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Supplemental information

Systemic administration of monovalent follistatin-like 3-Fc-fusion protein increases muscle mass in mice

Takayuki Ozawa, Masato Morikawa, Yasuyuki Morishita, Kazuki Ogikubo, Fumiko Itoh, Daizo Koinuma, Per-Åke Nygren, and Kohei Miyazono

Supplemental Information

Supplemental Figures

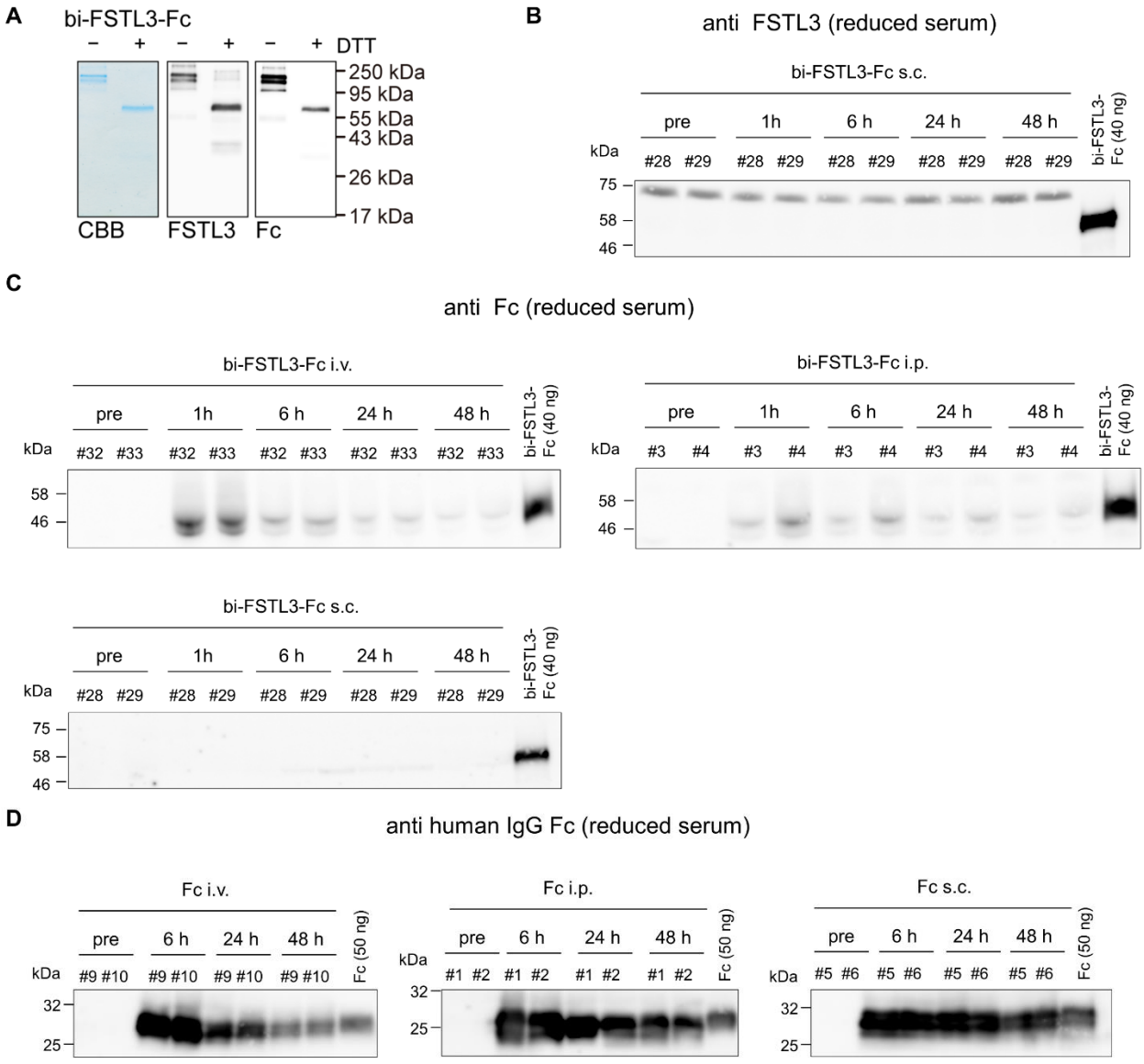


Figure S1. Bivalent FSTL3-Fc is efficiently cleared from mouse circulation, related to Figure 3.
(A) SDS-PAGE of purified recombinant bi-FSTL3-Fc. Results of Coomassie Brilliant Blue (CBB) staining and immunoblot analysis for FSTL3 and human IgG Fc are presented.
(B) Immunoblot analysis for FSTL3 in reduced serum taken from mice subcutaneously injected (s.c.) with bi-FSTL3-Fc (10 mg/kg) at the indicated time points (n=2 independent experiments; the identifying number represents each mouse).
(C) Immunoblot analysis for human IgG Fc in reduced serum taken from mice systemically injected with bi-FSTL3-Fc (10 mg/kg) at the indicated time points (n=2 independent experiments; the identifying number represents each mouse).
(D) Immunoblot analysis for human IgG Fc in reduced serum taken from mice systemically injected with control Fc (10 mg/kg) at the indicated time points (n=2 independent experiments; the identifying number represents each mouse).

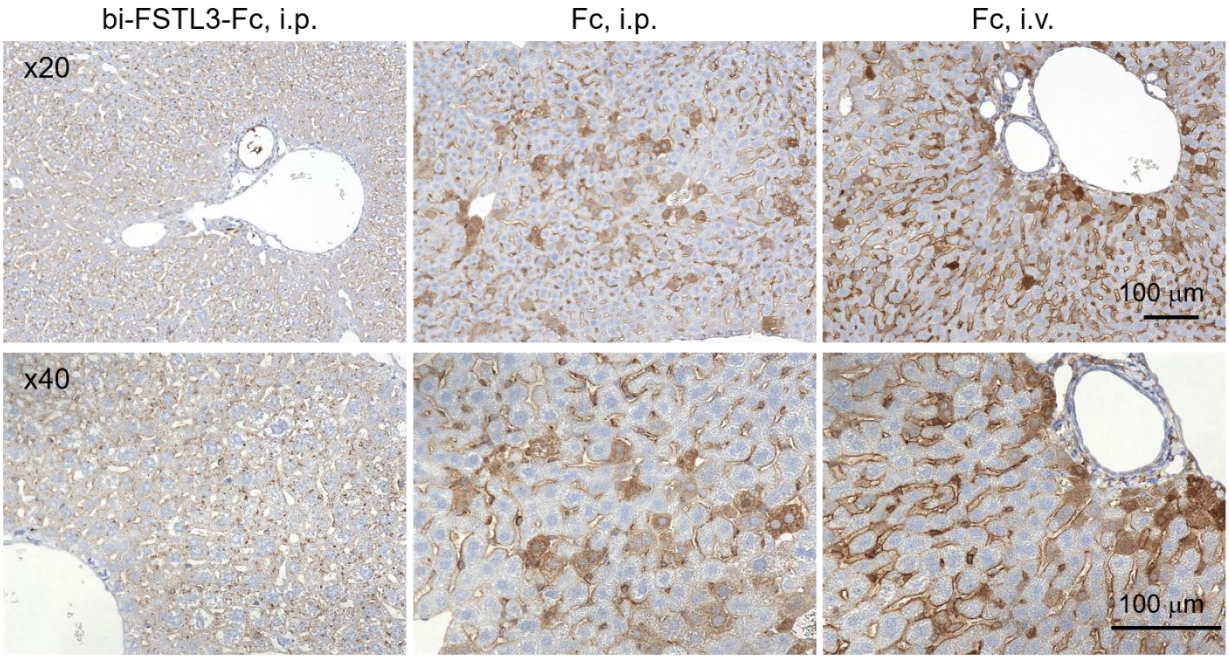


Figure S2. Immunohistochemistry in liver tissue reveals tissue distribution of bivalent FSTL3-Fc in mice, related to Figure 3.
Immunohistochemistry for human IgG Fc in mouse liver tissue 5 h after bi-FSTL3-Fc injection via i.p. or Fc injection via i.p. or i.v.. Images are representative of different experiments (n=2 independent samples), Scale bar: 100 µm.

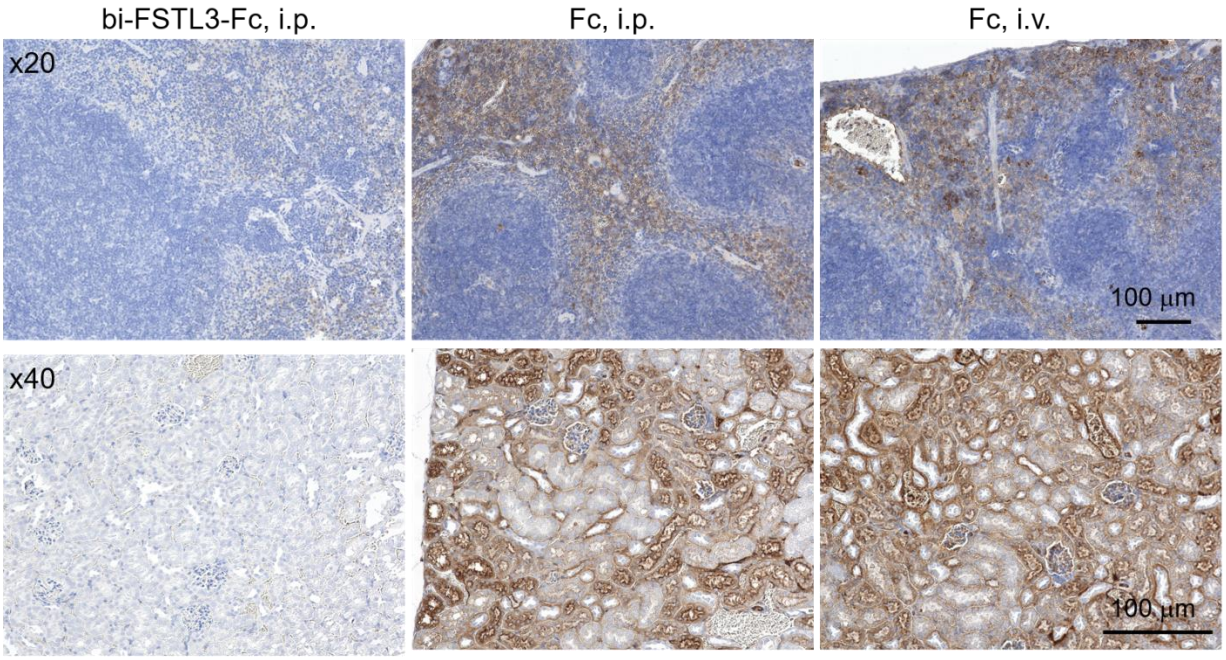


Figure S3. Immunohistochemistry in spleen tissue reveals tissue distribution of bivalent FSTL3-Fc in mice, related to Figure 3.

Immunohistochemistry for human IgG Fc in mouse spleen tissue 5 h after bi-FSTL3-Fc injection via i.p. or Fc injection via i.p. or i.v.. Images are representative of different experiments (n=2 independent samples), Scale bar: 100 µm.

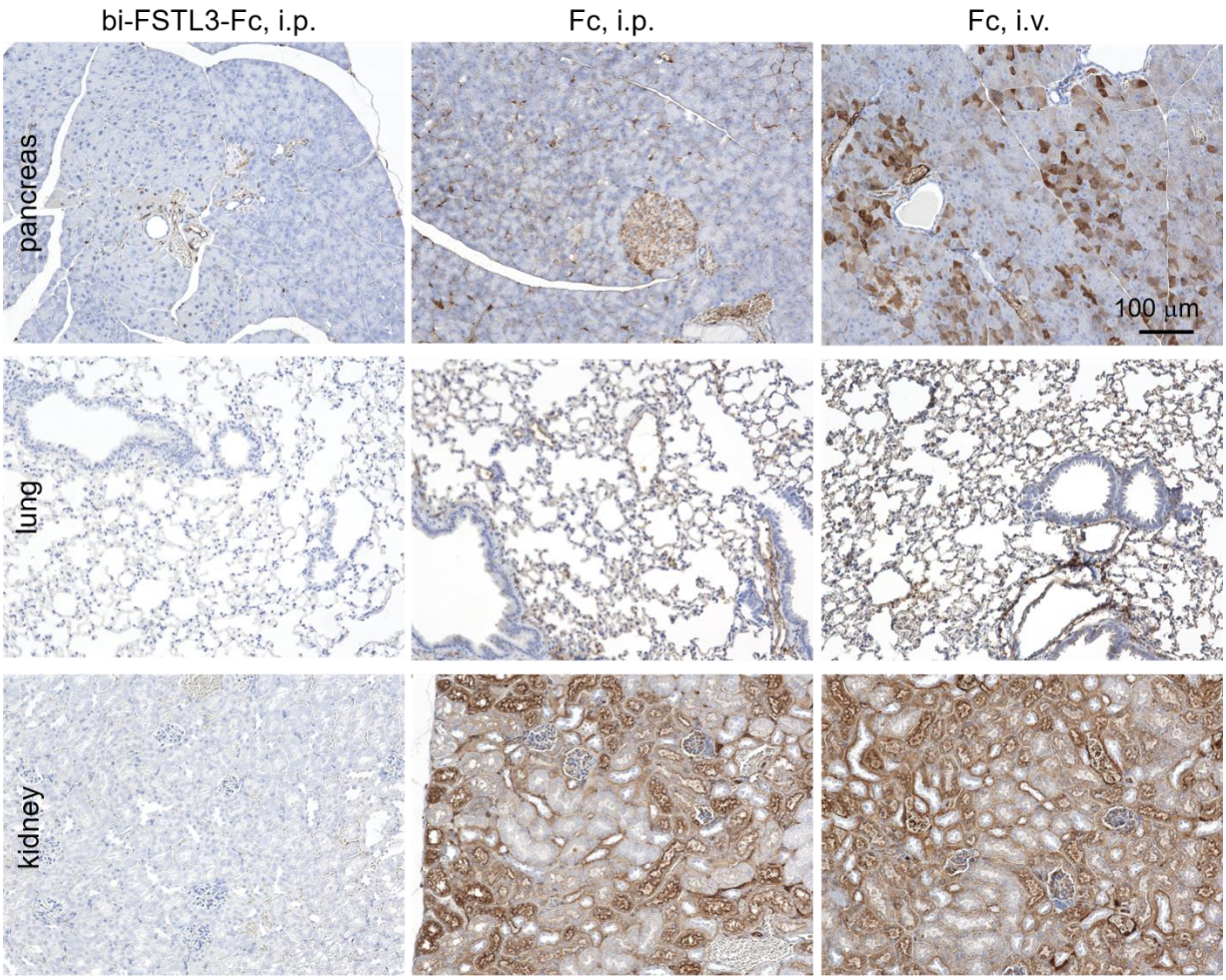
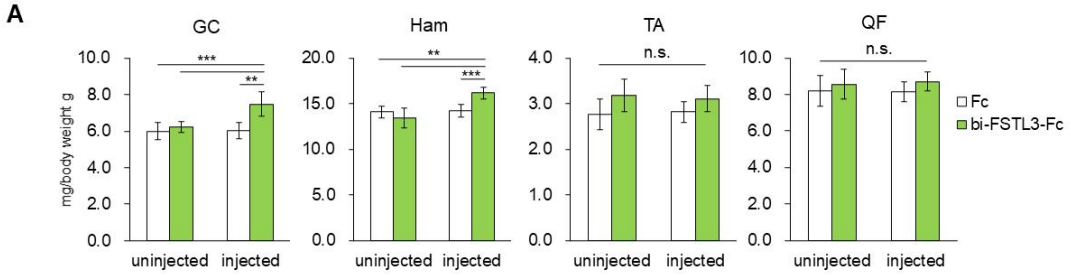
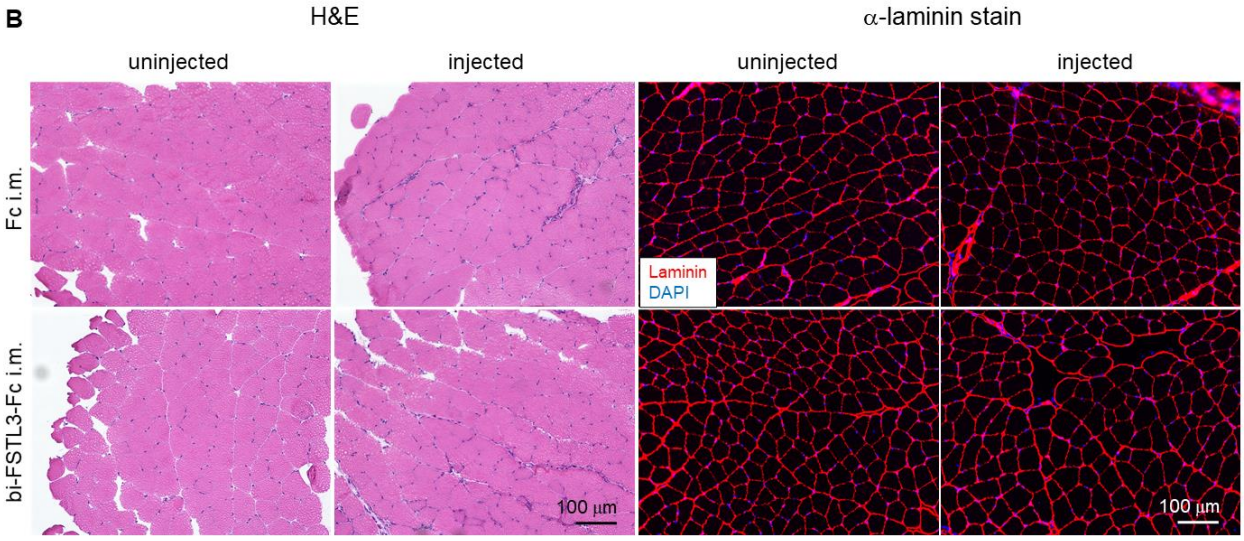
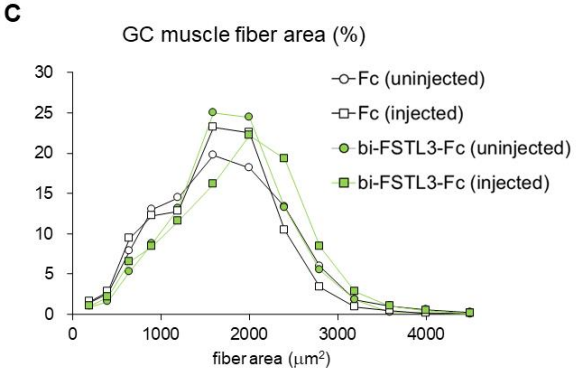


Figure S4. Immunohistochemistry reveals tissue distribution of bivalent FSTL3-Fc in mice, related to Figure 3.

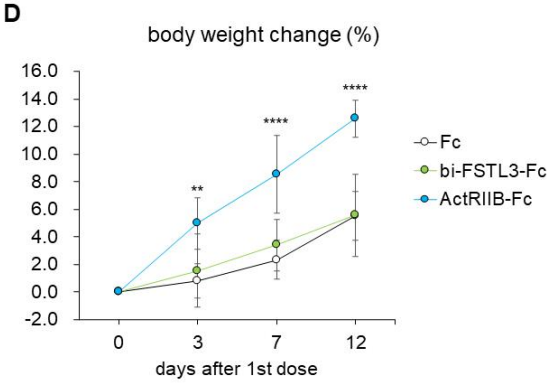
Immunohistochemistry for human IgG Fc in mouse tissues 5 h after bi-FSTL3-Fc injection via i.p. or Fc injection via i.p. or i.v.. Images are representative of different experiments (n=2 independent samples), Scale bar: 100 µm.







	Fc		bi-FSTL3-Fc	
	uninjected	injected	uninjected	injected
average (μm²)	1469.4	1525.4	1390.7	1644.1
median (μm²)	1416	1533	1413	1687
fiber number	3282	2737	3469	2817



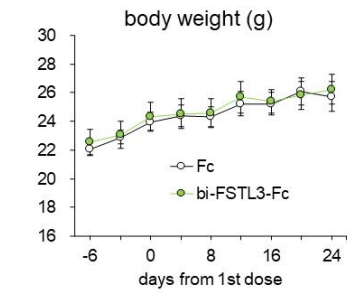


Figure S5. Local administration of bi-FSTL3-Fc increases muscle mass in healthy mice, related to Figure 4.
(**A**) Normalized muscle weights of mice intramuscularly injected with bi-FSTL3-Fc or control Fc into the right calf (hindlimb) twice weekly for 4 weeks. Muscle weight was normalized to the body weight. All mice were sacrificed 2 days after final injection (Data are means ± SD from n=6 for each group). Differences between the conditions were analyzed by ANOVA with Tukey-Kramer post hoc test for multiple comparison; n.s.; not significant, **p < 0.01, ***p < 0.001. GC: gastrocnemius, QF: quadriceps femoris, TA: tibialis anterior, and Ham: hamstrings, uninjected: left hindlimb, injected: right hindlimb
(**B**) Representative H&E and IHC images for laminin. Representative cross-sectional images of myofibers in the GC muscle excised from mice with local administration of bi-FSTL3-Fc or control Fc for 4 weeks. Scale bar: 100 μm.
(**C)** Quantification and distribution of muscle fiber cross-sectional area of (B). The differences in muscle fiber cross-section area were analyzed using Wilcoxon rank sum test, and p values were adjusted with Benjamini-Hochberg correction for multiple comparisons; ****p < 0.0001.
(**D,E**) Body weight of mice with local administration of bi-FSTL3-Fc, ActRIIB-Fc, or control Fc before and after the start of the injection for 2 weeks (D) or for 4 weeks (E). Data are means ± SD from n=10 (D) and n=6 (E) independent experiments. Differences between the conditions were analyzed by Tukey-Kramer post hoc test (D), or Welch's t-test (E); ***p < 0.001, ****p < 0.0001.

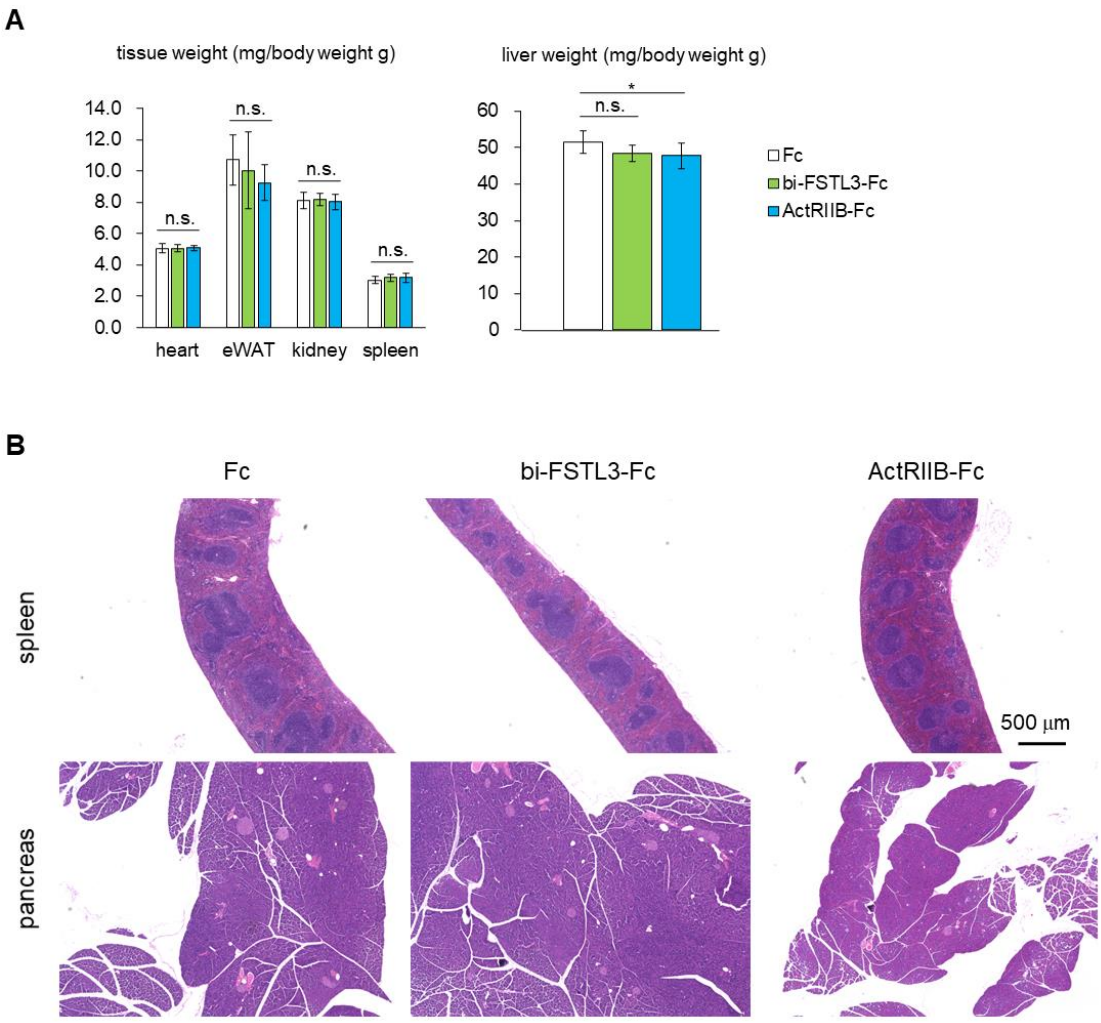


Figure S6. Local administration of bi-FSTL3-Fc does not cause macroscopic and histological changes in healthy mice, related to Figure 4.

(**A**) Normalized weight of tissue taken from mice with local administration of bi-FSTL3-Fc, ActRIIB-Fc, or control Fc for 2 weeks. Tissue weight was normalized to the body weight. Data are means ± SD from n=10 independent experiments. Differences between the conditions were analyzed by Tukey-Kramer post hoc test; n.s.; not significant, *$p < 0.05$.

(**B**) Representative images of H&E-stained sections of the spleen and the pancreas taken from mice with local administration of bi-FSTL3-Fc, ActRIIB-Fc, or control Fc for 2 weeks. Scale bar: 500 μm.

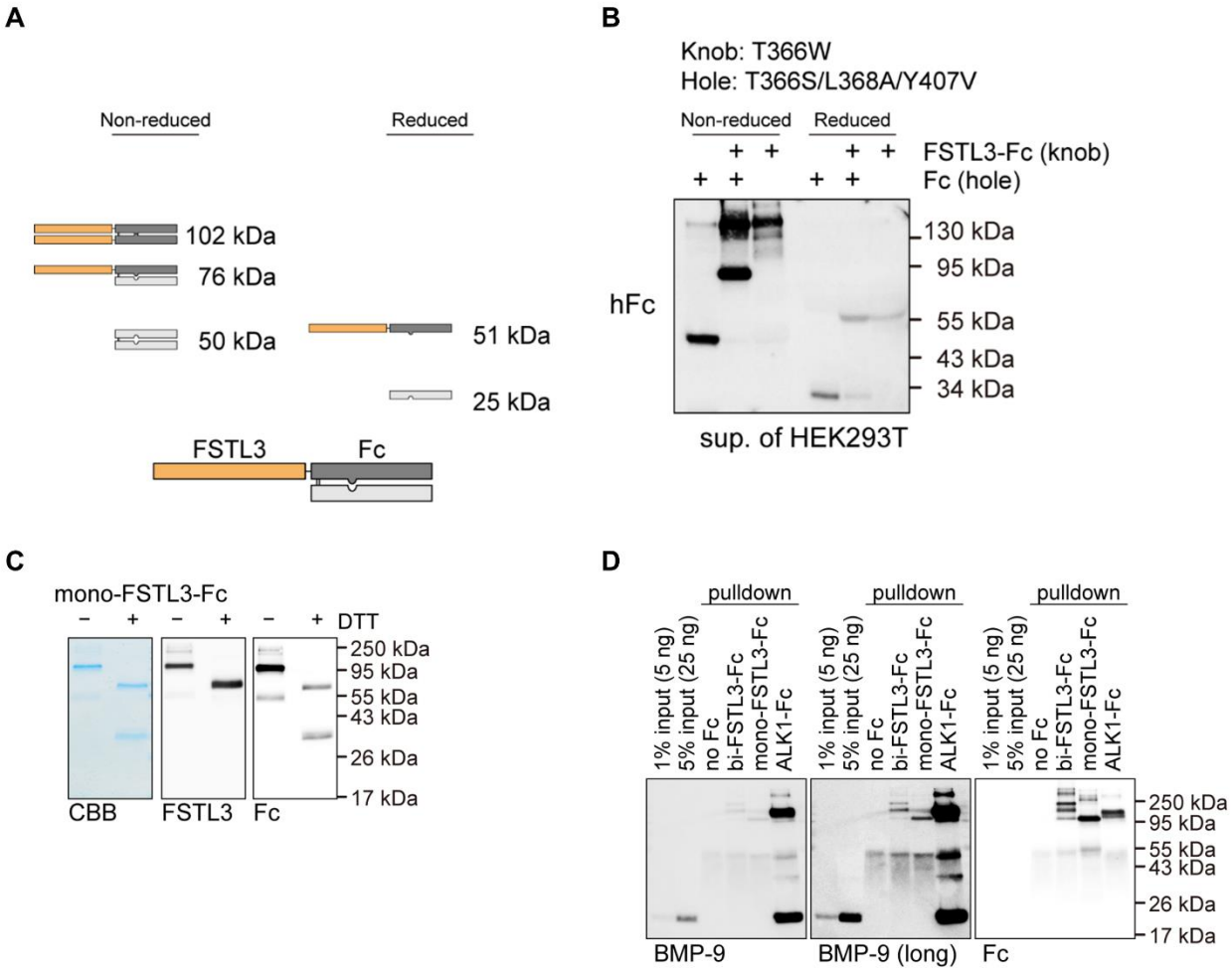


Figure S7. Monovalent FSTL3-Fc is generated with the knobs-into-holes technology, related to Figure 6.
(**A**) Schematic presentation of mono-FSTL3-Fc in either non-reduced or reduced conditions. The predicted protein molecular weight (MW) was calculated by the sum of the MW of all amino acids.
(**B**) Immunoblot analysis for human IgG Fc in the supernatant of HEK293T cells expressing indicated plasmids.
(**C**) SDS-PAGE of purified recombinant mono-FSTL3-Fc. Results of Coomassie Brilliant Blue (CBB) staining and immunoblot analysis for FSTL3 and human IgG Fc are presented. DTT: dithiothreitol.
(**D**) Protein pulldown assay for detection of association between BMP-9 and FSTL3-Fc. Biotin-free BMP-9 (500 ng) was subjected to pulldown assay in the absence or presence of 1 μg Fc-fused protein on beads. Since anti-BMP9 antibody recognizes non-reduced BMP9 homodimer, SDS-PAGE was performed under non-reduced conditions. long: long exposure.

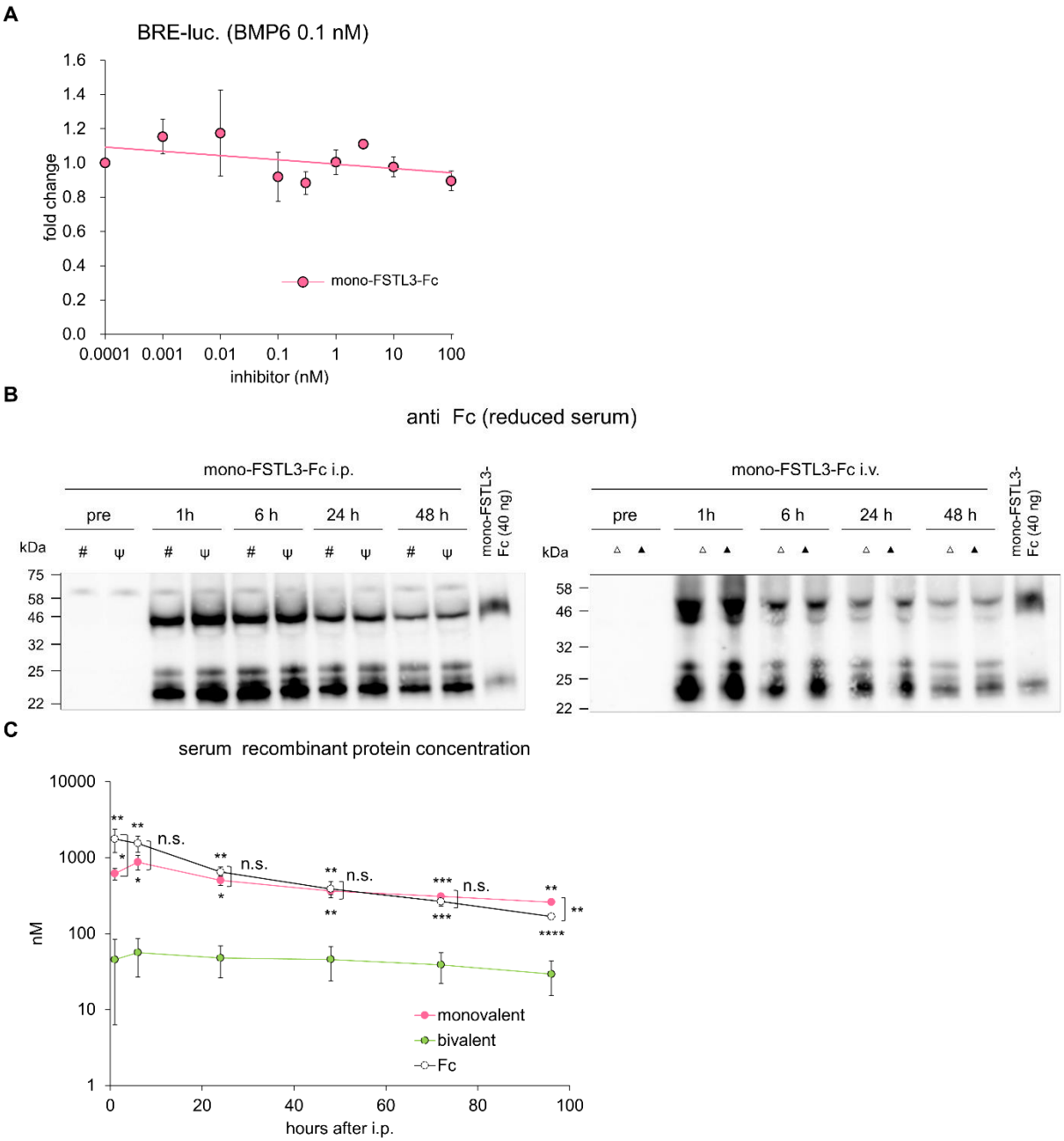


Figure S8. Monovalent FSTL3-Fc protein has longer serum half-life and increases muscle mass after systemic administration in mice, related to Figure 6.

(**A**) Ligand neutralization by mono-FSTL3-Fc, measured in HepG2-reporter cells with BRE-Luc for SMAD1/5/8. The data represent mean ± SD from n=3 independent experiments.

(**B**) Immunoblot analysis for human IgG Fc in reduced serum taken from mice at indicated time points after i.p. or i.v. injection with mono-FSTL3-Fc (10 mg/kg) (n=2 independent experiments. #, ψ, △, or ▲ represents each mouse). See also Fig. 6E.

(**C**) Time course of serum molar concentration of FSTL3-Fc or control Fc measured by anti-human IgG Fc ELISA. Concentration is presented as molar concentration (see also Fig. 6F, which presents mass concentration from the same experiment). Serum was taken at the indicated time points after injection with mono-FSTL3-Fc (128.7 nmol/kg), bi-FSTL3-Fc (97.3 nmol/kg) or control Fc (190.1 nmol/kg). The data represent mean ± SD from n=3 independent experiments, except for bi-FSTL3-Fc, at 96 h (n=2, because of anesthesia-related death). Differences between the conditions were analyzed using analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test for multiple comparison; n.s.; not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$.

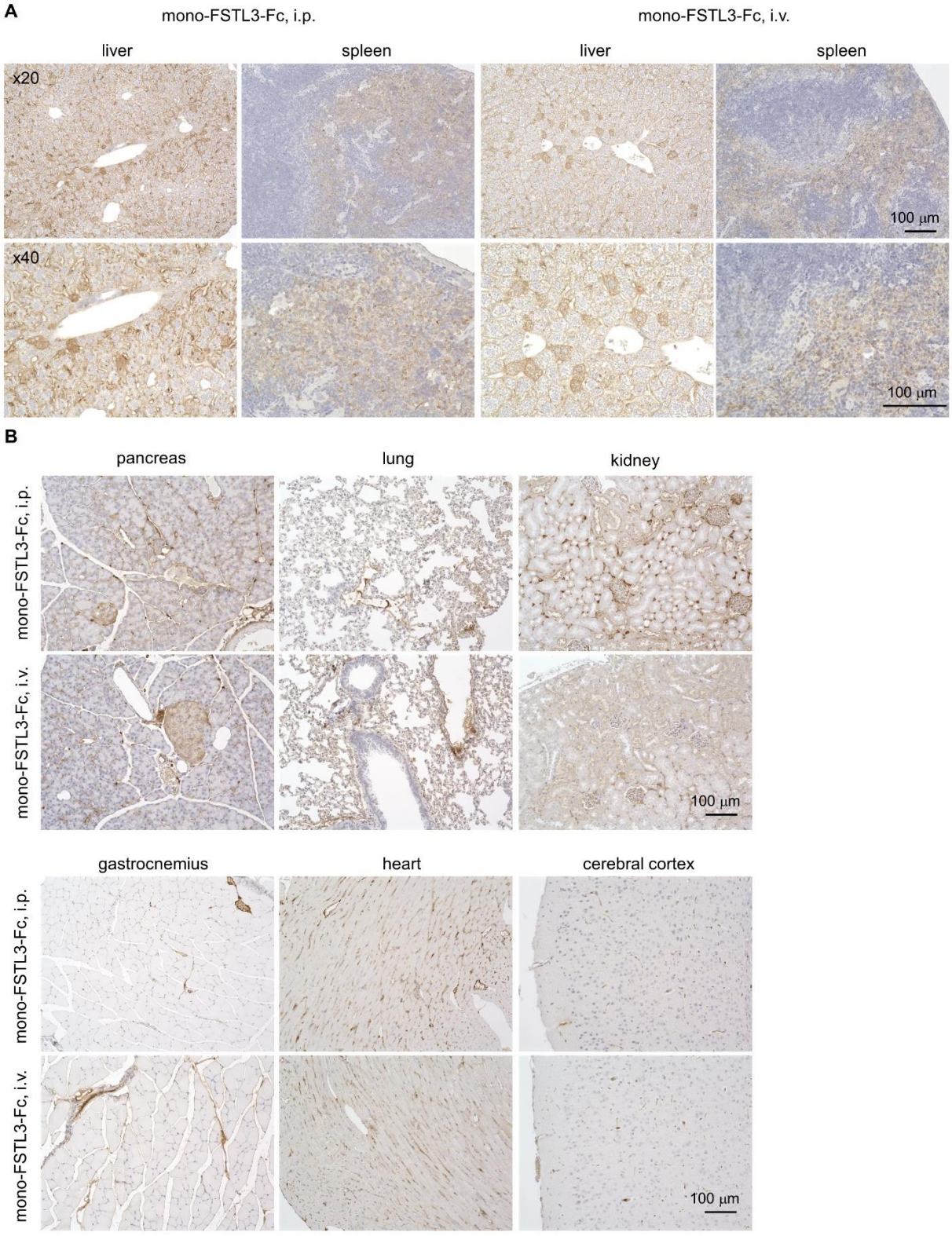


Figure S9. Immunohistochemistry reveals tissue distribution of monovalent FSTL3-Fc in mice, related to Figure 6.

(**A,B**) Immunohistochemistry for human IgG Fc in mouse tissues 5 h after mono-FSTL3-Fc injection via i.p. or i.v. (A; liver and spleen, B; pancreas, lung, kidney, gastrocnemius, heart and cerebral cortex). Images are representative of different experiments (more than n=2 independent samples), Scale bar: 100 μm.