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Supplemental Information

An Insulin-Sensitive Circular RNA that Regulates Lifespan in Drosophila

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Figure S1: Differentially expressed circRNAs in the fat body, gut and thorax of dilp 2-3,5 mutant flies, Related to Figure 1. (A) Global circRNA expression was only mildly increased during ageing in the fat body, unchanged in the gut and mildly decreased in the thorax of wDah wildtype flies. Global circRNA levels were up-regulated in fat body and gut of dilp 2-3,5 mutants (fat body: genotype p<0.05, interaction with age: p<0.0001; gut: genotype: p<0.0001, interaction with age: p<0.0001, 2-way ANOVA, n=3) and not significantly changed in the thorax of dilp2-3,5 mutants (n=3). (B) CircSfl was up-regulated in most tissues and at all ages in dilp2-3,5 mutants (p<0.05, beta-binomial test, n=3).
Figure S2: Verification and regulation of circSfl, Related to Figure 2. (A) RNase R treatment did not affect circSfl stability, but degraded linear Sfl transcripts as indicated by higher Ct values as measured by q-RT-PCR and normalized to input. (B) RNA sequencing on brains showed that Sfl RA was the predominantly expressed linear isoform in wildtype flies. In contrast, dilp 2-3,5 mutants expressed Sfl RA and RB at similar levels. (C) CircSfl was also up-regulated in male dilp 2-3,5 mutants (whole flies), while Sfl RA was decreased and Sfl RB increased compared to wDah control flies (Student’s t-test, n = 4). (D) CircSfl was up-regulated in young, middle-aged and old dilp2-3,5 mutant fly heads. While Sfl RA transcript levels were not increased in insulin mutant flies, Sfl RB transcript levels were increased at all investigated timepoints (*p<0.05, ***p<0.001, ****p<0.0001, 2-way ANOVA with Bonferroni post-hoc test). (E) Reduction of TOR signalling by application of rapamycin did
not affect circSfl or linear Sfl expression levels in fly heads (Student’s t-test, n=4). (F) Dietary restriction (1x SYA) did not affect transcript levels of circSfl, Sfl RA or Sfl RB in comparison to control fully fed female flies (2x SYA) (Student’s t-test, n = 4). (G) CircSfl expression was decreased in mth\textsuperscript{1} mutant female flies (Student’s t-test, n = 4).

Figure S3: Generation of circBtsz over-expression mutants, Related to Figure 3. (A) CircBtsz was strongly over-expressed in \textit{elav-Gal4} > \textit{UAS-circBtsz-inv} flies, but not in the other transgenic lines tested (**p<0.01, ***p<0.001, 1-way ANOVA with Tukey post-hoc test, n=3).
Figure S4: Overexpression of circSfl does not affect stress resistance, body weight or development, Related to Figure 3. (A) Gut-specific over-expression of circSfl by NP1-Gal4 did not affect female lifespan. (B) Ubiquitous over-expression of circSfl did not affect lifespan of male flies. (C-D) CircSfl over-expression mutants showed a normal response to (C) starvation (n~100) and (D) to oxidative stress (log-rank test, n~100). (E) CircSfl over-expression mutants showed normal climbing abilities (2-way ANOVA, n~60). (F) Body weight of circSfl over-expression mutants was not different to the UAS-circSfl/+ control (1-way ANOVA with Tukey post-hoc test, n=10). (G) Mean development time of circSfl over-expression mutants was not different compared to the da-Gal4/+ control (1-way ANOVA).
Figure S5: SiRNA mutants to knock-down circRNAs and weight and development of sflΔex2 mutants, Related to Figure 4. (A) Three different siRNAs were used to target the circRNA-specific backsplice junction. (B) circSfl expression levels were not significantly decreased in fly lines expressing siRNAs against the circSfl-specific splice junction. (C) CircBtsz expression was strongly reduced in flies expressing siRNAs targeting the circBtsz-specific backsplice junction, but not the linear Btsz transcript. (D) Weight of sflΔex2 mutants was similar to wDah control flies. The reduced weight of dilp 2-3,5 mutants was partially rescued in sflΔex2, dilp 2-3,5 double mutants (sflΔex2: n.s., dilp 2-3,5: ****p<0.0001, interaction: *p<0.05, 2-way ANOVA with Bonferroni post-hoc test, n = 10-15). (E) sflΔex2, dilp 2-3,5 double mutants were even more developmentally delayed as dilp 2-3,5 mutants (Mean
eclosion day: $sfl^{\Delta ex2}$, $dilp$ 2-3,5 vs. $dilp$ 2-3,5: $p<0.0001$, interaction between $sfl^{\Delta ex2}$ and $dilp$ 2-3,5: $p<0.0001$, 2-way ANOVA with Bonferroni post-hoc test, n ~ 60-130).

Figure S6: The 25kDa Sfl protein isoform does result from proteolytic cleavage of the full length Sfl protein, Related to Figure 5. (A) 10 different miRNA seed sequences were identified in the circSfl transcript. (B) Number of miRNA binding motifs in circSfl (green) compared to all other circRNAs in *Drosophila*. Note, the number of identified miRNA binding sites in circSfl is lower in this analysis compared to (A), as all circRNAs were analysed in parallel, which reduces the power to detect individual sites. (C) Low levels of circSfl were detected by polysome-profiling in wildtype flies indicative for low levels of circSfl translation.
(D) Over-expression of FLAG::circSfl-inv resulted in a single band of around 25 kDa similar to the smaller band observed in FLAG::Sfl knock-in flies. (E) Over-expression of an HA-tagged Sfl cDNA (elav-Gal4>UAS-sfl) increased linear Sfl transcript levels but not circSfl levels (**p<0.001, *p<0.05, 1-way ANOVA with Tukey post-hoc test, n =3). (F) Western blot analysis identified a single band of around 110 kDa corresponding to the full-length Sfl protein in HA-tag Sfl cDNA over-expressor flies. No protein was detected at the size of 25kDa.

Figure S7: Sfl over-expression mutants have a normal starvation response, climbing ability, weight and development, Related to Figure 6. (A) Over-expression of Sfl in the gut by NP1-Gal4 did not affect lifespan of female flies. (B) Neuron-specific over-expression of...
Sfl did not affect lifespan of male flies. (C) Female Sfl over-expression (elav-Gal4>UAS-sfl) mutants showed normal climbing ability. (D) The body weight of Sfl over-expression mutants was not different to UAS-sfl/+ controls (1-way ANOVA with Tukey post-hoc test, n=10). (E) Development time of Sfl over-expression mutants was not changed compared to both controls (1-way ANOVA). (F) Over-expression of Sfl in neurons did not alter starvation resistance. (G) Adult-onset knock-down of Sfl by RU induction shortened lifespan (da-GS>sfl RNAi uninduced vs. induced: p<0.0001, log rank test, n~200).

**Supplemental Tables**

**Table S1: Cloning strategy for vectors generated in this study including primers used., Related to STAR Methods.** Underlined bases highlight the restriction site used. **Fat** bases highlight siRNA or guideRNA sequences within the primers.

| Vector name         | Vector backbone | Primer names | Primer sequences                                      | Method                                      |
|---------------------|-----------------|--------------|------------------------------------------------------|---------------------------------------------|
| pUAST attb-circBtsz-exon | pUAST attb | CW113        | ATGAATTCGTATCAATTTG CCCACCGGCAGG                   | Restriction digest via EcoRI and NotI (EcoRI is naturally present) |
|                     |                 | CW114        | ATGCGGCGCGCATATTCTA GTGCCATCTCTGGCC                  |                                             |
| pUAST attb-circBtsz-1000 | pUAST attb | CW102        | AGTGAATTCTCATTGGAATT GGCCTTTAGA                      | Restriction digest via EcoRI and NotI (EcoRI is naturally present) |
|                     |                 | CW103        | ATGCGGCGCGCCGFCAACAA TTTCCCGCGCATATCA                |                                             |
| pUAST attb-circBtsz-inv | pUAST attb | CW102        | AGTGAATTCTCATTGGAATT GGCCTTTAGA                      | 1) Restriction digest via EcoRI + NotI (CW102 + 192) 2) Restriction digest via NotI (CW193 + 194) |
|                     |                 | CW192        | ATGCGGCGCGCCGFCTCGTGTA GATAATATGTGGCTCTAA           |                                             |
|                     |                 | CW193        | ATGCGGCGCGCTCATTAATG GTCCTTTAGA                      |                                             |
|                     |                 | CW194        | ATGCGGCGCGCCGCCCTACA ATCCCTGTTCATATTT                |                                             |
| pWalium20 circBtsz-1 | pWalium 20     | CW168        | CTAGCAGTCATAGAATAG ATGATCAATTTAGTTAT ATTCAAGCATAAAAATGAT ACATCATTTACTAGTGCG | pWalium cloning Ni et al. (2008) |
|                     |                 | CW169        | AATTCGCCACATAGAATGA TGTATCAATTTATGTGGTA ATATAACTAAAAATGGATAC ATCATCTAGTGCCTAG |                                             |
|                     |                 | CW187        | ATGAGCTTTTAAAAATGAG AAGCAAGGGAGAGCCG                  |                                             |
| pUAST               | pUAST           | CW090        | ATGCGGCGCGCATAAAGAG                                 | Restriction digest                          |


| attb-circSfl-exon | attb | ACGACCCACTGGGACG | with NotI and KpnI |
|------------------|------|------------------|------------------|
| pUAST attb-circSfl-1000 | pUAST attb | CW085 ATGCGGCGCCCAACATT GTTGGCCCATCAAACACT | Restriction digest via NotI and KpnI |
| CW086 ATGGTACCAGCTGCCCAACAC ATGAGTTTATTC | |
| pUAST attb-circSfl-inv | pUAST attb | CW085 ATGCGGCGCCCAACATT GTTGGCCCATCAAACACT | |
| CW088 ATGGTACCCTGCTCTCCACA TAGTTTCAAATCCCA | |
| CW087 ATGGTACCCCAACATTGGT GCCATCTAAACACT | |
| CW089 ATGCTACCTCTAATCAGGT CAAAAGGTGTAGCCT | |
| pUAST attb FLAG::circSfl-inv | pUAST attb-circSfl-inv | Gene Synthesis Add FLAG tag before circSfl ORF |
| 1) Restriction digest via NotI and KpnI (CW085 + 088) | 2) Restriction digest via KpnI (CW087 + CW089) |
| pCFD4 Sfl N Term | pCFD4 | CW224 TATATAGGAAAGATATCC GGGTGAACCTCGTGCTCT TGTATAGTCAATATAGGTC | pCFD4 Cloning Port et al. (2014) |
| CW225 ATTTTAACCTTCTATTTCT AGCTCTAAAACGACG TAAATTGAATAGTC | |
| pBS FLAG::Sfl Donor | pBluescript SK+ | CW265 ATGCTACCCGAAATGC CACCATCAATAGTCTAGAGCG | 1) Restriction Digest via KpnI + NotI |
| CW266 ATGCGGCGCCCAACCCAGAG CAGACTGAAGTGCATTG | 2) Insert Gene Synthesis Product via EcoRI and HindIII |
| pWalium20-circSfl-1 | pWalium 20 | CW164 CTAGCAGTGGTAAAAATGGATAAAGGACTAGTATATTTCAAGCATAGTCCCTCT TATCCATTAAACACGCG | pWalium cloning Ni et al. (2008) |
| CW165 AATTCCGCGAGTTAAAAATGG AATAAGAGGAATAGCCTG ATATACACTAGTCCCTCT TATCCATTAAACACGCG | |
| pWalium20-circSfl-2 | pWalium 20 | CW360 CTAGCAGTGGTAAAAATGGATAAAGGACTAGTATATTTCAAGCATAGTCCCTCT TATCCATTAAACACGCG | pWalium cloning Ni et al. (2008) |
| Transgenic fly line | Vector used | Injected into |
|--------------------|-------------|---------------|
| UAS-circBtsz-exon   | pUAST attb circBtsz-exon | y' w⁺; vas-phiC31; attP40 |
| UAS-circBtsz-1000  | pUAST-circBtsz-1000 | y' w⁺; vas-phiC31; attP40 |
| UAS-circBtsz-inv    | pUAST-circBtsz-inv | y’ w⁺; vas-phiC31; attP40 |
| pWalium20-circBtsz-1| pWalium20-circBtsz-1 | y’ w⁺; vas-phiC31; attP40 |
| UAS-circSfl-exon    | pUAST attb circSfl-exon | y’ w⁺; vas-phiC31; attP40 |
| UAS-circSfl-1000   | pUAST attb circSfl-1000 | y’ w⁺; vas-phiC31; attP40 |
| UAS-circSfl-inv     | pUAST attb circSfl-inv | y’ w⁺; vas-phiC31; attP40 |
| UAS-FLAG::circSfl-inv | pUAST attb FLAG::circSfl-inv | y’ w⁺; vas-phiC31; attP40 |
| pCFD4 Sfl N term    | pCFD4 Sfl N term | y’ v⁺; nanos-phiC31; attP40 |
| FLAG::Sfl           | pBS FLAG::Sfl | Progeny of nos-Cas9 x pCFD4 Sfl N term |
| pWalium20-circSfl-1 | pWalium20-circSfl-1 | y’ w⁺; vas-phiC31; attP40 |
| pWalium20-circSfl-2 | pWalium20-circSfl-2 | y’ w⁺; vas-phiC31; attP40 |
| pWalium20-circSfl-3 | pWalium20-circSfl-3 | y’ w⁺; vas-phiC31; attP40 |

Table S2: Transgenic flies generated in this study, Related to STAR Methods.
| Target gene | Forward primer | Sequence (for) | Reverse primer | Sequence (rev) |
|-------------|----------------|----------------|----------------|----------------|
| Rpl32       | SOL268         | ATATGCTAAGCTGTCGACAA ATGG | SOL269         | GATCCGTAACCGATGTGG GCA |
| circSfl RA  | CW061          | ATGTGATACGGGCCTTGGTTT | CW062          | CCAGACTGTCCACTGCAA T |
| circSfl RB  | CW170          | ATGGAAGCTGTCGATTGAGT | CW062          | CCAGACTGTCCACTGCAA T |
| Sfl all     | CW078          | CCACACGGGCTTATAGC | CW079          | CAGATCCTTCAGTGCCCTCG |
| circ-Btsz   | CW049          | AGTCGGTGAGGCAGAGAGAT | CW050          | CTGCTTTGCAGCCGAAATG |
| Btsz        | CW049          | AGTCGGTGAGGCAGAGAGAT | CW071          | GGTGTGGTGGGATCGAG |

Table S3: Oligonucleotides used for q-RT-PCR in this study, Related to STAR Methods.