFHS. Below monosome/disome tracks, positions of differential codon sites are depicted, with height corresponding to odds ratio values, and same color coding as in panel C. (E) Weight of “old” male mice in Figure 6A at termination. (F) Weight of “old” female mice in Fig. 6A at termination. (G) Intraperitoneal Insulin Tolerance Test (ipITT) in male mice from Fig. 4A. Statistical analysis is based on the area under the curve (AUC) for each experimental group. Data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-significant; *, p≤0.05; **, p≤0.01; in Welch’s t-test in (E, F) and Mann-Whitney U-test in (G).
Fig. S9. Livers from High Fat-High Sugar-fed mice contain high amounts of the ROS-marker 4-HNE. (A) Whole-mount scans of 4-HNE (brown) and hematoxylin (blue) – stained livers from male young, chow-fed, (B) male HFHS-fed and (C) male aged chow-fed WT mice (cohorts in Fig. 4A and Fig. 6A). Note the absence of localized 4-HNE staining in (A), focal aggregates in (B) and increased staining around portal veins (PV) and in steatotic areas in (C). Scale bars = 100 µm.
Fig. S10. Analysis of structure of brown adipose tissue in aging and obesity. (A) Whole-mount scans of hematoxylin and eosin (H/E) – stained BAT (brown adipose tissue) lobes from young (top), aged (middle) and HFHS-fed (bottom) male WT and ZAK\textsuperscript{−/−} mice from cohorts in Fig. 6A and Fig. 4A.
Fig. S11. Analysis of function of brown adipose tissue in obesity. (A) Segmentation of H/E-stained samples from Fig. S11A to demarcate individual lipid droplets (red). Genotype-associated lipid droplet size distribution of aged WT and ZAK<sup>-/-</sup> mice are shown in Fig. 6I. (B) Whole mount BAT scans of livers from a subset of HFHS-fed mice (cohort in Fig. 4A) were imaged and analysed as in (A) (n=6). (C) Average skin temperature surrounding BAT in mice from Fig. S7E. (D) Representative infrared thermal images used for quantification in (C). All data are plotted as mean and all error bars represent the standard error of the mean (SEM). ns., non-significant; *, p≤0.05; **, p≤0.01; ***, p<0.001, **** p≤0.0001 in a two-way ANOVA with Tukey post hoc test for > 2 groups.
Table S1  
Proteomic and phosphoproteomics analysis of mouse livers  
Protein and phospho-peptide intensities from experiment in Fig. 5A-C; Fig. S7A-C. Sheet (A). Log2-transformed protein intensities for the ZAK-KO samples. Sheet (B). Log2-transformed protein intensities for the WT samples. Sheet (C). Log2-transformed phosphorylation site intensities for the ZAK-KO samples. Sheet (D). Log2 phosphorylation site intensities for the WT samples. Data were filtered to contain only proteins/phospho-peptides with 3 valid values in one condition, then normalized and missing values were imputed. Phosphorylation site abundance (Sheet (C), (D)) were corrected for changes in total protein abundance.

Table S2  
Differential analysis of corrected phosphorylation site intensities from mouse livers  
Comparison of phosphorylation sites based on values in Table S1. Sheet (A). Statistical comparison of corrected phosphorylation site intensities for ZAK KO mice fed HFHS vs. chow diet. Sheet (B). Statistical comparison of corrected phosphorylation site intensities for WT mice fed HFHS vs. chow diet.

Table S3  
Per-gene quantification of total signal for monosome/disome reads and specific codon sites.  
Referring to Fig. 5G.
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