

1 Supplementary Information for:

2 **Alanine-based suppressor tRNA enables disease-agnostic nonsense**
3 **suppression across protein classes and inheritance patterns**

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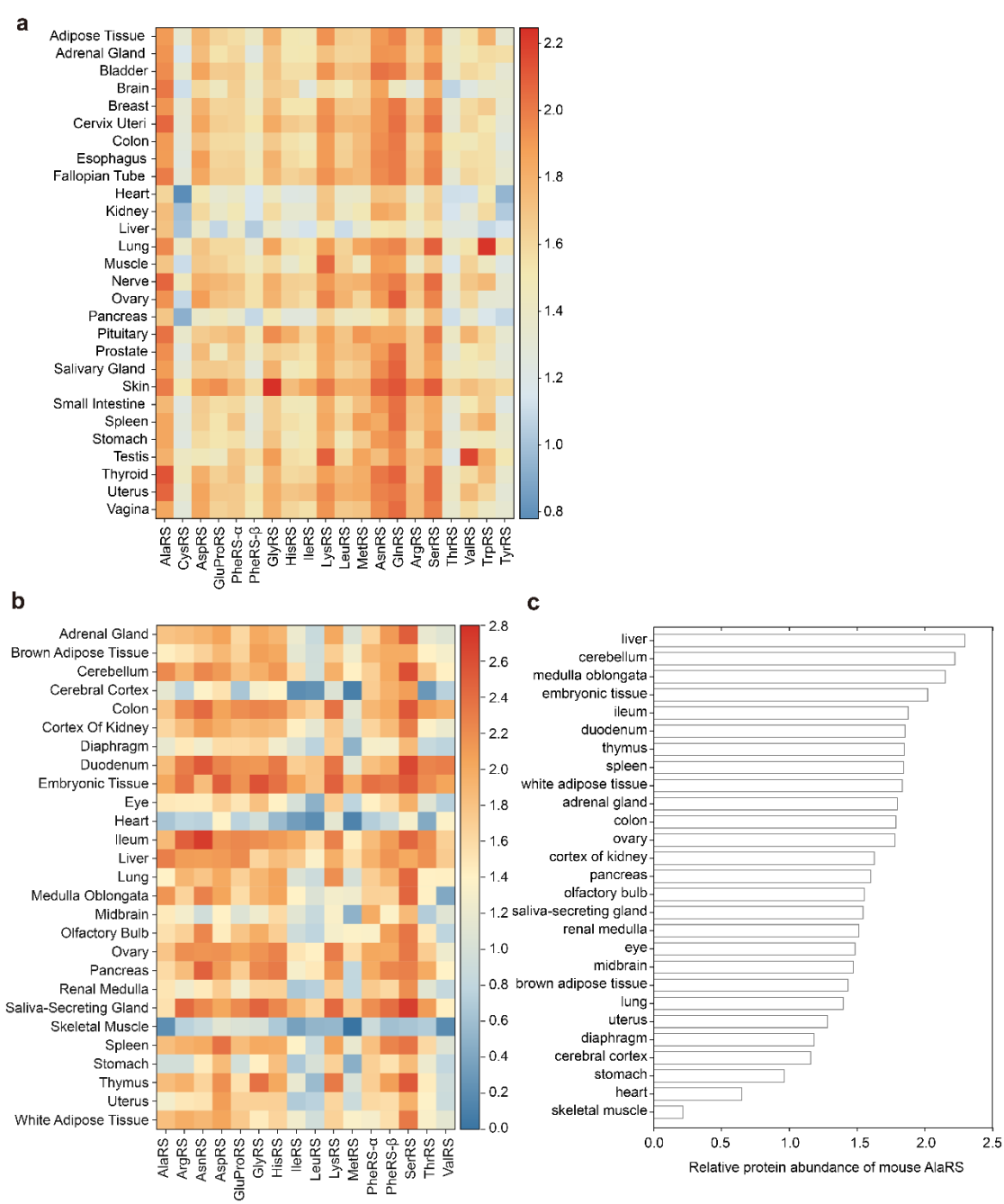
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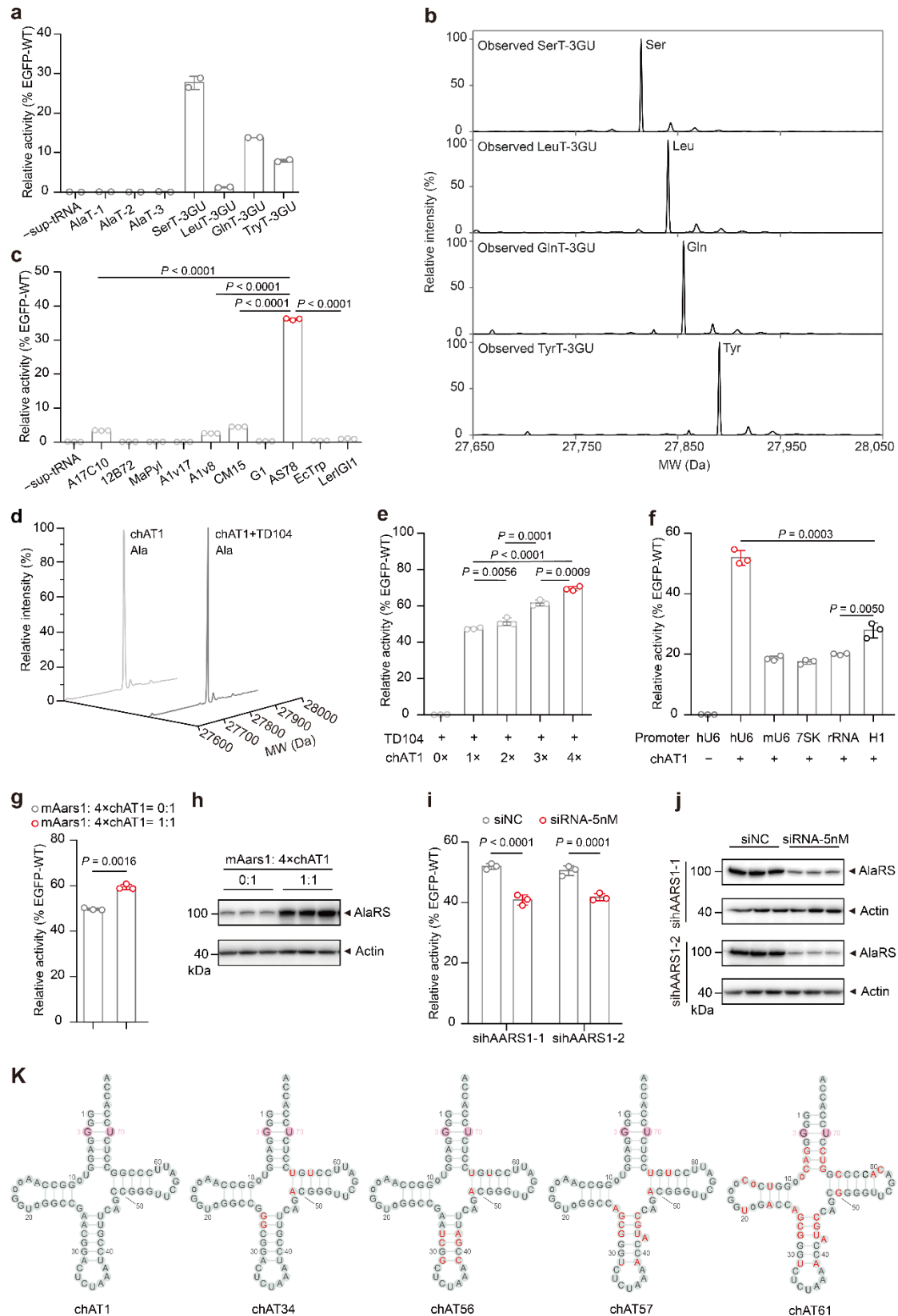
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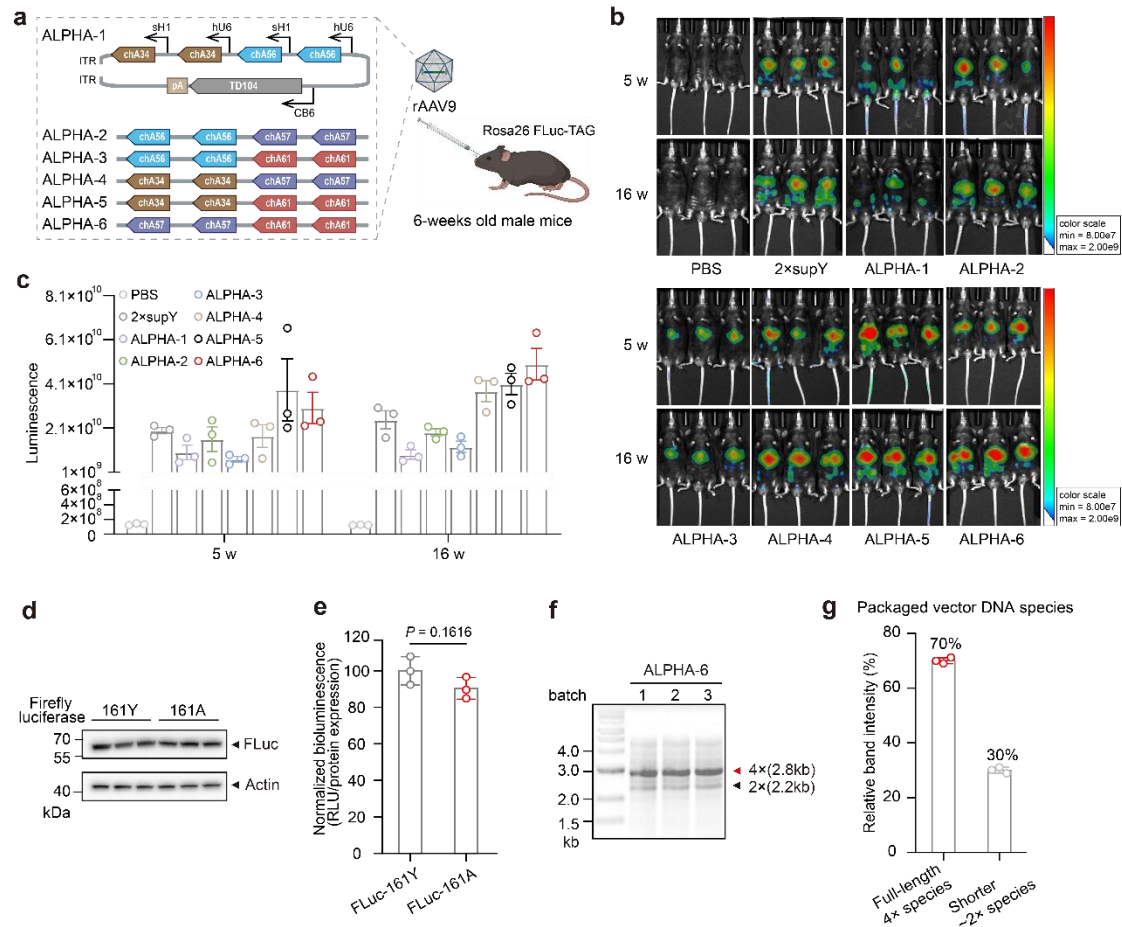
Supplementary Fig. S1. Tissue expression profiles of aaRSs in human and mouse. **a**, Heatmap of tissue-level aaRS-associated values across human organs. Each row corresponds to one tissue and each column to one of 20 cytosolic aaRSs. Color intensity reflects the relative gene expression level across tissues. **b**, Heatmap of tissue-level aaRS-associated values across mouse organs. Protein abundance of 16 cytosolic aaRSs across 27 adult mouse tissues, based on Expression Atlas dataset E-

50 PROT-11¹⁸. **c**, Relative protein abundance of AlaRS across the 27 mouse tissue
51 samples represented in E-PROT-11.
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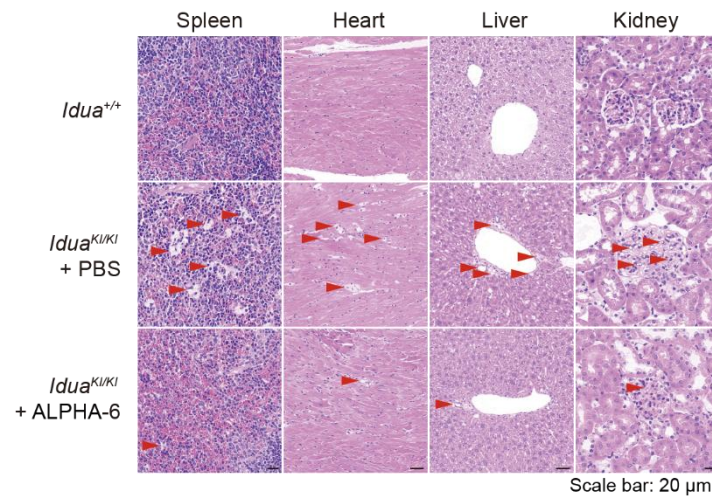
Supplementary Fig. S2. The engineering and cassette optimization of the ALPHA system. **a**, Readthrough efficiency of the tested G3:U70 tRNA variants, including three native tRNA^{Ala}-derived suppressor tRNAs and heterologous SerT-,

57 LeuT-, GlnT- and TyrT-derived scaffolds carrying an introduced G3:U70 wobble
58 pair. **b**, Mass spectrometry analysis showing the amino acid incorporation by the
59 active suppressor tRNA in (a). The observed MW for EGFP variants in presence of
60 these suppressor tRNA were 27814.0 Da, 27840.5 Da, 27855.5 Da and 27890.5 Da,
61 which were consistent with cognate amino acid incorporation, respectively. **c**,
62 Readthrough activity of various sup-tRNAs constructed by incorporating identity
63 element G3:U70 of tRNA^{Ala} into orthogonal tRNA scaffolds used in mammalian
64 cells. AS78-G3:U70 showed the highest TAG suppression activity and termed chAT1.
65 AS78 is an engineered variant of chPheT in our lab. **d**, Mass spectrometry analysis
66 confirming the high fidelity of alanine incorporation by the ALPHA system. The
67 predicted MW for EGFP incorporated with Ala at 190 site was 27797.3 Da, the
68 observed MW was 27797 Da. **e**, Effect of suppressor tRNA copy number on ALPHA
69 activity. Increasing the copy number of chAT1 in the presence of TD104
70 progressively enhanced TAG readthrough efficiency, with the 4-copy configuration
71 showing the highest reporter rescue. **f**, Evaluation of RNA polymerase III promoters
72 for ALPHA cassette expression. The indicated promoters were compared for their
73 ability to drive suppressor tRNA expression and support nonsense suppression. **g**,
74 Mouse AlaRS overexpression enhanced readthrough mediated by the ALPHA system.
75 **h**, Western blot analysis of AlaRS overexpression in (g). **i**, siRNA-mediated AlaRS
76 partial depletion reduced ALPHA-mediated TAG readthrough in HEK293T cells. **j**,
77 Western blot analysis of AlaRS knockdown in (i). **k**, Secondary structures of chAT1,
78 chAT34, chAT56, chAT57 and chAT61. The shared G3:U70 alanine-identity pair and
79 nucleotide substitutions relative to chAT1 are highlighted as indicated. For reporter-
80 based assays, matched reporter-only controls (–sup-tRNA) were included to
81 determine basal readthrough. Readthrough efficiency was quantified as relative
82 activity normalized to EGFP-WT. Error bars represent the mean $\pm$ SD from three
83 biologically independent experiments. Statistical analysis was performed by one-way
84 ANOVA followed by Dunnett's multiple comparisons test (c, e, f), and an unpaired
85 Student's t-test (g), and one-way ANOVA followed by Holm–Šídák's multiple-
86 comparisons test (i).

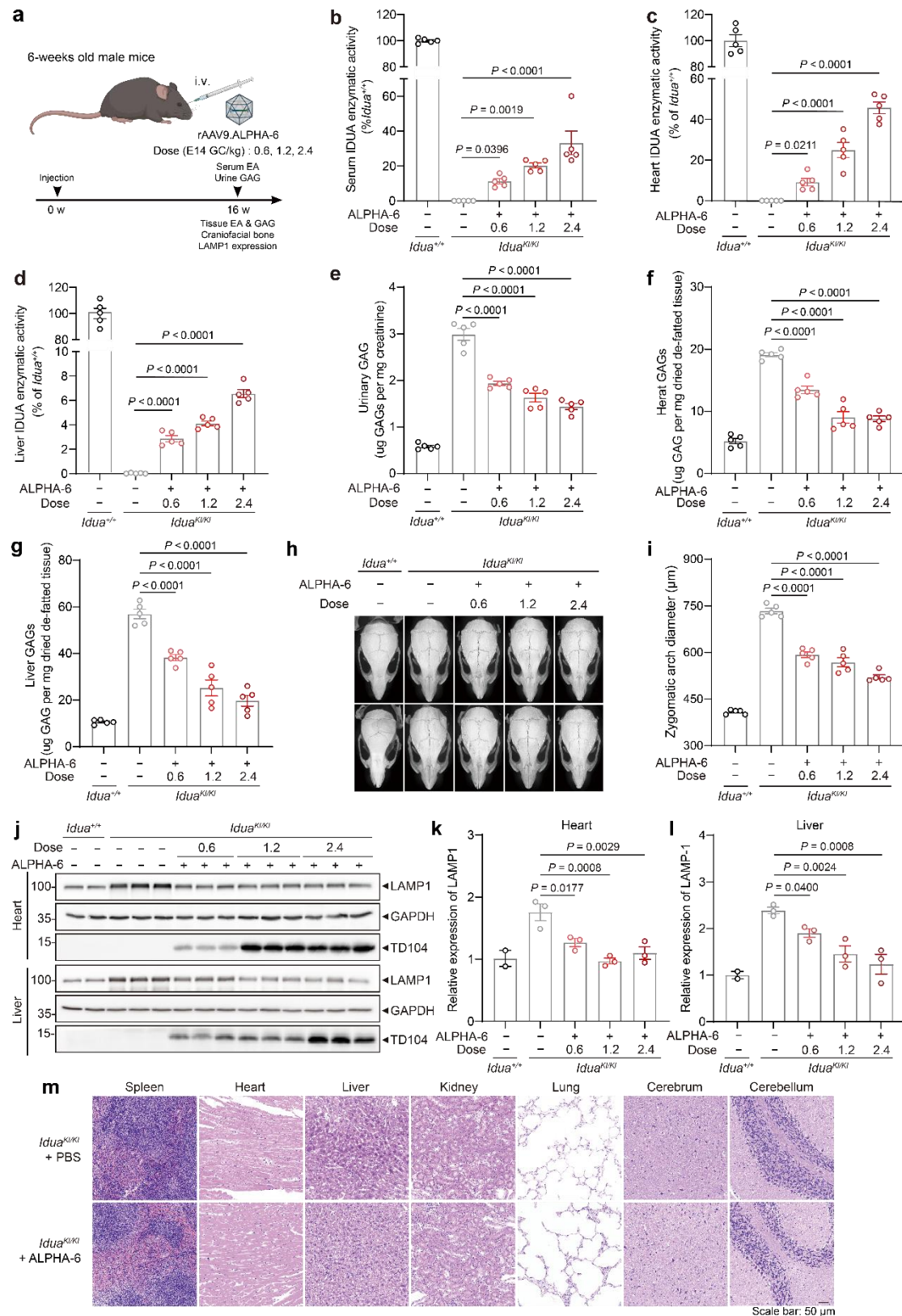


Supplementary Fig. S3. *In vivo* evaluation of ALPHA-mediated readthrough in Rosa26-Luciferase-161TAG mice and characterization of ALPHA-6 packaged genome integrity. **a**, Schematic of ALPHA system architecture and workflow for activity evaluation *in vivo*. ALPHA1-6 were injected through the orbital vein at dose of 0.75×10^{14} GC/kg. **b**, Representative bioluminescence images of mice injected with ALPHA-1, ALPHA-2, ALPHA-3, ALPHA-4, ALPHA-5, ALPHA-6, 2×supTyr or PBS via retro-orbital injection, acquired at 5 and 16 weeks after administration. **c**, Quantification of luminescence intensity in the indicated mice at 5-week post-injection and 16-week post-injection. Error bars are mean $\pm$ SEM (n= 3 mice per group). **d**, Western blotting analysis of the expression level of firefly luciferase variants in HEK293T cells. The proteins were expressed with the fusion of C-terminal FLAG-tag and visualized with anti-FLAG antibody. **e**, FLuc bioluminescence activity was shown with the normalization to the expression level. No significant difference was observed between FLuc-161A and FLuc-161Y (unpaired Student's t-test, $P = 0.1616$). **f**, Alkaline

102 agarose gel electrophoresis of packaged vector DNA extracted from three independent
103 ALPHA-6 production batches. **g**, Quantification of relative band intensity for the
104 packaged DNA species. Bars represent mean $\pm$ SD from three independent experiments
105 (e, g).
106



Supplementary Fig. S4. ALPHA-6 treatment alleviates histological abnormalities in MPS I mice. Representative H&E staining images of the spleen, heart, liver, and kidney 16 weeks post-injection. Scale bars: 20 μ m. Red arrows indicate vacuolation of GAG accumulation.



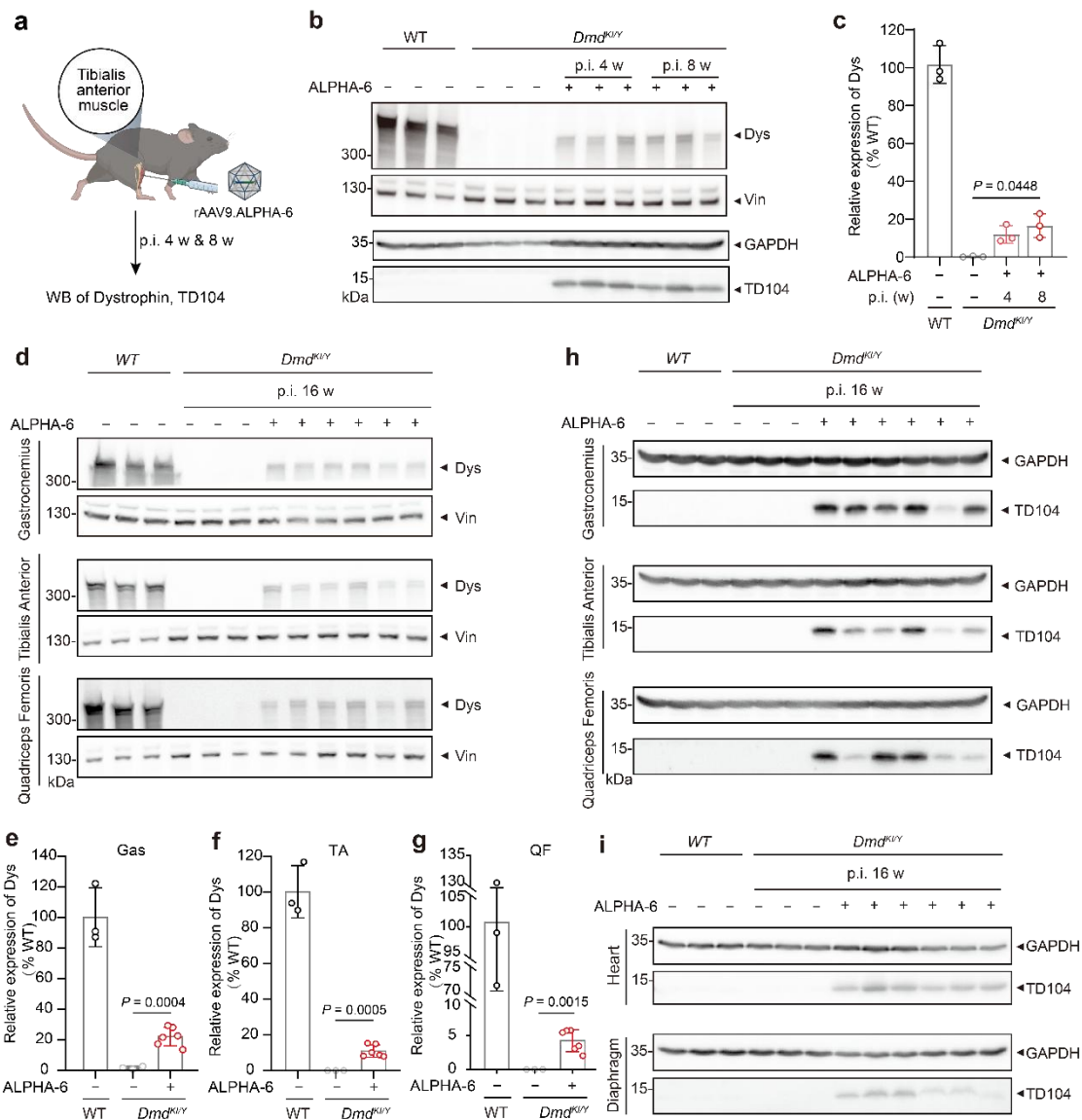
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114 **Supplementary Fig. S5. ALPHA-6 alleviates the MPS I symptoms after a single**

115 **injection in a dose-dependent manner. a. Workflow of *in vivo* assessment of the**

116 **therapeutic efficacy with different doses of ALPHA-6 in male *Idua*^{KI/KI} mice. b-c.**

117 Changes in serum IDUA enzyme activity (b) and urine GAG levels (c) at 16 weeks
118 post-injection. **d-g**, IDUA enzymatic activity in heart(d), liver(e) and GAG levels in
119 heart (f), liver (g) among different doses at 16 weeks after ALPHA-6 treatment. **h**,
120 Micro-CT scan of the mice head. **i**, Quantification of the zygomatic arch diameter of
121 mice in each group. **j-l**. Representative western blot images (j) and quantification
122 LAMP1 protein expression of mice heart (k) and liver (l) in each group. **m**,
123 Representative H&E-stained sections of the spleen, heart, liver, kidney, lung,
124 cerebrum and cerebellum collected at the 16-week endpoint from untreated *Idua*^{KI/KI}
125 mice and *Idua*^{KI/KI} mice treated with the highest dose of ALPHA-6 (2.4×10^{14} GC/kg).
126 No treatment-related pathological lesions or overt toxicity were observed in the
127 ALPHA-6-treated group. Scale bar, 50 μ m. Error bars represent the mean $\pm$ SEM (n=
128 5 mice per group for b-i). Statistical analysis (without *Idua*^{+/+}) was performed by one-
129 way ANOVA followed by Holm-Šídák's multiple comparisons test (b, d and e).
130 Statistical analysis was performed by one-way ANOVA followed by Dunnett's
131 multiple comparisons test (c, f, g, i, k and l).



Supplementary Fig. S6. Intramuscular delivery of ALPHA-6 enables *in vivo*

dystrophin restoration. **a**, ALPHA-6 (1×10^{11} GC/side) was administered to the

tibialis anterior muscle of *Dmd*^{KI/Y} mice via intramuscular injection at 6 weeks of age,

and dystrophin and TD104 protein levels were assessed at 4- and 8-week post-

injection. **b-c**. Western blot images (b) and densitometric quantification (c) of tibialis

anterior muscle collected at 4 and 8 weeks after ALPHA-6 injection in *Dmd*^{KI/Y} mice.

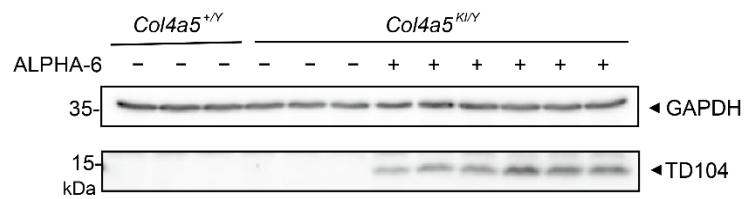
d-g, Representative western blots of dystrophin in the gastrocnemius, tibialis anterior

and quadriceps femoris muscles collected 16 weeks after systemic intraperitoneal

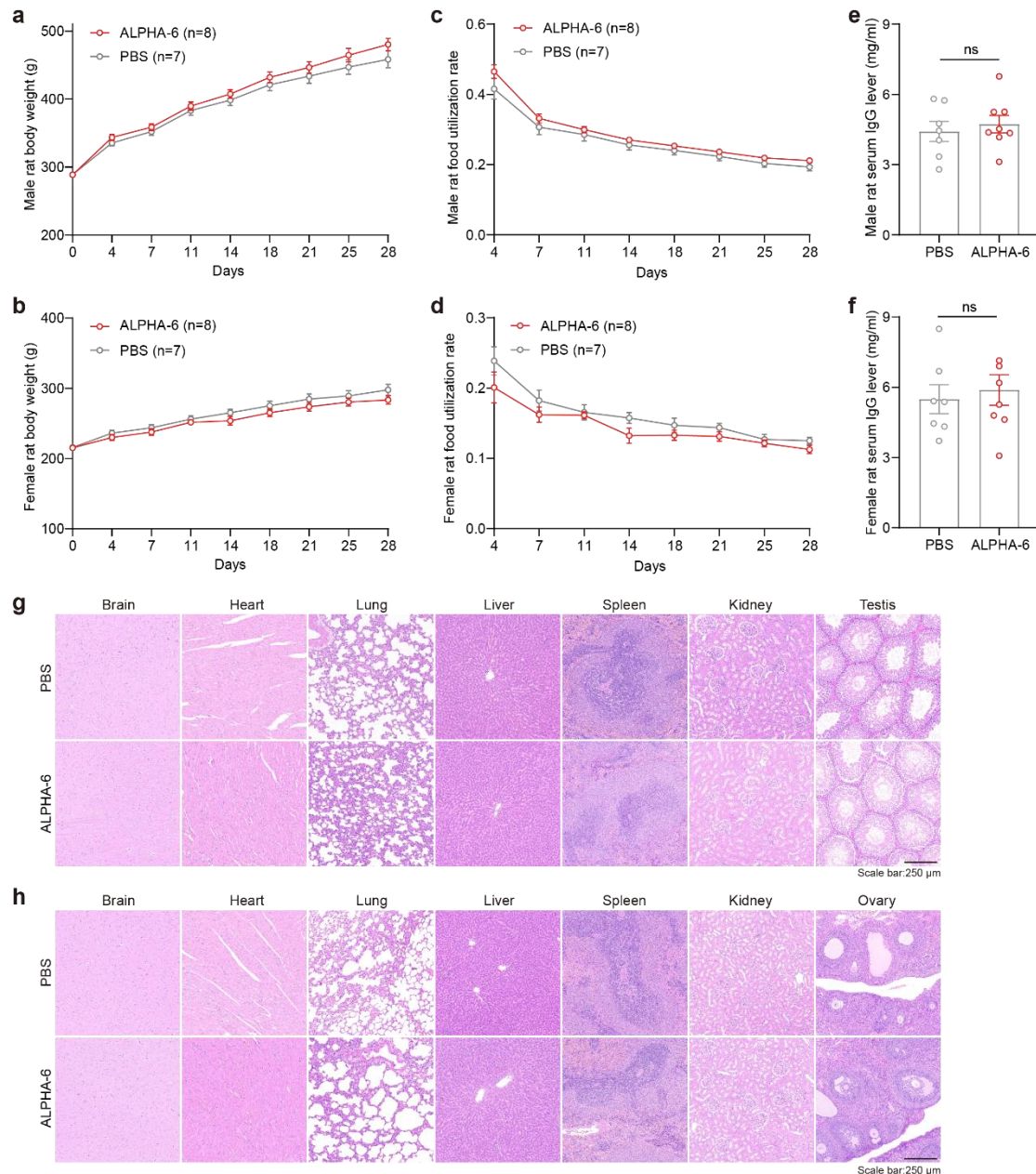
administration of ALPHA-6 to *Dmd*^{KI/Y} mice at 4 weeks of age (d), and the

corresponding quantification of dystrophin in Gas (e), TA (f) and QF (g). **h-i**,

144 Representative western blots of FLAG-tagged TD104 in Gas, TA, QF muscles (h),
145 and heart, diaphragm (i). The samples were collected 16 weeks after systemic
146 ALPHA-6 administration. Error bars represent the mean $\pm$ SEM (n = 3 or 6 mice per
147 group). Statistical analysis was performed using a two-sided unpaired t-test (c, e-g).
148



Supplementary Fig. S7. In vivo expression of TD104 in kidneys of *Col4a5*^{KI/Y} mice following ALPHA-6 administration. Representative western blots of FLAG-tagged TD104 in kidney lysates collected at the study endpoint. *Col4a5*^{+/Y}, wild-type control; untreated *Col4a5*^{KI/Y} mice and ALPHA-6-treated *Col4a5*^{KI/Y} mice are indicated.



Supplementary Fig. S8. *In vivo* safety evaluation of ALPHA-6 in male and female SD rats. a-f, Body weight changes (a, b), food utilization rate (c, d) and serum IgG level (e, f) in male (a, c, e) and female (b, d, f) SD rats. **g-h**, Representative H&E-stained sections of brain, heart, lung, liver, spleen, kidney, testis, or ovary from untreated (n = 7) and ALPHA-6-treated (n = 8) rats. Male SD rats (g), female SD rats (h). Rats were treated at 8 weeks of age and euthanized 4 weeks after injection. Scale bar, 250 μ m. Error bars represent mean $\pm$ SEM. Statistical analysis was performed using two-way ANOVA followed by Šidák's multiple comparisons test (a-d) and unpaired t tests (e, f). *P* value > 0.05 was considered as not significant (ns).

Supplementary Table S1. siRNA sequences used for AARS1 knockdown in HEK293T cells.

siRNA	sequence (5'–3')
siNC	UUCUCCGAACGUGUCACGUdTdT ACGUGACACGUUCGGAGAAdTdT
sihAARS1-1	CGAUGUCCAGAAACGAGUGUdTdT AACACUCGUUUCUGGACAUCGdTdT
sihAARS1-2	GCAGGCAUGAACCAGUUUAAAdTdT UUUAAACUGGUUCAUGCCUGCdTdT

Supplementary Table S2. Effect of ALPHA-6 on serum biochemical parameters in rats.

Parameters	Sex	PBS	ALPHA-6	Normal range
Total proteins (g/L)	M	66.971±1.351	62.986±1.466	26.880~48.800
	F	69.029±1.960	67.300±2.197	
Albumin (g/L)	M	46.657±0.767	43.743±1.087*	26.880~48.800
	F	50.900±1.057	49.333±2.312	
ALT (U/L)	M	53.700±2.853	48.600±1.712	
	F	43.500±2.021	39.367±1.858	
AST (U/L)	M	205.400±23.63	188.557±16.171	
	F	113.900±10.903	107.433±10.549	
Total bilirubin (μmol/L)	M	0.900±0.138	1.171±0.042	
	F	1.057±0.148	1.117±0.070	
Alkaline phosphatase (U/L)	M	150.857±11.415	164.429±14.816	
	F	101.571±8.853	94.667±8.808	
BUN (mmol/L)	M	6.1141±0.330	5.371±0.181	
	F	5.300±0.405	4.683±0.264	
Creatinine (μmol/L)	M	37.500±2.872	34.286±2.090	
	F	40.429±2.034	38.167±2.725	
Glucose (mmol/L)	M	5.129±0.702	6.011±0.727	
	F	7.516±0.619	6.033±0.499	
Potassium (mmol/L)	M	5.940±0.195	5.684±0.261	
	F	5.193±0.208	4.816±0.171	
Sodium (mmol/L)	M	159.000±1.746	152.286±1.569*	121.900~170.400
	F	161.000±1.852	154.000±0.945**	119.400~168.300
Chlorine (mmol/L)	M	108.929±2.008	104.386±0.932	100.800~106.200
	F	114.286±1.725	109.338±0.924*	
Triglycerides (mmol/L)	M	0.627±0.083	0.617±0.045	
	F	0.376±0.034	0.285±0.024	
Total cholesterol (mmol/L)	M	1.533±0.124	1.633±0.086	
	F	2.171±0.187	2.135±0.228	
CK (U/L)	M	1787.667±392.383	2445.857±733.379	
	F	722.857±177.141	814.833±217.808	

Values are expressed as mean ± SEM. *p<0.05, **p<0.01. M means male, F means female.

Supplementary Table S3. Effects of ALPHA-6 on relative organ weight in rats.

Organs	Sex	PBS	ALPHA-6
Heart	F	0.338±0.008	0.351±0.010
	M	0.369±0.012	0.356±0.015
Liver	F	2.932±0.043	2.851±0.078
	M	2.923±0.094	2.940±0.079
Spleen	F	0.162±0.010	0.169±0.005
	M	0.206±0.007	0.193±0.006
Lungs	F	0.404±0.027	0.378±0.012
	M	0.542±0.051	0.526±0.060
Kidney	F	0.394±0.013	0.365±0.011
	M	0.370±0.006	0.379±0.014
Brain	F	0.424±0.014	0.436±0.019
	M	0.640±0.021	0.689±0.028
Ovary	F	0.037±0.005	0.041±0.003
Testis	M	0.386±0.011	0.352±0.017

Data are expressed as mean ± SEM.

Supplementary Table S4. Serum biochemical parameters in *Idua^{KI/KI}* mice 16 weeks after treatment with high-dose ALPHA-6.

Parameter	<i>Idua^{KI/KI}</i> +PBS	<i>Idua^{KI/KI}</i> +ALPHA-6	<i>p</i> value	Normal range
ALT (U/L)	37.66±5.06	38.90 ± 4.82	0.70	
AST (U/L)	57.06 ± 4.96	63.82 ± 2.85	0.03*	50-150
ALP (U/L)	90.96 ± 4.64	87.66 ± 3.72	0.25	
TP (g/L)	43.90 ± 0.91	41.42 ± 1.41	0.01*	50-75
ALB (g/L)	27.94 ± 0.77	26.70 ± 0.60	0.02*	25-45
TBIL (mg/dL)	0.16 ± 0.02	0.16 ± 0.01	0.79	
UA (mg/dL)	1.55 ± 0.57	1.59 ± 0.04	0.87	
BUN (mg/dL)	30.70 ± 2.64	31.83 ± 2.97	0.54	
CRE (mg/dL)	0.20 ± 0.06	0.19 ± 0.02	0.66	

Values are expressed as mean ± SEM. **p*<0.05, ***p*<0.01.