

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

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Premature termination codons (PTCs) underlie approximately 11% of pathogenic variants across thousands of genetic disorders. Current suppressor tRNA approaches have shown promising activity, but their performance across different tissues, amino acid substitution contexts, and diverse disease-relevant targets remain incompletely characterized. Here we describe ALPHA, a programmable alanine suppressor tRNA platform designed on the basis of human genetic and transcriptomic analyses. Alanine substitution at PTC sites yields the highest predicted benign substitution rate, and human alanyl-tRNA synthetase exhibits uniformly high expression across tissues. AAV9-mediated delivery of an optimized ALPHA construct restored full-length protein in four mouse models representing autosomal-recessive (MPS I), X-linked recessive (Duchenne muscular dystrophy and Alport syndrome), and autosomal-dominant (familial adenomatous polyposis) inheritance. Rescue was achieved for a soluble enzyme, a large cytoskeletal scaffold, a secreted extracellular matrix component, and a dosage-sensitive signaling regulator, with corresponding biochemical, histological, and functional improvements, alongside a favorable high-dose safety profile in rats. These results establish that a rationally designed alanine suppressor tRNA can support durable nonsense suppression across genetically diverse PTC-driven disorders.

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INTRODUCTION

Premature termination codons (PTCs) introduce aberrant stop codons that produce truncated proteins and frequently trigger nonsense-mediated mRNA decay, accounting for approximately 11% of pathogenic variants in human genetic diseases¹. Although these mutations collectively impose a massive clinical burden, they are distributed across thousands of distinct rare disorders^{1,2}. This extensive genetic heterogeneity makes the development of bespoke and gene-specific therapies practically and economically prohibitive for most patients³.

Engineered suppressor tRNAs (sup-tRNAs) have emerged as a promising alternative. By promoting translational readthrough of PTCs, sup-tRNAs can restore full-length protein expression without genome editing⁴. Sup-tRNAs were originally discovered in microorganisms as naturally occurring genetic elements that decode stop codons to restore gene function⁵. Building on this principle, early work demonstrated that a human suppressor tRNA could restore full-length protein expression by decoding a PTC in mammalian cell lines⁶. More recent systematic screening and optimization have produced highly active sup-tRNAs engineered from human tRNA scaffolds, which have been validated in diverse

cell lines⁷⁻¹¹. Awawdeh *et al.* further demonstrated that engineering alanine identity elements into pyrrolysine tRNA scaffolds can generate functional alanine suppressor tRNAs in mammalian cells¹². In parallel, advances in *in vivo* delivery technologies, such as adeno-associated viral (AAV) vectors and lipid nanoparticles (LNPs), have enabled evaluation of these engineered sup-tRNAs in animal models⁴. Several breakthrough studies have now demonstrated meaningful therapeutic efficacy and favorable safety profiles in mouse disease models^{8,10,11,13}.

Despite this progress, important challenges continue to limit the broader applicability of suppressor tRNA therapy. First, sup-tRNAs based on endogenous human tRNA scaffolds often exhibit tissue-specific biases linked to variable aminoacyl-tRNA synthetase (aaRS) expression¹⁴. Second, a previous analysis of the suppressor tRNAs evaluated in that study estimated that the delivered amino acids would be functionally tolerated at only approximately 32% of disease-associated PTC variants¹⁰, limiting broad applicability. Third, *in vivo* validation has been confined largely to soluble enzymes or intracellular scaffolds; the capacity of sup-tRNA therapy to rescue structurally diverse proteins, including large cytoskeletal proteins, secreted extracellular-matrix components, and

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signaling hubs, or to address haploinsufficient disorders remains largely unexplored.

These limitations indicate that the therapeutic reach of sup-tRNAs will depend on both the biological properties of the introduced amino acid and the engineering of the underlying tRNA scaffold. We reasoned that a broadly applicable sup-tRNA platform should be guided by both molecular engineering considerations and the landscape of human PTC-associated diseases. Such a framework would prioritize amino acids that are broadly tolerated across pathogenic variants while enabling robust and tissue-independent aminoacylation. Coupling this disease-informed design strategy with advances in sup-tRNA engineering could provide a path toward translational platforms with expanded therapeutic scope.

Here, we combine large-scale human genetic analysis, sup-tRNA engineering, and *in vivo* gene delivery to develop ALPHA (Alanine-based Programmable tRNA for Haploinsufficiency and PTC-Associated diseases). To test whether a single ALPHA platform could support rescue across diverse genetic architectures, target organs, and protein classes, we evaluated its efficacy in four distinct PTC mouse models representing autosomal-recessive (mucopolysaccharidosis type I; MPS I), X-linked recessive (Duchenne muscular dystrophy (DMD) and Alport syndrome), and autosomal-dominant (familial adenomatous polyposis (FAP)) inheritance (Fig. 1f). In each model, AAV9-delivered ALPHA restored full-length protein expression and produced biochemical, histological, and functional improvements.

RESULTS

Defining the clinical and theoretical landscape of alanine-mediated suppression

To define the clinical landscape of PTC-driven pathologies, we integrated 66,876 pathogenic PTC mutations from the ClinVar database¹⁵ with Orphadata rare disease annotations, identifying 3,424 distinct PTC-associated rare diseases. These disorders span fundamentally different genetic architectures, with autosomal-dominant, autosomal-recessive, and X-linked recessive disorders collectively accounting for 85.7% of mapped disease entities (Fig. 1a; Supplementary Data 1). Despite this profound genetic heterogeneity, it remains unknown whether a single sup-tRNA strategy can support therapeutic rescue across such diverse genetic contexts.

To determine whether a single dedicated amino acid substitution could address a substantial portion of this heterogeneous landscape, we systematically evaluated structural tolerance using AlphaMissense pathogenicity predictions¹⁶. Analysis of 66,876 pathogenic PTC mutations indicated that alanine substitution at PTC sites yielded the highest fraction of predicted benign outcomes and the lowest fraction of predicted pathogenic outcomes (Fig. 1b; Supplementary Data 2). Among AlphaMissense-scored PTC-to-alanine substitutions, 30,515 of 66,070 variants were predicted to be benign. Analysis by stop-codon identity further showed that Ala-benign variants were distributed across TAG, TGA and TAA codons, supporting the potential applicability of alanine suppression across all three PTC classes (Fig. 1c). In parallel, transcriptomic analysis of the GTEx database¹⁷ showed that human alanyl-tRNA synthetase (AlaRS) was among the most highly expressed and uniformly expressed aaRSs across the

surveyed human tissues (Fig. 1d; Supplementary Fig. S1a). Quantitative proteomic data from adult mouse tissues¹⁸ also showed that cytosolic AlaRS was broadly available across 27 surveyed tissues, providing support for subsequent evaluation of engineered tRNA in mouse models (Supplementary Fig. S1b, c). Integrating aaRS expression breadth with predicted substitution tolerance further highlighted alanine as a favorable candidate for suppressor-tRNA engineering (Fig. 1e).

Engineering a highly active alanine suppressor tRNA

Although alanine emerged as a favorable rescue amino acid, engineering functional alanine sup-tRNAs in mammalian cells has proven difficult. The G3:U70 wobble base pair of tRNA^{Ala} is a major identity determinant for AlaRS recognition¹⁹, yet neither anticodon engineering of human natural tRNA^{Ala} nor transplantation of G3:U70 element into other human tRNAs generated an active alanine suppressor tRNA (Supplementary Fig. S2a, b), consistent with previous studies^{10,12}. We therefore sought a structurally compatible scaffold for alanine suppressor-tRNA engineering. We envisioned that orthogonal tRNA scaffolds developed for noncanonical amino acid incorporation (genetic code expansion) would tolerate anticodon mutation to a stop codon while maintaining structural integrity and minimal interference with endogenous translation²⁰⁻²⁵. Based on this design principle, ALPHA was engineered to incorporate alanine at PTC sites and thereby restore full-length protein production (Fig. 2a). We introduced the identity element (G3:U70)^{19,26} of tRNA^{Ala} into a panel of orthogonal tRNAs previously validated in mammalian cells. These engineered tRNAs were then evaluated for stop codon (TAG) readthrough efficiency with a dual fluorescence reporter assay in HEK293T cells (Fig. 2b). After screening, we identified a chimeric tRNA (A1-sup-tRNA^{Ala}) derived from an evolved chPheT scaffold²⁷ that achieved the highest TAG readthrough efficiency (36.3%) (Supplementary Fig. S2c).

To further increase the readthrough efficiency, we hypothesized that the co-expression of the cognate tRNA-binding domain (TD) could protect the tRNA and enhance its activity^{24,28}. Indeed, co-expression of the PylRS TD (residues 1–104) increased readthrough to 55.4% (Fig. 2c). We then applied a stepwise, modular engineering strategy to the tRNA body. The parental chAT1 scaffold was conceptually divided into four structural modules, including the acceptor stem, anticodon stem, D and T arms (Fig. 2d). In the initial screening phase, structural elements derived from highly active tRNA scaffolds and previously validated suppressor-enhancing mutations were introduced into these modules. Functional modules that conferred an increase in readthrough activity were subsequently recombined in iterative rounds of optimization. Through this rational design, we constructed and evaluated a focused library of 104 distinct tRNA variants (Supplementary Data 3), identifying several high-activity variants, including chAT34, chAT56, chAT57 and chAT61, whose activity exceeded that of the parental chAT1 scaffold (Fig. 2e; Supplementary Fig. S2k). The best-performing variants restored reporter expression to as high as 78.5% of wild-type EGFP levels under TAG readthrough conditions (Fig. 2f). These sup-tRNA^{Ala} variants also decoded TGA and TAA codons, albeit with lower efficiency (51.3% and 18.4%, respectively)

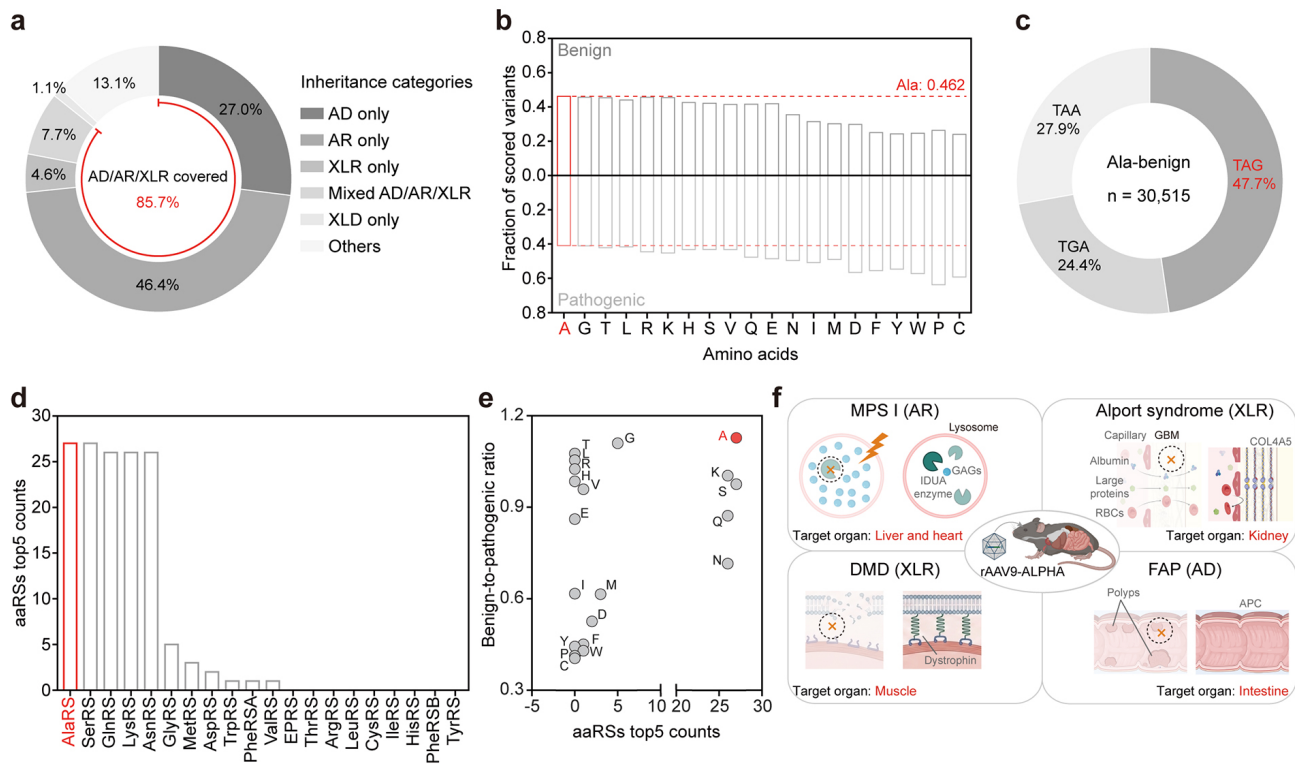


Fig. 1 Schematic of the suppressor tRNA^{Ala} platform for PTC-associated disease therapy. **a** Pie chart illustrates the inheritance landscapes of 3,424 rare diseases linked to genes harboring 66,876 pathogenic PTC mutations. These diseases were identified by cross-referencing genes carrying pathogenic PTC variants curated in ClinVar with established rare disease-associated genes. The autosomal dominant (AD), autosomal recessive (AR), X-linked recessive (XLR) associated diseases collectively accounted for 85.7% of all mapped disease entities. Diseases with non-standard inheritance modes or those lacking clear inheritance annotations are excluded from this analysis. **b** Distribution of missense mutation effect scores in PTC sites. For each amino acid, the fraction of scored variants classified as benign or pathogenic is shown. Alanine substitution yielded the highest fraction of predicted benign outcomes and the lowest fraction of predicted pathogenic outcomes. **c** Fractions of predicted benign outcomes for alanine substitution across three stop codons. **d** Tissue ranking of cytosolic aaRS expression. For each organ, aaRSs were ranked according to the expression level, and the bar plot shows how many times each aaRS appeared in the organ-specific top-five list. **e** Scatter plot showing the distribution of the benign-to-pathogenic ratio and the frequency of top five selection for each amino acid. Alanine exhibits the highest values in both metrics. **f** Schematic representation of the *in vivo* therapeutic strategy of ALPHA system. A recombinant AAV9 vector delivers an engineered sup-tRNA^{Ala} system to rescue disease phenotypes in four distinct PTC mouse models: mucopolysaccharidosis type I (MPS I, AR; IDUA-W392TAG), FAP, (AD; APC-L850TAG), X-ALport syndrome (XLR; COL4A5-R471TAG), and DMD (XLR; dystrophin-Q995TAG).

(Fig. 2f). Liquid chromatography-mass spectrometry (LC-MS) confirmed ~100% fidelity of alanine insertion at the PTC site, both with and without the TD104 domain (Supplementary Fig. S2d). Furthermore, to examine whether AlaRS availability can influence suppressor activity, we modulated AlaRS expression in HEK293T cells. AlaRS overexpression increased sup-tRNA^{Ala} mediated readthrough, whereas siRNA-mediated partial depletion of endogenous AlaRS reduced readthrough efficiency (Supplementary Fig. S2g–j). Although these experiments do not directly establish a tissue-to-tissue correlation between endogenous AlaRS expression and suppressor activity, they provide experimental support for the rationale of selecting an alanine suppressor tRNA based on the broad tissue availability of its cognate synthetase.

Optimizing the AAV cassette for durable *in vivo* readthrough

Efficient nonsense suppression in animals requires high intracellular tRNA abundance, which is dictated by the architecture of the expression cassette, specifically the choice of tRNA species, promoter and tRNA copy number. However, for AAV-mediated delivery, cassette design is strictly constrained by

the packaging capacity²⁹ and the high risk of genomic recombination induced by repetitive sequence arrays⁸. To navigate these inherent trade-offs, we evaluated cassette parameters to achieve durable single-dose expression, an advantage over LNP-based approaches that typically require repeated administration.

Although tRNA copy number and promoter choice have previously been shown to influence suppressor tRNA activity³⁰, we further evaluated these parameters in the context of our system. Dosage testing of 1 to 4 tRNA copies revealed that a 4-copy configuration yielded the highest efficiency (Supplementary Fig. S2e). Concurrently, profiling of various RNA polymerase III promoters identified human U6 and H1 as the most potent drivers of tRNA expression (Supplementary Fig. S2f). For *in vivo* evaluation, we then constructed a panel of multiplexed AAV vectors (Supplementary Fig. S3a). Each vector contained, in the same genome, a CB6-driven FLAG-tagged TD104 cassette and a 4-copy array of two distinct high-activity sup-tRNA^{Ala} variants driven by alternating U6 and H1 promoters.

Because repetitive multi-tRNA arrays are susceptible to

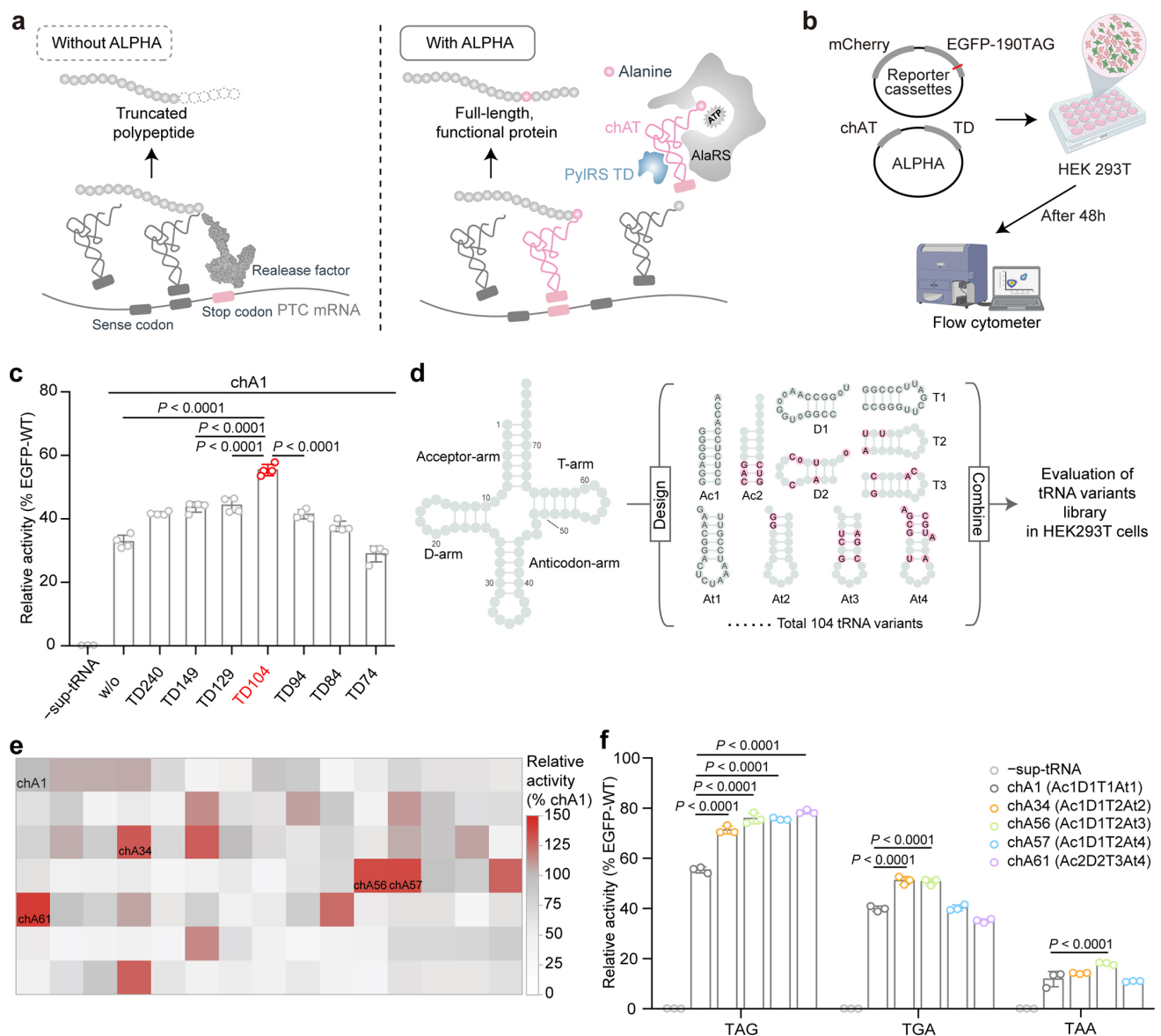


Fig. 2 The engineering and optimization of the ALPHA system. **a** Schematic of ALPHA-mediated nonsense suppression. In the absence of ALPHA, translation terminates at the PTC, producing a truncated and nonfunctional polypeptide. In the presence of ALPHA, alanine is incorporated into PTC sites and restores full-length, functional protein production. **b** Schematic of the cell-based screening workflow for identifying active sup-tRNA^{Ala}. The nonsense suppression efficiency was quantified by flow cytometry. **c** The readthrough efficiency of A1-sup-tRNA^{Ala} (chA1) was enhanced by the co-expression of cognate TD from PylRS. The segment of TD104 showed the highest performance and was therefore selected for subsequent analyses. “w/o” denotes chA1 without TD. **d** Schematic of the modular tRNA engineering strategy. The sup-tRNA^{Ala} was divided into four structural parts, which were individually optimized and then recombined to generate 104 different chimeric variants for activity screening in HEK293T cells. **e** Screening of engineered sup-tRNA^{Ala} variants for high readthrough activity. Relative activities of individual chAT variants are shown as percentages normalized to chA1. Top-performing variants, including chA34, chA56, chA57, and chA61, were identified for further characterization. **f** The readthrough efficiency of engineered sup-tRNA^{Ala} variants for TAG, TGA and TAA PTCs in EGFP. Error bars represent the mean $\pm$ SD from three or four biologically independent experiments. Reporter only (–sup-tRNA) is included to define basal reporter readthrough and was not included in the inferential comparisons. Statistical analysis was performed by one-way ANOVA followed by Dunnett’s multiple comparisons test (c), and two-way ANOVA followed by Dunnett’s multiple comparisons test (f).

recombination during AAV packaging and a previous 4-copy sup-tRNA^{Tyr} construct showed substantial recombination⁸, we next assessed the genome integrity of our 4-copy ALPHA vectors by alkaline agarose gel electrophoresis. Across three independent production batches, the full-length 4-copy band and a shorter species consistent with a 2-copy configuration accounted for approximately 70% and 30% of the total band intensity, respectively, with no other predominant lower-molecular-weight bands (Supplementary Fig. S3f, g). These

results indicate that the majority of packaged ALPHA genomes retained the intended 4-copy configuration. These optimizations were then evaluated *in vivo* using a newly generated Rosa26-targeted luciferase reporter mouse model harboring a TAG PTC, allowing direct quantification of readthrough efficiency and durability in a whole-animal setting. Intravenous administration of rAAV9.ALPHA-1-6 at 0.75×10^{14} genome copies per kilogram of body weight (GC/kg) restored luciferase expression *in vivo* (Supplemen-

tary Fig. S3b), with one construct (ALPHA-6) showing the highest and most sustained signal, yielding approximately twofold higher bioluminescence signal than the previously reported sup-tRNA^{Tyr} control at both 5 and 16 weeks (Supplementary Fig. S3c). To exclude an amino-acid-dependent effect on reporter activity, protein-normalized activities of FLuc-161A and FLuc-161Y were compared and found to be similar (Supplementary Fig. S3d, e). ALPHA-6 was therefore selected for all subsequent disease-model studies.

Broad-spectrum efficacy across diverse protein classes and inheritance patterns

To assess the generality of the ALPHA platform, we tested rAAV9.ALPHA-6 in four PTC mouse models deliberately chosen to span distinct protein architectures and inheritance modes, thereby addressing the narrow protein-class validation of prior sup-tRNA studies.

Evaluation of the ALPHA system in the Hurler syndrome model (MPS I, autosomal recessive)

We first selected a well-established mouse model of Hurler syndrome³¹. This model harbors a W392TAG mutation in the IDUA (α -L-iduronidase) protein (homozygous *Idua*^{W392TAG/W392TAG}, hereafter *Idua*^{KI/KI}). Loss of IDUA enzymatic activity leads to systemic accumulation of glycosaminoglycans (GAGs), resulting in progressive and multi-organ pathology, most notably profound skeletal dysplasia that manifests behaviorally as characteristic gait abnormalities and anatomically as pronounced zygomatic arch thickening^{31,32}. This model provides a stringent test of whether alanine substitution can restore enzymatic function in a multi-organ storage disease.

Six-week-old male *Idua*^{KI/KI} mice received a single intravenous injection of rAAV9.ALPHA-6 at 0.6×10^{14} GC/kg (Fig. 3a). At 4 weeks post-injection, the treatment restored serum IDUA enzyme activity to approximately 13.1% of wild-type levels, accompanied by a reduction in urinary GAG excretion (Fig. 3b, c). Both enzymatic activity and GAG reduction were sustained over the entire 16-week observation period (Fig. 3b, c). Gait analysis at the 16 weeks revealed a significant increase in hind-base width compared with PBS-treated controls (Fig. 3d, e). Micro-CT quantification of craniofacial morphology showed a reduction in zygomatic-arch diameter (Fig. 3f, g), indicating partial correction of bone abnormalities.

To further characterize the rescue, we quantified tissue-specific biochemical parameters at the 16-week endpoint. IDUA enzymatic activity was restored to 7.4% of wild-type in heart and 2.6% in liver (Fig. 3h, i), with significant GAG reduction in both tissues (Fig. 3j, k). Western blotting showed marked attenuation of LAMP1 (lysosome-associated membrane protein 1) overexpression (Fig. 3l–n), a well-established marker of lysosomal storage in heart and liver³³. Finally, we assessed whether these molecular improvements translated to structural preservation at the tissue level. Hematoxylin and eosin (H&E) staining confirmed widespread reduction in vacuolar pathology across spleen, heart, liver, and kidney (Supplementary Fig. S4).

We then performed a dose-response study using rAAV9.ALPHA-6 (Supplementary Fig. S5a). Dose-response studies using 0.6 , 1.2 and 2.4×10^{14} GC/kg further demon-

strated titratable rescue. Higher doses yielded greater restoration of serum and tissue IDUA activity with corresponding reduction in urinary and tissue GAG levels (Supplementary Fig. S5b–g). Notably, IDUA activity in heart approached heterozygous levels at the highest dose (Supplementary Fig. S5c). We also observed a dose-responsive reduction in zygomatic-arch thickening (Supplementary Fig. S5h, i), alongside proportional attenuation of LAMP1 in heart and liver (Supplementary Fig. S5j–l). Collectively, these results demonstrate that the ALPHA system enables durable and titratable restoration of IDUA enzymatic activity, leading to coordinated improvement across biochemical, histological and phenotypic endpoints in the Hurler syndrome mouse model.

Evaluation of the ALPHA system in DMD mouse model (X-linked myopathy)

To challenge the platform with a massive intracellular structural protein that forms an essential cytoskeletal scaffold, we generated DMD harboring Q995TAG mutation (*Dmd*^{KI/Y}) mice modeling DMD. This X-linked myopathy is caused by loss-of-function of dystrophin and leads to progressive muscle degeneration^{34,35}. This mouse model provides a stringent test of ALPHA because suppression of the Q995TAG mutation must enable completion of the exceptionally large dystrophin coding sequence and generate a stable, correctly localized, and functional full-length protein.

As an initial assessment, 6-week-old male mice received an intramuscular injection of rAAV9.ALPHA-6 (1×10^{11} GC/site) into the tibialis anterior (TA) muscle (Supplementary Fig. S6a). Western blotting analysis confirmed robust, time-dependent restoration of full-length dystrophin, reaching 11.9% and 16.6% of wild-type levels at 4 and 8 weeks post-injection, respectively (Supplementary Fig. S6b, c). FLAG-tagged TD104 was detected in the same TA lysates at both time points, confirming *in vivo* co-expression with the suppressor-tRNA cassette (Supplementary Fig. S6b).

For systemic evaluation, 4-week-old *Dmd*^{KI/Y} mice received a single intraperitoneal injection of rAAV9.ALPHA-6 (1×10^{14} GC/kg), and therapeutic outcomes were comprehensively evaluated 16 weeks post-injection (Fig. 4a). In addition to skeletal muscle, the primarily affected tissue in DMD, we also examined the heart and diaphragm because cardiomyopathy and respiratory failure caused by diaphragm weakness are major causes of mortality in DMD patients. Western blotting analysis at the endpoint revealed substantial accumulation of full-length dystrophin in skeletal muscle, heart and diaphragm (Fig. 4b–d; Supplementary Fig. S6d–g). FLAG-tagged TD104 was also detected in skeletal muscle, heart and diaphragm lysates (Supplementary Fig. S6h, i). H&E staining demonstrated markedly preserved muscle architecture across diaphragm (Diam), gastrocnemius (GAS), TA and quadriceps femoris (QF) muscle, alongside an increased proportion of morphologically healthy myofibers (Fig. 4e, f). Immunofluorescence showed widespread sarcolemmal dystrophin restoration in GAS, TA, and QF muscles (Fig. 4g). Consistent with protection against myofiber injury, treated mice exhibited reduced serum creatine kinase (CK) levels (Fig. 4h). These molecular and histological improvements translated into functional gains, including increased grip strength (Fig. 4i, j),

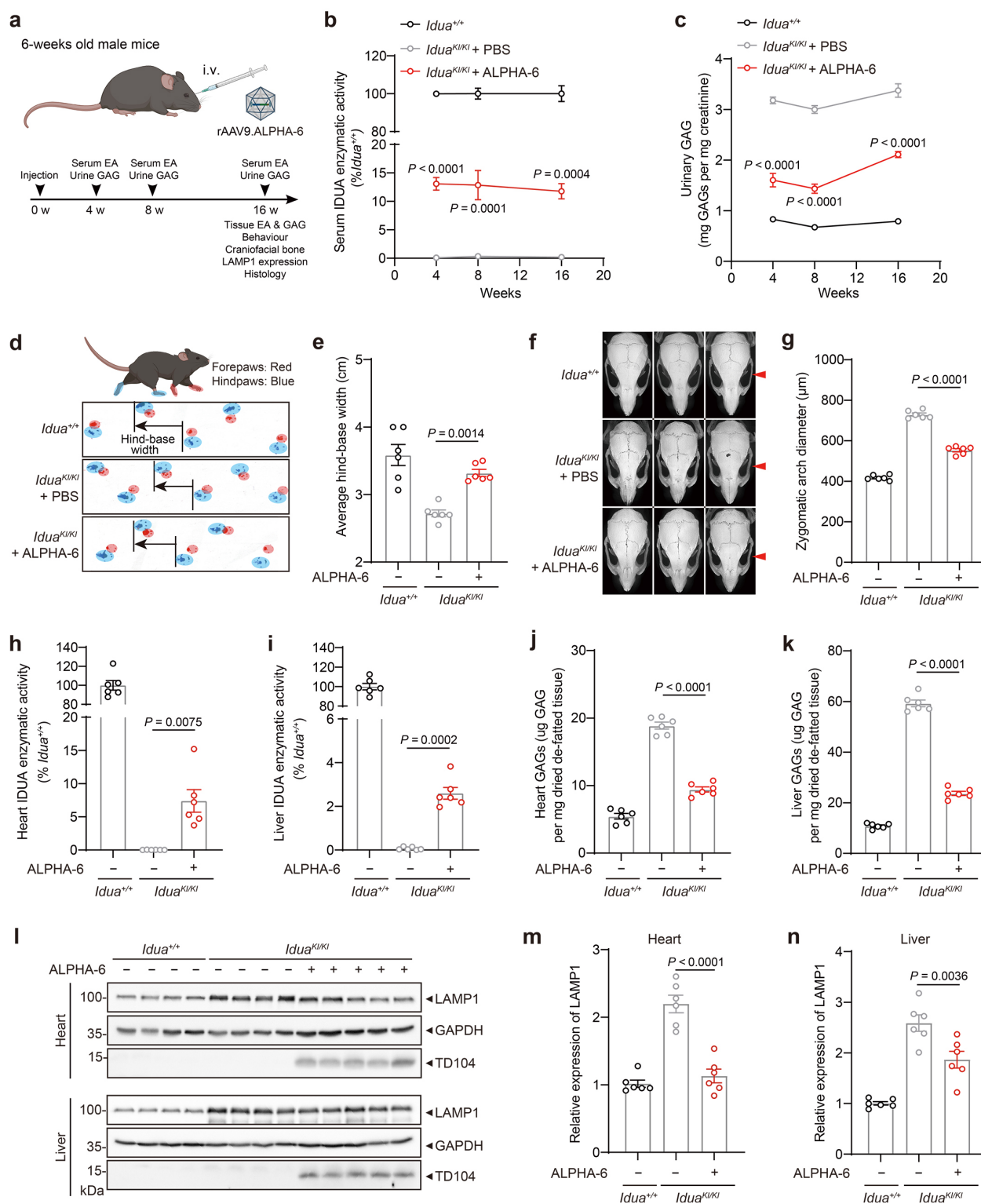


Fig. 3 ALPHA system treatment alleviates the MPS I symptoms in *Idua*^{KI/KI} mice. **a** Workflow of *in vivo* assessment of the therapeutic efficacy for ALPHA-6 by AAV delivery in male *Idua*^{KI/KI} mice. ALPHA-6 was injected through the orbital vein at a dose of 0.6×10^{14} GC/kg. **b**, **c** Serum IDUA enzymatic activity (**b**) and urine GAG levels (**c**) in *Idua*^{KI/KI} mice with or without ALPHA-6 treatment at the 4, 8 and 16 weeks after injection. **d**, **e** Footprint analysis of mice was performed at the 16 weeks after injection. Representative images of the footprint analysis (**d**) and the average hind-base width from the left hindpaws to the right hindpaws (**e**) were shown. **f**, **g** Micro-CT scans of mouse heads at the 16 weeks after treatment. Representative images (**f**) and quantification of zygomatic arch diameter across groups (**g**) were shown. **h**, **i** Heart (**h**) and liver (**i**) IDUA enzymatic activity for mice at the endpoint of treatment. **j**, **k** Heart (**j**) and liver (**k**) GAG levels for mice at the endpoint of treatment. **l**–**n** Western blot images (**l**) and quantification (**m**, **n**) of LAMP1 protein expression in heart (**m**) and liver (**n**) for mice at the 16 weeks after treatment. Error bars represent the mean $\pm$ SEM ($n = 6$ mice per group). Statistical analysis was performed by unpaired Student's *t*-test (**h**, **i**). Statistical analysis was performed by two-way ANOVA followed by Dunnett's multiple comparisons test (**b**, **c**), and one-way ANOVA followed by Dunnett's multiple comparisons test (**e**, **g**, **j**, **k**, **m**, **n**).

improved rotarod motor coordination time (Fig. 4k, l) and enhanced treadmill exercise endurance distance and running time (Fig. 4m, n).

Evaluation of the ALPHA system in the X-linked Alport syndrome mouse model (COL4A5 mutation)

While the platform successfully restored the intracellular enzyme and structural protein, we next tested whether it could rescue PTCs in genes encoding secreted proteins that undergo complex extracellular assembly. We selected X-linked Alport syndrome caused by a mutation in COL4A5 on the X chromosome^{36,37}. Loss of the type IV collagen $\alpha 5$ chain disrupts the highly organized glomerular basement membrane (GBM) architecture, invariably leading to end-stage renal disease^{36,38}. We generated a male COL4A5^{R471TAG/Y} (COL4A5^{KI/Y}) mouse model for this disease³⁹.

Four-week-old male mice received a single intraperitoneal injection of rAAV9.ALPHA-6 (1×10^{14} GC/kg) (Fig. 5a). FLAG-tagged TD104 was detected in kidney lysates at the study endpoint, consistent with vector expression in the kidney (Supplementary Fig. S7). The treatment attenuated the age-dependent progression of urinary albumin-to-creatinine ratio from 4 to 22 weeks of age (Fig. 5b) and produced robust reductions in endpoint serum blood urea nitrogen (BUN) and creatinine levels (Fig. 5c, d), bringing these markers close to the non-diseased wild-type range. Histopathological evaluation showed markedly attenuated glomerular hyalinosis and preserved glomerular architecture on H&E staining (Fig. 5e, g). Substantial reduction in renal fibrosis area was observed by Masson's trichrome staining (Fig. 5f, h). Moreover, immunofluorescence confirmed restoration of full-length COL4A5 protein expression (Fig. 5i, j). These data demonstrate that ALPHA-6 administration can slow progression of structural nephropathy.

Evaluation of the ALPHA system in the haploinsufficient mouse tumor model (FAP, autosomal dominant)

Haploinsufficient disorders⁴⁰, which affect more than 300 monogenic conditions^{41,42}, present a particular therapeutic challenge because conventional gene-replacement strategies frequently result in supra-physiological protein levels that can disrupt tightly regulated signaling pathways and cause toxicity^{4,43}. In contrast, tRNA-mediated nonsense suppression offers the distinct advantage of restoring expression directly from the endogenous transcript under native transcriptional and post-transcriptional control. To address haploinsufficient disorders in which precise physiological protein dosage is critical, we utilized the classic *Apc*^{Min/+} mouse model of FAP^{44,45}, which harbors an L850TAG nonsense mutation leading to adenoma formation and systemic complications⁴⁶. This autosomal dominant model tests whether ALPHA can restore native regulatory control without the overexpression risk of conventional gene replacement.

Eight-week-old *Apc*^{Min/+} mice received a single intraperitoneal injection of rAAV9.ALPHA-6 (1×10^{14} GC/kg) (Fig. 6a). At 12 weeks post-injection, macroscopic examination of the mid-small intestine revealed a reduction in visible adenomatous lesions (Fig. 6b). Quantification across the entire intestinal tract confirmed significant decreases in both total tumor counts and overall tumor load (Fig. 6c, d). The treatment also

reversed tumor-associated anemia, restoring red blood cell counts (Fig. 6e), hemoglobin concentration (Fig. 6f), and haematocrit levels (Fig. 6g). Gross pathological analysis further demonstrated attenuated splenomegaly in ALPHA-6-treated *Apc*^{Min/+} mice, as evidenced by representative spleen images (Fig. 6h) and a marked reduction in spleen coefficient (Fig. 6i). H&E staining and β -catenin immunohistochemistry (IHC) demonstrated reduced adenoma burden and normalization of aberrant Wnt signaling (Fig. 6j). These data demonstrate that ALPHA-6-mediated readthrough normalized aberrant Wnt signaling, reduced intestinal adenoma burden, and ameliorated tumor-associated systemic abnormalities in *Apc*^{Min/+} mice.

Preclinical safety and tolerability

To evaluate preclinical safety at a dose exceeding therapeutic levels, we intravenously administered rAAV9.ALPHA-6 (1×10^{14} GC/kg) to wild-type rats. Over the 4-week observation period, body weight kinetics and food consumption rates in both male and female rats remained indistinguishable from PBS-treated controls (Supplementary Fig. S8a–d). Furthermore, serum IgG levels were not elevated (Supplementary Fig. S8e, f). Comprehensive histopathological evaluation of major organs (including brain, heart, lung, liver, spleen, kidney, testis, and ovary) revealed no overt structural abnormalities, inflammatory infiltration, or cellular toxicity (Supplementary Fig. S8g, h). Serum biochemical parameters (Supplementary Table S2) and relative organ weights (Supplementary Table S3) were also within normal ranges. These results indicate that systemic ALPHA expression is well tolerated at the high dose tested.

To complement the 4-week safety study in wild-type rats, we evaluated the high-dose ALPHA-6 cohort in *Idua*^{KI/KI} mice at the 16-week endpoint. Serum indicators of hepatic and renal function did not show treatment-related abnormalities between untreated and ALPHA-6-treated *Idua*^{KI/KI} mice, including those animals receiving the highest dose (Supplementary Table S4). Histopathological examination similarly revealed no treatment-related abnormalities in the examined organs (Supplementary Fig. S5m). Thus, ALPHA-6 was well tolerated over the 16-week observation period, with no overt dose-limiting toxicity at the highest dose tested.

DISCUSSION

A major unresolved question in suppressor tRNA therapies is whether a single translational rescue strategy can be broadly applicable across the extraordinary diversity of PTC-associated diseases. Here, we have developed ALPHA, an engineered alanine suppressor tRNA platform that achieves efficient and durable nonsense suppression *in vivo*. By transplanting the minimal G3:U70 identity element of tRNA^{Ala} into an orthogonal scaffold and reinforcing it with the PyIRS TD, we generated sup-tRNAs with high readthrough activity (up to 78.5% for TAG) and ~100% alanine fidelity.

The central advance is the demonstration that alanine replacement is broadly tolerated across structurally diverse proteins and inheritance patterns. In four independent mouse models chosen to represent soluble lysosomal enzymes, massive cytoskeletal scaffolds, secreted extracellular matrix components, and signaling hubs, ALPHA restored functional

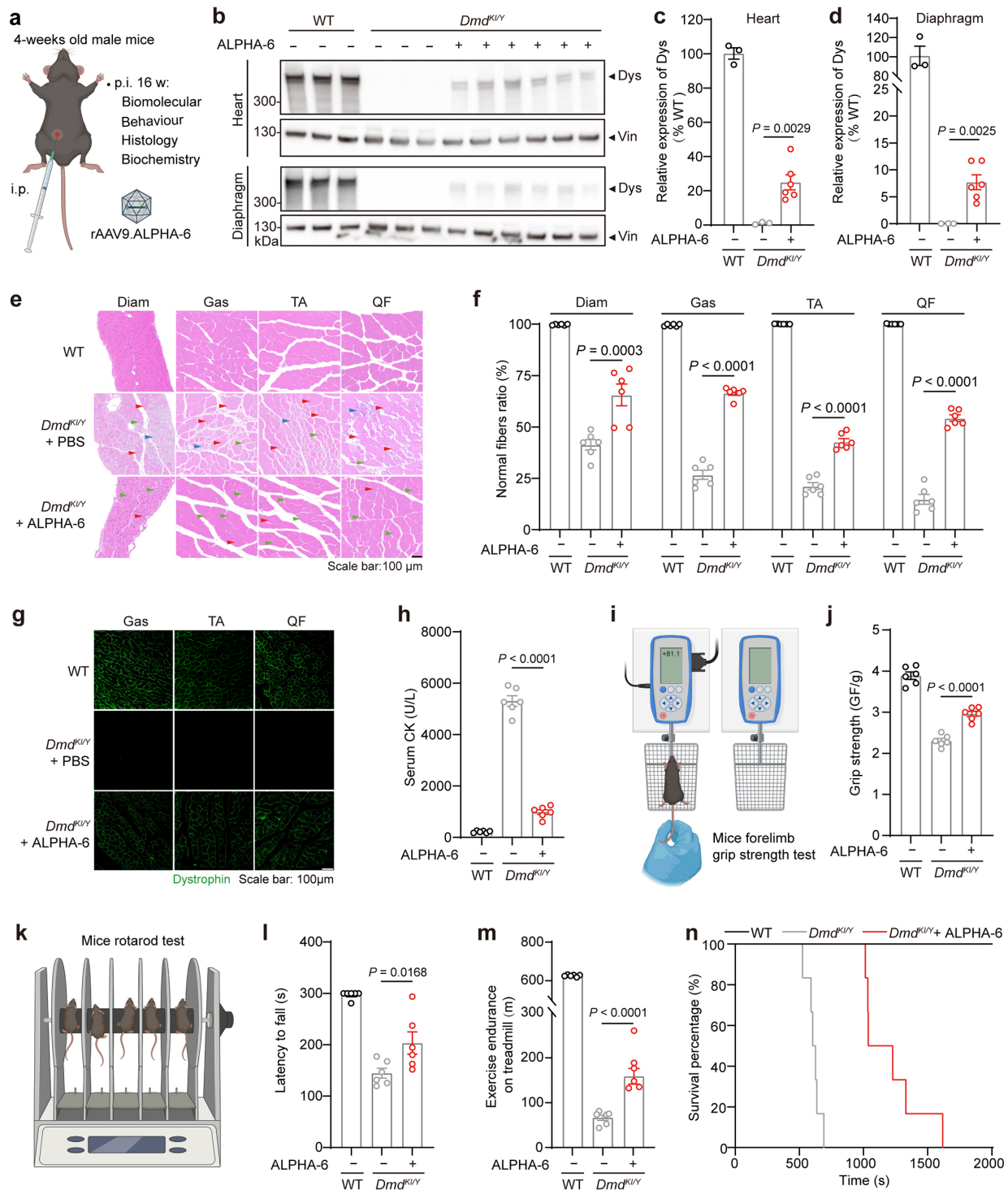


Fig. 4 Injection of ALPHA-6 alleviates disease symptoms in $Dmd^{KI/Y}$ mice. **a** Schematic of the workflow used to assess *in vivo* efficacy of ALPHA-6 in male $Dmd^{KI/Y}$ mice. ALPHA-6 was administered via intraperitoneal injection at a dose of 1×10^{14} GC/kg at 4 weeks of age. **b–d** Western blot images (**b**) and quantification of mouse dystrophin (Dys) protein expression in the heart (**c**) and diaphragm (**d**) of $Dmd^{KI/Y}$ mice following intraperitoneal ALPHA-6 injection. Vinculin (Vin) was used as the loading control for tissues. **e, f** H&E staining showing histological improvement and reduction of dystrophic features in ALPHA-6-treated mice. The representative image (**e**) and quantification of normal fiber ratio (**f**) were shown. The red arrow indicates abnormal fibers, the blue arrow indicates an area of inflammation, and the green arrow indicates normal fibers. **g** Immunostaining of dystrophin in GAS muscle, TA muscle and QF muscle of $Dmd^{KI/Y}$ mice, with wild-type C57BL/6J mice as control. Scale bar, 100 μ m. **h** Serum CK levels, a diagnostic marker for muscle injury, were measured and shown. **i** Schematic diagram of the forelimb grip strength test apparatus and operation in mice. **j** Grip strength was measured at 16 weeks post-injection to reflect muscle function. **k** Schematic diagram of the rotarod test apparatus. **l** The rotarod test was performed in the three groups at 16 weeks post-injection to evaluate physical coordination. **m** Exercise endurance was assessed at 16 weeks post-injection using a treadmill. **n** Survival percentage curve of mice in the exercise endurance test. Error bars represent the mean $\pm$ SEM ($n = 6$ mice per group). Statistical analysis was performed by a two-sided unpaired Student's *t*-test (**c, d**) or one-way ANOVA followed by Dunnett's multiple comparisons test (**f, h, j, l, m**).

protein expression while addressing the tissue bias and limited amino-acid tolerance of previous approaches. Quantitative analysis indicates that alanine substitution is predicted to be benign in 46.2% of AlphaMissense-scored PTC-to-alanine substitutions, expanding the theoretical scope of sup-tRNA therapy.

A particularly informative finding was the successful treatment of a haploinsufficient disorder (FAP). Most previous sup-tRNA studies have focused on classical loss-of-function diseases in which restoration of protein expression is the

primary therapeutic objective. In contrast, haploinsufficient disorders require recovery of protein abundance within a relatively narrow physiological range⁴⁰. Conventional gene-replacement approaches frequently rely on constitutive transgene expression and may risk supra-physiological expression. Because ALPHA restores protein levels under native regulatory control, it offers a fundamentally different therapeutic mechanism. The efficacy observed in the FAP model therefore extends the potential scope of sup-tRNA therapies beyond traditional loss-of-function disorders^{41,42}.

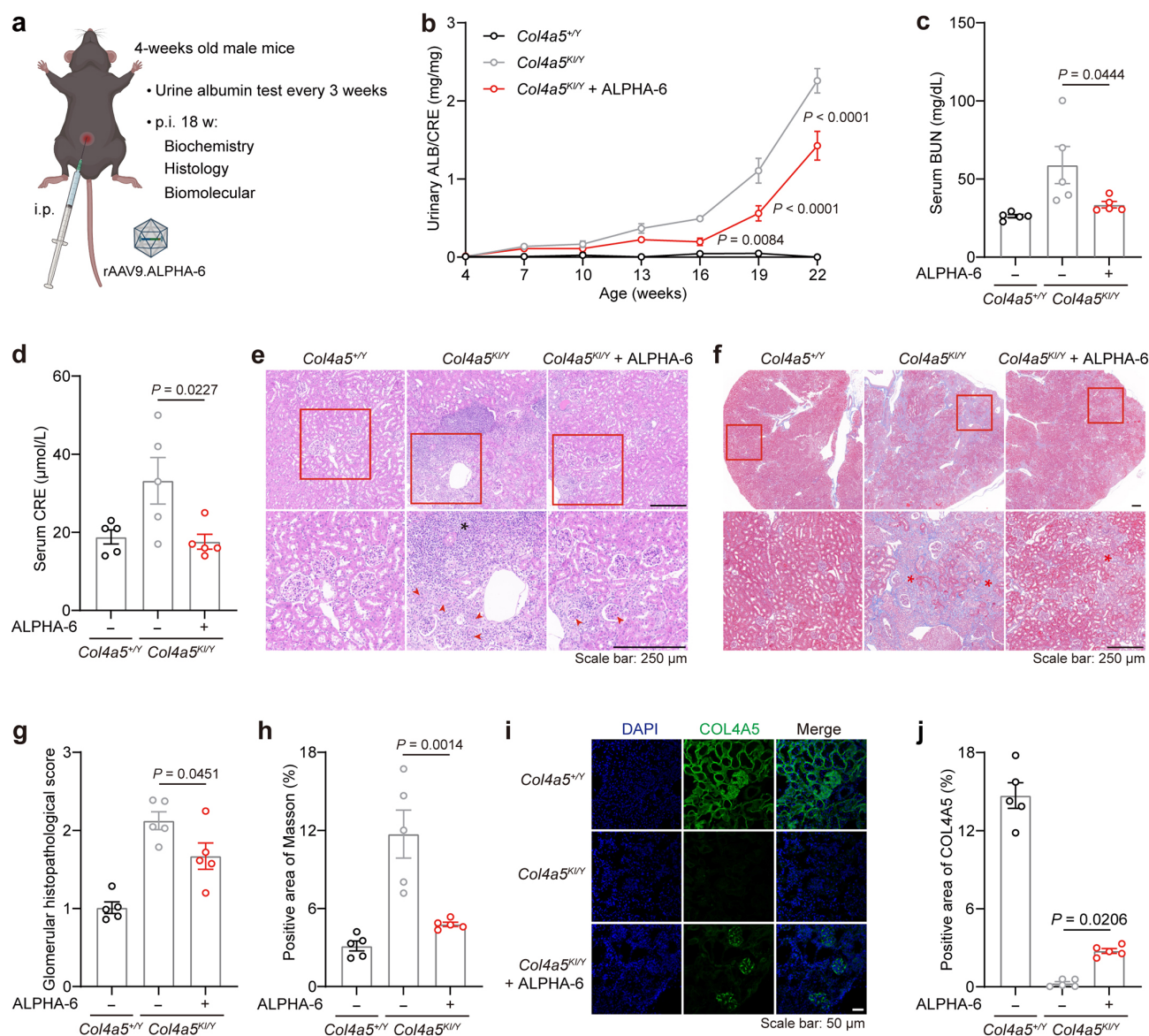


Fig. 5 ALPHA-6 treatment rescues the Alport syndrome in $Col4a5^{KI/Y}$ mice. **a** The workflow used to assess the efficacy of the ALPHA system in male $Col4a5^{KI/Y}$ mice. Four-week-old male mice were injected intraperitoneally with ALPHA-6 (1×10^{14} GC/kg). **b** Progression of the urinary albumin-to-creatinine ratio (ALB/CRE) from 4 to 22 weeks of age in mice. **c**, **d** Serum levels of BUN and creatinine evaluated at the study endpoint of treatment. **e** Representative H&E staining of kidney sections from the three groups. The bottom panels show magnified views of the red boxed region in the top panels. Asterisks and arrows indicate morphological signs of renal damage, such as inflammatory cell infiltration and glomerular hyalinosis, which are visibly reduced in the treated group. Scale bar: 250 μm . **f** Representative Masson's trichrome staining of kidney sections to assess renal fibrosis. The bottom panels are magnified views of the red boxed areas. Red asterisks indicate focal fibrosis. Scale bar: 250 μm . **g** Quantification of glomerular histopathological scores based on H&E staining evaluations. **h** Quantification of renal fibrosis area based on Masson's trichrome staining. **i**, **j** Representative immunofluorescence images of kidney sections (**i**) and quantification of fluorescence-positive area (**j**), and the scale bar is 50 μm . Statistical analysis in **b** was performed using two-way ANOVA followed by Dunnett's multiple comparisons test. In **c**, **d**, **g**, **h**, and **j**, statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test. Error bars represent the mean $\pm$ SEM ($n = 5$ mice per group).

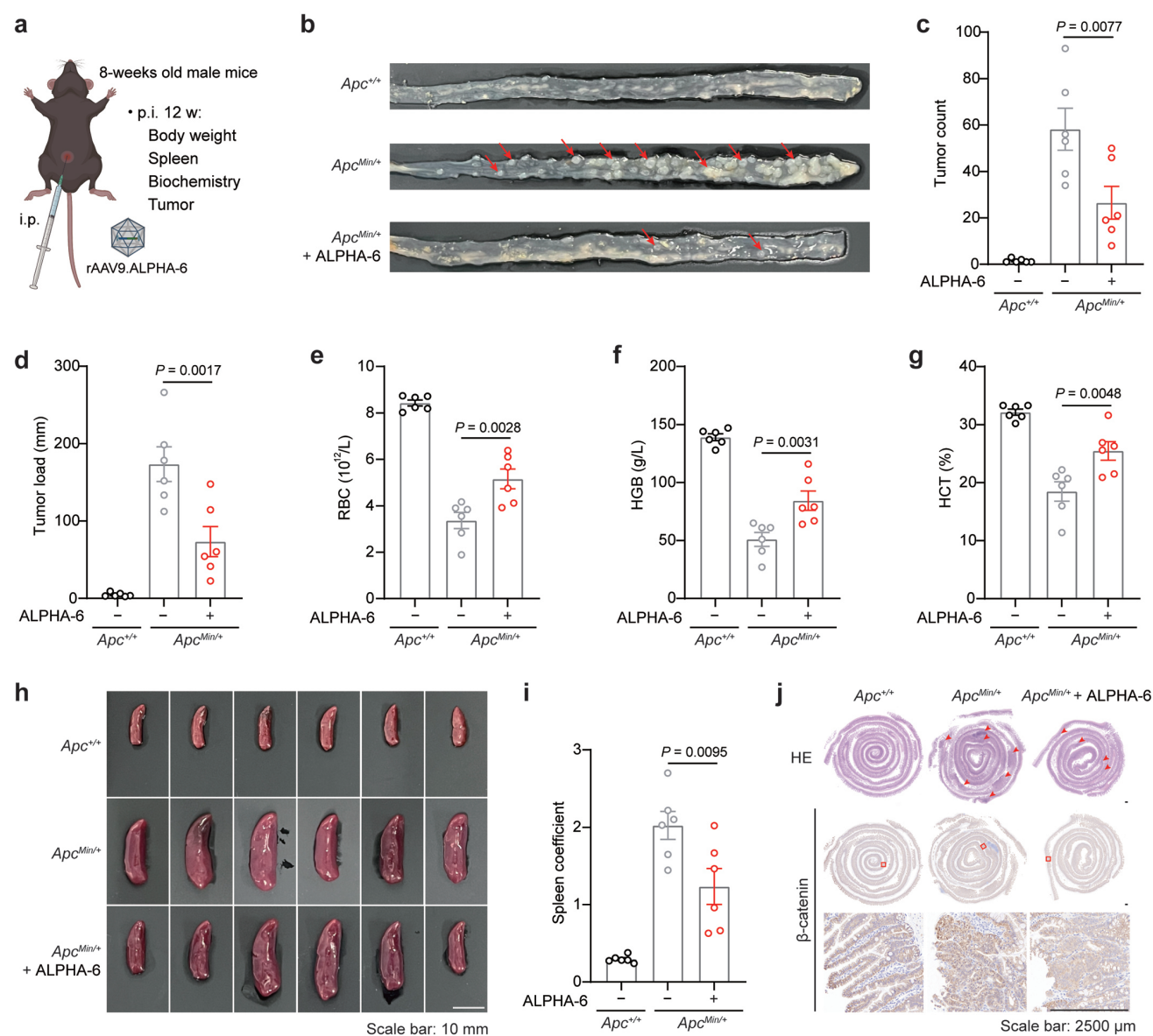


Fig. 6 ALPHA-6 treatment alleviates FAP in *Apc^{Min/+}* mice. **a** Schematic diagram of the experimental design: 8-week-old male *Apc^{Min/+}* mice were intraperitoneally injected with PBS or ALPHA-6 (1×10^{14} GC/kg). The relevant indicators were detected at 12 weeks post-injection. **b-d** Representative images of tumors (**b**), tumor count (**c**) and tumor load (the sum of the diameters of all tumors) (**d**) in each group were shown. **e-g** Complete blood count test, red blood cell (RBC) count (**e**), hemoglobin (HGB) concentration (**f**) and hematocrit (HCT) (**g**) in each group were shown. **h, i** The spleen morphology (**h**) and coefficient (**i**) of the mice spleen at 12 weeks post-injection were shown. **j** H&E staining and β -catenin IHC in the small intestines of each group were shown. Error bars represent the mean $\pm$ SEM ($n = 6$ mice per group). Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple-comparisons test.

Beyond therapeutic performance, our results establish orthogonal scaffold engineering as a viable alternative strategy for the development of sup-tRNAs with high efficiency. Recent studies have shown that aminoacylation identity is governed by complex structural determinants extending beyond the anticodon, creating inherent challenges for endogenous tRNA reprogramming¹⁰. The successful transplantation of alanine identity into orthogonal scaffolds demonstrates that transferable recognition elements can be leveraged to construct highly active suppressor tRNAs while maintaining amino-acid fidelity. This strategy provides a programmable route for engineering additional suppressor-tRNA systems and expanding the available suppression toolbox.

Several findings further support the translational potential of ALPHA. AAV-mediated delivery enabled durable suppressor activity for at least 16 weeks in mice, supporting the feasibility of long-term nonsense suppression *in vivo*. The availability of multiple alanine sup-tRNAs with comparable activity but substantial sequence divergence also facilitated the construction of multiplexed expression cassettes while minimizing repetitive sequence content, an important consideration for vector stability and manufacturing. High-dose rat toxicology showed no detectable systemic toxicity, consistent with recent ribosome-profiling data indicating that natural termination codon readthrough by engineered sup-tRNAs is minimal^{7,8}. Nevertheless, although TD104 lacks the catalytic domain of PylRS, its sustained expression as a non-human-

derived protein may be immunogenic. Long-term studies will therefore be required to evaluate TD104-specific immune responses before clinical translation.

We acknowledge several remaining limitations. Although alanine exhibits the highest predicted benign substitution frequency, it will not be universally tolerated at every pathogenic PTC site. Furthermore, suppression efficiency remains intrinsically stop-codon dependent, with TAA codons exhibiting lower responsiveness than TAG or TGA codons. Future innovations in sup-tRNA architecture, non-viral delivery vehicles, and mutant transcript stabilization strategies will likely further expand the therapeutic window of this approach. Collectively, our findings position ALPHA as a scalable, disease-agnostic framework with potential to address a substantial fraction of PTC-associated genetic disorders.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6J mice were purchased from Vital River Laboratories (Beijing, China). Sprague-Dawley rats were purchased from Shanghai Model Organisms. PTC disease mouse models (including W392TAG mutation in the IDUA protein (IduaKI/KI) mice, Q995TAG mutation in the DMD protein (DmdKI/Y) mice, R471TAG mutation in the COL4A5 protein (COL4A5KI/Y) mice, L850TAG mutation in the APC protein (*Apc*^{Min/+}) mice and Rosa26-targeted luciferase reporter mouse model harboring a TAG PTC) were generated by Shanghai Model Organisms using CRISPR/Cas9-mediated homology-directed repair.

All rodents were reared in-house (temperature: 20–25 °C, humidity: 40–70%) in a 12-h light/dark cycle under specific pathogen-free conditions. All animals had free access to food and sterilized water. All animal procedures were in accordance with the guidelines of, and approved by, the Institutional Animal Care and Use Committee of Zhejiang University.

Cell culture and transfection

HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, C11995500BT) supplemented with 10% (v/v) fetal bovine serum (Bio-Channel, BC20250314) and 1% penicillin-streptomycin (Gibco, 15140122). Cell lines were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C and passaged every 2 days up to 20 passages. Transient transfections of HEK293T cells were performed using PEI (Polysciences, 23966-1).

AlphaMissense-based pathogenicity assessment for amino acid substitution

Based on the 66,876 pathogenic PTC variants collected from ClinVar, the original amino acid affected by each variant was first identified. For each site, the original amino acid was then computationally substituted with each of the other 19 possible amino acids, generating a complete set of hypothetical missense variants for evaluation. These simulated missense substitutions were subsequently analyzed using AlphaMissense to predict their impact. For each substituted amino acid, the proportions of variants predicted to be pathogenic or benign were calculated across all generated missense variants. The resulting amino-acid-specific pathogenicity and benignity rates were used for downstream comparative anal-

yses and prioritization.

Collection and annotation of pathogenic PTC variants and rare disease genes

Pathogenic PTC variants were collected from the ClinVar database. After filtering for pathogenic variants, a total of 66,876 disease-causing PTC variants were retained for analysis. These variants were mapped to 3,570 unique genes based on the corresponding gene symbols. The gene list was further matched with rare disease genes curated in Orphadata. Rare disease information was integrated using the rare diseases natural history file. Gene matching was performed by exact comparison of gene symbols after standardization, including trimming of whitespace and conversion to uppercase. Through this procedure, 3,424 distinct rare diseases were identified as being associated with the ClinVar-derived PTC gene set. Inheritance pattern information was subsequently extracted and summarized from the Orphadata natural history dataset for downstream analyses.

Mouse tissue proteome analysis

Mouse tissue proteomic data were obtained from the Expression Atlas baseline proteomics experiment E-PROT-11, derived from the quantitative SILAC mouse proteome reported by Geiger *et al.*¹⁸. The Expression Atlas version contains 27 tissue samples from female C57BL/6J mice. Sixteen cytosolic aaRS proteins detected in the dataset were included. The legacy database gene symbol Aars was annotated as the current cytosolic gene symbol Aars1. Relative protein-abundance values supplied by Expression Atlas were transformed as $\log(x + 1)$, where x is the reported relative abundance value. No row-wise Z-score transformation was applied. For the heatmap, identical color scaling was used across all genes and tissues.

Plasmid construction

Plasmids for evaluating tRNA-mediated readthrough efficiency in HEK293T cells were constructed using Gibson assembly. tRNA sequences carrying the desired mutations were generated by PCR assembly using two overlapping primers and inserted into the pCMV vector to generate recombinant pCMV-U6-tRNA plasmids with or without CMV-TD104. For AAV packaging, AAV2/9.ALPHA1-6 constructs were generated as follows: the CB6 promoter and FLAG-tagged TD104 sequence were first inserted into the AAV2/9-GOI backbone by Gibson assembly, followed by insertion of the commercially synthesized 4xtrRNA cassette through restriction enzyme digestion and ligation (*EcoRI* and *SpeI*) to generate the recombinant plasmids AAV2/9.ALPHAx ($x = 1-6$)-CB6-TD104. Three native tRNAAla isoacceptors were modified by replacing their native anticodons with anticodons for stop codons. For heterologous scaffold testing, the G3:U70 pair was introduced into TyrT-, SerT-, GlnT- and LeuT-derived tRNA variants carrying a CUA anticodon. DNA sequences encoding the tRNA variants are provided in Supplementary Data 3.

Flow cytometric readthrough assay

HEK293T cells were seeded in 12- or 24-well plates and transiently transfected using PEI with the indicated suppressor-

tRNA expression plasmids and either the PTC-containing dual-fluorescence reporter plasmid (mCherry-T2A-EGFP-190TAG) or the matched WT reporter plasmid (mCherry-T2A-EGFP-WT). For experiments involving TD104, the TD104 expression plasmid was included as indicated. For reporter-only controls (–sup-tRNA), cells were transfected with the corresponding PTC reporter in the absence of suppressor-tRNA plasmid. Empty vector was added where necessary to maintain identical total DNA input across conditions. 24–36 h after transfection, cells were harvested and analysed by flow cytometry. Readthrough efficiency was calculated as the EGFP-to-mCherry fluorescence ratio of the PTC reporter normalized to that of the matched WT reporter and is presented as relative activity (% EGFP-WT). Reporter-only controls (–sup-tRNA) were used to determine basal reporter readthrough. Data represent 3 or 4 biologically independent experiments.

AlaRS overexpression and knockdown experiments

HEK293T cells were used to assess the effect of AlaRS dosage on ALPHA-mediated TAG readthrough. For overexpression experiments, cells received a fixed amount of pEGFP-mCherry-T2A-EGFP-190TAG-ALPHA-4×T reporter plasmid together with or without an equal amount of mouse Aars1 expression plasmid (1:1 relative to the reporter). Empty vector was added to maintain a constant total plasmid amount. Readthrough activity was measured 24–36 h after transfection using the flow-cytometric assay described above. For knockdown experiments, cells were transfected with non-targeting control siRNA or siRNAs targeting human AARS1 at the indicated concentrations, with control siRNA added to maintain a constant total siRNA amount. Twenty-four hours later, cells were transfected with the ALPHA reporter plasmid. Readthrough activity was measured 24 h later using the flow-cytometric assay described above. AARS1 knockdown was verified by immunoblotting, with protein abundance normalized to β -actin and the control siRNA condition set to 100%. siRNA sequences are listed in Supplementary Table S1.

Protein expression and purification in mammalian cells

For expression of EGFP containing alanine at the PTC site, HEK293T cells were seeded in 10-cm dishes and co-transfected with plasmids encoding tRNA with or without the TD104, together with a plasmid encoding EGFP harboring the UAG codon at a 1:1 ratio. Specifically, cells were transfected with 10 μ g of pCMV-U6-tRNA-CMV-TD104 and 10 μ g of pEGFP-mCherry-T2A-EGFP-190TAG-6×His. At 6 h post-transfection, the medium was replaced with fresh DMEM.

After expression for 48 h, cells were collected and resuspended in 1 mL of modified RIPA lysis buffer (25 mM triethanolamine, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1× PMSF) and lysed at RT for 10 min. The resulting lysate was centrifuged at 12,000× *g* for 15 min at 4 °C to collect the supernatant. The supernatants were incubated with 60 μ L of Ni²⁺-NTA bead slurry (SA004100, Smart-Life Sciences) for 4 h at 4 °C. The Ni²⁺-NTA beads were collected by centrifugation (1,000× *g*, 1 min, 4 °C) and washed three times with 500 μ L of wash buffer_1 (50 mM Tris, 200 mM NaCl, 25 mM imidazole, 1× PMSF, pH 8), followed by three washes with 500 μ L of wash buffer_2 (50 mM Tris, 200 mM NaCl, 50 mM imidazole, 1× PMSF, pH 8).

Finally, the proteins were eluted with 500 μ L of elution buffer (50 mM Tris, 200 mM NaCl, 250 mM imidazole, 1× PMSF, pH 8). The eluted proteins were desalted using a 10 kDa ultrafiltration tube with ddH₂O and concentrated to a final volume of 100 μ L. The purified proteins were subjected to LC-MS analysis.

LC-MS analysis

LC-MS analysis of EGFP was performed on a Waters Xevo G3 QTOF mass spectrometer equipped with a Waters ACQUITY UPLC H-Class PLUS system. The purified samples (as described above) were separated on a Waters ACQUITY UPLC Protein BEH C4 column (300 Å, 1.7 μ m, 2.1 mm × 50 mm) for 10 min at a flow rate of 300 μ L/min and mobile phase A/phase B gradient from 95% to 5% (v/v, mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile). The eluted samples were then injected via an electrospray ionization (ESI) source. Data were acquired in positive ion mode with a range of 400–4000 *m/z*. Mass spectral deconvolution was performed using MassLynx software (Waters, version 4.2).

AAV particle production and purification

AAV particles were produced in HEK293T cells and purified by iodixanol gradient ultracentrifugation according to the previous protocol⁴⁷. The AAV2/9-GOI backbone, pRep2/Cap9 and pHelper plasmids were kindly provided by Prof. Shengda Lin at Zhejiang University. Each 5-layer cell culture flask was transfected with 35 μ g of AAV2/9.ALPHAx-CB6-TD104, 35 μ g of Rep/Cap plasmid (pRep2/Cap9), and 100 μ g of adenoviral helper plasmid (pHelper) using 510 μ L PEI (23966-1, Polysciences, 1 mg/mL). The culture medium was replaced with DMEM supplemented with 1% penicillin-streptomycin without fetal bovine serum at 22–24 h after transfection. At 84 h post-transfection, cells and supernatants were collected. AAV particles were purified by one round of iodixanol step-gradient ultracentrifugation using 17%, 25%, 40%, and 60% iodixanol layers, followed by ultracentrifugation at 53,000× *g* for 10 h at 14 °C. The AAV-containing fraction (25% layer) was then harvested and concentrated in PBS supplemented with 0.001% Pluronic F-68 (24040032, Gibco) using an Amicon Ultra-15 centrifugal filter (100 kDa MWCO, UFC9100, Millipore).

Droplet digital PCR (ddPCR)

The titers of AAV genome were quantified using a customized TaqMan assay targeting TD104 (forward primer: GACAA-GAAGCCTCTGGACGTGC; reverse primer: GCTGTTGTTGAC-GACGAGGTGA; probe: GGCACTCTGCACAAGATCAAGC). Prior to ddPCR, AAV samples were treated with DNase I (EN0521, ThermoFisher Scientific) at 37 °C for 30 min. DNase I was subsequently inactivated by addition of EDTA to a final concentration of 20 mM. Samples were then lysed by addition of an equal volume of 0.2% SDS and incubation at 95 °C for 30 min, and subsequently diluted with EASY Dilution (Takara, 9160). The ddPCR reactions were prepared using ddPCR Supermix for Probes (No dUTP; 186-3023, Bio-Rad), and droplets were generated and analyzed on a QX200 ddPCR system (Bio-Rad) according to the manufacturer's instructions. AAV genome titers were calculated based on positive

droplet counts.

Characterization of rAAV vector genome integrity by denaturing agarose gel electrophoresis

Agarose gel (1%; TSJ001, Tsingke) was boiled with ultra-pure water and cooled to 55 °C, to which 0.1 volume of 10× alkaline gel electrophoresis buffer (500 mM NaOH and 10 mM EDTA) was added. 1 µL of 1 kb DNA ladder (N3232S, NEBio-labs) was diluted to a final volume of 20 µL with ddH₂O. Separately, 2.5 µL of AAV (1 E11 GC/µL) was diluted to 20 µL using ddH₂O. To each diluted sample, 5 µL of 50 mM EDTA and 5 µL of 6× alkaline gel loading buffer (ml833269, Milbio) were added. The mixtures were heated at 95 °C for 10 min, chilled on ice. Electrophoresis was performed at a current of 300 mA on ice for 6 h. Then, the gel was soaked in neutralization solution (0.5 M Tris-HCl, pH 8.0) for 45 min at room temperature. The neutralized gel was stained with GelRed (GR501-01, Vazyme, 1:10,000) in TAE buffer for 45 min and imaged using an SH 523 Chemiluminescence Imaging System (SHST, Hangzhou Shenhua Science Technology, Hangzhou, China). After background subtraction, the intensities of bands corresponding to full-length and truncated vector genomes were quantified using ImageJ.

IDUA enzymatic activity

Mouse tissues were collected immediately after transcardial perfusion with ice-cold PBS, snap-frozen in liquid nitrogen, and stored at -80 °C until analysis. Liver and heart tissues were lysed in ice-cold T-PER protein extraction reagent (78510, ThermoFisher Scientific) supplemented with protease inhibitor (Roche, 4693159001) using a TissueLyser (Jingxin, Shanghai; JXFSTPRP-24L). Lysates were centrifuged at 13,000× g for 15 min at 4 °C, and the supernatant was collected for total protein quantification using the BCA Protein Assay Kit (CW0014S, Cwbio). The α-l-iduronidase enzyme activity assay was performed in a total reaction volume of 100 µL, containing no more than 50 µg of total protein or 10 µL of serum, along with sodium formate buffer (pH 3.5, 130 mM), Triton X-100 (0.05%, v/v), NaCl (150 mM), d-saccharic acid 1,4-lactone monohydrate (0.42 mg/mL, Sigma-Aldrich, S0375), and 4MU-iduronic acid (0.12 mM, sc-220961, Santa Cruz Biotechnology). The reaction was incubated at 37 °C for 20 h and quenched with glycine buffer (0.2 M, pH 10.8). The fluorescence of released 4MU (excitation wavelength: 358 nm; emission wavelength: 438 nm) was detected using a multi-mode microplate reader (BioTek Synergy Neo2) and values were normalized to a standard curve generated with 4MU (M1381, Sigma-Aldrich). Specific enzyme activity was calculated as pmoles of 4MU released per mg of total protein per hour.

GAG assay

Urine was collected by gently holding mice over a 96-well plate, transferred to Eppendorf tubes, and centrifuged at 13,000 rpm for 15 min. Clarified urine samples were stored at -80 °C until analysis. Sulfated glycosaminoglycan (sGAG) levels in urine were measured using the Blyscan Sulfated GAG Assay Kit (B3000, Biocolor) according to the manufacturer's instructions. Tissues were homogenized in a chloroform:methanol mixture (2:1, v/v) using a TissueLyser and dried in a Savant DNA120 SpeedVac concentrator (ThermoFisher Scien-

tific) to remove fat. Dried, defatted tissues were weighed and digested with papain (P3125, Sigma-Aldrich) at 65 °C for 3 h. The resulting supernatants were used for GAG quantification with the Blyscan Sulfated GAG Assay Kit according to the manufacturer's instructions. GAG levels were shown as µg GAG per mg dried, defatted tissue.

Urine albumin-to-creatinine ratio

Urine was collected between 9:00 a.m. and 12:00 p.m. by gently holding mice over a 96-well plate, transferred to Eppendorf tubes, and centrifuged at 3,000 rpm for 15 min. The resulting supernatants were harvested, and the urinary albumin-to-creatinine ratio (ALB/CRE) was measured using a urinary microalbumin assay kit (E038-1-1, Nanjing Jiancheng) and a creatinine assay kit (C011-2-1, Nanjing Jiancheng) according to the manufacturer's instructions.

Serum biochemical analysis

Blood was collected from the orbital vein into 1.5 mL Eppendorf tubes and allowed to clot at room temperature for 2 h. After centrifugation at 3,000 rpm for 15 min at 4 °C, serum was collected, snap-frozen in liquid nitrogen, and transported on dry ice to the Center for Drug Safety Evaluation and Research, Zhejiang University, for analysis. Serum CK, BUN, and CRE levels were measured using automated analyzers (Roche Cobas C501 and TECOM TEK8500 VET) according to the manufacturers' instructions. All analyses were performed under a rigorous quality control system.

Serum IgG

Serum IgG levels were measured using a Rat IgG ELISA Kit (e-el-r0518, Elabscience) according to the manufacturer's instructions.

Western blotting analysis

Approximately 50 mg of tissue was lysed in 500 µL of ice-cold T-PER or strong RIPA buffer (BL504A, Biosharp) supplemented with protease inhibitor cocktail (4693159001, Roche). Homogenates were centrifuged, and protein concentrations were determined by the BCA Protein Assay Kit. Lysates were mixed with SDS loading buffer and boiled at 100 °C for 10 min, separated by SDS-PAGE, and transferred onto nitrocellulose membrane (10600001, Cytiva). The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies: rat anti-LAMP1 (553792, BD Pharmingen, 1:2,000), rabbit anti-FLAG (F7425, Sigma, 1:1,000), mouse anti-Dystrophin (D8168, Sigma, 1:500), mouse anti-Vinculin (66305-1-Ig, Proteintech, 1:2,000), rabbit anti-AlaRS (17394-1-AP, Proteintech, 1:1000), rabbit anti-Beta Actin (20536-1-AP, Proteintech, 1:2,000), and mouse anti-GAPDH (60004-1-Ig, Proteintech, 1:100,000). HRP-conjugated goat anti-mouse IgG (SA00001-1, Proteintech, 1:5,000), goat anti-rabbit IgG (SA00001-2, Proteintech, 1:5,000), or HRP-conjugated goat anti-rat (Biosharp, BL002A) was used as the secondary antibody, as appropriate. Chemiluminescence signals were captured by a Multifunctional Imager (SH-523, ShenhuaBio) and quantified with ImageJ software.

Luciferase activity

HEK293T cells were transfected with equal amounts of plas-

mids encoding full-length Firefly luciferase carrying either Ala or Tyr at residue 161 and a C-terminal FLAG tag. 24 h after transfection, cells were lysed and Firefly luciferase bioluminescence was measured using the One-Lite Luciferase Assay System (DD1203, Vazyme) according to the manufacturer's instructions. Background-subtracted luminescence values were obtained for each biological replicate. Matched lysate aliquots were analysed by immunoblotting using anti-FLAG and anti- β -actin antibodies. FLAG-firefly luciferase band intensity was quantified using ImageJ. Protein-normalized luciferase activity was calculated by dividing the corresponding background-subtracted luminescence value by the FLAG-luciferase abundance.

Intestinal polyp scoring

The entire intestinal tract was isolated from each mouse. The small intestine was divided equally by length into three segments, defined as the proximal small intestine (adjacent to the stomach), middle small intestine, and distal small intestine (adjacent to the cecum), respectively. From the large intestine, only the colorectum was collected. The four intestinal segments were opened longitudinally, spread flat on a black background plate, and gently rinsed with ice-cold sterile saline to remove luminal contents and facilitate visualization of intestinal adenomas, taking care not to damage the intestinal wall. Excess surface liquid around the tissue was carefully removed, and a steel ruler was placed alongside the tissue as a scale reference. Images were then captured at a fixed height. Adenoma number, adenoma diameter, and adenoma burden were quantified using ImageJ software. Adenoma burden was defined as the sum of the diameters of all adenomas in each sample.

Histology and IHC

Tissues were fixed in 4% paraformaldehyde (G1101-500ML, Servicebio), dehydrated, embedded in paraffin, and sectioned at a thickness of 4–5 μ m. H&E staining and Masson's trichrome staining were performed by Servicebio. For *Apc*^{Min/+} mouse samples, β -catenin IHC was performed (GB150016, Servicebio, 1:1,000), and sections were scanned using a bright-field microscope.

Immunofluorescence

Freshly dissected kidney and muscle tissues were embedded in OCT compound, rapidly frozen, and sectioned at a thickness of 6–8 μ m using a cryostat. Frozen sections were mounted on positively charged glass slides and fixed in pre-chilled methanol at –20 °C for 20 min. For kidney sections, slides were air-dried, treated with urea/glycine buffer (6 M urea, 0.1 M glycine, pH 3.5) for 30 min at room temperature, and washed three times with PBST (0.2% Tween-20 in PBS). Sections were blocked with 5% fetal bovine serum in PBST and incubated overnight at 4 °C with anti-COL4A5 antibody (05005, Bcell, 1:200). For muscle sections, slides were air-dried, hydrated in PBS for 10 min, blocked with 5% fetal bovine serum in PBST, and incubated with anti-Dystrophin antibody (D8168, Sigma, 1:500) overnight at 4 °C. After primary antibody incubation, sections were washed three times with PBST and incubated overnight at 4 °C with DyLight 488-conjugated goat anti-mouse IgG (A23210, Abbkine,

1:500). Sections were then washed three times with PBST, mounted with DAPI-containing mounting medium (ab104139, Abcam), and imaged using an LSM880 microscope (Zeiss).

Gait analysis

Mice were acclimated for 30 min before testing. The forepaws and hindpaws were painted with red and blue non-toxic dyes, respectively. Hind-base width was then measured from the hindpaw footprints.

Forelimb grip strength

Forelimb grip strength was measured using a digital force meter (XR501, Xinruan). During the test, mice were allowed to grasp the grid with their forelimbs and were then gently pulled backward horizontally until release. Care was taken to maintain a consistent pulling speed and angle throughout the procedure. Each mouse was tested in six consecutive trials with adequate rest between trials to minimize fatigue. The peak force from the six trials was averaged and normalized to body weight.

Rotarod test

Motor coordination and balance were evaluated using a rotarod apparatus (XR6C, Shanghai Xinruan). Before formal testing, mice were habituated to the rotating apparatus at 7 rpm for 15 min per day for 3 consecutive days. On the test day, mice were acclimated in the testing room for 30 min before formal testing. During the test, each mouse was placed on the rotating rod, which started at 4 rpm and gradually accelerated to 40 rpm over 2 min, then maintained at 40 rpm for an additional 3 min. The latency to fall onto a soft pad was recorded. Each mouse underwent three consecutive trials with a 15-min rest period between trials, and the maximum fall latency among the three trials was adopted for subsequent data analysis.

Treadmill test

The exercise tolerance test was performed using a treadmill (XR-PT-10B, Shanghai Xinruan). Mice were first trained for three consecutive days before the test to acclimate to the apparatus (10 min per day at 9 m/min with 0.5 mA electrical stimulation). For the test, each mouse was placed in a dedicated lane and allowed to run at an initial speed of 5 m/min for 3 min. The treadmill speed was then increased by 2 m/min every 3 min until reaching 13 m/min. Mice were considered exhausted when they remained immobile for 10 s despite continuous mild electrical stimulation. Total running distance and running time were recorded automatically by the treadmill system.

Micro-CT

Mice were anesthetized with 4% isoflurane and positioned in the scanner. The microstructure of the craniofacial bone was analyzed using a micro-CT scanner (U-CT-XUHR, MILabs). The scanner was set with the following settings: tube voltage 55 kV, tube current 0.17 mA, exposure time 75 ms, step angle 0.25°, and scan mode set to "Accurate" with ultra-focus magnification. Raw projection data were reconstructed into three-dimensional volumes using MILabs Rec. Reconstructed images were exported and analyzed in ITK-SNAP software to

measure zygomatic arch diameter.

***In vivo* luminescent imaging**

For Rosa26 TAG mice, 100 μ L of D-luciferin (40901ES03, Yeasen, 15 mg/mL in sterile PBS) was injected intraperitoneally. Ten minutes later, mice were anesthetized with 4% isoflurane and imaged using an Ami HT *in vivo* imaging system (Spectral Instruments Imaging). Bioluminescent signals were quantified using Aura software according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10. Data are presented as mean $\pm$ SD for cell-based experiments and mean $\pm$ SEM for animal studies. The value of *n* denotes biologically independent experiments or animals. For comparisons between two groups, an unpaired Student's *t*-test was used. Two-way ANOVA with Dunnett's multiple comparisons test was used for time-course studies. Multi-group comparisons were analyzed by one-way or two-way ANOVA, as appropriate, followed by Dunnett's or Holm-Šídák's multiple-comparisons test, as indicated. Reporter-only (–sup-tRNA) and 0 $\times$ conditions were used as background controls where indicated and were excluded from the corresponding inferential comparisons. *P* < 0.05 was considered statistically significant.

DATA AVAILABILITY

All data necessary to evaluate the conclusions of this study are provided in the main text and Supplementary Information. Additional data and materials are available upon request from the corresponding authors.

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AUTHOR CONTRIBUTIONS

S.L. and W.D. conceived the idea and supervised the study. C.F. and L.W. conducted most experiments and analysed the data. L.H. assisted with all animal experiments. Y.F. and L.L. assisted with cell experiments. X.-H.F. provided technical advice. S.L., W.D., C.F. and L.W. wrote the manuscript. All authors commented on the final draft of the manuscript.

COMPETING INTERESTS

L.H. and S.L. are listed as inventors on patent applications describing the development and application of alanine-based suppressor tRNA. S.L. is a founder and shareholder of Edipro Pharmaceutical Technology Inc. L.H. is an employee of Edipro

Pharmaceutical Technology Inc. The other authors declare no competing interests.

REFERENCES

1. Mort, M., Ivanov, D., Cooper, D.N. & Chuzhanova, N.A. A meta-analysis of nonsense mutations causing human genetic disease. *Hum. Mutat.* **29**, 1037–1047 (2008).
2. Vitale, E. et al. Nonsense mutations in rare and ultra-rare human disorders: an overview. *IUBMB Life* **77**, e70031 (2025).
3. Yingling, N., Sena-Esteves, M. & Gray-Edwards, H.L. A paradox of the field's own success: unintended challenges in bringing cutting-edge science from the bench to the market. *Hum. Gene Ther.* **35**, 83–88 (2024).
4. Coller, J. & Ignatova, Z. tRNA therapeutics for genetic diseases. *Nat. Rev. Drug Discov.* **23**, 108–125 (2024).
5. Hirsh, D. Tryptophan transfer RNA as the UGA suppressor. *J. Mol. Biol.* **58**, 439–458 (1971).
6. Temple, G.F., Dozy, A.M., Roy, K.L. & Kan, Y.W. Construction of a functional human suppressor tRNA gene: an approach to gene therapy for β -thalassaemia. *Nature* **296**, 537–540 (1982).
7. Lueck, J.D. et al. Engineered transfer RNAs for suppression of premature termination codons. *Nat. Commun.* **10**, 822 (2019).
8. Wang, J.M. et al. AAV-delivered suppressor tRNA overcomes a nonsense mutation in mice. *Nature* **604**, 343–348 (2022).
9. Albers, S. et al. Engineered tRNAs suppress nonsense mutations in cells and in vivo. *Nature* **618**, 842–848 (2023).
10. Pierce, S.E. et al. Prime editing-installed suppressor tRNAs for disease-agnostic genome editing. *Nature* **648**, 191–202 (2025).
11. Xu, M.Y. et al. An engineered UGA suppressor tRNA gene for disease-agnostic AAV delivery. *Nat. Biotechnol.* <https://doi.org/10.1038/s41587-025-02982-5> (2026).
12. Awawdeh, A. et al. Efficient suppression of premature termination codons with alanine by engineered chimeric pyrrolysine tRNAs. *Nucleic Acids Res.* **52**, 14244–14259 (2024).
13. Shi, N.N. et al. Restoration of dystrophin expression in mice by suppressing a nonsense mutation through the incorporation of unnatural amino acids. *Nat. Biomed. Eng.* **6**, 195–206 (2022).
14. Sangha, A.K. & Kantidakis, T. The aminoacyl-tRNA synthetase and tRNA expression levels are deregulated in cancer and correlate independently with patient survival. *Curr. Issues Mol. Biol.* **44**, 3001–3017 (2022).
15. Landrum, M.J. et al. ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic Acids Res.* **46**, D1062–D1067 (2018).
16. Cheng, J. et al. Accurate proteome-wide missense variant effect prediction with AlphaMissense. *Science* **381**, eadg7492 (2023).
17. The GTEx Consortium. The GTEx Consortium atlas of genetic regulatory effects across human tissues. *Science* **369**, 1318–1330 (2020).
18. Geiger, T. et al. Initial quantitative proteomic map of 28 mouse tissues using the SILAC mouse. *Mol. Cell. Proteomics* **12**, 1709–1722 (2013).
19. Naganuma, M. et al. The selective tRNA aminoacylation mechanism based on a single G•U pair. *Nature* **510**, 507–511 (2014).
20. Sakamoto, K. et al. Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. *Nucleic Acids Res.* **30**, 4692–4699 (2002).
21. Wu, N., Deiters, A., Cropp, T.A., King, D. & Schultz, P.G. A genetically encoded photocaged amino acid. *J. Am. Chem. Soc.* **126**, 14306–14307 (2004).
22. Chen, P.R. et al. A facile system for encoding unnatural amino acids in mammalian cells. *Angew. Chem. Int. Ed.* **48**, 4052–4055 (2009).
23. Italia, J.S. et al. An orthogonalized platform for genetic code expansion in both bacteria and eukaryotes. *Nat. Chem. Biol.* **13**, 446–450 (2017).
24. Ding, W.L. et al. Chimeric design of pyrrolysyl-tRNA synthetase/tRNA pairs and canonical synthetase/tRNA pairs for genetic code expansion. *Nat. Commun.* **11**,

- 3154 (2020).
25. Osgood, A.O., Huang, Z.Y., Szalay, K.H. & Chatterjee, A. Strategies to expand the genetic code of mammalian cells. *Chem. Rev.* **125**, 2474–2501 (2025).
 26. Ramos, A. & Varani, G. Structure of the acceptor stem of *Escherichia coli* tRNA^{Ala}: role of the G3-U70 base pair in synthetase recognition. *Nucleic Acids Res.* **25**, 2083–2090 (1997).
 27. Ding, W.L. et al. Rare codon recoding for efficient noncanonical amino acid incorporation in mammalian cells. *Science* **384**, 1134–1142 (2024).
 28. Zhao, H.X. et al. Directed-evolution of translation system for efficient unnatural amino acids incorporation and generalizable synthetic auxotroph construction. *Nat. Commun.* **12**, 7039 (2021).
 29. Colella, P., Ronzitti, G. & Mingozzi, F. Emerging issues in AAV-Mediated *in vivo* gene therapy. *Mol. Ther. Methods Clin. Dev.* **8**, 87–104 (2018).
 30. Specht, C. et al. An engineered glutamic acid tRNA for efficient suppression of pathogenic nonsense mutations. *Nucleic Acids Res.* **53**, gkaf532 (2025).
 31. Wang, D. et al. Characterization of an MPS I-H knock-in mouse that carries a nonsense mutation analogous to the human *IDUA-W402X* mutation. *Mol. Genet. Metab.* **99**, 62–71 (2010).
 32. Higuchi, T. et al. Enzyme replacement therapy (ERT) procedure for mucopolysaccharidosis type II (MPS II) by intraventricular administration (IVA) in murine MPS II. *Mol. Genet. Metab.* **107**, 122–128 (2012).
 33. Meikle, P.J. et al. Diagnosis of lysosomal storage disorders: evaluation of lysosome-associated membrane protein LAMP-1 as a diagnostic marker. *Clin. Chem.* **43**, 1325–1335 (1997).
 34. Duan, D.S., Goemans, N., Takeda, S., Mercuri, E. & Aartsma-Rus, A. Duchenne muscular dystrophy. *Nat. Rev. Dis. Primers* **7**, 13 (2021).
 35. Yiu, E.M. & Kornberg, A.J. Duchenne muscular dystrophy. *J. Paediatr. Child Health* **51**, 759–764 (2015).
 36. Bekheirnia, M.R. et al. Genotype-phenotype correlation in X-linked Alport syndrome. *J. Am. Soc. Nephrol.* **21**, 876–883 (2010).
 37. Jais, J.P. et al. X-linked Alport syndrome: natural history and genotype-phenotype correlations in girls and women belonging to 195 families: a "European community Alport syndrome concerted action" study. *J. Am. Soc. Nephrol.* **14**, 2603–2610 (2003).
 38. Harvey, S.J. et al. Role of distinct type IV collagen networks in glomerular development and function. *Kidney Int.* **54**, 1857–1866 (1998).
 39. Hashikami, K. et al. Establishment of X-linked Alport syndrome model mice with a *Col4a5* R471X mutation. *Biochem. Biophys. Res. Commun.* **17**, 81–86 (2019).
 40. Johnson, A.F., Nguyen, H.T. & Veitia, R.A. Causes and effects of haploinsufficiency. *Biol. Rev. Camb. Philos. Soc.* **94**, 1774–1785 (2019).
 41. Huang, N., Lee, I., Marcotte, E.M. & Hurles, M.E. Characterising and predicting haploinsufficiency in the human genome. *PLoS Genet.* **6**, e1001154 (2010).
 42. Sarel-Gallily, R., Golan-Lev, T., Yilmaz, A., Sagi, I. & Benvenisty, N. Genome-wide analysis of haploinsufficiency in human embryonic stem cells. *Cell Rep.* **38**, 110573 (2022).
 43. Morrill, S.A. & Amon, A. Why haploinsufficiency persists. *Proc. Natl. Acad. Sci. USA.* **116**, 11866–11871 (2019).
 44. Galiatsatos, P. & Foulkes, W.D. Familial adenomatous polyposis. *Am. J. Gastroenterol.* **101**, 385–398 (2006).
 45. Leoz, M.L., Carballal, S., Moreira, L., Ocaña, T. & Balaguer, F. The genetic basis of familial adenomatous polyposis and its implications for clinical practice and risk management. *Appl. Clin. Genet.* **8**, 95–107 (2015).
 46. Moser, A.R. et al. *Apc^{Min}*: a mouse model for intestinal and mammary tumorigenesis. *Eur. J. Cancer* **31A**, 1061–1064 (1995).
 47. Grieger, J.C., Choi, V.W. & Samulski, R.J. Production and characterization of adeno-associated viral vectors. *Nat. Protoc.* **1**, 1412–1428 (2006).

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.15302/vita.2026.10.0079>.

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