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Supplementary Information for

Impairment of the neurotrophic signaling hub B-Raf contributes to motoneuron degeneration in Spinal Muscular Atrophy

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Supplementary Methods

Gene-Ontology (GO) enrichment, hierarchical clustering and network analysis

GO-enrichment was performed with the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System (version 14.1) (1-3) using the total detectable proteins on the array as a reference and Fisher's exact t-test (4). Again, raw p-values were adjusted to control a false discovery rate of 5 %. The Perseus software (version 1.6.0.7) (5) was used for the hierarchical clustering analysis which was constraint to a maximal number of 10 clusters. The (phospho)-proteins were subjected to a network analysis using the functional enrichment tool (FunRich, version 3.1.3). The connections between the nodes were drawn based on three maintained databases (BioGRID, Intact, Human Proteinpedia) providing both, physical and functional interactions (6) .

Western blot

Tissue samples were lyzed in ice-cold RIPA-buffer (137 mM NaCl, 20 mM Tris-HCl pH 7, 525 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 1% (w/v) sodium desoxycholate, 1% (v/v), Triton-X-100, protease inhibitor cocktail by Roche, 11873580001) and phosphatase inhibitor cocktail (Roche, 04906837001)) with the TissueLyser LT (Qiagen) for 1 min at 50 Hz using Tungsten carbide beads (Qiagen, 69997). Tissue debris was removed by centrifugation at 22,000 g and 4 °C for 15 min. The cleared protein lysates were incubated for 10 min at 95 °C together with Laemmli buffer. Proteins were separated by SDS-PAGE and blotted on a nitrocellulose membrane as described previously (7). The membranes were blocked with 2.5% (w/v) bovine serum albumin (BSA) in Tris buffered saline (TBS) supplemented with 0.1% (v/v) Tween for the incubation with anti-phospho-antibodies and with milk-powder instead of BSA for the incubation with non-phospho antibodies and secondary antibodies. A list of all antibodies is provided in the Supplement **(Table S1)**. Densitometry was performed with the program LabImage 1D. We normalized the band intensities of an immunodetection by the geometric mean of the band intensities thereby generating values which group around one as the centroid. This was done with the protein of interest (e.g. B-Raf) and the housekeeping (e.g. GAPDH).

This normalized protein of interest values were divided by the corresponding housekeeping values from the same blot. These relative expression values were divided by the mean of the control relative expression and multiplied with 100 to get [% of control mean].

Immunohistochemistry and immunocytochemistry

Primary spinal cord cultures were grown in 24-wells on coverslips and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature. After washing with PBS, cells were blocked with 3% (v/v) normal goat serum (NGS) and 0.3% Triton-X100 (v/v) in PBS for 1 h at

room temperature. The blocking solution was used for dilution of the primary antibodies (4 °C overnight) after which the cells were washed with PBS twice. Secondary antibodies were diluted in blocking solution and the cells were incubated for 1 h at room temperature. Cells were washed with PBS five times with DAPI (1:1000) for nuclear staining in the third washing step. A detailed list of the antibodies is provided in the Supplement (Table S1). Spinal cord samples (L1-5) were fixed in 4% (w/v) paraformaldehyde in PBS for three hours and dehydrated with 30% (w/v) sucrose in water overnight. The samples were embedded in Tissue-Tek (Sakura) before cryo-sectioning in 10 µm thin sections. Subsequently, samples were washed with PBS and blocked with 3% BSA (w/v), 0.3% Triton-X100 (v/v) in PBS for 1 h. The antibodies were diluted in 1% BSA (w/v) and 0.3% Triton-X100 (v/v) in PBS and incubated overnight at 4 °C. The spinal cord sections were rinsed with PBS three times and incubated for 1 h with the secondary antibodies in blocking solution at room temperature. The sections were washed three times with PBS, stained with DAPI (1:2000 in PBS for 10 min) and washed again with PBS for three times and one time with H_20. Finally, the sections were mounted in Mowiol® and analyzed with an Olympus BX60 epifluorescence microscope equipped with 40x and 100 x objectives. Microphotographs of iPSC-derived motoneurons were acquired with LEICA SP8 confocal microscope with sequential acquisition setting at four random fields each sample. B-Raf fluorescence intensity was measured in the cytoplasm of SMI32-positive motoneurons using Fiji ImageJ analysis software. Microphotographs of primary motoneurons were acquired with a Zeiss LSM 980 confocal microscope (Fig. 2 H) or with an Olympus BX60 epifluorescence microscope equipped with a 40x objective (Fig. 2 I). For quantification of B-Raf in primary motoneurons, five random fields were selected and automatically analyzed with CellProfiler (8). The CellProfiler pipeline file is available from the Center for Open Science (https://osf.io) repository with the following Research Resource Identifier RRID: doi 10.17605/OSF.IO/6PZYK (https://doi.org/10.17605/OSF.IO/6PZYK). Briefly, B-Raf mean intensity was detected within SMI32 positive areas and normalized by the surrounding background intensity. All experimenters were blinded to the experimental groups.

C. elegans genetics

The wild type *C. elegans* strain used was N2, Bristol variety. The genetic mutants used were: LM99 *smn-1(ok355) I/hT2 [bli-4(e937) qIs48]* (I;III); MT8666 *mek-2(n1989) I*. N2, LM99 and MT8666 animals were provided by the *Caenorhabditis* Genetic Center (CGC) funded by NIH Office of Research Infrastructure Programs (P40OD010440). The transgenic strains used were: NA1330 *gbIs4[GBF109 punc-25::smn-1(RNAi), GB301 pchs-2::GFP] III*; NA1719 *juIs76[punc-25::GFP, lin-15(+)] II*; NA1721 *juIs76 II, gbIs4 III* (9); UP687 *csIs42[phsp-16.41::lin-45^{wt}, unc-119(+)]*; UP707 *csIs34[phsp-16.41::lin-45^{S312A}, unc-119(+)] X* (10). UP687 and UP707 were kindly provided by Prof. M. Sundaram (Penn University, USA). Genetic crosses were made to transfer *lin-45* inducible transgenes in *smn-1(RNAi)* background; the strains obtained were: NA2084 *juIs76 II, gbIs4 III, csIs34 X*; NA2085 *gbIs4 III, csIs34 X*; NA2108 *juIs76 II, gbIs4 III, csIs42*; NA2114 *gbIs4 III, csIs42;* NA2086 *juIs76 II, csIs34 X*; NA2111 *juIs76 II, csIs42*. Genetic crosses were made to transfer *mek-2(n1989)* missense mutation in *smn-1(RNAi); csIs34* background; the

strain obtained was: NA2277 *mek-2(n1989) I; gbIs4 III; juIs76 II; csIs34 X.* In all strains, the presence of the transgenes *gbIs4* and *juIs76* was verified by phenotyping the clonal F2s using a dissecting microscope equipped with epifluorescence; the presence of the transgenes *csIs34* and *csIs42* and of *mek-2(n1989)* point-mutation were verified by polymerase chain reaction (PCR) (primers listed in Supplementary **table S2**). At least two independent isogenic clones were examined after each crossing. All strains were kept at 20°C unless otherwise noted. *csIs34* and *csIs42* bear heat-inducible transgenes which could be slightly leaky at 20°C and therefore were kept at 15°C. In order to express *csIs34* and *csIs42* heat-inducible transgenes we performed a continuative heat-treatment maintaining *C.elegans* at 25°C and the phenotypes were analyzed after at least 2 generations. Moreover, we performed an upshift of heat treatment consisting in keeping animals at 15°C and moving to 25°C at different stages **(Figure 5 D)**. To this end, synchronized animals were grown at 15°C until the desired developmental stage (eggs, L1, L2 and L4 larval stages) and then transferred and grown at 25°C until the young adult stage, when the phenotype was scored.

***C. elegans* transgenesis**

The construct GBF377 (*punc-47::lin-45*^{wt}) for MN-*lin-45*^{wt} expression was created by two-step PCR fusion (11) of the *unc-47* promoter with *lin-45* gene, both amplified from the genome of wild type strains. The transgenic construct GBF378 (*punc-47::lin-45*^{hyp}) for MN-*lin-45*^{hyp} expression was similarly created by PCR fusion of *unc-47* promoter with *lin-45*^{S312A} mutated gene. This time a three step PCR fusion approach was used: in the first stage the 5'-end and the 3'-end of *lin-45* gene were separately amplified from wild type genomic sequences, using adequate overlapping primers carrying a T>G substitution in the position +2578 of the *lin-45* gene (isoform *c.1*); then we created the *lin-45*^{S312A} mutated version of the gene by fusing these two fragments with nested primers and the nucleotide change was confirmed by Sanger sequencing; finally, in the third stage, the amplicon with the promoter and the amplicon with the mutated gene were fused as above using nested primers. All primer sequences are listed in Supplementary table 2. Germline transformation was accomplished as described (12), by injecting a DNA mixture containing the constructs at 2 ng/µl (lower concentration, LC) and 20 ng/µl (higher concentration, HC) into the gonads of NA1721 [*juIs76, gbIs4*] (9) adult animals together with a phenotypic marker for the selection of the transgenic progeny. For this purpose we used plasmid pJM371 *pelt-2*::RFP, a kind gift of Prof. J.D. McGhee (University of Calgary), as co-injection marker at 30 ng/µl. This plasmid allows to recognize transgenic animals through RFP expression in the nuclei of intestinal cells (13). At least three independent transgenic lines were examined for each concentration, except for *gbEx683*: *gbEx679a-c[GBF377 punc-47::lin-45*^{wt}*LC, pJM371 pelt-2::RFP]; gbEx678a-c[GBF377 punc-47::lin-45*^{wt}*HC, pJM371 pelt-2::RFP]; gbEx682a-c[GBF378 punc-47::lin-45*^{hyp}*LC, pJM371 pelt-2::RFP]; gbEx683a-b[GBF378 punc-47::lin-45*^{hyp}*HC, pJM371 pelt-2::RFP].* Genetic crosses were performed to obtain strains without *juIs76* for locomotion assays: NA2273 *gbIs4 III, gbEx679c;* NA2276 *gbIs4 III, gbEx678b*; NA2274 *gbIs4 III, gbEx682a*; NA2275 *gbIs4 III,*

gbEx683a. In all strains, the presence of the transgenes was verified by phenotyping the clonal F2s using a dissecting epifluorescence microscope.

RNA isolation and quantitative real-time PCR

500 L2 animals (wild type*, smn-1 KO)* or pellets of young adult animals (wild type*, smn-1(RNAi)*, *smn-1(RNAi) i-lin-45*^{wt}*, smn-1(RNAi) i-lin-45*^{hyp}) grown at 15° and 25°C were flash-frozen three times using dry-ice/ethanol and thawing at 37°C, before proceeding with RNA extraction in TRI Reagent® (Merck), according to the TRI Reagent® protocol. RNA was treated with DNase I-RNase-free (Thermo Scientific). In transgenic lines *csIs34* and *csIs42 (i-lin-45*^{wt} and *i-lin-45*^{hyp}*) lin-45* has been cloned as cDNA, hence mRNA is indistinguishable from gDNA (10). Therefore, to ensure that gDNA was completely eliminated from transgenic strains, 1µg of RNA was incubated with 2U of DNase for 2h at 37°C. Then complete gDNA removal was verified by PCR analysis using specific oligonucleotides designed to amplify the promoter region of the transgene. Reverse transcription was performed with SuperScript Reverse Transcriptases (Invitrogen) and the steady-state mRNA abundance was determined using the Power SYBR Green PCR Master Mix (Applied Biosystems) on the 7900HT Fast Real Time PCR System (Applied Biosystems), following standard procedures. Expression levels were normalized to the average level of the housekeeping gene *act-1*. Each experiment assay was performed in triplicate in three independent experiments. The oligonucleotide sequences for transcript analysis are listed in Supplemeentary Table 2.

***C. elegans* phenotypic analysis and treatment with MEK/ERK inhibitors**

For microscopy analysis young adult animals were immobilized with 0.01% tetramisole hydrochloride (Sigma-Aldrich) on 4% agar pads. The degenerative phenotype was scored counting the number of visible D-type motoneurons expressing GFP, normally nineteen, per animal using a motoneuron specific promoter (*punc-25::GFP*) and a Zeiss Axioskop microscope equipped with epifluorescence and DIC Nomarski optics with a 40x objective. For each data set the number of visible neurons among the total expected (nineteen) was scored. The number of animals analyzed each time (n) has been added to graphs. Confocal images were collected with Leica TCS SP8 AOBS microscope, using 20x objective. To test GABAergic motoneuron functionality a locomotion assay was performed blindly, on NGM agar plates, 3 cm in diameter, seeded with *Escherichia coli* strain *OP50*. Using an eyelash, the animals were gently touched first on the tail to induce a forward movement and then on the head to induce a backward movement. A defect in backward movement was scored when animals were unable to fully move back. The locomotion was evaluated for increments of ten animals each replicate and the percentage of normal locomotion was calculated, with a total number of animals scored of 100. For each data set the percentage of animals with normal locomotion among the total of tested animals was shown. For treatments with MEK (U0126, Calbiochem®) and ERK (FR180204, Calbiochem®) inhibitors, *i-lin-45*^{hyp}*;smn-1(RNAi)*, wild type and *smn-1(RNAi)* animals have been grown at 25°C (**Figure 5 D,F**), control strains at 20°C (**Figure S4 F**). Animals were synchronized by bleaching

and when they reached L2 stage (after ~28h at 20°C or ~20h at 25°C) we transferred 40 animals on plates seeded with dead *OP50* bacteria and 20µM of each inhibitor in DMSO 1%. DMSO 1% was used as mock. Motoneuron death has been scored at YA stage and treatments were performed in triplicates.

Statistical analyses

Statistical analyses were performed with GraphPad Prism (version 8.3.0). We avoided pseudo-replication by analyzing biological independent replicates only which were used as statistical n as follows: n≙indivdual mice (Fig. 1 E-I, Fig. 2 A-F, Fig. S3), n≙embryo preparations (Fig. 2 J, K), n≙cell passages (Fig 3, B, C), n≙iPSC lines (Fig. 3E), n≙worm preparations (Fig. 4 A, Fig. S4 A), n≙individual worms (Fig. 4 C, Fig. 5 B, E, F, Fig. S4 B, D, E, F), n≙integer of ten worms (Fig. 4D, Fig. 5 C, Fig. S4 D). Different litters were used for the detection of protein expression at postnatal day 5 by array (Fig. 1A-D, Fig. S1, Fig. S2), at P5 by Western blot (Figure 1 E-I), and by Western blots during development (Fig. 2 A-F). Student's t-tests were used for the comparison of two groups with an unpaired experimental design. Two groups in paired design (e.g. passages prepared at the same day) were compared by a paired ratio t-test. The Kruskal-Wallis test was combined with Dunn's multiple comparison test for the comparison of more than two groups. The significance level for all tests was α=5%.

Figure S1

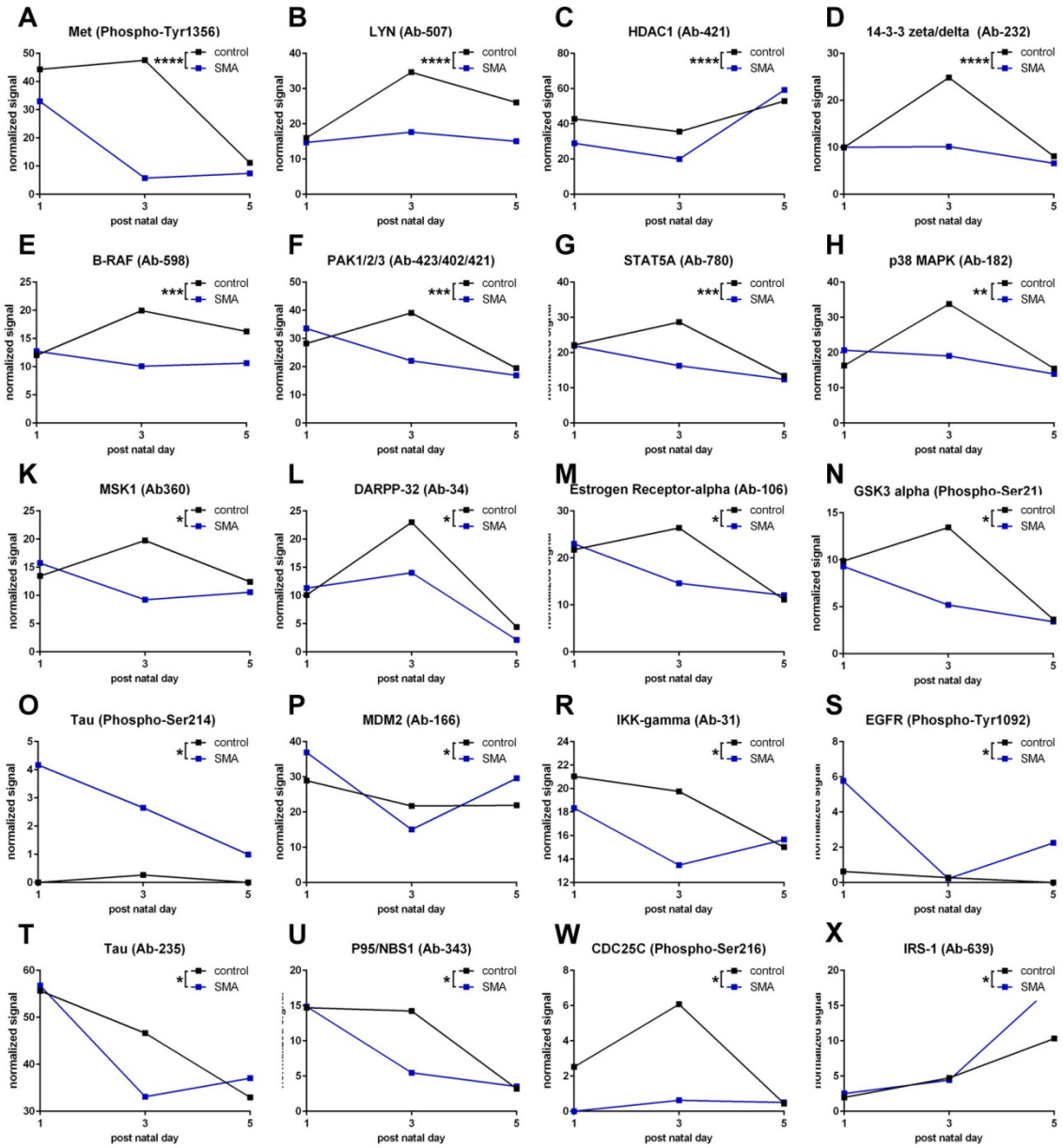

Figure S1: Developmentally dysregulated targets. The targets (proteins and phospho-proteins) were quantified via an antibody array with each data-point giving the mean of two technical replicates. A multiple t-test was performed for a statistical comparison of the control datapoints with the SMA datapoints with *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.001 (n=3).

Figure S2

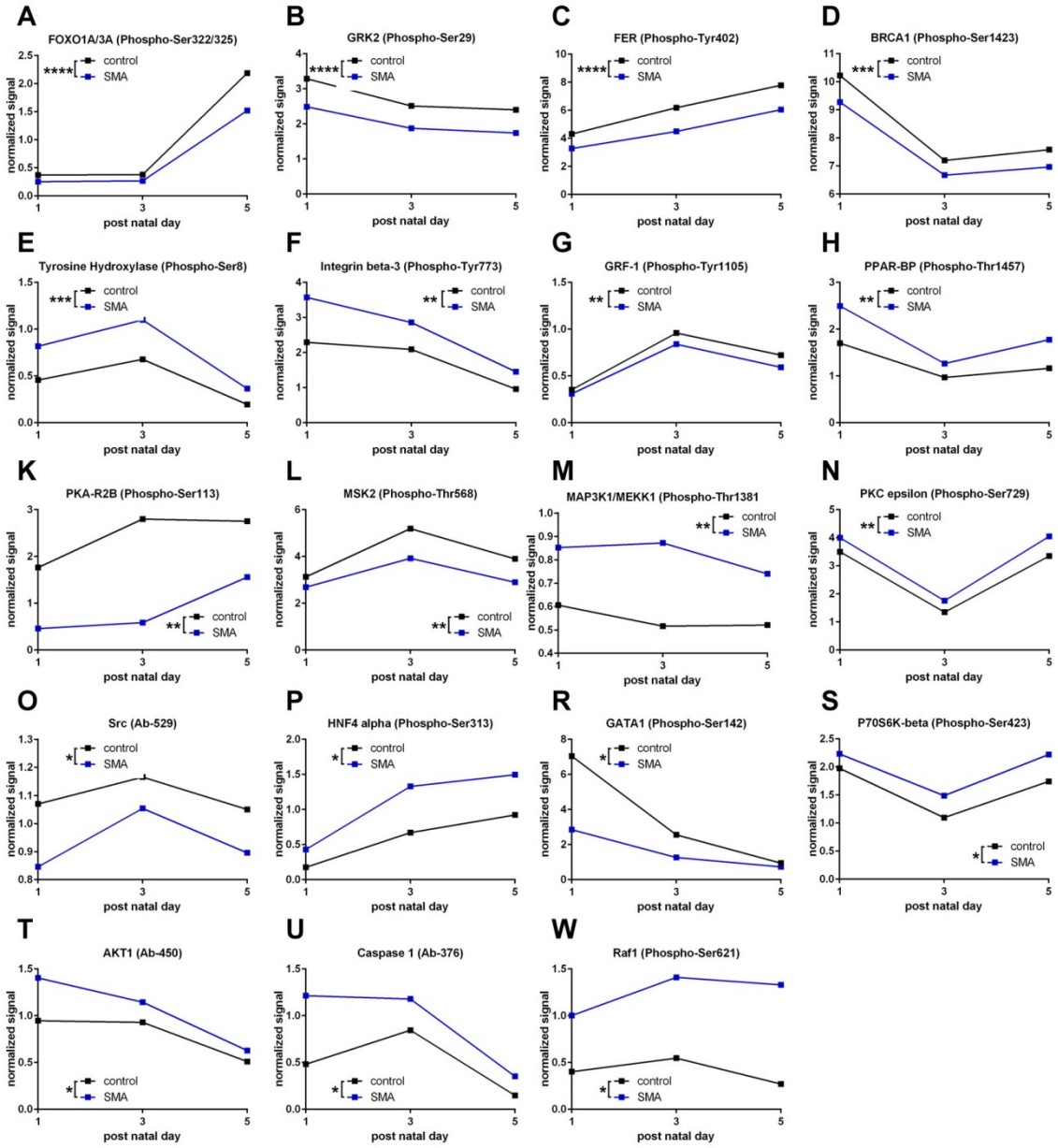

Figure S2: Persistently dysregulated targets. The targets (proteins and phospho-proteins) were quantified and the fold change (SMA-signal divided by control signal) was calculated. A multiple t-test was performed for a statistical comparison of the SMA-Fold changes compared to a constant abundance with *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.001 (n=3).

Figure S3

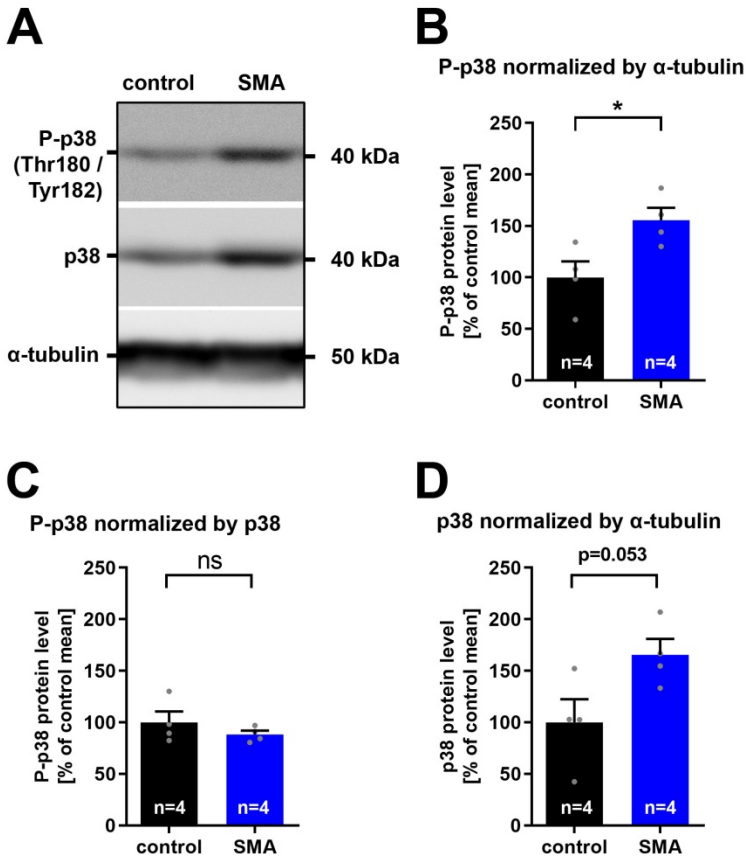

Figure S3: p38 regulation in SMA mice spinal cord. (A) Representative Western blot of Th3-13 spinal cord samples of P5 SMA mice and heterozygous control littermates of phosphorylated p38 (P-p38) and total p38. **(B)** Densitometric quantification of P-p38 normalized by α-tubulin **(C)** normalized by total p38 and **(D)** total p38 normalized by α-tubulin. Note that the increase of P-p38 is accompanied by increased total p38 levels indicating an expressional mechanism behind this effect. All bar graphs show mean + SEM with n=4. Significance was evaluated with a Student's t-test with *p≤0.05 or ns=non significant.

Figure S4

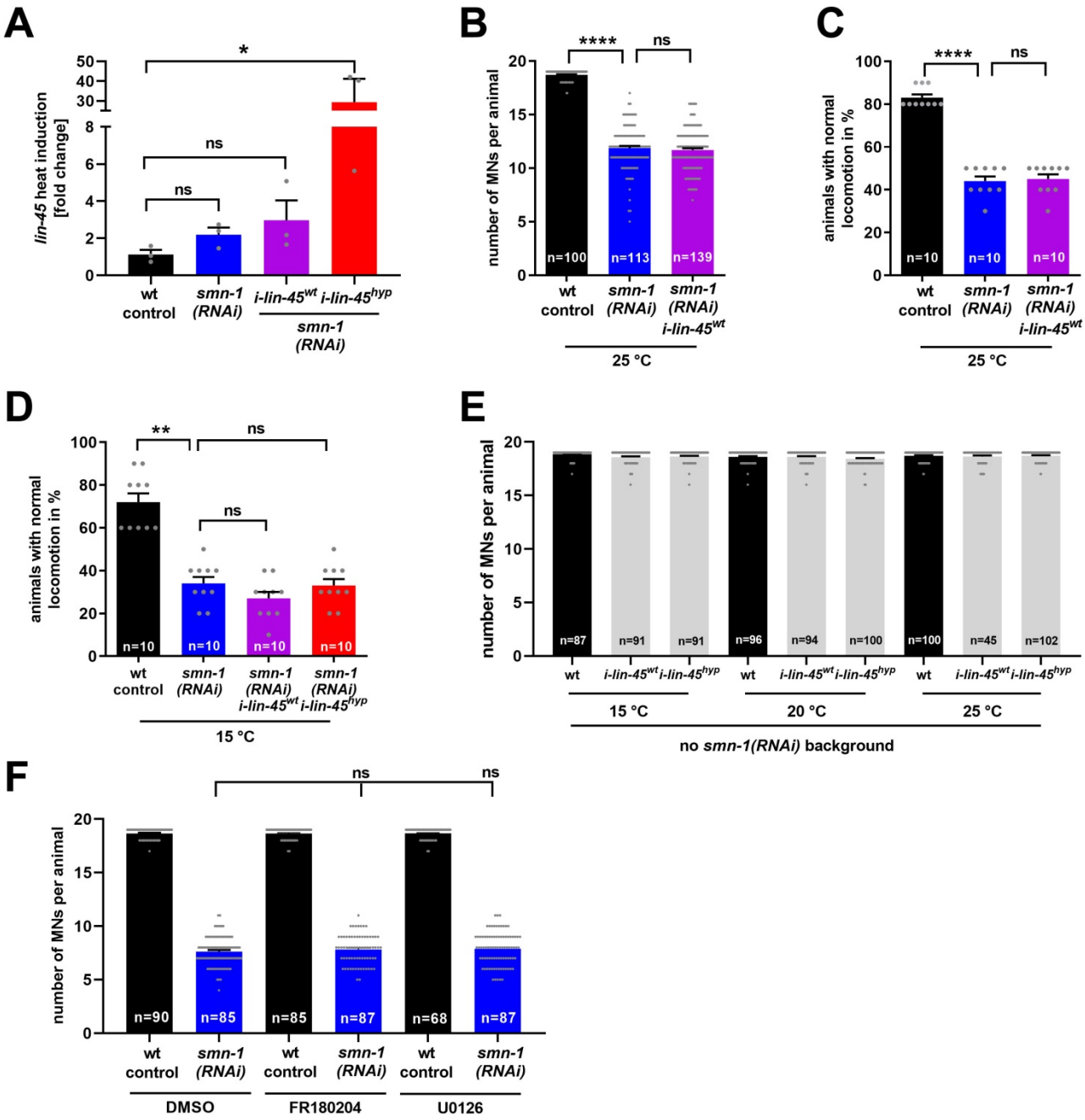

Figure S4: Control experiments on *C. elegans* with *lin-45* expressing inducible transgenes. **(A)** Fold changes of the *lin-45* temperature-induction (25°C expression divided by 15°C expression) measured by qRT-PCR in heat inducible strains (*i-lin-45*^{wt} and *i-lin-45*^{hyp}), with *smn-1* silencing in the background and in *smn-1(RNAi)* mutant and wild type animals as controls. Note, that *smn-1(RNAi)*; *i-lin-45*^{wt} animals do not show the expected heat-induction of *lin-45*^{wt}. **(B)** Motoneurons were identified as distinct GFP expressing cells and quantified for each animal. **(C-D)** The backward locomotion was evaluated at different temperatures and the percentage of normal locomotion was calculated. As expected, there is no induction of gene expression for any of the genotypes at 15°C. **(E)** The motoneurons were identified as distinct GFP expressing cells and quantified for each animal. At 15°C and 20°C there should be no induction of gene

expression which is obtained at 25°C. **(F)** Motoneuron number in inhibitor treated smn-1(RNAi) and wild type control worms. MEK (U0126) and ERK (FR180204) inhibitors were used at 20µM. All Graphs show individual data points as scatter plots with bars and whiskers displaying mean + SEM. Kruskal-Wallis test was combined with Dunn's multiple comparison test with ****p≤0.0001 and ns (non-significant) (B-F) and One-Way ANOVA with Dunnett's multiple comparison test with *p≤0.05 and ns (non-significant) (A).

Supplemental table S1

Application	Antigen	Origin	Type	Size [kDa]	Dilution	Manufacturer
Western blot	14-3-3 ζ/δ (D7H5)	rabbit	polyclonal	28	1:1000	Cell Signaling Technolog (7413)
	A-Raf	rabbit	polyclonal	68	1:1000	Cell Signaling Technology (4432)
primary antibodies	B-Raf (D9T6S)	rabbit	monoclonal	86	1:1000	Cell Signaling Technology (14814)
	Phospho-B-Raf (Ser445)	rabbit	polyclonal	86	1:1000	Cell Signaling Technology (2696)
	GAPDH (FL-335)	rabbit	polyclonal	36	1:1000	Santa Cruz Biotechnology (sc-25778)
	SMN	mouse	monoclonal	38	1:4000	BD Biosciences (610647)
	Phospho-p38 (Thr180/Tyr182)	rabbit	monoclonal	41	1:1000	Cell Signaling Technology (9215)
	p38	rabbit	polyclonal	41	1:1000	Cell Signaling Technology (9212)
Western blot	anti-mouse IgG Peroxidase	sheep	-	-	1:4000	Amersham plc (NA 931)
secondary antibodies	anti-rabbit IgG Peroxidase	donkey	-	-	1:5000	Amersham plc (NA934)
Immuno-chemistry	B-Raf	rabbit	polyclonal	-	1:50 (1:10 in cryo IHC)	Thermo Scientific (PA514926)
primary antibodies	DAPI, dilactate	-	-	-	1:1000	Sigma Life Sciences
	SMI32	mouse	monoclonal	-	1:1000	Sternberger Monoclonal Inc.
	NeuN	mouse	monoclonal	-	1:500	Millipore (MAB377)
	SMN	mouse	monoclonal	-	1:400	BD Biosciences (610647)
Immuno-chemistry	anti-mouse Alexa 555	goat	-	-	1:500	Molecular Probes, Invitrogen (A21422)
	anti-rabbit Alexa 488	goat	-	-	1:500	Molecular Probes, Invitrogen
secondary antibodies	anti-rabbit Alexa 647	goat	-	-	1:500	Molecular Probes, Invitrogen

Supplemental table S2

Application	Primer name	Sequence 5'-3'
lin-45 expression level by real-time PCR	ActRTfw	CCCAATCCAAGAGAGGTATCCT
	ActRTrev	ATATCATCCCAGTTGGTGACGAT
	Fw RT lin-45	GATGCACAACGACCCAGGCC
	Rev RT lin-45	CGGTACCGAAGGATCCCGAACC
	Fw hsp-16.41	CCAACACGCTTTGTTCTAGTGC
	Rev lin-45f gDNA	GAGGAGTTGAAGGACTTGCC
mek-2(*n1989)* genotyping	Fw mek-2	GTTTGTCGGACGAATCTCGG
	Rev mek-2 wt	CATCGAATCAATCAACATTCAAG
	Rev mek-2 n1989	CATCGAATCAATCAACATTCAAA
csIs34 and *csIs42* genotyping	Fw hsplin-45	GAGGAAGCCAACACGCTTTG
	Rev hsplin-45	CAATTTCGAAATGGCATCCCG
MN specific expression of *lin-45*^{wt} and *lin-45*^{hyp}	Fw punc-47Ext	CTGTCAAAGGGGTACAGCATG
	Rev punc-47	CTGTAATGAAATAAATGTGACG
	Fw punc-47Lin-45	GCGTCACATTTATTTCATTACAGATGTCTGCAATGGCTGTACTG
	Rev Exinj lin-45	CAAATACTGTGGTGCAGCTTC
	Rev oMS104	GATATTCGGAGCAGCAGAGCTCCGATC
	Fw oMS103	GATCGGAGCTCTGCTGCTCCGAATATC
	Rev lin-45 Ext2	CCATTCGTCCGACATCCTGTGG
	Fw punc-47 Int	CCCGGAACAGTCGAAAGTCG
	rev Inj lin-45	GAAGAAACAGGAAGTCAGGCG
	Rev lin-45 Seq	GCCACCTGGAGGTGATGAACAT
	Fw lin-45 Seq	GGCCCAGATAGATCAGTTGCCG

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