Dietary restriction (DR) during adulthood can greatly extend lifespan and improve metabolic health in diverse species. However, whether DR in mammals is still effective when applied for the first time at old age remains elusive. Here, we report results of a late-life DR-switch experiment using 800 mice. Female mice aged 24 months were switched from an ad libitum (AL) diet to DR or vice versa. Strikingly, the switch from DR to AL acutely increases mortality, whereas the switch from AL to DR causes only a weak and gradual increase in survival, suggesting the body has a memory of earlier nutrition. RNA sequencing in liver and brown and white adipose tissue (BAT and WAT, respectively) demonstrates a largely refractory transcriptional and metabolic response in fat tissue to DR after an AL diet, particularly in WAT, and a proinflammatory signature in aged preadipocytes, which is prevented by chronic DR feeding. Our results provide evidence for a 'nutritional memory' as a limiting factor for DR-induced longevity and metabolic remodelling of WAT in mammals.
refractory to late-life DR, which coincided with major age-related shifts in white adipose progenitor cells. Both switch-resistant genes and lipidomic profiles of WAT pointed to impaired membrane lipogenesis and mitochondrial biogenesis in response to late-life DR. A nutritional memory thus limited both increased survival and metabolic remodelling of WAT in response to DR imposed late in life.

**Results**

**Acute mortality shift in response to late-onset ad libitum, but not late-onset dietary restriction, in mice.** We conducted a diet-switch experiment in mice (Fig. 1a) using 800 females of the B6D2F1 hybrid strain, which show a robust lifespan extension under DR feeding.22,24. This large number of animals enabled profiling of age-specific mortality. Animals were bred in three breeding rounds. DR began when mice were at 12 weeks of age, with stepwise restriction over 4 weeks until DR mice reached 40% of the food intake of AL controls. A subset of the AL and DR animals was subjected to a diet switch after 20% of the AL-fed animals had died, corresponding to 24 months of age at the onset of the diet switch (721–746 d, depending on breeding cohort); we estimated that this subset would have >95% power to detect a reversal in mortality rates over a 6-month period and maximized statistical power to detect changes in mortality rate. Half of the AL cohort was subjected to stepwise DR over 4 weeks (late-onset DR; ALDR), while half of the chronic-DR-fed...
mice received a reciprocal food increase back to the level of AL controls (late-onset AL; DRAL). Both diet switches caused weight gain and loss equivalent to what was seen with chronic AL or DR feeding, respectively, reaching the level of the chronic diet groups within 8 months following the switch (Fig. 1b). The rate of weight gain of old DRAL mice was highly similar to that observed in young AL animals (Extended Data Fig. 1a). While absolute food intake was comparable between old AL and DRAL mice (Extended Data Fig. 1b,c) relative to their lower body weight, DRAL animals exhibited a slightly higher food intake (Extended Data Fig. 1d). Thus, 21 months of DR feeding did not permanently lower the endogenous food intake target of these mice.

Animals that switched to DR showed only a delayed and incomplete reduction in mortality rate compared with chronic DR mice (Fig. 1c–e and Extended Data Fig. 1b). For the first 7 months after the switch, during which their median lifespan was passed, ALDR mice showed no significant improvement in mortality (Fig. 1d,e). When analysing survival data for the whole duration of the experiment, two out of three breeding cohorts showed no significant response to the ALDR switch. (Extended Data Fig. 1b,c). Therefore, late-onset DR caused no measurable increase in survival in a large fraction of old animals. In stark contrast, the reciprocal switch from DR to unrestricted feeding caused an acute increase in mortality in all three breeding cohorts (Fig. 1d,f and Extended Data Fig. 2b,c).

Fig. 2 | Post-switch weight change correlates with survival outcome under late-onset DR. a, Scatterplot representation of mouse-specific rates of weight change versus mouse-specific age at death for both ALDR and DRAL switch cohorts. Linear regression analysis found a significant association for weight change and age at death for the ALDR switch cohort. Animals with a weight loss higher than 0.2 g d\(^{-1}\) are marked with an X. b, Scatterplot representation of mouse-specific weights at switch date versus mouse-specific age at death for both ALDR (green) and DRAL (orange) switch cohorts. Linear regression found no significant association. Animals with a weight loss higher than 0.2 g d\(^{-1}\) are marked with an X. n = 45 biologically independent animals per diet.

Preservation of body weight associates with late-onset dietary restriction outcome. The strong effect of a history of AL feeding on subsequent mortality under DR could indicate the presence of a physiological memory that may impede the molecular changes mediating the benefits of DR. Interestingly, there was a significant inverse association between the animal-specific rate of weight change and age at death for the ALDR but not DRAL cohort (Fig. 2a). There was, however, no association between the absolute weight pre-switch and lifespan in either of the switch groups (Fig. 2b). In agreement with previous findings on chronic DR regimens\(^{20,21}\), preservation of weight and, specifically, fat may thus increase the responsiveness of survival to late-onset DR, implicating a role for lipid metabolism.

Brown and white adipose tissue, but not liver, show a transcriptional memory of ad libitum feeding. In light of a possible memory effect of AL feeding on lipid metabolism, we next investigated the molecular memory of AL feeding in liver, BAT and gonadal WAT, which fulfil key functions in lipid turnover and storage. Tissues were sampled 2 months post-switch, when the effects on mortality were the most disparate between the two switch diets (compare Fig. 1e–g).

In contrast, body (Fig. 1b) and adipose weights (Fig. 3b,c) indicated that the two diet switches already had caused comparable changes in fat tissue mass at this time point (Fig. 3a). RNA sequencing (RNA-seq) profiling revealed high transcriptional similarity between DRAL mice and chronic AL controls in all three tissues, indicating that late-onset AL feeding induced a transcriptional profile similar to chronic AL feeding. Similarly, hepatic transcriptional profiles from ALDR mice clustered with the DR controls. In strong contrast, in BAT, and even more in WAT, ALDR profiles clustered with those of the AL diet (Fig. 3c,d), and were thus resistant to the diet switch. Chronic DR caused significant gene expression changes in 3,569 genes in liver, 2,412 in BAT and 3,296 in WAT, when compared with expression in AL controls. Of these, only 62 genes (~2%) in the liver, but 866 genes (~35%) in the BAT and a total of 1,609 genes (~50%) in the WAT, were still differentially expressed between DR and ALDR mice 2 months post-switch (Fig. 3e). These ‘switch-resistant’ genes in the adipose tissues are candidates for a transcriptional memory of AL feeding. In DRAL switch mice, we detected only 22 (0.8%) switch-resistant genes in the liver, 19 (0.08%) in BAT and 423 (~13%) switch-resistant genes in the WAT (Fig. 3i).

To analyse the transcriptional similarity between chronic and newly DR animals, we focused on genes that were differentially up- or downregulated under chronic DR (Fig. 3g). Plotting for each gene the scaled expression in response to chronic or late-onset DR confirmed an almost complete transcriptional adaptation to the ALDR switch in the liver, while both adipose tissues remained largely refractory (Fig. 3g). Corresponding to the acute rise in mortality, DRAL mice broadly adopted the expression profile of chronic AL fed animals across tissues, as did ALDR mice in the liver. (Fig. 3g). In addition, unsupervised hierarchical clustering revealed that ALDR switch-resistant genes in adipose tissues were not resistant in general, because their expression adopted an AL-like pattern under the reciprocal DRAL switch (Fig. 3h and Extended Data Fig. 3a). Thus, the liver transcriptome remained acutely responsive to either diet change, whilst the adipose tissue was specifically unresponsive to late-onset DR.

The incomplete reprogramming of RNA expression in response to late-onset DR could simply indicate that the adipose tissues respond slowly to DR, which would argue against the presence of a specific memory of AL feeding. To investigate this possibility, we repeated the experiment in young mice by switching AL-fed
animals to DR (young ALDR), starting when mice were aged 12 weeks (Fig. 3). As in the experiment in old mice, tissues were collected 2 months post-switch for expression profiling. Strikingly, there was a complete transcriptional reprogramming in all three tissues (Fig. 3) and Extended Data Fig. 3c,d). Genes that were resistant to the switch in old ALDR mice showed full sensitivity in young ALDR mice. Thus, the adipose tissue transcriptome was highly responsive to newly DR switches in young mice, but this transcriptional flexibility markedly declined with age, in particular in the WAT.

Taken together, switching mice from DR back to full feeding caused a rapid loss of DR-related RNA expression patterns, implying no or only a weak memory of a prior DR regimen. In contrast, chronic AL feeding caused the formation of an adipose-tissue-specific gene expression memory over time. This reduced transcriptional flexibility of BAT and WAT in response to DR mirrored the resistance of age-specific mortality to late-onset DR.

**Chronic ad libitum feeding causes a proinflammatory expression pattern in preadipocytes.** Age-related changes have been previously identified to impair the differentiation of WAT preadipocytes into brite adipocytes under cold exposure in aged mice, and we hypothesized that a similar process could contribute to the reduced transcriptional flexibility of the WAT in old AL mice. To test this hypothesis, we obtained publicly available single-cell (sc) RNA-seq data of the gonadal stromal-vascular-fraction (SVF) from the Tabula muris consortium, comprising cells isolated from 3- and 24-month-old AL-fed mice. Indeed, preadipocytes from old mice exhibited profound transcriptional shifts, including decreased expression of growth and differentiation factors and elevated expression of inflammatory response genes and secretable cytokines, such as C–C motif chemokine ligand 2 (CCL2) and (CCL7) (Extended Data Fig. 4a–d). We further found a significant, inverse association between the gene expression changes in aged preadipocytes and those observed under DR with bulk RNA-seq. This association was still significant when limited to ALDR switch-resistant genes, which were functionally enriched for inflammatory response genes and chemokines (Extended Data Fig. 4e,f and Supplementary Table 1). Many of these genes were found to be particularly, or even exclusively, expressed in preadipocytes (Extended Data Fig. 4g,h). Thus, expression patterns in bulk RNA-seq may indeed reflect those found by scRNA-seq. Our analysis thus strongly suggests that preadipocytes lower the expression of growth and differentiation factors associated with WAT plasticity while acquiring an inflammatory phenotype during ageing under AL-diet conditions. This phenotype appears to be strongly prevented by DR, but not by late-onset DR. The transcriptional memory of AL diet may thus be, in part, rooted in preadipocytes.

**Mitochondrial biogenesis in white adipose tissue is impaired under late-onset dietary restriction.** We next investigated the molecular pathways affected by the transcriptional memory of AL feeding because these could point to mechanisms that improve health under chronic DR. Whereas analysis of switch-resistant genes in the liver showed no major gene ontology enrichment (Extended Data Fig. 5a), the corresponding analysis in BAT revealed increased lipid transporter activity and largely downregulated mitochondrial function, including markers of uncoupling-dependent thermogenesis (Fig. 3k, Extended Data Fig. 6a–d and Supplementary Table 2). Switch-resistant genes in WAT showed the strongest functional enrichment for various mitochondria-related pathways, fatty-acid metabolism and phospholipid biosynthesis, all of which were upregulated under chronic and young ALDR but not old ALDR feeding (Fig. 3k and Supplementary Table 3). In addition, we identified a switch-resistant reduction of inflammatory response and interferon-gamma-related genes. The increase in mitochondrial gene expression in WAT contrasts with the downregulation of the same gene set in BAT, indicating that the declining transcriptional flexibility acts in a tissue-specific manner.

To test whether the shifts in gene expression could be correlated with functional consequences, we characterized the predicted switch-resistant upregulation of mitochondrial function in the WAT because this tissue exhibited the most switch-resistant genes and strongest functional enrichment. Indeed, genes associated with mitochondria in WAT (both nuclear and mitochondrially encoded genes) showed globally increased expression under chronic DR compared with that under either switch diet (Fig. 4a). Furthermore, expression of the transcription factor peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), a key driver of mitochondrial biogenesis, was significantly increased in chronic DR and in young ALDR mice, but was resistant to the ALDR switch (Fig. 4b). Consistent with this observation, chronic DR increased abundance of mitochondrial DNA (mtDNA), protein levels of mitochondrial complex I and IV subunits (mtCO1; NDUF9) and key mitochondrial metabolites (propionyl- and succinyl-CoA), all of which were resistant to the ALDR switch (Fig. 4c–e and Extended Data Fig. 6e). Of note, expression levels of various thermogenic processes such as UCP1 (uncoupling), CKMT1 (creatine cycling) and SERCA2b (Ca2+ cycling) were unaffected by diet (Fig. 4f,g and Extended Data Fig. 6 f–h). Remarkably, all parameters of mitochondrial activity reverted back to the level of AL controls in the DRAL switch (Fig. 4b–e), in line with only weak messenger RNA expression differences between chronic and late-onset AL and only a small fraction of the DR-related transcriptional program still being active (Fig. 3f–h, Extended Data Fig. 6l and Supplementary Table 4). Long-term AL feeding thus interfered with elevated, uncoupling-independent mitochondrial biogenesis and activity in the WAT in response to late-onset DR.

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**Fig. 3 | Detection of an age- and tissue-specific transcriptional memory of prior AL feeding.** a. Organ weight of gonadal fat pads at dissection time point (805 d). One-way ANOVA followed by two-sided post-hoc Tukey test; n=10 biologically independent animals per diet. Means±s.e.m. b–d. Principal component analysis plot of RNA-seq data in liver (b), BAT (c) and gonadal WAT (d). e, f. Venn diagrams depicting the overlap of differentially expressed genes in liver (left), BAT (middle) and WAT (right) under DR feeding (e), relative to the AL or ALDR group; and under AL feeding (f), relative to the DR or DRAL groups. Switch-resistant genes are highlighted in red. g. Boxplot representation of scaled expression levels of differentially up- and downregulated genes under chronic DR as opposed to chronic AL controls in all three tissues. Boxplots indicate expression levels of ALDR switch-resistant genes. Whiskers represent the 1st and 5th quartiles, box edges represent the 2nd and 4th quartiles and the centre line represents the third quartile/median. h. Heatmap of unsupervised clustering of expression changes for ALDR switch-resistant genes in WAT; colour bar represents z score range. i. Schematic representation of the DR switch experiment in young mice. j. Boxplot representation of scaled expression levels of differentially up- and downregulated genes under chronic DR as opposed to levels in chronic AL controls in BAT and WAT of ALDR switch mice at a young age (5 months, 5m). Boxplot components are defined in g, k. Representative GO enrichment of DR switch-resistant genes in BAT (left) and WAT (right). Lengths of bars represent negative In-transformed P using two-sided Fisher’s exact test. Colours indicate gene-wise log, fold-changes (log, FC)) between DR and ALDR mice. Numbers beside bars indicate differentially expressed genes in that GO category. The complete list of enriched GO terms can be found in Supplementary Tables 2 and 3. Biologically independent animals used for RNA-seq: liver: n=3 (AL, DR, ALDR, DRAL, ALDR 5m, AL 5m); BAT: n=3 (AL, DR, DRAL, ALDR, ALDR 5m, AL 5m) n=5 (ALDR); WAT: n=3 (AL, DR, ALDR, AL 5m) n=5 (ALDR, DRAL). **P < 0.0001.
De novo lipogenesis in white adipose tissue exhibits the strongest memory of prior ad libitum feeding. In order to find putatively causal mediators of the transcriptional memory in the WAT, we plotted for each gene the log2-fold expression change in response to chronic or late-onset DR relative to chronic AL and ranked each gene’s influence on the resulting linear fit. The 50 switch-resistant genes with the highest influence (as determined via Cook’s distance; Fig. 5a,b) were enriched primarily for lipid metabolism, including fatty acid (FA) biosynthesis, TG metabolism and phospholipid biosynthesis (Fig. 5c). They included the key lipogenic transcription factor SREBF1 (ref. 34) and several of its corresponding downstream targets (Fig. 5d,e), AclY, Acaca (Acc), Fasn, Scd1 and Elovl6, which...
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Fig. 4 | WAT-specific impairment of mitochondrial biogenesis under late-onset DR. a, Distribution of gene-wise expression changes in WAT under chronic DR and switch diets relative to chronic AL feeding for genes associated with the GO term ‘Mitochondrion’ (n=1299 genes). Whiskers represent the first and fifth quartiles, box edges represent the second and fourth quartiles and the centre line represents the third quartile/median. Two-sided Wilcoxon rank-sum test, adjusted for multiple testing. b, Ppargc1a (encoding PGC1α) mRNA expression (RNA-seq, normalized (norm.) counts) in WAT. Two-sided Wald test, adjusted for multiple testing. c, mtDNA copy number in WAT. One-way ANOVA, two-sided Tukey post hoc test. d, Western blot analysis of mtCO1 and NDUF9A in WAT, with α-tubulin used as a loading control. One-way ANOVA, two-sided Tukey post hoc test. e, Propionyl- and succinyl-CoA levels in WAT. One-way ANOVA, two-sided Tukey post hoc test. f, mRNA expression (RNA-seq) of thermogenic marker genes in WAT. Two-sided Wald test, adjusted for multiple testing. g, Propionyl-CoA and Succinyl-CoA. h, Lipid level (pg per mg tissue). One-way ANOVA, two-sided Tukey post hoc test. Biologically independent animals used: RNA-seq: n = 3 (AL, DR, ALDR, AL 5m); western blot, n = 3 (AL, DR, ALDR, AL 5m); Lipidomics: n = 4 per diet. Data are means ± s.e.m., ***p < 0.0001.

code for key enzymes in FA synthesis, desaturation and elongation45. Their expression was strongly upregulated (some more than ten-fold, (Figs. 4g and 5c) in chronic DR and in young ALDR mice, but they remained largely refractory to the ALDR switch at old age.

We next explored the metabolic consequences of these changes in gene expression, by full liquid chromatography–tandem mass spectrometry (LC–MS/MS) profiling of the WAT lipidome. This allowed quantification of 516 lipid species and 32 different classes with a dynamic range of ~5x10^6, including the major neutral lipid, (lyso-)phospholipid and sphingolipid classes46. Our dataset thus permitted a global and unbiased analysis of cellular lipid dynamics (Supplementary Table 5). Consistent with transcriptional upregulation of de novo lipogenesis, we detected elevated levels of free FAs in chronic DR and young ALDR mice, including intermediate metabolites of FA synthesis, palmitate and palmitoleate (Fig. 5f–h). In contrast, late-onset DR did not cause a similar increase in FAs, while DRAL mice had FA levels lowered to those of chronic AL controls. These results suggest that imperfect activation of lipogenesis is a direct consequence of the transcriptional memory in the WAT of old ALDR mice.

Chronic dietary restriction causes white adipose tissue autonomous reprogramming of phospholipid synthesis. To better understand the possible functions of newly synthesized FAs in the WAT...
Fig. 5 | WAT-specific transcriptional memory predicts impaired activation of de novo lipogenesis under late-onset DR. a, Scatterplots depicting the expression change for each gene under DR or ALDR feeding (relative to AL) in WAT. The top 50 switch-resistant genes are highlighted in red. b, Gene-wise Cook’s distances from weighted linear regression analysis of expression changes under DR or ALDR feeding (relative to AL) in WAT. Genes were given an arbitrary number. c, GO enrichment of the top 50 ALDR switch-resistant genes. Colours indicate gene-wise log fold-changes (log2(FC)) between DR and ALDR mice. Lengths of bars represent negative ln-transformed P values, determined using two-sided Fisher’s exact test. Numbers beside bars indicate numbers of differently expressed genes in that GO category. d, Srebf1 mRNA expression (RNA-seq). e, mRNA expression (RNA-seq) of key de novo synthesis genes in WAT. Two-sided Wald test, adjusted for multiple testing. f–h, Abundance of the total pool of free fatty acids (f), palmitate (g) and palmitoleate (h) in WAT. One-way ANOVA followed by two-sided post-hoc Tukey test. i, Heatmap of unsupervised clustering of abundance changes for measured lipid classes in WAT; color bar represents z score range. Black box highlights lipids with induction specifically in young ALDR mice. FACN, fatty acyl carnitine; SM, sphingomyelin; PI, phosphatidylinositol; MG, monoglyceride; FA CoA, fatty acyl coenzyme A; CE, ceramides; aTG, alkyltriglyceride. Biologically independent animals used for RNA-seq: n = 3 (AL, DR, ALDR, AL 5m) n = 5 (ALDR, DRAL). The RNA-seq experiment was done once. Biologically independent animals used for lipidomics: n = 4 per diet. Data are means ± s.e.m., ***q < 0.0001.
In agreement with previous studies\(^2\), DR-fed mice appeared to use DR thus instigated broad-spectrum changes in the WAT lipidome.

Levels of neutral TGs were markedly reduced (Figs. 5i and 6a). Consistent with the major transcriptional reprogramming, this process remained refractory to the ALDR switch at old age.

Newly synthesized FAs to build various types of membrane lipids, de novo synthesis results in generally shorter and less saturated FAs, given that rapid de novo synthesis results in generally shorter and less saturated FAs, we thus analysed the relative TG profile for global shifts in elongation and desaturation. Indeed, chronic and short-term DR at young age decreased the chain length of TG-associated FAs, a shift not observed under late-onset DR (Extended Data Fig. 7a,b).

To experimentally verify that the observed phospholipid profiles were a consequence of tissue-specific changes in lipid utilization and not confounded by lipid import from other tissues (such as the liver), we conducted an ex vivo pulse–chase experiment (Fig. 6b). To this end, we employed highly water-soluble phosphatidylglycerol (PG) with fluorophor-labeled fatty acyl groups (NBD-PG), which, in contrast to most lipid classes, is readily taken up and used by mammalian cells in culture\(^3\). Adipose tissue explants from freshly isolated WAT of chronically DR- or AL-fed 24-month-old mice were a consequence of tissue-specific changes in lipid utilization and not confounded by lipid import from other tissues (such as the liver), we conducted an ex vivo pulse–chase experiment (Fig. 6b). To this end, we employed highly water-soluble phosphatidylglycerol (PG) with fluorophor-labeled fatty acyl groups (NBD-PG), which, in contrast to most lipid classes, is readily taken up and used by mammalian cells in culture\(^3\). Adipose tissue explants from freshly isolated WAT of chronically DR- or AL-fed 24-month-old mice were seeded for 24 h, before the tissue cultures were incubated with NBD-PG and a transfection agent for 48 h. Subsequent lipid extraction and thin-layer chromatography (TLC) visualized the distribution of fluorescent fatty acyl groups among several lipid classes, representing new lipid molecules that were synthesized by turnover of NBD-PG (Fig. 6b,c and Extended Data Fig. 8a,b).

Lipid extracts of both DR- and AL-derived adipocytes showed a clear and equally strong fluorescent PG band, indicating potent...
Chronic, but not late-onset, DR reprograms lipid synthesis to promote mitochondrial membrane synthesis. a, Analysis of lipid pathway activity in the WAT of DR-fed mice relative to AL controls. Red and blue arrows show reactions with positive and negative activity, respectively. Coloured circles indicate relative log2-transformed abundance of lipid classes. Green arrows indicate the major predicted lipid flux across the network. PS, phosphatidylserine; LPS, lysophosphatidylserine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPG lysophosphatidylglycerol. b,c, Active (b) and inactive (c) pathways under DR and switch diets relative to AL control (dashed line indicates significance threshold). One-sided Student’s t tests (forward or reverse reaction) were used to calculate P values before z transformation. P values above the bar graph indicate significance for the pathway relative to DR. Pathway activity was predicted on the basis of lipidomics data, with n = 4 biologically independent animals per diet. d, mRNA expression (RNA-seq) of key genes in PC synthesis (Cept1) and transport (Stard7), which map to differentially active pathways. Two-sided Wald test, adjusted for multiple testing. e, Cardiolipin levels in WAT of chronic or switch diet fed mice (lipidomics). One-way ANOVA followed by two-sided post hoc Tukey test. f, Schematic representation of the reprogrammed transcriptome and lipidome in the WAT of chronically DR-fed mice. Processes with impaired activation as a result of prior AL feeding are indicated. Biologically independent animals used: RNA-seq: n = 3 (AL, DR, ALDR, AL 5m) n = 5 (ALDR, DRAL); Lipidomics: n = 4 per diet. Data are means ± s.e.m., ***q < 0.0001, **q < 0.01.

Fig. 7 | Chronic, but not late-onset, DR reprograms lipid synthesis to promote mitochondrial membrane synthesis. a, Analysis of lipid pathway activity in the WAT of DR-fed mice relative to AL controls. Red and blue arrows show reactions with positive and negative activity, respectively. Coloured circles indicate relative log2-transformed abundance of lipid classes. Green arrows indicate the major predicted lipid flux across the network. PS, phosphatidylserine; LPS, lysophosphatidylserine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPG lysophosphatidylglycerol. b,c, Active (b) and inactive (c) pathways under DR and switch diets relative to AL control (dashed line indicates significance threshold). One-sided Student’s t tests (forward or reverse reaction) were used to calculate P values before z transformation. P values above the bar graph indicate significance for the pathway relative to DR. Pathway activity was predicted on the basis of lipidomics data, with n = 4 biologically independent animals per diet. d, mRNA expression (RNA-seq) of key genes in PC synthesis (Cept1) and transport (Stard7), which map to differentially active pathways. Two-sided Wald test, adjusted for multiple testing. e, Cardiolipin levels in WAT of chronic or switch diet fed mice (lipidomics). One-way ANOVA followed by two-sided post hoc Tukey test. f, Schematic representation of the reprogrammed transcriptome and lipidome in the WAT of chronically DR-fed mice. Processes with impaired activation as a result of prior AL feeding are indicated. Biologically independent animals used: RNA-seq: n = 3 (AL, DR, ALDR, AL 5m) n = 5 (ALDR, DRAL); Lipidomics: n = 4 per diet. Data are means ± s.e.m., ***q < 0.0001, **q < 0.01.

Cardiolipin metabolism links lipogenesis with mitochondrial biogenesis under late-onset dietary restriction. Finally, we determined whether specific membrane lipids were affected by the restructuring of the lipidome in WAT of chronic DR mice. We employed a lipid-reaction analysis approach38 to gauge the activity of whole pathways on the basis of steady-state metabolite levels as measured by lipidomics. Strikingly, chronic DR, but not late-onset DR, caused widespread reprogramming of almost the entire lipidome to promote the synthesis of phospholipids, especially PC and cardiolipin (CL) (Fig. 7a,b and Extended Data Fig. 9a,b). Simultaneously, pathways that degrade membrane lipids or convert them to triglycerides were significantly less active (Fig. 7c). This shift in lipid use from storage fat to phospholipids concurs with results from the ex vivo experiment (compare Fig. 6c,d), which validates our pathway analysis. Switch-resistant expression patterns of key genes involved in TG lipolysis (patatin-like phospholipase domain-containing 2 (Pnpla2, also known as Atdfl), lipase e, hormone sensitive type (Lipe, also
known as Hsl19-20), PA and PC synthesis (1-acylglycerol-3-phosphate O-acyltransferase 1 (Agrp1)–Agrp3, choline phosphotransferase-1 (Chpt1)21-24) and re-acetylation of lyso-phospholipids (lyso phosphatidylcholine acyltransferase 3 (Lptc3))21, were in line with the predicted pathway activity (Fig. 7d and Extended Data Fig. 9c). The transcriptional memory of prior AL feeding was thus paralleled by a metabolic memory. In contrast, metabolic consequences of long-term DR were rapidly reversed when DR mice were switched back to AL feeding (Fig. 7b,c and Extended Data Fig. 8b,c).

CL is usually synthesized from PG and is almost exclusively located in the membranes of mitochondria25. Interestingly, CL levels showed a four-fold induction in chronic-DR-fed and in young ALDR-fed mice, the strongest increase of all lipid classes (Fig. 7e), although findings from both the ex vivo experiments and network analysis indicated no significant differences between CL synthesis and PG (Fig. 7a,c and Extended Data Fig. 7b). Instead, lipid pathway analysis suggested that CL levels became increasingly dependent on PC under chronic DR feeding. CL levels are proportional to mitochondrial mass, which depends on phospholipid supply to the organelle, including trafficking of PC26-28. PC is the most abundant lipid species in mitochondria29 and must be imported from the endoplasmic reticulum (ER) via its exclusive transport protein STARD7 (refs. 30-32). The gene encoding this protein was also identified as a switch-resistant gene (Fig. 7d), thus suggesting that PC synthesis and PC transport from the ER to mitochondria was impaired. Our results therefore link DR-related mitochondrial biogenesis in the WAT (Fig. 4) with increased synthesis of membrane lipids, which would be required during the expansion of mitochondrial mass (Fig. 7f). In this model, the strong transcriptional memory of past AL feeding for lipogenesis, membrane lipid remodelling and downstream cardiolipin synthesis would pose a bottleneck for mitochondrial biogenesis (Fig. 7f).

In summary, we have demonstrated a strong dependence of age-specific mortality on past AL feeding, paralleled by strongly age-related changes in preadipocytes and formation of a strong gene-expression and metabolic memory in adipose tissues, which impeded the coordinated reprogramming of lipid metabolism and mitochondrial activity under late-onset DR. Long-term DR-fed mice, however, retained only a weak memory of past nutrition and responded acutely to changes in diet.

Discussion
Potential DR-related therapies applicable for humans would ideally function in the older people, as they experience the greatest burdens of age-related metabolic pathologies, including type 2 diabetes1. Furthermore, it is important to understand whether over-nutrition in early adulthood can be completely overcome by subsequent diet. We have therefore performed a systematic assessment of prior diet effects on mortality, tissue-specific gene expression and lipidome dynamics in young and old mice.

Previous studies of late-onset DR have yielded inconclusive results. Onset of DR at 17 or 24 months was first reported to have no or even a worsening effect on survivorship of male, single-housed mice in a 3-month follow-up period1. However, this study instigated DR without an adaptation period, monitored survival only over a period of 90 days and did not specify the switch cohort size. A further study of group-housed male mice suggested that there was a strong improvement of survival when DR was initiated at 19 months of age, before mice of the control cohort started to die10. However, the absence of a chronic DR control precluded any conclusion about the completeness of the survival effect relative to chronic DR. Neither study employed cohort sizes appropriate for profiling of age-specific mortality, and therefore could not probe for acute effects owing to the cumulative nature of survival data.

We have investigated the consequences of late-life diet changes with large cohorts of control and switch diet groups, and followed all animals until death. We chose the switch time point a priori on the basis of statistical power and the cohorts’ mortality. However, even with this large group of mice, conclusions on age-specific mortality are reliable only in the first 6–9 months post-switch, before too few AL mice were left for statistically valid comparisons. We further recognize that, owing to the comparatively small number of weighed animals, the statistically significant association between weight loss and survival in the ALDR cohort is predominantly driven by a few mice. Even though these few low-responders showed no evidence of being sick, exhibited average weights at the beginning of the switch, and were present among all cohorts, a larger and more targeted study will be necessary to assess our initial observation. Nevertheless, the robustness of our findings is supported by consistent results across breeding cohorts, with lifespans of AL and DR mice showing statistically insignificant differences. We saw a significant response of mortality to the ALDR switch only in breeding cohort 2. Such batch-specific variation has also been seen in the changes in mouse lifespans in response to rapamycin33.

Our study demonstrates that long-term DR can lead to a partial, lasting protective effect when returning to full feeding, as the mortality of late-onset AL mice remained below that of chronic AL-fed mice. A similar, albeit weaker, protective effect was observed for female flies, and the magnitude of the effect increased with the duration of prior DR feeding34. This suggests an evolutionarily conserved function of long-term DR, which may have implications for humans too. A chronically maintained, healthy lifestyle may thus confer some benefits even when changing nutritional behaviour late in life. However, our results also demonstrate that many health benefits of DR can be lost upon returning to full feeding. Furthermore, profiling of liver, BAT and WAT implied that the lasting benefits are unrelated to the effects of DR on metabolic health, as these were acutely reversed to the level of chronically AL-fed mice. Our findings thus suggest that other mechanism or tissues mediate the long-term protective effect of DR. For example, DR reduces the occurrence of neoplasia across tissues35-37, and delayed cancer onset may thus keep mortality lowered after refeeding, as fatal tumours would require time to develop.

There may be multiple, non-mutually-exclusive explanations of the refractoriness to the mortality of old mice to newly imposed DR, including accumulation of damage to DNA and genomic instability, senescent cells and irreversible pathologies. We have identified an adipose-tissue transcriptional and metabolic memory that impedes metabolic reprogramming under DR and that could thus limit the capacity of the mice to reduce their mortality. This phenomenon appears to be independent of genomic context, as we observed switch-resistant genes coding for mitochondrial processes in BAT and WAT yet regulated in opposite directions. In contrast to adipose tissues, and in agreement with previous studies19, the hepatic transcriptome retained plasticity and was able to respond to dietary changes at late age. Tissue-independent mechanisms could be involved in formation of the metabolic memory. For instance, DR remodels the gut microbiome, which causally contributes to metabolic reprogramming in liver and WAT38-40. Loss of DR-essential microbiome species during ageing under AL feeding could thus render mice refractory to late-onset DR.

As another candidate, tissue-specific mechanism, we discovered major transcriptional shifts in WAT preadipocytes of old AL-fed mice, suggesting that stem cell and/or precursor exhaustion contributes to the WAT memory. This is interesting, since tissue-resident stem/progenitor cells show differing transcriptome shifts during ageing, with, for example, neuronal stem cells exhibiting few intrinsic expression shifts compared with hematopoietic stem cells41,42. Given that pre-adipocyte differentiation is strongly influenced by immune-derived factors and chemokines43-45, the increased inflammatory signature and lowered growth factor secretion could indicate a compromised differentiation potential and thus loss of
transcriptional flexibility during ageing. Although we only had access to scRNA-seq profiles of AL-fed mice, the switch-resistant repression of inflammatory genes under DR (as well as interferon response genes) could, in this model, represent a way of maintaining plasticity in the adipose tissue through prevention of sterile inflammation. Interestingly, the WAT of AL-fed mice exhibits—prior to any other tissue—major age-related expression shifts, most notably of inflammatory genes, at 15 months of age. It is noteworthy that ex vivo WAT explant cultures from DR-fed mice still retained clear differences in TG and membrane lipogenesis after 72 h incubation in a medium with all nutrients in abundance and without exposure to the systemic DR environment, thus supporting the role of tissue- and/or cell-type-specific effects as mediators of the transcriptional memory.

We found that the impaired transcriptional activation of key mitochondrial and lipid metabolism pathways under late-onset DR accurately predicted compromised mitochondrial biogenesis, de novo lipogenesis and phospholipid dynamics in the WAT. Taking advantage of our extensive lipidomics dataset and published pathway databases, we successfully applied a new reaction-dynamics analysis, yielding ex vivo validated predictions based on measurements of steady-state levels at just a single time point. Considering that lipogenesis is strongly downregulated in the liver of DR-fed mice, and that TGs in the WAT contain FAs with shorter chain length and fewer double bonds—an indicator of rapidly de novo synthesized FAs—the switch-resistant lipid dynamics are likely a result of tissue-autonomous changes. Consistent with our findings, DR is known to promote lipogenesis in the WAT and to increase mitochondrial biogenesis. Although mitochondrial biogenesis after 4 weeks of DR was shown to promote non-shivering thermogenesis in WAT, we found no evidence for UCP1-mediated thermogenic activity in mice treated for 2 or 22 months with DR, suggesting this to be a transient phenotype or one that is dependent on environmental and/or husbandry conditions. We also found no evidence for thermogenic activity through UCP1-independent mechanisms such as Ca²⁺ or creatine cycling. Notably, rodents, monkeys and humans show reduced core body temperature in response to long-term DR, suggesting lowered thermogenic activity under DR. Consistently, we observed evidence for ‘whitening’ of the BAT in the lifelong DR cohort in our RNA-seq dataset, with downregulation of mitochondrial genes, including that encoding UCP1, suggesting decreased mitochondria-dependent thermogenesis in the BAT of DR-fed mice. DR-induced whitening of BAT has previously been shown in a rat model of type 2 diabetes, but was not seen in a recent study of male, single-housed mice. This might reflect gender differences in the response of the BAT to DR or could be caused by different husbandry conditions. In contrast to single-housed animals, group-housed DR mice may be able to reduce energy-demanding thermogenesis in the BAT by behavioural adaptation, for example increased huddling. Notwithstanding, we cannot preclude altered activity of yet unknown thermogenic processes, and thus future studies should assess thermogenic capacity on a functional level by measuring respiration of adipose tissue directly. Our analysis further suggests a role for WAT-specific synthesis of new FAs in order to provide membrane lipids, such as PC and CL, during expansion of mitochondrial biomass. However, we cannot exclude that low lipogenesis in ALDR mice is a reaction to impaired mitochondrial biogenesis to prevent an overflow of lipids due to low beta-oxidation capacity. Also, newly synthesized FAs themselves might have additional roles. The FA palmitoleate, for example, which was strongly induced under chronic and early-life-onset DR but not late-onset DR, is secreted from the WAT and can act as a bioactive lipidokine to remodel metabolism in liver and muscle. Moreover, synthesis of CL is essential for mitochondrial biogenesis during cold exposure. Finally, whole-body depletion of the lipogenic transcription factor SREBF1b abrogates lipogenesis and mitochondrial biogenesis in the WAT of DR-fed mice, thus strongly implicating SREBF1-driven lipid synthesis in adipose tissue as a limiting element for mitochondrial dynamics and, potentially, essential for improved survival under DR. In line with this hypothesis, induction of mitochondrial biogenesis by the PGC1α homologue spargel in the fat body, an organ functionally analogous to the mammalian liver and WAT, is sufficient to extend lifespan in Drosophila. Given the important endocrine role of the adipose tissue, DR-related remodelling of the WAT may lead to differential secretion of critical endocrine signals that coordinate the systemic response to DR. It will thus be a key task in the future to assess the dependence of reduced mortality under DR on lipogenesis and or mitochondrial biogenesis in the WAT specifically. This could lead to new strategies to maintain the effectiveness of DR when applied late-onset, or could even partially replicate the physiological benefits of reduced food intake under unrestricted feeding.

Methods

Mouse husbandry and DR protocol. The DR study was performed in accordance with national regulations, recommendations and guidelines of the Federation of the European Laboratory Animal Science Association (FELASA), with all protocols approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany (reference no. 8.87–50.10.07.176 and 84-02.04.2015.A437) (Nature Research Reporting Summary). Female F1 hybrid mice (C3B6F1) were generated in-house by crossing C3H/HeJ males with C57Bl/6NBr females (strain code numbers 626 and 027, respectively, Charles River Laboratories). Experimental animals were generated in 3 breeding batches with 300, 280 and 220 animals in breeding round F1, F2 and F3, respectively (Nature Research Nature Research Reporting Summary). Lifespans of chronic DR and AL mice from the F1 breeding round were previously published. Litter size was adjusted to a maximum of 8 pups by removing male pups within 3 d of birth. Pups were weaned at 3–4 weeks of age and were randomly assigned to cages upon weaning (Nature Research Reporting Summary). Animals were housed in groups of 5 females in individually ventilated cages under specific-pathogen-free conditions with constant temperature (21°C), 50–60% humidity and a 12-hour light–dark cycle. For environmental enrichment, mice had constant access to nesting material and chew sticks. All mice received commercially available rodent chow (sniff R/M-H autoclavable, sniff Spezialdiäten) and were provided with acidified water ad libitum. Food consumption of the AL group was measured weekly, and DR animals received 60% of the food amount consumed by AL animals. To avoid developmental effects, chronic DR treatment was started at 12 weeks of age. Late-life ALDR and DRAL diet switches were introduced when 20% of AL animals of the respective control cohort had died, corresponding to ~24 months of age. DR was introduced stepwise, by reducing the food delivered by 10% per week over 4 consecutive weeks. DR animals were fed once per day, and all animals were checked daily for their well-being and any deaths. Fifteen animals per cohort (3 cages) were weighed weekly up to the age of 6 months, then again weekly following the diet switch. In groups of 12 months, 23 months, and then again weekly following the diet switch. In groups of mice per diet group of the F3 cohort were euthanized at the ages of 5 and 27 months, corresponding to 2 months (short-term) and 24 months (long-term) DR treatment. All mice were killed within a period of 3 h prior to the regular feeding time of the DR mice. Mice were killed by cervical dislocation, and tissues were rapidly collected and snap-frozen using liquid nitrogen.

Post-switch lifespan and mortality analysis. Animals that had died before the diet switch were eliminated from the mortality analysis. Cox regression of post-switch survival curves was performed using custom RStudio (https://www.rstudio.com/) scripts and the following packages: survival, survminer and flexsurv (Nature Research Reporting Summary). Survival data were modelled with two factors, diet and breeding cohort (= diet + cohort; diet factor levels: AL, DR, ALDR, DRAL; cohort factor levels: F1, F2, F3). Schoenfeld residuals were analysed to confirm that the data underlying the Cox regression in Fig. 1c met the proportionality assumption. Contrasts were used to compare the hazard ratios between ALDR relative to AL and between DRAL relative to DR (ALDR vs. DRAL switched diet). We repeated the analysis with altered contrasts to analyse the hazard ratio difference between ALDR relative to DR and the DRAL relative to AL (ALDR vs. DRAL switched versus new diet). In order to directly compare the effects of each diet switch relative to their previous diet, we further introduced a contrast to subtract the hazard ratios of ALDR from DRAL [hazard ratio difference, Fig. 1g]. Analyses were repeated for each cohort separately, for which the ‘cohort’ factor was omitted. To determine if the results of the cohort-wise Cox regression could be accounted for by the larger cohorts of animals under DR and DRAL feeding, which
could alone have produced more significant differences for comparisons involving those groups, we repeated each analysis 1,000 times while randomly downsampling the DR and DRAL groups to match the number of AL- and ADLR-fed mice. The resulting distributions of P values for each analysis were plotted as boxplots (Extended Data Fig. 1c).

To test for acute effects of either switch diet, we performed Cox regression for the first 2 months post-switch, censoring all mice that were still alive at the end of that period. We repeated the Cox regression and iteratively extended the analysed time interval by 1 month. P values and hazard ratios for ALDR, DRAL and the hazard ratio difference were recorded for each iteration (Fig. 1d,e).

Visualizing age-specific mortality rate. Events of death were summarized in bins of 10 d. Mortality (px) was estimated as μx = ln (px) (where px is the probability of an individual alive at age x – 1 surviving to age x). Data for Fig. 1f,e were smoothed by averaging μ over 3 10-d bins. Mortality trajectories were truncated when n < 40 AL-fed mice (equivalent to 25% survival).

Calculating rate of weight change. We monitored the weight of 15 mice per diet group and breeding cohort. We used generalized additive modelling to determine the inflection point of the weight gain/loss curve, which we found at day 889 (equivalent to roughly 14.5 d on new diet). Rate of weight change for each animal was estimated by linear regression over the weight trajectory for this interval. We thereby limited the analysis to the interval before the trajectories plateaued.

In order to compare the weight increase between chronic and late-onset AL mice, we performed linear modelling of the weight change in young chronic-AL-fed mice, starting at the same average body weight as the DRAL cohort. To allow comparability, we included only the period for the AL-fed mice reached the same weight as DRAL animals at the inflection point.

Analysis of food-intake quantification. Average food intake of chronic or late-onset AL fed mice was monitored through food consumption per cage. In order to normalize food consumption to body weight, we estimated the average body weight from the weighing group.

RNA sequencing and analysis. We isolated RNA from liver, BAT and epididymal WAT of AL, DR, ALDR and DRAL female mice at old age (27 months), as well as AL and ALDR female mice at young age (5 months). For liver tissue, we profiled the transcriptome of three biological replicates per treatment and age group. For BAT, we profiled four biological replicates per treatment and age group, with two extra replicates for old ALDR mice. In case of WAT, we profiled three biological replicates per treatment and age group, with two extra replicates for old ALDR and DRAL mice from the same cohort (three to five). RNA was isolated using Trizol Reagent (no. 15996018, Thermo Fisher Scientific, Germany) according to the manufacturer’s protocol before samples were treated with DNase using the TURBO DNA-free kit (Thermo Fisher Scientific). RNA quality was measured using the Agilent TapeStation system (Agilent Technologies). RNA-seq library preparation and sequencing were performed by the Max Planck Genome Centre Cologne, Germany (http://mpg.mpmp.mpmp.de/home/). According to the facility’s procedure, stranded TrueSeq RNA-seq libraries were prepared as described in using 3 µg of RNA isolated RNA as input for liver and WAT, and 1 µg of enriched RNA for BAT. Multiplexed libraries were sequenced with 2×10 million, 100-bp paired-end reads on an Illumina HiSeq2500 (Illumina) liver and WAT, and 1×25 million, 130-bp reads for BAT. Liver RNA-seq data for young ALDR, young AL, old DR and old AL mice were previously published and are publicly available under the Gene Expression Omnibus (GEO) ID GSE92486. Liver RNA-seq data for old ALDR and DRAL-fed mice and RNA-seq data from BAT and WAT are available under GEO ID GSE124772.

Raw sequence reads were trimmed to remove adaptor contamination and poor-quality reads using TrimGalore! (v0.3.7, parameters: --paired --length 25). Trimmed sequence were aligned on TopHat2 (ref. [7]) (v2.0.14, parameters: --no-mixed --library-type stranded) using paired-end reads on an Illumina HiSeq2500 (Illumina) liver and WAT, and 1×25 million, 130-bp reads for BAT. Liver RNA-seq data for young ALDR, young AL, old DR and old AL mice were previously published and are publicly available under the Gene Expression Omnibus (GEO) ID GSE92486. Liver RNA-seq data for old ALDR and DRAL-fed mice and RNA-seq data from BAT and WAT are available under GEO ID GSE124772.

QTL-PCR analysis. QTL-PCR was conducted on tissues that were derived from the same tissue collection group (but not identical mice) as the ones used for RNA-seq. In order to isolate total RNA from WAT for QTL-PCR analysis, samples were homogenized in Trizol (ThermoFisher Scientific), incubated 5 min at RT and then centrifuged at full-speed (16,100g) for 10 min at 4 °C in a tabletop centrifuge. To avoid carry-over of the resultant fat layer, the Trizol subnatant was carefully transferred to a fresh tube, mixed with 200 µl chloroform (no. 356927-100, Sigma Aldrich) and incubated for 10 min at RT prior to centrifugation at 12,000g for 10 min at 4 °C. The supernatant was removed, and the pellet was washed twice with 500 µl ice-cold 70% ethanol and centrifuged at 7,500g for 5 min at 4 °C. Pellets were air-dried for 15 min at RT and re-suspended in 50 µl diethyl pyrocarbonate (DEPC)-treated, autoclaved double-distilled water followed by DNase treatment to remove genomic DNA contaminations using the DNase-free DNA Removal Kit (Ambion) and incubated for 10 min at RT followed by centrifugation at 12,000g for 10 min at 4 °C. The aqueous RNA-containing phase was transferred to a fresh tube, mixed with 500 µl isopropanol, 50 µl 3 M sodium acetate and 15 µl GlycolBlue coprecipitant (Ambion), incubated 20 min at RT and then centrifuged at 12,000g for 10 min at 4 °C. The supernatant was removed, and the pellet was washed twice with 500 µl ice-cold 70% ethanol and centrifuged at 12,000g for 10 min at 4 °C. Total RNA was quantified using a Janus Automated Workstation (PerkinElmer). qRT-PCR was done on a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific) in 20 µl reaction mix (10 µl Taqman Gene Expression Master mix (no. 4369106, Applied Biosciences) and the following Taqman probes (ThermoFisher Scientific): Steat (Mm01138344_m1), Acaca (Mm01304257_m1), Elovl6 (Mm00851223_s1), Fasn (Mm00662319_m1), Pobr2 (Mm01176661_g1), Ucp1 (Mm01244861_m1). Pipetting was carried out using a Janus Automated Workstation (PerkinElmer). qRT-PCR was done on a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher Scientific) in 20 µl reaction mix (10 µl Taqman Gene Expression Master mix (no. 4369106, Applied Biosciences) and the following Taqman probes (ThermoFisher Scientific): Steat (Mm01138344_m1), Acaca (Mm01304257_m1), Elovl6 (Mm00851223_s1), Fasn (Mm00662319_m1), Pobr2 (Mm01176661_g1), Ucp1 (Mm01244861_m1). Pipetting was carried out using a Janus Automated Workstation (PerkinElmer).
Protein purification and western blotting. Protein purification and western blotting were conducted on tissues that were derived from the same tissue collection group (but not identical mice) as the ones used for RNA-seq. For western blot analysis, WT samples were homogenized in Pierce RIPA Lysis and Extraction Buffer buffer (no. 89900, ThermoFisher Scientific) supplemented with PhosSTOP phosphatase inhibitor cocktail (no. 4906837001, Roche) and cComplete, Mini, EDTA-free Protease Inhibitor Cocktail (no. 1183716001, Roche). Homogenates were incubated for 10 min on ice and then sonicated for 5 min. After centrifugation for 15 min at 16,000 g, the supernatant was collected, and a table top centrifuge, protein extracts were transferred to fresh tubes, and protein concentrations were quantified using the Pierce BCA assay (no. 23225, ThermoFisher Scientific). We separated 25 μg of protein extract per sample on 12% acrylamide gels (no. 567804, Criterion TGX Stain-Free Protein Gel, Bio-Rad) and blotted these on polyvinylidene difluoride (PVDF) membranes (Immobilon-FL IPFL00100, Merck) for 1 h at 100 V on ice. Membranes were blocked for 1 h at RT in Odyssey Blocking Buffer (TBS) (927-50000 L W Biocore Biosciences), followed by overnight incubation in the following primary antibodies diluted in Odyssey Blocking Buffer: NDUFA9 (1:1,000, AB_301431, Abcam), mCO1 (1:1,000, AB_208418, Abcam), α-tubulin 1H10 (1:1,000, AB_261966, Cell Signaling Technology). Blots were washed 4 times with TBS 0.2% Tween (TBS-T), incubated with fluorescently labelled secondary antibodies (1:15,000, IRDye 680RD, (AB_10956166, LI-COR Biosciences), IRDye 800CW (1:15,000, AB_621842, LI-COR Biosciences)) diluted in Odyssey Blocking Buffer. At 1 h for RT after following 4 washing steps with TBS-T at RT. Image acquisition was done using infrared imaging and Odyssey Imaging System (LI-COR Biosciences). For the western blot analysis of UCPI, samples were blotted on Amersham Hybond PVDF membranes (GE10600023, Merck), blocked for 1 h at RT in 5% non-fat dry milk powder (A0830, 1000 PanReac AppliChem) and washed 3 times in TBS-T. Membranes were incubated O VN with the following primary antibodies diluted in sterile filtered 5% fatty-acid-free bovine serum albumin (RP9704100, Fisher Scientific) in TBS-T: UCPI (1:1,000, AB_2687530) or α-tubulin 1H10 (1:1,000, AB_2619646) both obtained from Cell Signaling Technology. Blots were washed three times in TBS-T and incubated with anti-rabbit HRP-coupled secondary antibodies (1:10,000, AB_2536330, ThermoFisher Scientific) diluted in 5% milk in TBS-T for 1 h at RT, which was followed by 3 washing steps in TBS-T before incubation in 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibodies (Pierce RCEL Plus Western Blotting Substrates 21312, ThermoFisher Scientific) and image acquisition on a ChemiDoc XRS S System (Bio-Rad). After UCPI image acquisition, blots were stripped in 0.5 M sodium hydroxide (80405-500G, Sigma) for 1 h at RT, washed 3 times in TBS-T before ECL incubation and image acquisition to control for residual UCPI signal. Blots were washed 3 times in TBS-T before blocking and incubation in primary α-tubulin antibody as described before.

Protein bands were quantified using the Fiji software package with α-tubulin as loading control. Samples were normalized against the respective AL control.

Analysis of mtDNA copy number. mtDNA copy-number quantification was conducted on tissues that were derived from the same tissue collection group (but not identical mice) as the ones used for RNA-seq. To analyse mtDNA copy number, total DNA of WT samples was isolated using the DNA Blood and Tissue kit (69506, Qiagen) with an additional centrifugation step at 200 g for 5 min after lysis in ATL buffer. DNA concentrations were quantified using the Qubit Tissue kit (69506, Qiagen) with an additional centrifugation step at 200 g for 5 min. DNA amplification was done using 5 ng of total DNA per reaction. Specific Taqman Universal PCR Master Mix (Applied Biosystems). Reactions were run in 384-well plates with the positive and negative electrospray ionization75.

Distribution of the fluorescent signal was analysed with Fiji software (GE Healthcare). Thin-layer chromatography plates were stained with 470 mM CuSO4 in 8.5% phosphoric acid and subsequently incubated for 10 min at 180 °C. Thin layer chromatography fluorescence signal analysis. Determination of fluorescent signal was analysed with Fiji. First, the distribution and width of each fluorescent band was quantified by vertical paths run through the centre of each lane using 'integrated density' as measurement. Since there was no fluorescent signal in WT cells incubated with NBD-PG, it can be assumed that the signal obtained for fluorescent signal in the NBD-transfected cells was originally PG. Thus, the sample-wise relative distribution of fluorescent signal (that is relative conversion rate of PG into other lipid species) can be obtained by normalising against the total sum.
of the integrated signal, thereby removing potential differences in cell number or PG uptake. Data from technical replicates were averaged after quantification. Fluorescent and non-fluorescent lipid standards were run in parallel to identify individual lipid classes and to estimate the influence of the fluorescent label on the retention behaviour of the TLC. TG band was identified as the top most running band after CuSO₄ staining and scanning.

Next, the fluorescence signal of each band was measured using the ‘regions of interest’ option in Fiji. For each band (TG, CL, PG, and so on) the selected area was of equal size across all samples. Measured values were normalised against the sample-wise, total fluorescent signal as quantified by a bin spanning all bands together. Data from technical replicates were averaged after quantification.

**Lipid reaction network analysis.** Reaction network analysis was performed as described previously in ref. 1. This method calculates statistical z scores for all possible lipid pathways in order to predict whether a particular pathway is active or inactive in DR– as compared with AL-fed mice. Reactions with higher z scores were classified as active. First, we retrieved all publicly annotated reactions and lipid pathways from Reactome77 to construct a network of reactions. Using the lipodomics database, we calculated the molecular concentrations for each lipid species and class before computing for each reaction the so-called reaction weight (ω) as a ratio of product over substrate. Next, we performed, for each reaction, one-sided Student’s t tests using the weights observed under chronic DR or AL feeding, to identify reactions with differential activity. Resulting P values were converted to z scores using the qnorm function (call: qnorm(1 – P)) provided by the R package ‘stats’. We chose the significance level (P < 0.05, corresponding to a z > 1.645) to be determined as significantly active under DR as opposed to AL for visualization. We multiplied the z score with –1 for cases where the reaction was significantly more active under AL as opposed to DR.

Finally, we calculated an average z score for each possible combination of reactions (that is pathways) to detect consistent changes in the flux across multiple reaction steps. With A(A₁,A₂,…,Aₙ) being the pathway of interest, where Aₙ (i=1,2,…,n) are metabolites, we calculate the average z score Zₐ of the pathway as follows:

\[
Z_{A} = \frac{1}{\sqrt{k-1}} \sum_{i=1}^{k-1} Z_i
\]

Zₐ represents the z score for each reaction involved in the pathway. As shown in ref. 2, Zₐ follows a normal distribution. To determine if a pathway A was significantly more active in DR– compared with AL-fed mice, we chose the significance level (P) to be 0.05, corresponding to a z > 1.645. For visualization, we multiplied the Zₐ with –1 for cases where the pathway was significantly more active under AL as opposed to DR. We repeated the analysis correspondingly for the ALDR and DRAL groups with AL-fed mice used as reference.

**Quantitative and statistical analysis.** Statistical analysis and sample sizes. RStudio (https://www.rstudio.com/) and Deseq2 (ref. 3) were used for statistical analysis. Data are expressed as mean± s.e.m. P values were calculated using the following tests: Deseq2’s Wald test (RNA-seq data), Fisher’s exact test (enrichment analysis). We chose the significance level (P) to be 0.05, corresponding to a z > 1.645. For visualization, we multiplied the Zₐ with –1 for cases where the pathway was significantly more active under AL as opposed to DR. We assumed that the pathway A follows a normal distribution. To determine if a pathway A’s retention behaviour of the TLC. TG band was identified as the top most running band after CuSO₄ staining and scanning.

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Author contributions
S.G., A.B., M.J.O.W. and L.P. designed the experiments and drafted the manuscript together with O. Hahn. S.G., A.B., M.J.O.W. and L.P. designed the experiments and drafted the manuscript. O. Hahn conceptualized and performed the lipidome network analysis. A.N. conceptualized and performed the in vitro pulse–chase experiments. T.T. and T.L. designed and conducted the in vitro pulse–chase experiments. T.T. and T.L. designed and conducted the in vitro pulse–chase experiments, and guided the mortality experiments. S.P. conceptualized and performed power analyses to determine the required number of animals for the switch experiments, and guided the mortality experiments. S.G., A.B., M.J.O.W. and L.P. designed the experiments and drafted the manuscript together with O. Hahn. S.G., A.B., M.J.O.W. and L.P. designed the experiments and drafted the manuscript. O. Hahn performed most of the lifespan and bioinformatic analyses. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | Food intake of AL and DRAL mice. Body weights for chronic and switch AL cohorts. Solid lines indicate when chronic DR and diet switches were applied. The dashed lines indicates linear fit of weight gain for both cohorts. Slope of linear fits are indicated below. \( n = 45 \) biologically independent animals per diet. b, Averaged daily food intake per mouse. Each point represents the values from one cohort, encompassing 6 cages each. \( n = 3 \) cohorts per diet. c, Average daily food uptake for 3- and 5-weeks post-switch, split by cohorts and individual cage. \( n = 6 \) biologically independent cages per diet and cohort. Two-sided Wilcoxon rank-sum test, adjusted for multiple testing. d, Averaged daily food intake per mouse after normalizing against average body weight. Each point represents the values from one cohort, encompassing six cages. \( n = 3 \) cohorts per diet. Means ± s.e.m.
Extended Data Fig. 2 | Demography of dietary restriction for each of the three breeding cohorts. **a**, Pre- and post-switch weight curves for chronic and switch diet mice from the 3 breeding cohorts (± 95% confidence intervals). Solid and dashed lines indicate the time point for diet switch and tissue collection, respectively. Tissues were collected from the F3 cohort only. The number above the graph indicates total cohort size at birth. n = 15 biologically independent animals per diet and cohort. **b**, Cohort-specific post-switch KM survival curves for chronic and switch diet cohorts. Cox regression (dashed line) was used to avoid making assumptions about the shape of the trajectories. **c**, Cohort-specific distribution of P values as computed from n = 1,000 Cox regression analyses with random down-sampling of DRAL and DR cohorts to match the size of AL/ALDR cohorts. Analyses were run relative to the pre- and post-switch control. The dashed line indicates significance threshold. Whiskers represent 1st and 5th quartiles, box edges represent 2nd and 4th quartiles, and the centre line represents the 3rd quartile/median. Outliers are marked by points. **b**, c, Biologically independent animals per cohort at start of switch: F1: n = 58 (AL), n = 69 (DR), n = 72 (DRAL), n = 57 (ALDR); F2: n = 55 (AL), n = 69 (DR), n = 69 (DRAL), n = 56 (ALDR); F3: n = 44 (AL), n = 52 (DR), n = 53 (DRAL), n = 44 (ALDR). Means ± s.e.m.
Extended Data Fig. 3 | Transcriptional reprogramming in response to early-onset DR and late-onset AL. 

**a**, Heatmap of unsupervised clustering of expression changes for ALDR switch-resistant genes in BAT (n = 3–5 per group; colour bar represents z score range). 

**b**, Boxplot representation of scaled expression levels of differentially up- and downregulated genes under chronic DR as opposed to chronic AL controls in livers of ALDR switch mice at a young age. Whiskers represent 1st and 5th quartiles, box edges represent 2nd and 4th quartiles, and the centre line represents the 3rd quartile/median.

**c, d**, Heatmap of expression changes for ALDR switch-resistant genes in BAT (c) and WAT (d) of young ALDR switch mice (n = 3 per group; colour bar represents z score range). Biologically independent animals used for RNA-seq: Liver: n = 3 (AL, DR, ALDR, DRAL, ALDR 5m, AL 5m); BAT: n = 3 (AL, DR, DRAL, AL 5m, ALDR 5m) n = 5 (ALDR); WAT: n = 3 (AL, DR, ALDR, AL 5m) n = 5 (ALDR, DRAL).
Extended Data Fig. 4 | see figure caption on next page.
Extended Data Fig. 4 | scRNA-seq profiling of the stromal–vascular fraction in young and old WAT. a, t-SNE visualization of scRNA-seq data (FACS Smart-seq2) from the GAT stromal–vascular fraction, split by age. Preadipocytes as annotated by the Tabula Muris Consortium are coloured by age. b, Scatterplot representation of average expression levels of genes of young and old preadipocytes. Differentially expressed genes (DEGs) are indicated in blue. c, Representative GO enrichment of the top 300 differentially expressed genes between old and young preadipocytes. Lengths of bars represent negative ln-transformed P values, calculated using two-sided Fisher’s exact test. d, t-SNE visualisation of scRNA-seq data coloured by expression of two regulated genes, Ccl7 and Tgfβ3. e, Scatterplot of expression differences between old and young preadipocytes (by scRNA-seq) versus expression differences between DR and AL (by bulk RNA-seq). The number of common DEGs in each quadrant is indicated in blue, and the number of ALDR switch-resistant genes is indicated in red. There was a significant inverse association as determined by two-sided Fisher’s exact test for common DEGs (P = 0.0026) and when analysis was limited to ALDR switch-resistant genes (P = 0.003). f, Representative GO enrichment of 91 switch-resistant genes following the inverse association in e. Lengths of bars represent negative ln-transformed P values, calculated using two-sided Fisher’s exact test. The complete list of enriched GO terms can be found in Supplementary Table 1. g, Violin plot representing expression of selected genes across all profiled cell types. Points indicate cell-wise expression levels, and the violin indicates average distribution of expression split by age. h, mRNA expression (RNA-seq) of the same genes WAT. Scd1 expression is shown in Fig. 5. Two-sided Wald test, adjusted for multiple testing. All scRNA-seq data represents cells that were derived and processed from n = 4 biologically independent mice per age group, encompassing a total of n = 1,962 biologically independent cells. Biologically independent animals used for bulk RNA-seq: n = 3 (AL, DR, ALDR, AL 5m) n = 5 (ALDR, DRAL). Means ± s.e.m., ***P < 0.0001.
Extended Data Fig. 5 | Extended functional enrichment analysis of liver. Representative GO enrichment of ALDR switch-resistant genes in the liver. Lengths of bars represent negative ln-transformed $P$ values, calculated using two-sided Fisher's exact test. Biologically independent animals used for RNA-seq; $n = 3$. 

- Exogenous drug catabolism: 3
- Drug transport: 4
- PI3K signaling: 3
- Intrinsic apoptotic signaling: 5
- Triglyceride metabolism: 3
- Receptor protein tyrosine kinase signaling: 5
- Lipid metabolism: 11
Extended Data Fig. 6 | see figure caption on next page.
Extended Data Fig. 6 | Thermogenic marker expression in WAT and BAT. a, Distribution of gene-wise expression changes in BAT under chronic DR and switch diets relative to chronic AL feeding for genes associated with the GO term ‘Mitochondrion’ (n = 1299 genes). Whiskers represent 1st and 5th quartiles, box edges represent 2nd and 4th quartiles, and the centre line represents the 3rd quartile/median. Two-sided Wilcoxon rank-sum test, adjusted for multiple testing. b, mRNA expression (RNA-seq) of thermogenic marker genes in BAT. c, mRNA expression (RNA-seq) of marker for thermogenesis and mitochondrial biogenesis in BAT. d, Apoe mRNA expression (RNA-seq) in BAT. Two-sided Wald test, adjusted for multiple testing. e, Whole LICOR western blot image of Fig. 4d. f, Western blot analysis of UCP1 in WAT, with α-tubulin as loading control. Tissue extract from one BAT sample (very right lane) was included as positive control for the UCP1 antibody. g, h, mRNA expression (RNA-seq) of uncoupling-independent, thermogenic marker genes in WAT for creatine cycling (g) and Ca\(^{2+}\) cycling (h). Two-sided Wald test, adjusted for multiple testing. i, DRAL switch-resistant genes in WAT. Lengths of bars represent negative ln-transformed P values using two-sided Fisher’s exact test. The complete list of enriched GO terms can be found in Supplementary Table 4. Biologically independent animals used for RNA-seq: BAT: n = 3 (AL, DR, DRAL, AL 5m, ALDR 5m) n = 5 (ALDR); WAT: n = 3 (AL, DR, ALDR, AL 5m) n = 5 (ALDR, DRAL). The Western blot analysis was done once using tissues of n = 4 biologically independent animals per diet that were derived from the same cohort but were not identical to the mice used for RNA-seq. Means ± s.e.m., *** p < 0.0001.
Extended Data Fig. 7 | Triglyceride composition in WAT. a, Distribution of TG species for the switch at young (left) and old (right) age classified according to the number of carbon atoms as proxy for TG-associated chain length. Values represent normalized relative abundances (0–100%) on a logarithmic scale. b, Selected TG groups classified according to associated chain length. Values are identical to the ones in a. One-way ANOVA followed by two-sided post-hoc Tukey test. c, Distribution of TG species for the switch at young (left) and old (right) age, classified according to the number of double bonds in TG-associated chains. Values represent normalized relative abundances (0–100%) on a logarithmic scale. d, Selected TG groups classified according to the number of double bonds. Values are identical to the ones in c. One-way ANOVA followed by two-sided post-hoc Tukey test. Biologically independent animals used for Lipidomics: n = 4 per diet. Means ± s.e.m., ***P < 0.001.
**Extended Data Fig. 8 | Fluorescence signal analysis of pulse–chase experiment outcome.**

**a**, Cellular uptake profiles of exogenously supplied NBD-PG by explant-cultured adipocytes. Lipids were separated by TLC and analysed by fluorescence scanning. The TLC analysis was done once with lipid extracts from \( n = 3 \) biologically independent mice per diet (indicated above), with \( n = 3 \) technical replicates each. For each biological replicate, two technical replicates were co-incubated with NBD and one with just the transfection agent. The dashed line represents the paths used to quantify fluorescent signal distribution in Fig. 6d. Dashed boxes represent the areas used to quantify individual bands. Lipid species with low polarity run on top, with TGs being represented by the top band. Fluorescent lipids run slightly lower than non-fluorescent lipids. Standard phospholipids allowed the identification of lipid spots representing TG, DG and PG levels (the asterisks indicate unidentified lipid species). Applied non-fluorescent standard lipids involve: Tetra-oleoyl CL (TO-CL); CL-rich phospholipid-extract from heart; palmitoyl-oleoyl-DG (PODG); di-oleoyl-PG (DOPG); di-oleoyl-PA (DOPA). Fluorescent NBD-labelled lipids involve: PG, PA, PC, PE, PS.  

**b**, Relative fluorescent signal in each of the major bands. \( n = 3 \) biologically independent, 24-month-old animals per diet; technical replicates were averaged prior analysis. Two-sided t test. Data for the TG band are shown in Fig. 6e.  

**c**, Non-fluorescent scans of identical TLC plate after staining with CuSO\(_4\). Due to high abundance of TGs (upper band) in adipocytes, no phospholipids can be observed. Standard phospholipids allowed the identification of lipid spots.
Extended Data Fig. 9 | Lipid reaction analysis in ALDR and DRAL mice. 

a, b, Analysis of lipid pathway activity in WAT of ALDR (a) or DRAL (b) mice relative to AL control. Red and blue arrows show reactions with positive and negative activity, respectively. Coloured circles indicate relative log2-transformed abundance of lipid classes involved.

c, mRNA expression (RNA-seq) of key genes mapping to differentially active pathways in Fig. 7a. Two-sided Wald test, adjusted for multiple testing. Biologically independent animals used: RNA-seq: n = 3 (AL, DR, ALDR, AL 5m) n = 5 (ALDR, DRAL); Lipidomics: n = 4 per diet. Means ± s.e.m., ***q < 0.0001.
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Odyssey Infrared Imaging System. Application software version 3.0.30, LI-COR Biosciences
QuantStudio Software v1.3. ThermoFisher Scientific
ImageQuant TL 8.2 software. GE Healthcare
ChemiDoc™ XRS+ System + Image Lab 5.1 software, BioRad.

Data analysis

Raw sequence reads were trimmed using Trimm Galore! (v0.3.7).
Trimeq sequences were aligned using TopHat2 (v2.0.14).
RStudio was used for statistical analysis. The following, publicly-available R packages were used: Deseq2, survival, survminer, flexsurv, applet2, topGO, org. Mm. eg.db, Seurat (version 3).
Data visualization and analysis were performed using SeqMonk.
The Fiji software package was used to quantify protein bands in western blot and fluorescent intensity in thin layer chromatography.

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All the data generated or analysed during this study are include in the published article and its Supplementary Information files, and are available from the
corresponding authors. Raw RNA-sequencing data are available under accession numbers GSE92486 and GSE124772 on the NCBI Gene expression Omnibus database. Analysed lipidomics data are available under Table S3. Correspondence and requests for material should be addressed to S.G. and I.P.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size for RNA-seq and lipidome profiling were based on accepted guideline papers (e.g. Conesa et al., 2016; Genome Biology) and prior, published studies by our lab investigating the effects of dietary restriction (DR) on hepatic transcriptome and lipidome (Hahn et al., 2017; Genome Biology and Hahn et al., 2019; Plos Genetics).
To investigate the sample size needed to obtain robust results as to whether late-onset DR results in an acute reversal of mortality, we used actual data from dietary restriction experiments. To objectively determine the impact of acute DR on mortality dynamics after a dietary switch, we used standard survival analysis techniques (including log-rank and Cox regression) as well as methods of mortality analysis developed by co-author Scott Pletcher (Pletcher et al., 2000). We followed the assumptions that (i) Gompertz mortality dynamics apply (i.e., death rates increase exponentially throughout life); (ii) life-long DR produces a 25% extension of lifespan); and (iii) a substantial time-lag would be required for mortality rates in the switched cohorts to reach levels seen in the life-long DR cohort. Our power analyses identified that, for all conditions that were investigated, at least 100 animals per cohort would be sufficient to detect mortality reversal over 90% of the time (i.e., statistical power > 0.90). Based on our calculations of statistical power, we proposed to age 200 animals per treatment (ad libitum, DR, and switch from ad libitum to DR) and execute the switch to DR following the death of 20% of the ad libitum cohort.

| Data exclusions | No data was excluded.

| Replication | We validated findings from RNA-seq profiling (switch-resistant mitochondrial biogenesis, switch-resistant lipogenesis) with orthogonal wet lab methods, such as qPCR profiling of mtDNA content, RNA expression levels of selected genes and protein abundance as measured by western blot. Shifts in lipid turnover as predicted by the lipidome analysis were verified using ex vivo pulse chase experiments.

| Randomization | Upon weaning female mice of each breeding cohort were randomly assigned to cages and were fed AL. DR was introduced at the age of 12 weeks in half of the cages of each breeding cohort. Before the late-life diet switch we randomized cages in the rack. This was done to avoid reallocating old mice to new cages.

| Blinding | DR treatment requires daily feeding of mice with a defined amount of food, therefore, blinding of DR animals was not possible. However, scoring of dead animals in the lifespan analysis was done by mouse care takers, who were not aware of the study design, thus preventing bias. Lipidome, ex vivo pulse chase and western blot analysis were done under blinded conditions.

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | n/a | Involved in the study |
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| - Palaeontology                |    | X                     |
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| - ChIP-seq                      |    | X                     |
| - Flow cytometry                |    | X                     |
| - MRI-based neuroimaging        |    | X                     |

Antibodies

Antibodies used
- NDUF10, 1:1000, AB_301431, Abcam
- mCO1, AB_2084810, Abcam
- α-Tubulin 13H10, AB_2615646, Cell Signaling Technology
- IRDye 680RD, AB_2095166, LI-COR Biosciences
- IRDye 800CW, AB_621842, LI-COR Biosciences
- UCP1, AB_2687530, Cell Signaling Technology
Animals and other organisms

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Laboratory animals
Mus musculus. Female F1 hybrid mice (C3H/HeOuJ females with C57BL/6 NCrI males [strain codes G26 and G27, respectively, Charles River Laboratories]). Experimental animals were generated in three breeding batches with 300, 280 and 220 animals in breeding round F1, F2 and F3, respectively. Animals were monitored from birth to death. Median life spans [F1, F2, F3 in days]: AL [858, 866, 843], DR [1057, 1074, 1098], AL-DR [863, 914, 833], DR-AL [1024, 1051, 1013]. The oldest animal (DR, F3) died at the age of 1453 days.

Wild animals
Our study did not involve any wild animals.

Field-collected samples
Our study did not involve any field-collected samples.

Ethics oversight
The DR study was performed in accordance with the recommendations and guidelines of the Federation of the European Laboratory Animal Science Association (FELASA), with all protocols approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany [reference numbers: 8.87-50.16.37.09.17/6 and 84-02.04.2015.A437].

Note that full information on the approval of the study protocol must also be provided in the manuscript.