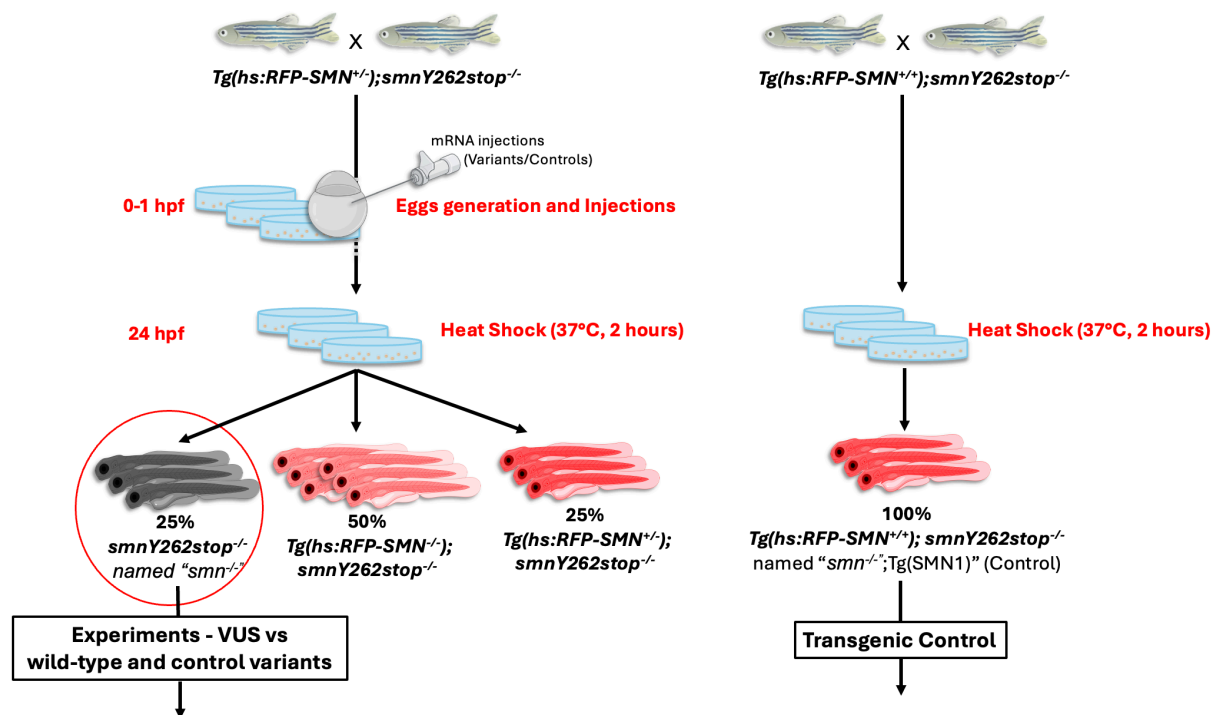


Clinical relevance of zebrafish for gene variants testing.

Proof-of-principle with *SMN1*/SMA.

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Appendix Figure S1. Methodological framework to produce maternal zygotic *smn1* knockout mutants depleted in SMN protein. Zebrafish embryos/larvae named *smn^{-/-}* in this study are produced from *Tg(hs:RFP-SMN^{+/+}); smnY262stop^{-/-}* zebrafish (PMID 19592581 & 23459934). Incrossing this line produces 100% maternal zygotic *smnY262stop^{-/-}* mutants, of which 25% do not inherit the rescue transgene *Tg(hs:RFP-SMN)*, so do not express any form of SMN protein. To select these 25% maternal zygotic animals *smnY262stop^{-/-}* (named *smn^{-/-}* in this manuscript) from their transgenic siblings *Tg(hs:RFP-SMN); smnY262stop^{-/-}*, the clutches were heat-shocked at 24 hpf (hours post fertilisation) for 2 hours at 37°C and subsequently identified by the absence of detectable red fluorescence.

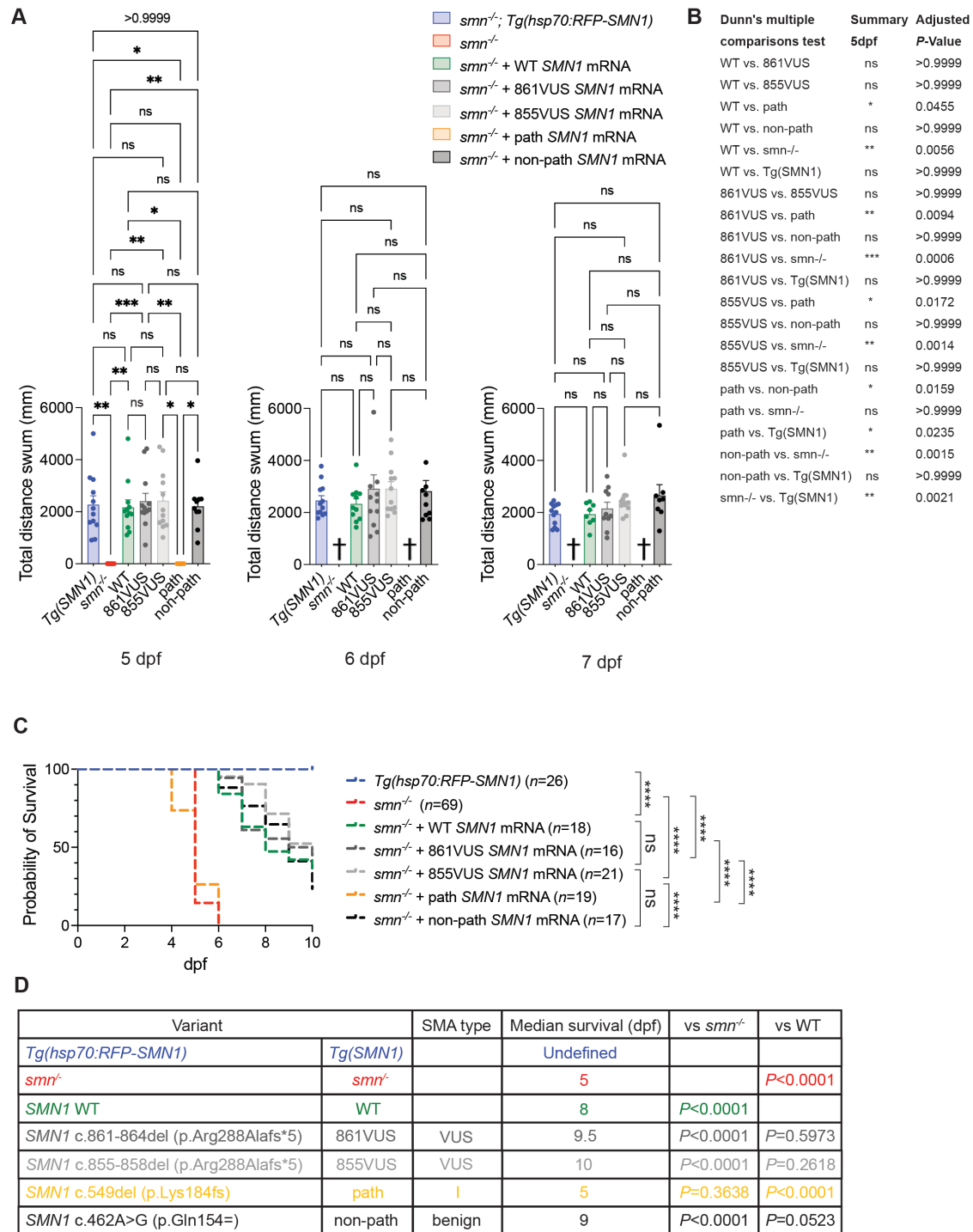
Appendix Table S1. Plasmids created for *SMN1* VUS analysis.

Plasmid name	Purpose
249-pME-hsa_SMN1-wt	contains <i>SMN1</i> wild-type cDNA
250-pME-c.861_864_SMN1	contains <i>SMN1</i> variant cDNA (861VUS)
252-pME-c.855_858_SMN1	contains <i>SMN1</i> variant cDNA (855VUS)
253-pME-c.549del_SMN1	contains <i>SMN1</i> variant cDNA (path)
254-pME-c.462A>G_SMN1	contains <i>SMN1</i> variant cDNA (non-path)
260-pT3-hsa_SMN1-WT_polyA	<i>in vitro</i> transcription of <i>SMN1</i> wild-type mRNA
261-pT3-c.861_864_hsa_SMN1_polyA	<i>in vitro</i> transcription of <i>SMN1</i> 861VUS mRNA
263-pT3-c.855_858_hsa_SMN1_polyA	<i>in vitro</i> transcription of <i>SMN1</i> 855VUS mRNA
264-pT3-c.549del_hsa_SMN1_polyA	<i>in vitro</i> transcription of <i>SMN1</i> path mRNA
265-T3-c.462A>G_hsa_SMN1_polyA	<i>in vitro</i> transcription of <i>SMN1</i> non-path mRNA
279-hsa-SMN1c.5C>G(p.Ala2Gly)	contains <i>SMN1</i> variant cDNA A2G
280-hsa-SMN1c.5C>T(p.Ala2Val)	contains <i>SMN1</i> variant cDNA A2V
281-hsa-SMN1c.84C>T(p.Ser28=)	contains <i>SMN1</i> variant cDNA S28= (non-path 2)
282-hsa-SMN1c.131A>T(p.Asp44Val)	contains <i>SMN1</i> variant cDNA D44V
283-hsa-SMN1c.734C>T(p.Pro245Leu)	contains <i>SMN1</i> variant cDNA P245L
284-hsa-SMN1c.785G>T(p.Ser262Ile)	contains <i>SMN1</i> variant cDNA S262I
285-hsa-SMN1c.821C>T(p.Thr274Ile)	contains <i>SMN1</i> variant cDNA T274I
287-pT3-hsa_SMN1_A2G	<i>in vitro</i> transcription of <i>SMN1</i> variant A2G mRNA
288-pT3-hsa_SMN1_A2V	<i>in vitro</i> transcription of <i>SMN1</i> variant A2V mRNA
289-pT3-hsa_SMN1_S28=	<i>in vitro</i> transcription of <i>SMN1</i> variant S28= mRNA
290-pT3-hsa_SMN1_D44V	<i>in vitro</i> transcription of <i>SMN1</i> variant D44V mRNA
291-pT3-hsa_SMN1_P245L	<i>in vitro</i> transcription of <i>SMN1</i> variant P245L mRNA
292-pT3-hsa_SMN1_S262I	<i>in vitro</i> transcription of <i>SMN1</i> variant S262I mRNA
293-pT3-hsa_SMN1_T274I	<i>in vitro</i> transcription of <i>SMN1</i> variant T274I mRNA
298-pT3-hsa_SMN1:c.43C>T (p.Gln15Ter)	<i>in vitro</i> transcription of <i>SMN1</i> variant Q15* mRNA (path 2)

Appendix Table S2. Q-PCR primers for measuring *hsa-SMN1* mRNA expression.

Primer name	Reference	Sequence
310-dre_eef1a-qF1	PMID: 32728128	5'-GGGCAAGGGCTCCTTCAA-3'
311-dre_eef1a-qR1	PMID: 32728128	5'-CGCTCGGCCTTCAGTTTG-3'
312-hsa_SMN1-qF1	This study	5'-TCTTTTCTCCCTCCACCACC-3'
313-hsa_SMN1-qR1	This study	5'-CAGAAGGAAATGGAGGCAGC-3'

Replicate 1

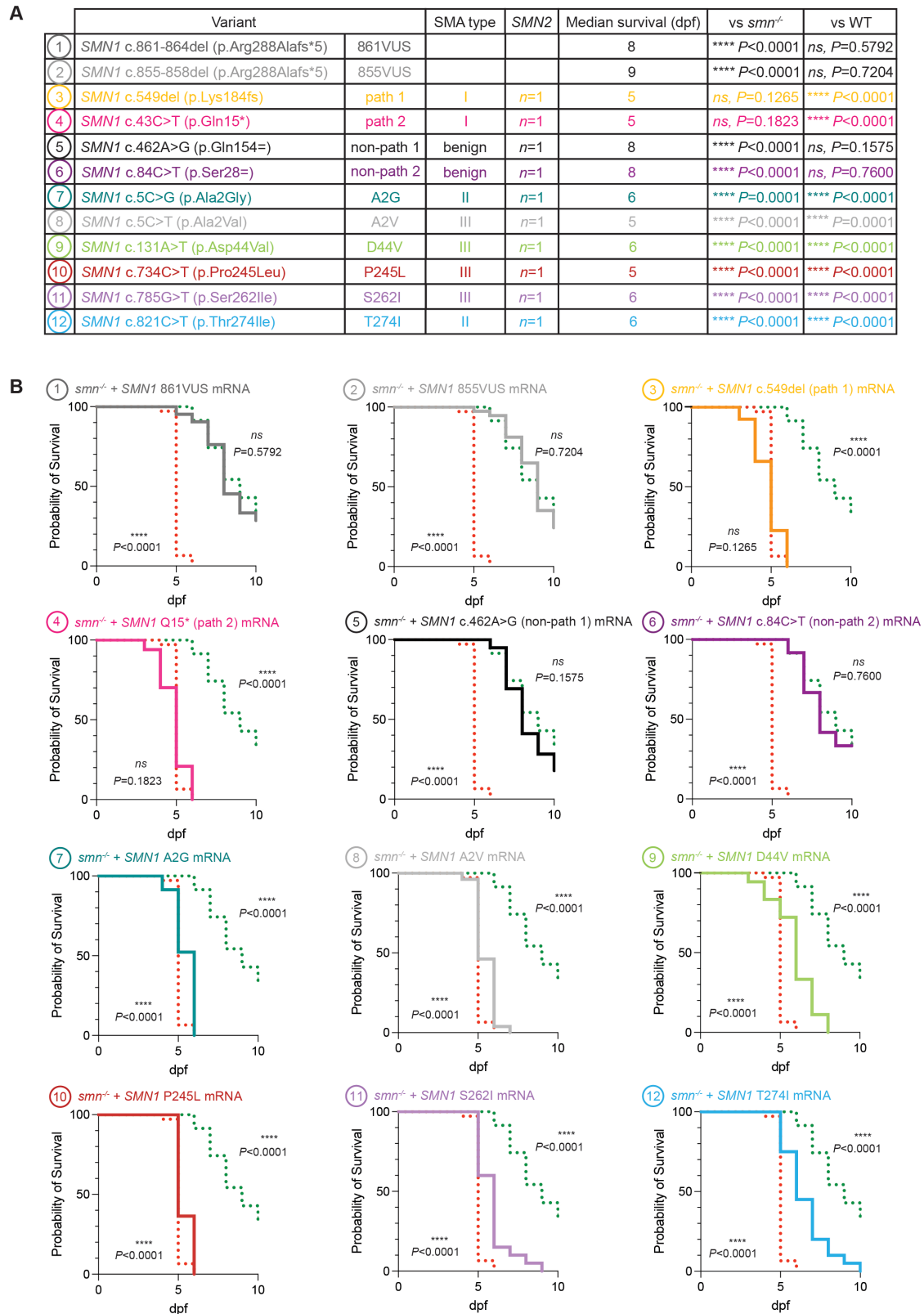


Appendix Figure S2. 861VUS and 855VUS mRNA injections rescue SMN-deficient *smn*^{-/-} zebrafish movement and survival (Details/Statistics for Figure 3, Replicate 1). First of three replicate experiments assessing the ability of injected *SMN1* mRNAs to rescue the swimming ability and survival of SMN-deficient *smn*^{-/-} fish. **(A)** SMN-deficient *smn*^{-/-} zebrafish present dramatic motor function loss at 5 dpf and die by 6 dpf. Human wt-*SMN1* transgenic ubiquitous expression (*Tg(SMN1)*) or injected wt-*SMN1* mRNA (WT) rescue this motor function loss. The non-pathogenic *SMN1* variant (non-path) and both 861VUS and

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855VUS efficiently restore motor function with no detectable significant difference. In contrast, mRNA from the pathogenic *SMN1* variant (path) failed to improve motor function with no difference from negative control (*smn*^{-/-}). Graphs represent comparisons of the total distance swum by each cohort of fish over 24 minutes at 5, 6 and 7 dpf. Each data point represents one fish. Cross symbols indicate 100% mortality. Error bars represent standard error of the mean. Statistical significance was evaluated using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant. Source data are available online for this Figure. **(B)** Actual P -values for the comparisons shown in (A). **(C)** SMN-deficient *smn*^{-/-} zebrafish (*smn*^{-/-}, red dashed line) had a median survival of 5 days. Injected mRNA from the known pathogenic *SMN1* variant (path, orange dashed line) did not have any effect on survival. In contrast, demonstrating restoration of Smn/SMN function, injected mRNA from 861VUS (dark grey dashed line) and 855VUS (light grey dashed line) extended survival of *smn*^{-/-} larvae similar to wt-mRNA (WT, green dashed line) and non-pathogenic variant (non-path, black dashed line) animals. ****, $P < 0.0001$; ns, not significant. Statistical significance was evaluated using the log-rank (Mantel-Cox) test. **(D)** Exact P -values and median survival data for the analysis shown in (C). Source data are available online for this Figure.

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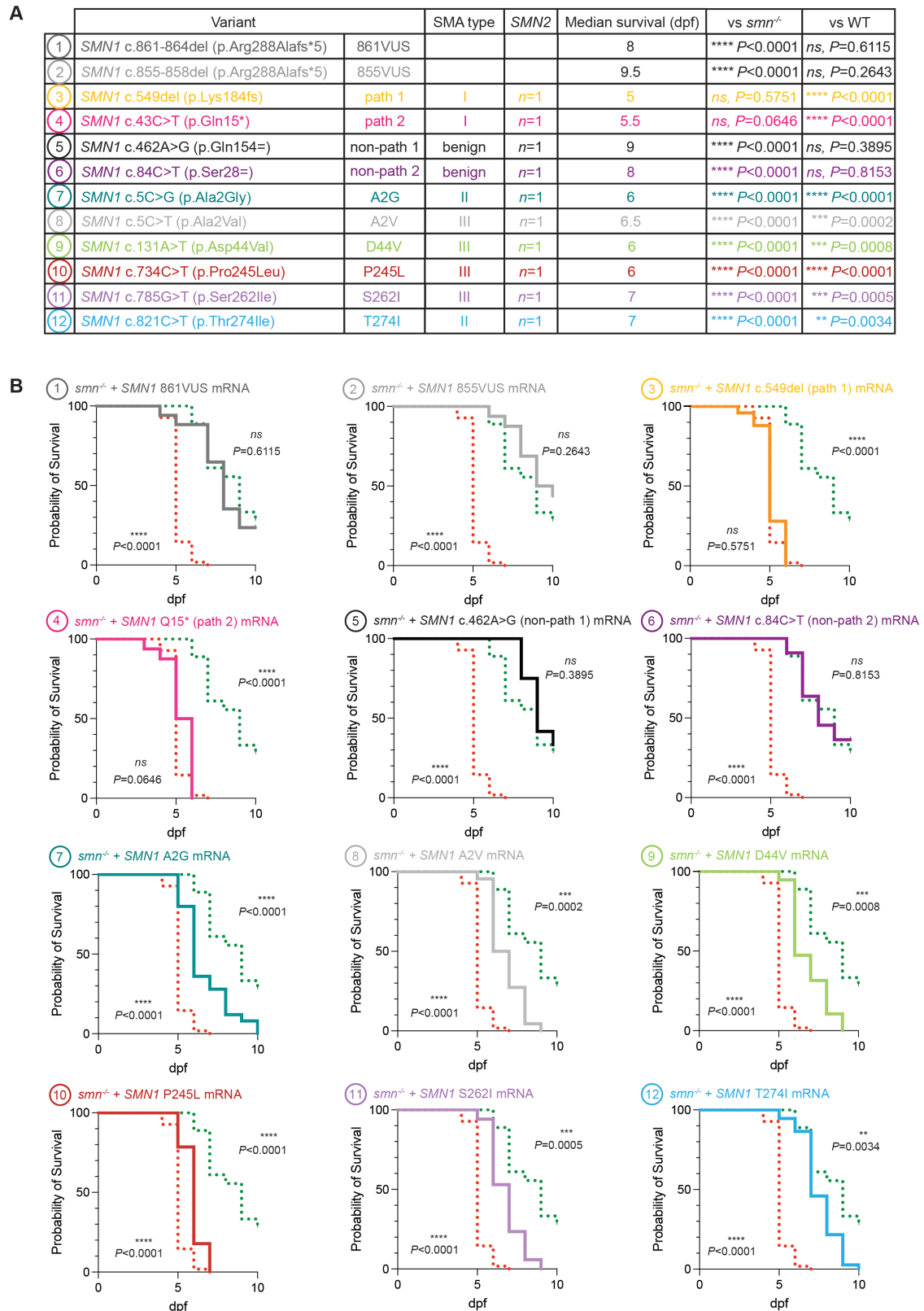


Appendix Figure S5. mRNA injections of *SMN1* hypomorphic variants extend the survival of SMN-deficient zebrafish, but only partially compared with wild-type or non-pathogenic controls (Related to Figure 5, second of three replicate experiments). (A) Median survival of *smn*^{-/-} larvae injected with each of the *SMN1* variants associated with SMA type II or III, was compared with both VUS and with known pathogenic and non-pathogenic

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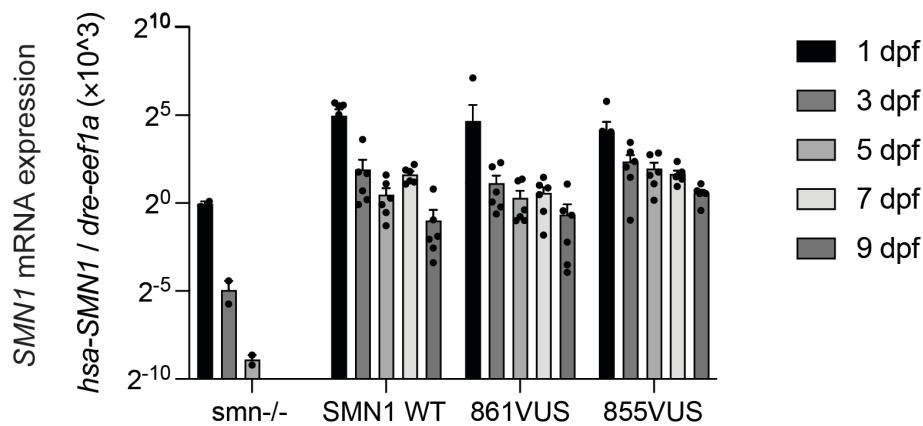
SMN1 variants. Kaplan-Meier analysis was used to determine median survival. Statistical significance, compared to *smn*^{-/-} mock-injected control fish (*smn*^{-/-}) and wt-*SMN1* mRNA injected animals (WT), was determined by log-rank (Mantel-Cox) test. ****, $P < 0.0001$; ns, not significant. Exact P -values are also shown. **(B)** Survival curves for each of the tested *SMN1* variants (solid coloured line) shown relative to those for *smn*^{-/-} mock-injected control fish (dotted red line) and wt-*SMN1* mRNA-injected animals (dotted green line). P -values are as in (A). All tested missense variants, associated with mild forms of the disease, significantly extended the survival of SMN-deficient *smn*^{-/-} animals, though none matched the rescue efficiency of wt-*SMN1* mRNA, suggesting that the presented methodology would also be useful to detect hypomorphs. These findings, combined with the data presented in Figure 3 and 4, support that the tested 861VUS and 855VUS variants are neither pathogenic nor hypomorphic.

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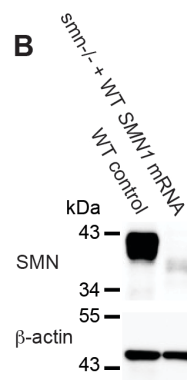


Appendix Figure S6. mRNA injections of *SMN1* hypomorphic variants extend the survival of SMN-deficient zebrafish, but only partially compared with wild-type or non-pathogenic controls (Related to Figure 5, third of three replicate experiments). (A) and (B), as in Appendix Figure S5.

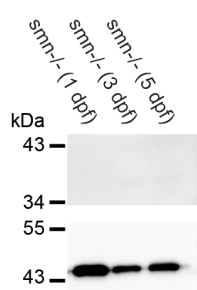
A



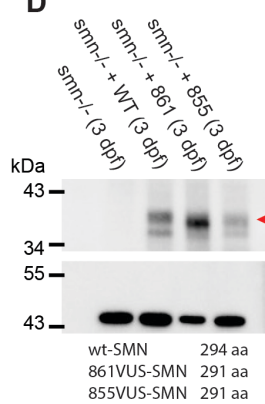
B



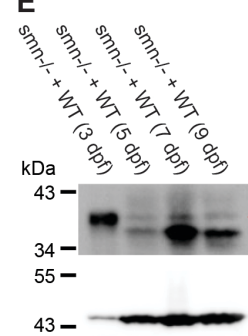
C



D

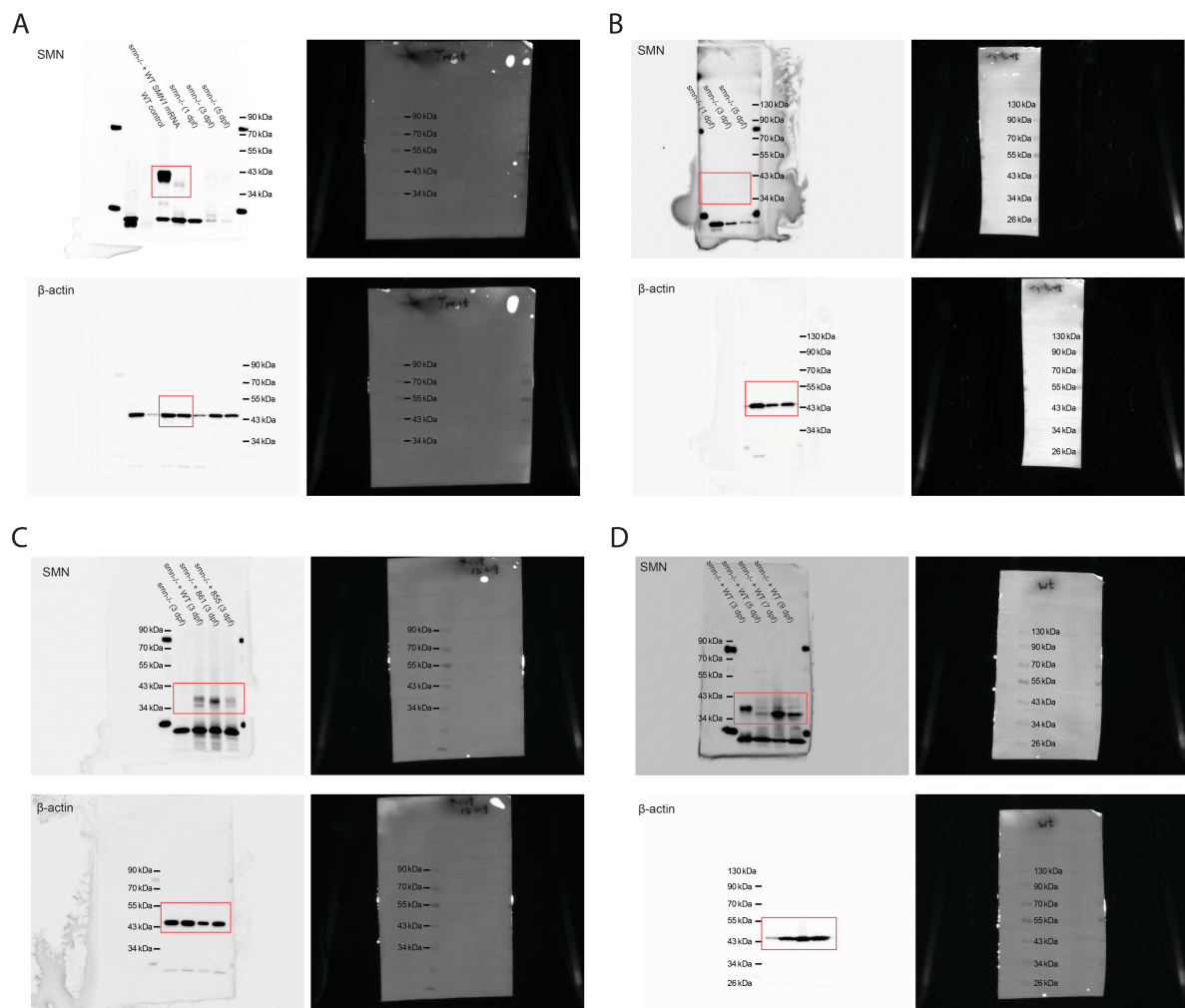


E



Appendix Figure S7. RT-qPCR and western blot analysis following injections of wt, 861VUS and 855VUS mRNAs in zebrafish. **A**, RT-qPCR analysis showing *SMN1* mRNA expression relative to endogenous *dre-eef1a* mRNA. Expression from wt, 861VUS, and 855VUS mRNAs shows a sharp decline between 1 and 3 dpf but remains detectable at low levels up to 9 dpf. Low levels of human *SMN1* mRNA are also detected in uninjected *smn*^{-/-} control animals (without associated detectable protein on western blots; see panels C and Hao *et al.*, 2013, Fig. 3). This is likely due to minor maternal deposition of transgenic human *SMN1* mRNA from the incross of maternal zygotic *Tg(hs:RFP-SMN);smn*^{Y262stop/-}. *n*=2 fish for *smn*^{-/-}, 1 fish per time point from 2 biological replicates; *n*=6 fish for *SMN1* WT and *SMN1* 861VUS, 3 fish per time point from 2 biological replicates. **B**, Western blot analysis of wild-type control and maternal zygotic *smn*^{-/-} animals complemented with 250 pg of human *SMN1* mRNA at 3 dpf. Black arrowhead, Zebrafish SMN. Red arrowhead, human SMN from mRNA injections. Human SMN has a predicted molecular weight of 32 kDa with an apparent weight of 38 kDa on the gels (<https://dshb.biology.uiowa.edu/MANSMA12-2E6>). **C**, Western blot showing absence of detectable SMN protein in maternal zygotic *smn*^{-/-} controls across all analysed time points. **D**, Western blot comparing *smn*^{-/-} control and *smn*^{-/-} animals injected with 250 pg of wt, 861VUS or 855VUS at 3 dpf, showing detectable expression of the respective SMN proteins. The shorter length of 861VUS and 855VUS proteins results in a slight shift in migration. **E**, Temporal analysis of SMN protein levels after 250 pg mRNA injections. SMN levels decrease sharply after 3 dpf but remain detectable at low levels until at least 9 dpf. Protein loading was 15 µg per lane, except for wt-mRNA at 5, 7, and 9 dpf, where 100 µg was loaded per lane. Protein extractions were performed using 40 embryos at 1 dpf, 10 larvae at 3 dpf, and 20 larvae at 5, 7 and 9 dpf.

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Appendix Figure S8. Native western blot snapshots associated with Appendix Figure S2. A, blots associated with Appendix Figure S2B. B, blots associated with Appendix Figure S2C. C, blots associated with Appendix Figure S2D. D, blots associated with Appendix Figure S2E.