Appendix A

	Analytical Report 2
Report to	Dr Tanja Davis Department of Physiological Sciences Mike de Vries Building c/o Merriman and Bosman Street Stellenbosch 7600
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1. Background and samples submitted

Fourty-four mouse tail tissue samples (listed in Table 1) were submitted for genotyping of transgenic mice. The samples were submitted along with nucleotide sequences of the genotyping primers which were to be ordered by the CPGR.

2. Analysis

2.1. DNA yield and quality

The yield and purity of the extracted DNA samples were assessed using the NanoDrop-8000 spectrophotometer (Table 1) and the integrity of the samples was assessed using agarose electrophoresis (Fig. 1).

Table 1. DNA concentrations, OD ratios ($A_{260/280}$ and $A_{260/230}$) for DNA extractions from the mouse tail tissues.

#	Sample	Conc.	Units	260/280	260/230
1	MT01	84.14	ng/ul	1.84	2.02
2	MT02	91.41	ng/ul	1.9	2.16
3	MT03	48.52	ng/ul	1.82	2.05
4	MT04	41.68	ng/ul	1.82	2.03
5	MT05	41.09	ng/ul	1.82	1.74
6	MT06	96.48	ng/ul	1.9	2.13
7	MT07	63.96	ng/ul	1.9	2.12
8	MT08	65.77	ng/ul	1.92	1.98
9	MT09	67.17	ng/ul	1.91	1.79
10	MT10	126.9	ng/ul	1.89	1.97
11	MT11	32.43	ng/ul	1.84	2.47
12	MT12	30.49	ng/ul	1.96	2
13	MT13	66.57	ng/ul	1.91	2.01

#	Sample	Conc.	Units	260/280	260/230
14	MT14	172.2	ng/ul	1.91	2.17
15	MT15	55.57	ng/ul	1.92	1.64
16	MT16	49.28	ng/ul	1.88	2.04
17	MT17	33.65	ng/ul	1.91	2.16
18	MT18	99.41	ng/ul	1.91	2.08
19	MT19	67.89	ng/ul	1.87	2.36
20	MT20	75.15	ng/ul	1.94	2.12
21	MT21	103.2	ng/ul	1.96	2.22
22	MT22	95.51	ng/ul	1.89	2.17
23	MT23	64.42	ng/ul	1.92	2.25
24	MT24	91.69	ng/ul	1.89	2.06
25	MT25	252.3	ng/ul	1.95	2.22
26	MT26	133.8	ng/ul	1.95	2.21
27	MT27	123.5	ng/ul	1.94	2.03
28	MT28	123.4	ng/ul	2	1.81
29	MT29	113.3	ng/ul	1.97	2.32
30	MT30	80.33	ng/ul	1.94	2.2
31	MT31	77.91	ng/ul	2.02	2.22
32	MT32	122.2	ng/ul	1.95	2.21
33	MT33	89.23	ng/ul	1.93	2.14
34	MT34	96.29	ng/ul	1.94	2.22
35	MT35	78.81	ng/ul	1.96	2.12

#	Sample	Conc.	Units	260/280	260/230
36	MT36	99.96	ng/ul	1.97	2.08
37	MT37	119.2	ng/ul	1.95	2.21
38	MT38	125	ng/ul	1.93	2.16
39	MT39	88.48	ng/ul	1.97	1.87
40	MT40	115.5	ng/ul	1.95	2.15
41	MT41	75.59	ng/ul	1.96	2.03
42	MT42	118.6	ng/ul	1.9	2.07
43	MT43	92.99	ng/ul	2.01	1.9
44	MT44	98.44	ng/ul	1.97	2.22

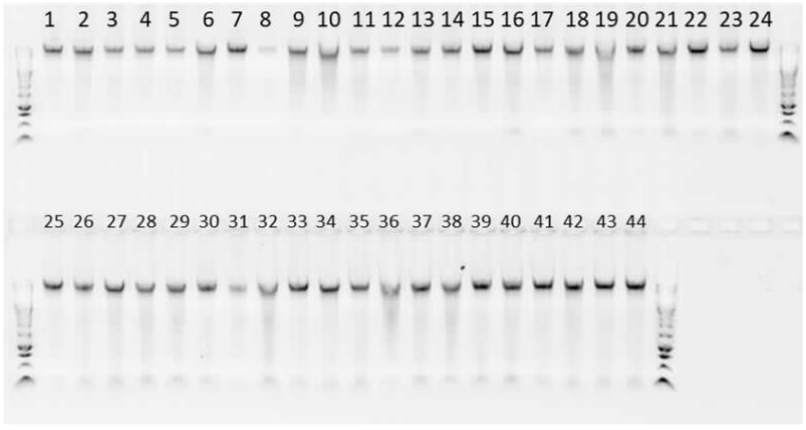

Figure 1. 1% Agarose gel electrophoresis images of high molecular weight genomic DNA extracted from mouse tails.

2.2. Genotyping of mouse tails

The 303 bp fragment was amplified in all 44 DNA samples tested (Fig. 2).

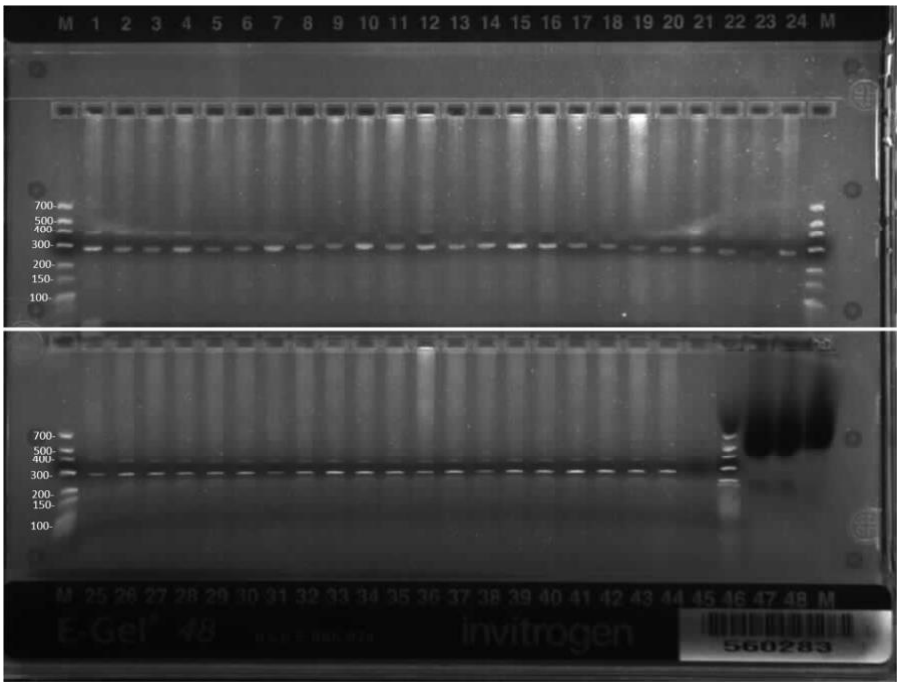

Figure 2. Genotype identification by PCR amplification of mouse tail DNA samples. Amplification of the ~303 bp target fragment was amplified in PCR reactions using 150ng of each DNA sample (lanes 1-44). An NTC (no-template control) was included (lane 45) as a negative control. The Thermo Scientific GeneRuler Low Range DNA Ladder was used as a size standard.

3. Experimental procedures

3.1. PCR primers

The three PCR primers were sourced from Inqaba Biotech and were resuspended in TE buffer (10mM Tris, pH 8.0, 1mM EDTA) buffer to a 100µM stock concentration, as per manufacturer instructions. Working stocks of 10µM were prepared and used for the PCR reactions.

3.2. DNA extractions

Genomic DNA extraction from the mouse tails was automated using the QIAamp® DNA Kit (QIAGEN) on the QIAcube and eluted in 100 µl. The quality and quantity of the extracted DNA was measured using the NanoDrop-8000 spectrophotometer. Ratio of the absorbance at A_{230}, A_{260} and A_{280} were estimated to determine the quality and the DNA concentration was measured based on A_{260} values. Ten µL of each DNA sample was analysed on a 1% agarose gel stained with ethidium bromide.

3.3. Genotyping

For PCR, the three primers were mixed (0.3 µM for each primer) with 150 ng each DNA sample and amplified with the KAPA HiFi PCR Kit in a 25 µL total reaction volume. The cycling protocol was: 1 cycle of 95°C for 3 min; 35 cycles of 98°C for 20 sec, 60°C for 15 sec, and 72°C for 30 sec. PCR products were separated by electrophoresis using E-Gel® 48 4% gels.

4. Deviations

No deviations to protocols or standard methods employed or deviations from the agreed study plan.

5. Enquiries

Your application specialist on this project is Aubrey Shoko; do not hesitate to contact him on 021 447 5669 or aubrey.shoko@cpgr.org.za for additional discussion or information on this report.

Appendix B

RNA EXTRACTION, CDNA SYNTHESIS AND CYCLE SEQUENCING

Report date: 2022-03-18 *Reported by: AA Vorster*

CAF Central Analytical Facilities
STELLENBOSCH UNIVERSITY

BACKGROUND

The biological specimen, EO771, was received for RNA extraction and mRNA enrichment on the 8th of February 2022.

MRNA ENRICHMENT

mRNA was isolated from 2.5 x 10^4 cells using the Dynabeads®mRNA DIRECT™Micro Kit (ThermoFisher, Waltham, MA, USA) according to the manufacturer's protocol for "*mRNA isolation from cultured cells and cell suspensions*, *p10*" (Revision 004, Revision Date: 14 May 2012). Briefly, cells were washed in 250ul phosphate-buffered saline and resuspended in 100ul Lysis/Binding Buffer. The lysate was mixed with Dynabeads® Oligo (dT)25 to allow mRNA with poly(dA) tails to anneal to the beads. Unbound RNA was washed from the supernatant using wash buffer A and B. The bound mRNA was subsequently eluted in 100ul ice-cold 10mM Tris-HCl. The procedure was performed in duplicate.

CDNA SYNTHESIS

The SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher Scientific) was used to convert expressed RNA transcripts into a representative cDNA library according to the manufacturer's protocol (Doc. Part No. 100002284 Pub. No. MAN0000749 Rev. A.0). Briefly, 10ul enriched mRNA (8.5ng/ul) was transcribed with the addition of 4ul 5X VILO™ Reaction Mix and 2ul 10X SuperScript™ Enzyme Mix in a final reaction volume of 20ul. cDNA was synthesized at 42°C for 1 hr, followed by inactivation at 85°C at 5 minutes.

QUALITY CONTROL

The total RNA samples were assessed for RNA integrity (RINe) on the TapeStation 4150, using the RNA ScreenTape assay (Agilent Technologies, Waldbronn, Germany) according to the protocol, G2991-90021 Rev. B. The quality and quantity of mRNA yielded from the isolation method was considered sufficient for first-strand cDNA synthesis. The concentration of the cDNA was determined with fluorometry on the Qubit 4.0 using the Qubit™ssDNA Assay Kit (ThermoFisher Scientific).

Table 1. RNA quality control assessment

Sample	RINe	RNA conc.	cDNA conc.
EO771_Replicate1	8.9	8.5 ng/ul	72.4 ng/ul
EO771_Replicate2	8.7	11 ng/ul	65.0 ng/ul

PCR AMPLIFICATION

For PCR amplification of single-plex SAA targets, 50ng cDNA was added to a final reaction volume of 20ul; consisting of 10 pmol of each sense and antisense primer pair, 250uM of each dNTP, 1x Phusion HF buffer and one unit Phusion™ High-Fidelity DNA Polymerase. Amplification of the cDNA template was performed using a touch-down PCR (Green & Sambrook, 2018), where the target template was dissociated at 98°C for the first 10 amplification cycles, followed by 25 cycles of amplification with a lower DNA dissociation temperature of 89°C. Primer annealing temperatures for SAA1, SAA2 and SAA3 was 55 °C, whilst the SAA4 primers were annealed at 60°C. The yield and specificity of the amplified products was verified on the PerkinElmer LabChip® GXII Touch (PerkinElmer, Waltham, MA, USA), using the X-mark chip and HT DNA NGS 3K reagent kit according to the manufacturer's protocol; CLS145098 Rev. E.

POST-PCR PURIFICATION

Post-PCR purification was performed with a 1.8x volume Agencourt™ AMPure™ XP (Beckman Coulter, Brea, CA, USA) reagent and eluted in 20ul nuclease-free H_2O.

SEQUENCING

Direct-cycle sequencing was performed according to standard protocols.

REFERENCES

Green MR, Sambrook J. Touchdown Polymerase Chain Reaction (PCR). Cold Spring Harb Protoc. 2018 May 1;2018(5). doi: 10.1101/pdb. prot095133. PMID: 29717053.

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Fig. B.1 Materials and methods of EO771 SAA1/2/3/4 mRNA expression analysis by the Stellenbosch University Central Analytical Facilities (CAF).

Table B.1 Primers sourced from Integrated DNA Technologies (IDT) and used for PCR amplification and direct-cycle Sanger sequencing.

	Forward primer (5' – 3')	Reverse primer (5' – 3')
SAA1	CAT TTG TTC ACG AGG CTT TCC	GTT TTT CCA GTT AGC TTC CTT CAT GT
SAA2	TCT TCT GCT CCC TGC TCCT	AGC CAG CTT CCT TCA TGT CA
SAA3	ACA GCC AAA GAT GGG TCC AG	CTG GCA TCG CTG ATG ACT TT
SAA4	TGTCCTCTGTTCTTTGTTCCTG	TGGTCTGCATTTTGGTAATTAGC

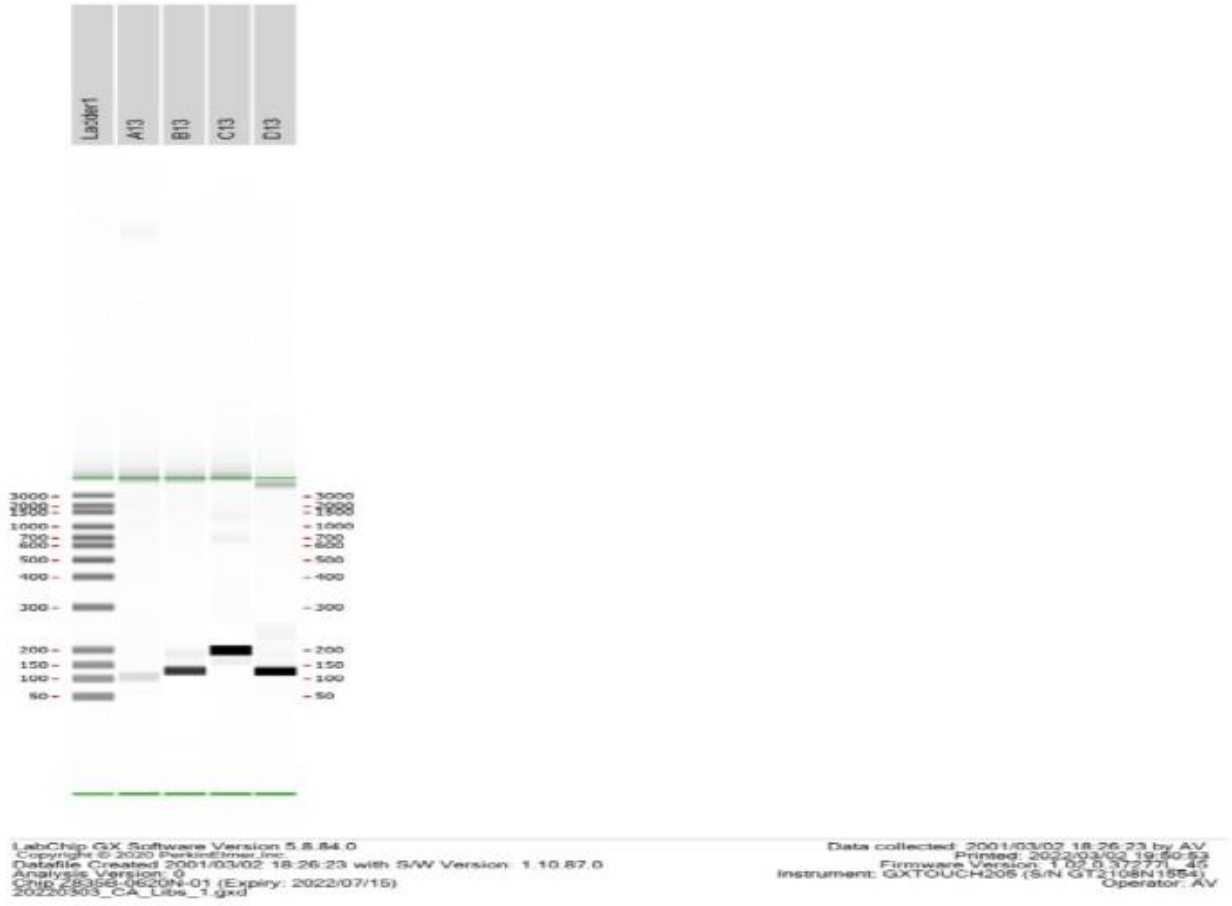

Fig. B.2 Virtual electrophoretic gel of SAA1/2/3/4 PCR amplicons. Lane A13, SAA1 (~101 bp); Lane B13, SAA2(~129 bp); Lane C13, SAA3 (~198 bp); Lane D13, SAA4 (~125 bp).

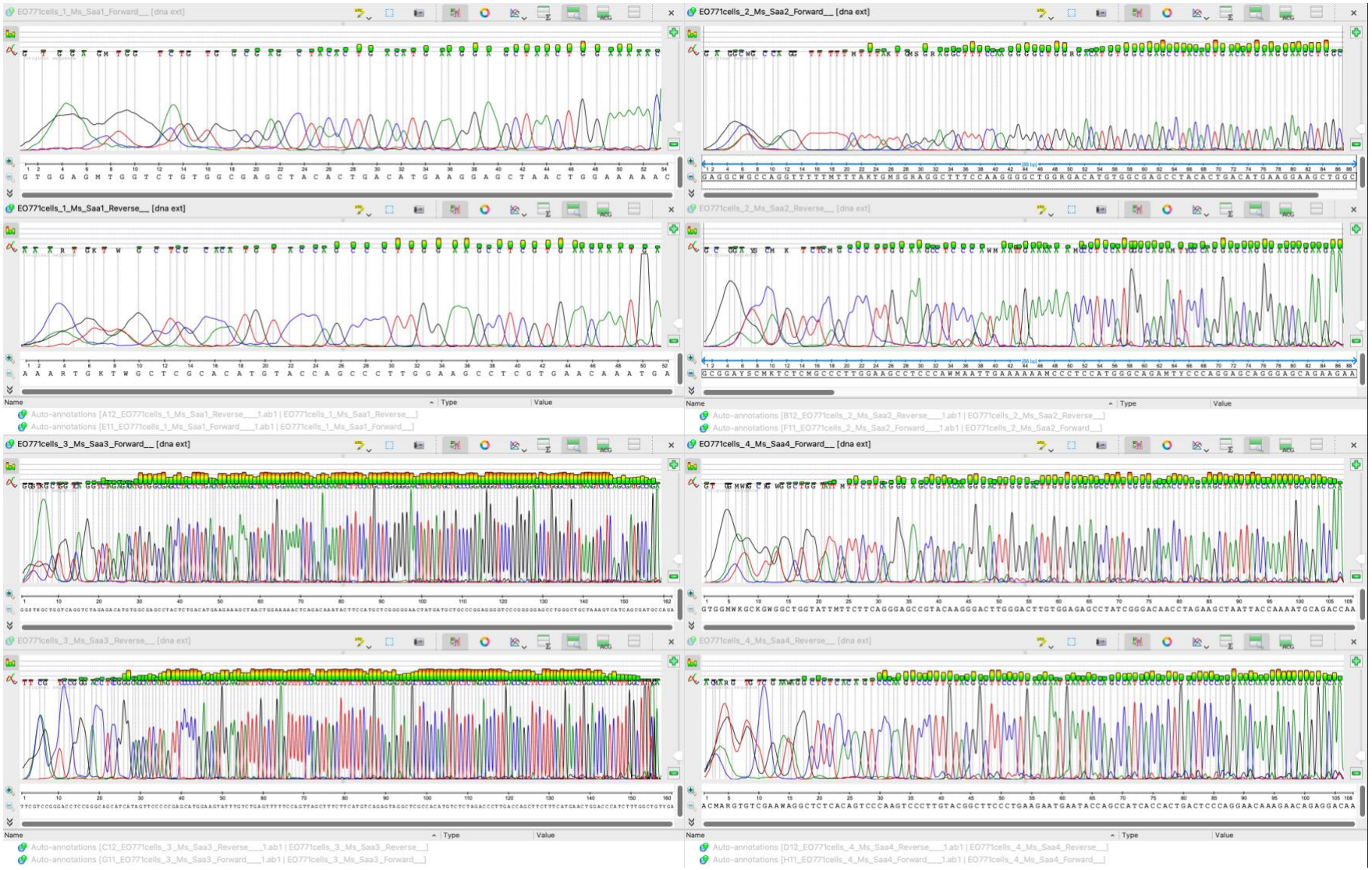

Fig. B.3 Electropherograms of PCR amplicon direct-cycle Sanger sequencing.

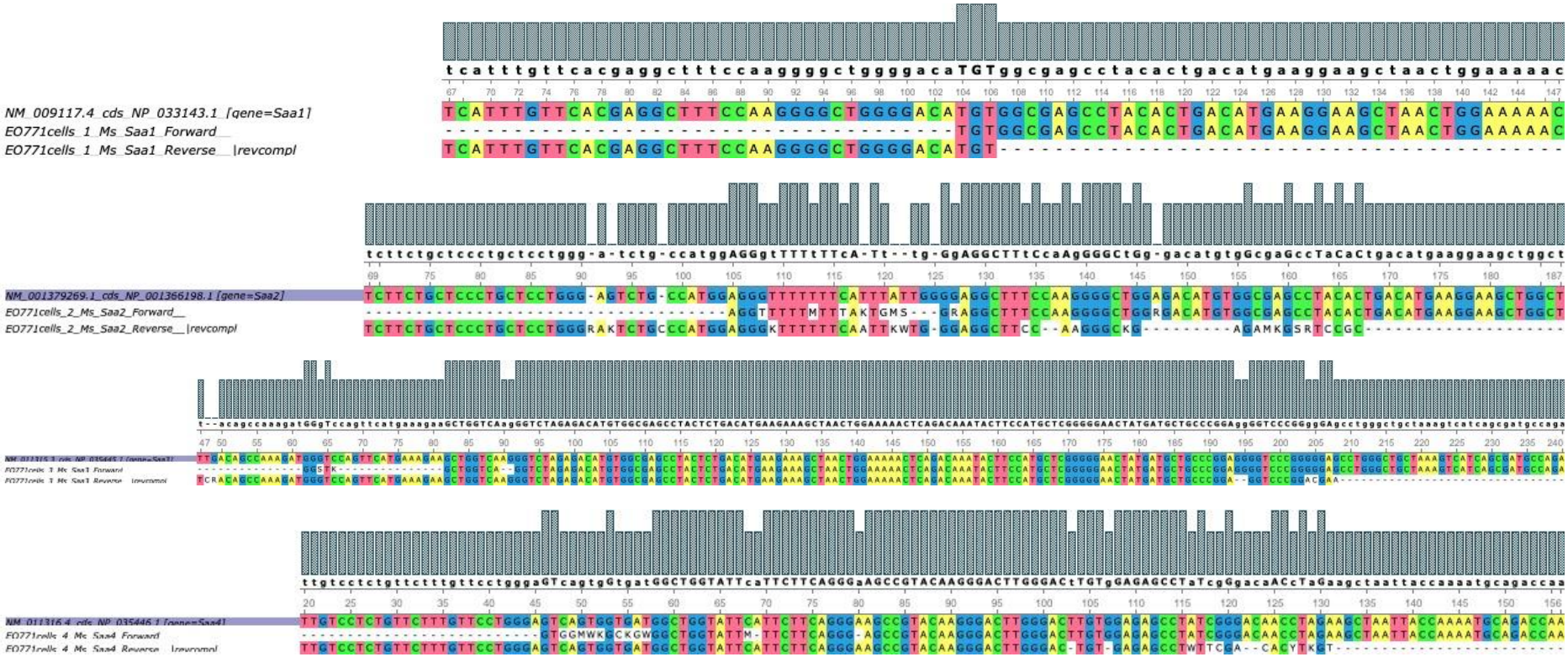

Fig. B.4 MUSCLE (Unipro UGENE) forward and reverse sequence alignments of direct-cycle Sanger sequence results to NM_009117.4 (SAA1), NM_001379269.1 (SAA2), NM_011315.3 (SAA3), and NM_011316.4 (SAA4) coding sequences. From top to bottom: SAA1, SAA2, SAA3, and SAA4.

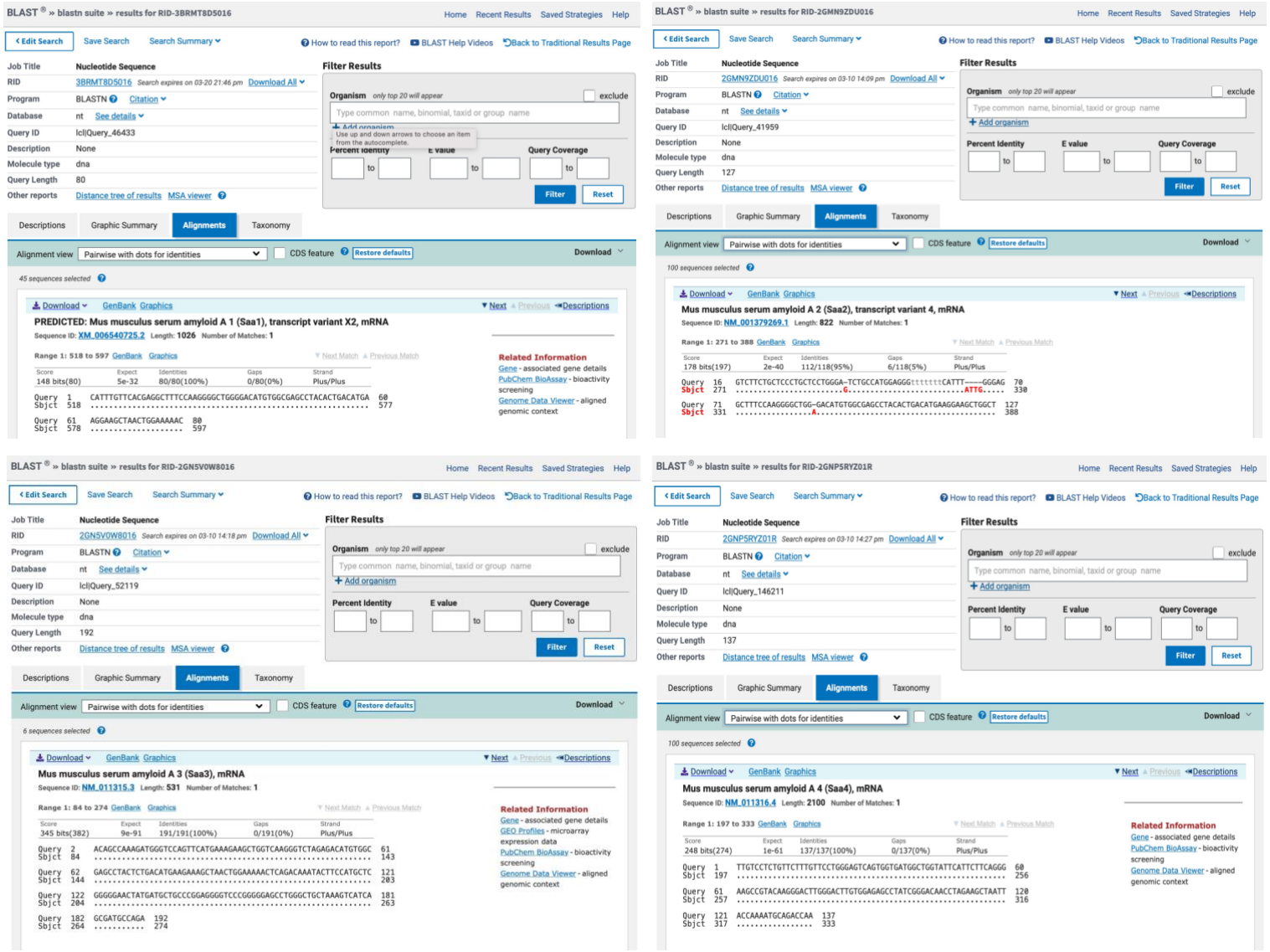

Fig. B.5 BLAST results of SAA1/2/3/4 consensus sequences determined from Fig. B.5.

Appendix C

Table C.1 Antibodies used in Western blotting

Primary antibody	MW (kDa)	Dilution	Time	Secondary antibody	Dilution	Time (min)
SAA antisera	~ 12	1:1000	1 Day	Anti-mouse HRP	1:10 000	60
β-catenin	~ 92	1:1000	1 Day	Anti-rabbit HRP	1:1000	60
Laminin 1β	~ 210	1:200	1 Day	Anti-rat HRP	1:5000	60
Vimentin	~ 57	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
E-cadherin	~ 135	1:1000	1 Day	Anti-mouse HRP	1:10 000	60
Snail	~ 29	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
αSMA	~ 42	1:1000	1 Day	Anti-mouse HRP	1:10 000	60
tCAS3 & cCAS3	~ 19, 30	1:1000	3 Days	Anti-rabbit HRP	1:10 000	60
tCAS7 & cCAS7	~ 20, 35	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
tCAS8 & cCAS8	~ 18, 57	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
tCAS9 & cCAS9	~ 35, 47	1: 500	1 Day	Anti-mouse HRP	1:10 000	60
Cytochrome C	~ 14	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
tPARP & cPARP	~ 89, 116	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
MCM2	~ 125	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
p53	~ 53	1:2500	1 Day	Anti-mouse HRP	1:10 000	60
p16	~ 16	1:2500	1 Day	Anti-rabbit HRP	1:10 000	60
NLRP3	~ 110	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60
NFκB & p-NFκB	~ 65	1:1000	1 Day	Anti-rabbit HRP	1:10 000	60

SAA, serum amyloid a; α-SMA, α-smooth muscle actin; tCAS, total (full length) caspase; cCAS, cleaved caspase; tPARP, total (full length) poly (ADP-ribose) polymerase (PARP); cPARP, cleaved PARP; MCM2, DNA mini-chromosome maintenance protein 2 or DNA replication licensing factor 2; p53, tumor protein 53; p16, cyclin-dependent kinase inhibitor 2A; NLRP3, nucleotide-binding oligomerization domain-like receptor with pyrin domain 3; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; p-NFκB, phosphorylated NFκB.

De Beer laboratory: Mouse SAA antisera; Abcam: Laminin 1β (ab44941), tCAS8/cCAS8 (ab25901), MCM2 (ab108935), p53 (ab26), p16 (ab189034), α-SMA (ab7817), Anti-rat HRP (ab205720); Cell Signalling Technology: β-actin (4967), β-catenin (19807), Vimentin (5741), E-cadherin (14472), Snail (3879), tCAS3/cCAS3 (9661, 9662), tCAS7/cCAS7 (12827), tCAS9/cCAS9 (9508), Cytochrome C (11940), tPARP/cPARP (9532), NLRP3 (15101), NFκB (8242), p-NFκB (3033), Anti-rabbit HRP (7074), Anti-mouse HRP (7076).

Appendix D

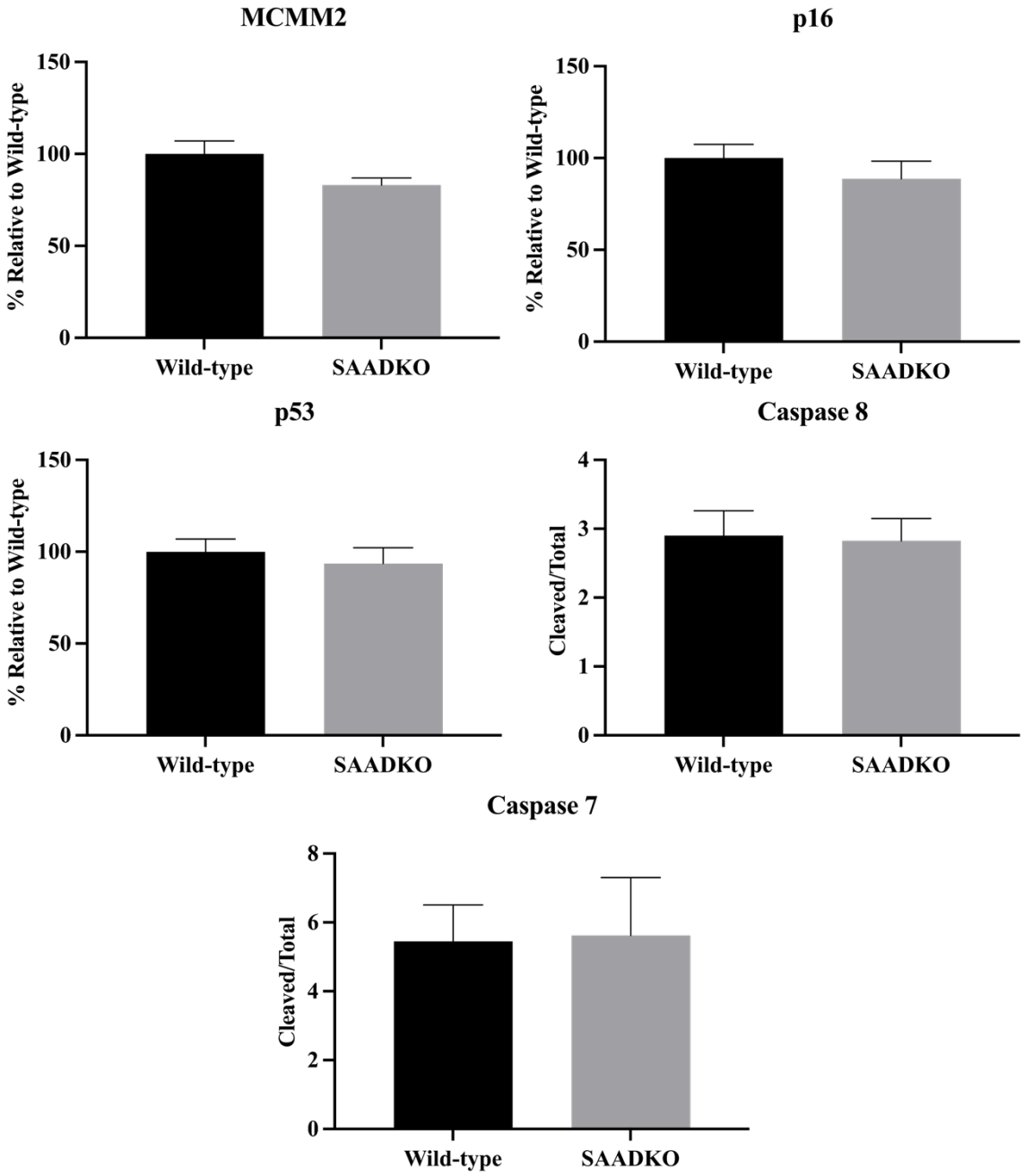

Fig. D.1 Tumor apoptotic (caspase 7, caspase 8) and cell cycle markers (p53, p16, MCM2) that were not influenced by systemic SAA1/2-deficiency. Caspase 7, caspase 8, p53, and p16 were analyzed with an uncorrected unpaired t-test. MCM2 was analyzed with an unpaired t-test with Welch's correction. Data show the mean ± SEM (N = 7). Representative western blot images can be found in Fig. D.5.

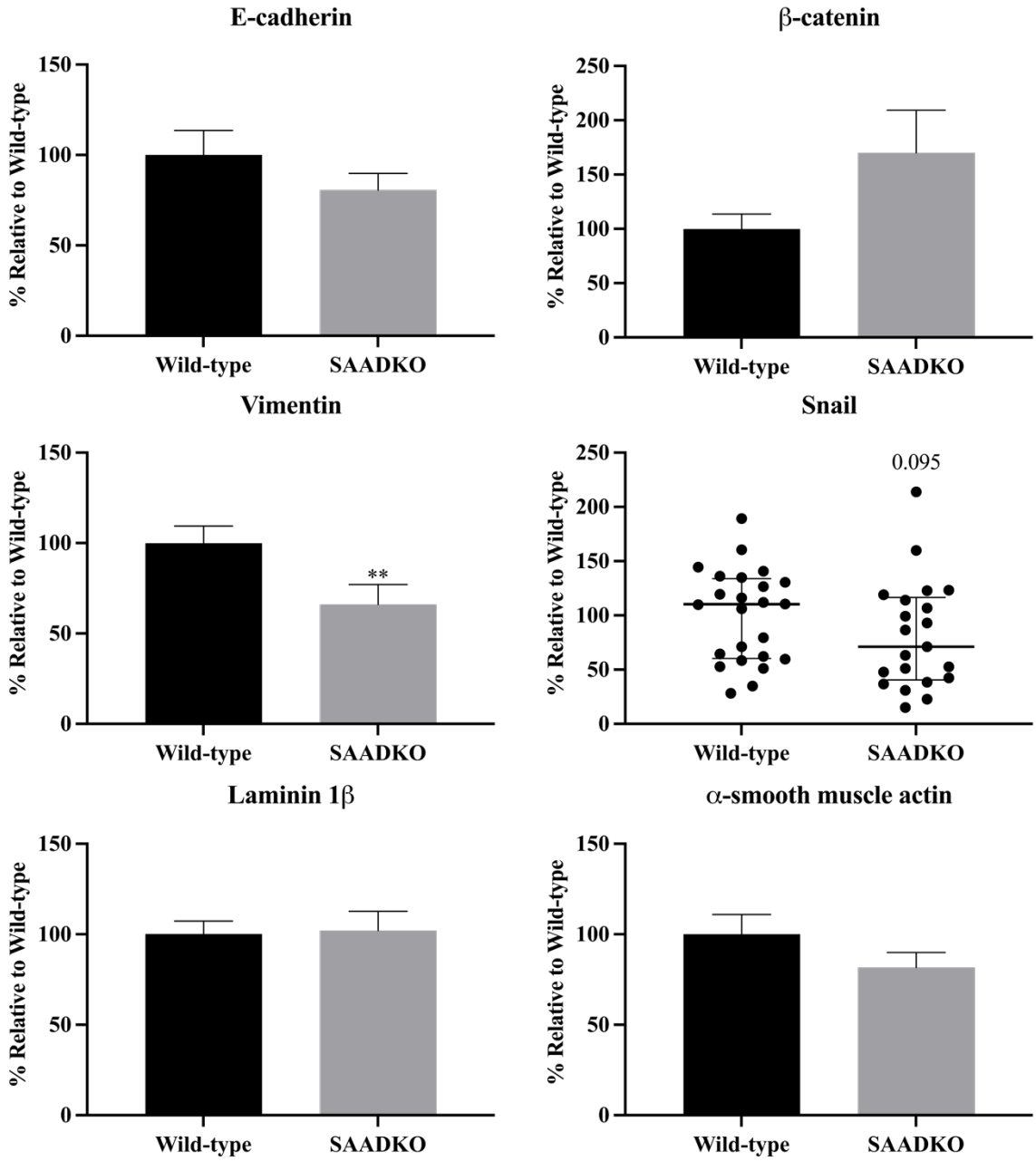

Fig. D.2 SAADKO tumors only express lower levels of the EMT marker, vimentin. E-cadherin, Laminin 1β, and α-smooth muscle actin were analyzed with an uncorrected unpaired t-test. Vimentin and β-catenin were analyzed with an unpaired t-test with Welch's correction. Snail was analyzed with a Mann-Witney test. E-cadherin, β-catenin, vimentin, laminin 1β, and α-smooth muscle actin data show the mean ± SEM, whereas Snail data show the median with IQR (N = 7). ** $p < 0.01$ WT vs. SAADKO. Representative western blot images can be found in Fig. D.5.

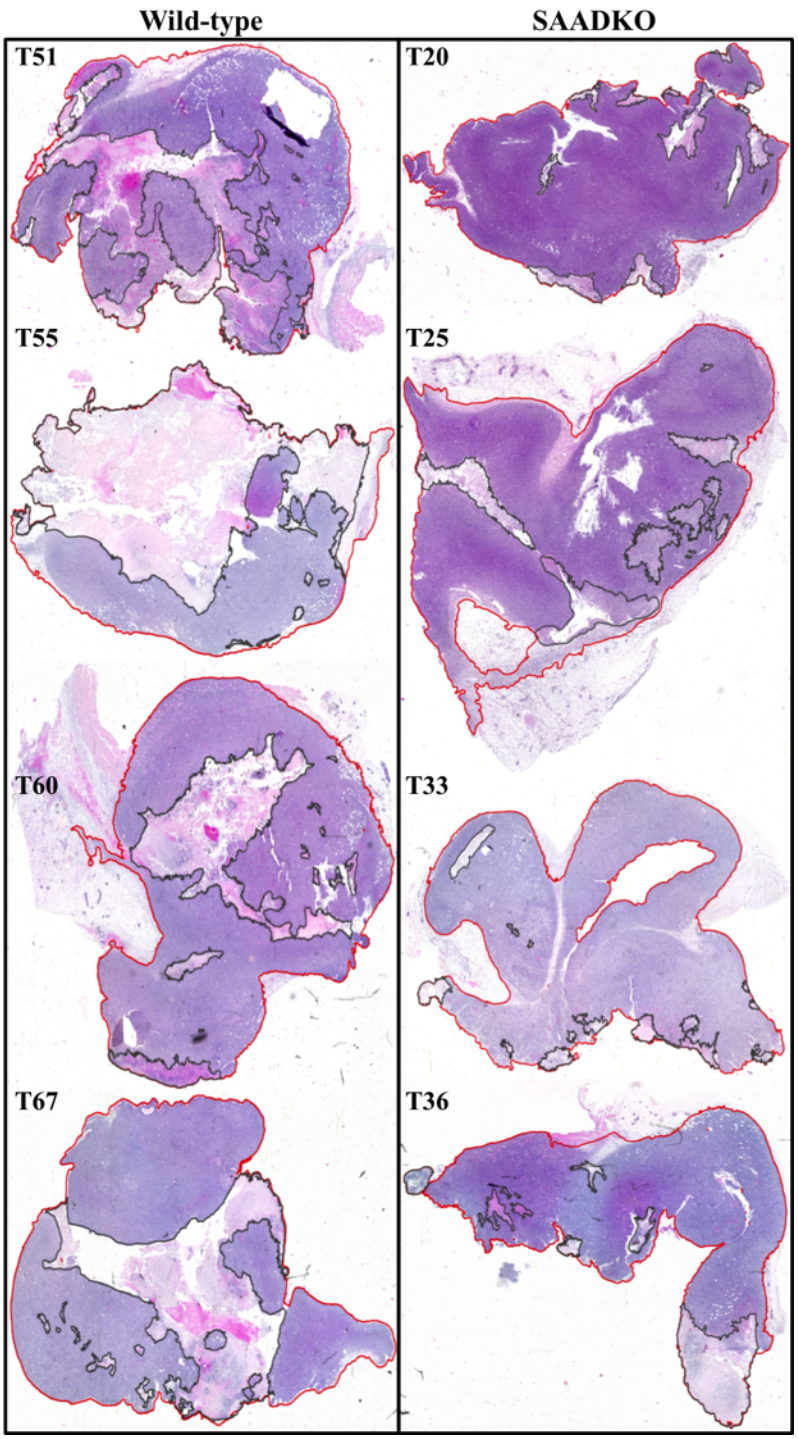

Fig. D.3 Representative photomicrographs (H&E, 400X) of WT and SAADKO tumors assessed with QuPath. The total tumor area assessed is demarked in red, while necrotic regions are shown in black. T, Tumor number

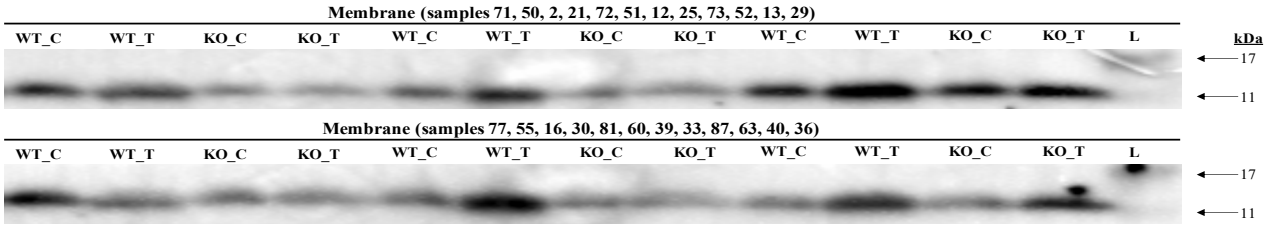

Fig. D.4 Representative western blot images for plasma SAA from WT control, WT tumor, SAADKO control, and SAADKO tumor mice. Blots were cropped to the left of WT_C (samples 71 and 77) to remove the internal standard. Sample numbers indicated above each membrane correspond to the order of loading. Representative total protein (stain-free) images are shown in Fig. D.5. WT_C, Wild-type control; WT_T, Wild-type tumor; KO_C, SAA1/2 double-knockout control; KO_T, SAA1/2 double-knockout tumor; L, molecular weight marker.

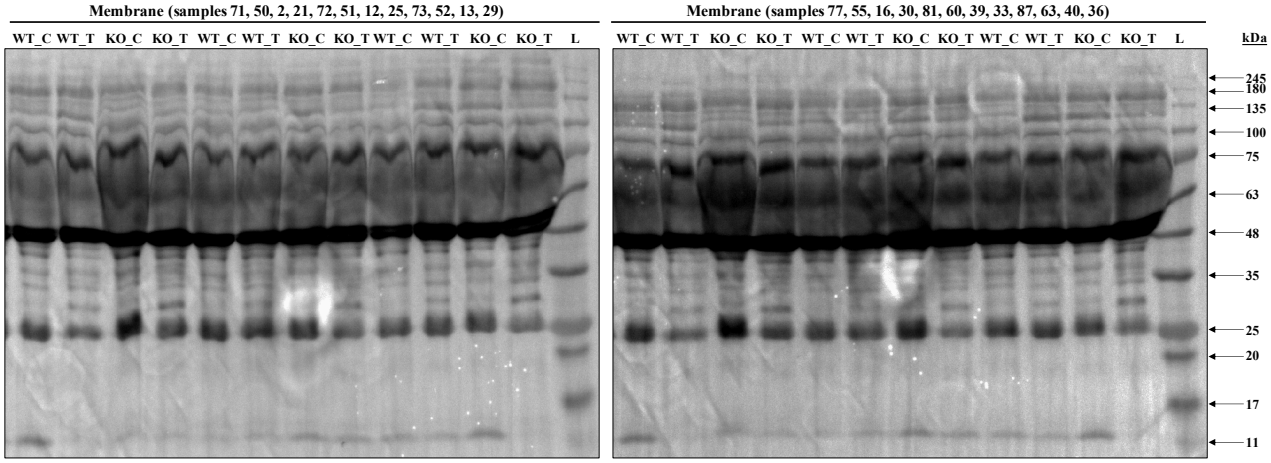

Fig. D.5 Representative total protein (stain-free) images for plasma of WT control, WT tumor, SAADKO control and SAADKO tumor mice. Gels were cropped to the left of WT_C (samples 71 and 77) to remove the internal standard. Sample numbers indicated above each membrane correspond to the order of loading. WT_C, Wild-type control; WT_T, Wild-type tumor; KO_C, SAA1/2 double-knockout control; KO_T, SAA1/2 double-knockout tumor; L, molecular weight marker.

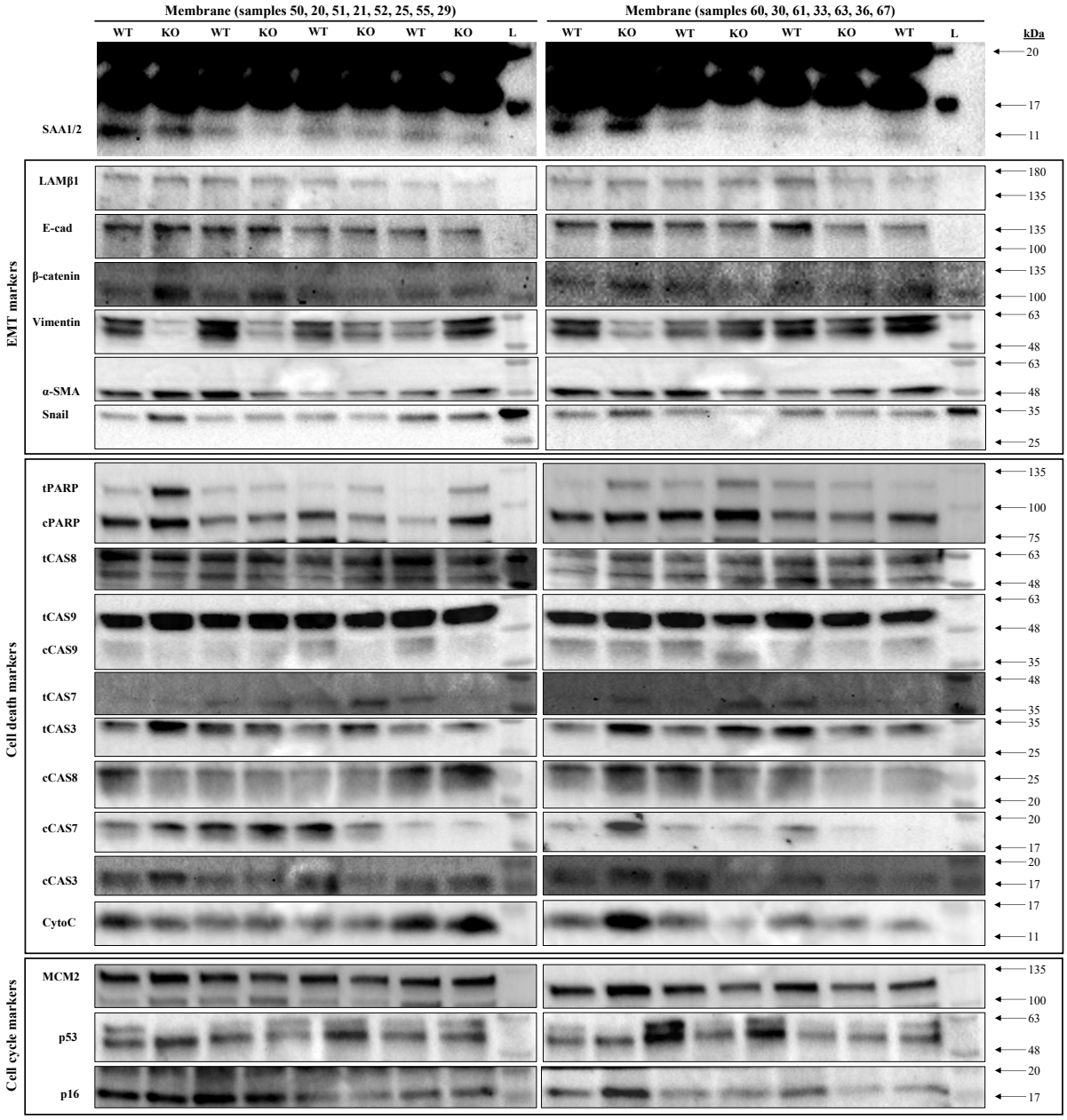

Fig. D.6 Representative western blot images of proteins blotted for WT and SAADKO tumor lysates. Blots were cropped to the left of WT (samples 50 and 60) to remove the internal standard. Sample numbers indicated above each membrane correspond to the order of loading. Representative total protein (stain-free) images are shown in Fig. D.7. WT, Wild-type; KO, SAA1/2 double-knockout; LAMβ1, laminin 1β; E-cad, E-cadherin; α-SMA, α-smooth muscle actin; cCAS, cleaved caspase; tCAS, total caspase; cPARP, cleaved PARP; tPARP, total PARP; CytoC, cytochrome C; L, molecular weight marker.

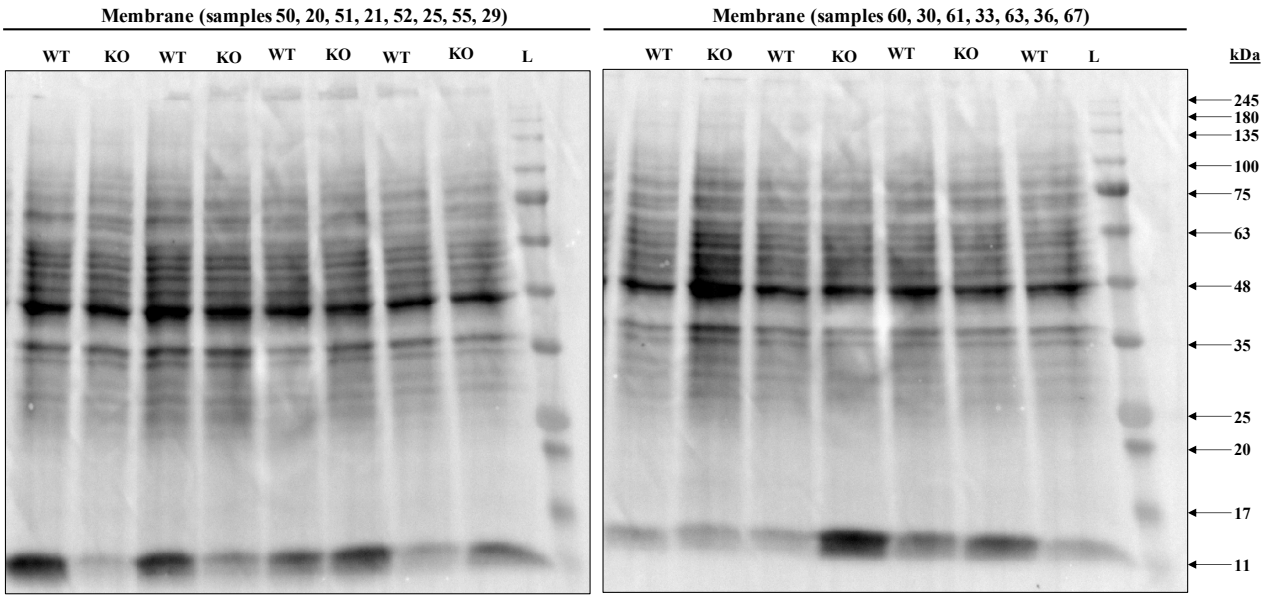

Fig. D.7 Representative total protein (stain-free) images for tumor lysates of WT and SAADKO mice. Gels were cropped to the left of WT (samples 50 and 60) to remove the internal standard. Sample numbers indicated above each membrane correspond to the order of loading. WT, Wild-type; KO, SAA1/2 double-knockout; L, molecular weight marker.

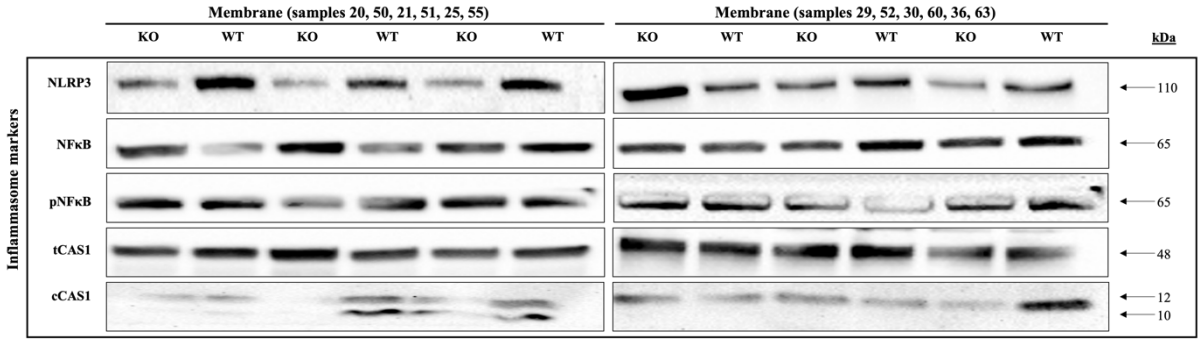

Fig. D.8 Representative western blot images of proteins blotted for WT and SAADKO tumor lysates. Blots were cropped to the left of KO (samples 20 and 29) to remove the internal standard. Sample numbers indicated above each membrane correspond to the order of loading. Representative total protein (stain-free) images are shown in Fig. D.9. WT, Wild-type; KO, SAA1/2 double-knockout; NLRP3, nucleotide-binding oligomerization domain-like receptor with pyrin domain 3; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; p-NFκB, phosphorylated NFκB; cCAS, cleaved caspase; tCAS, total caspase.

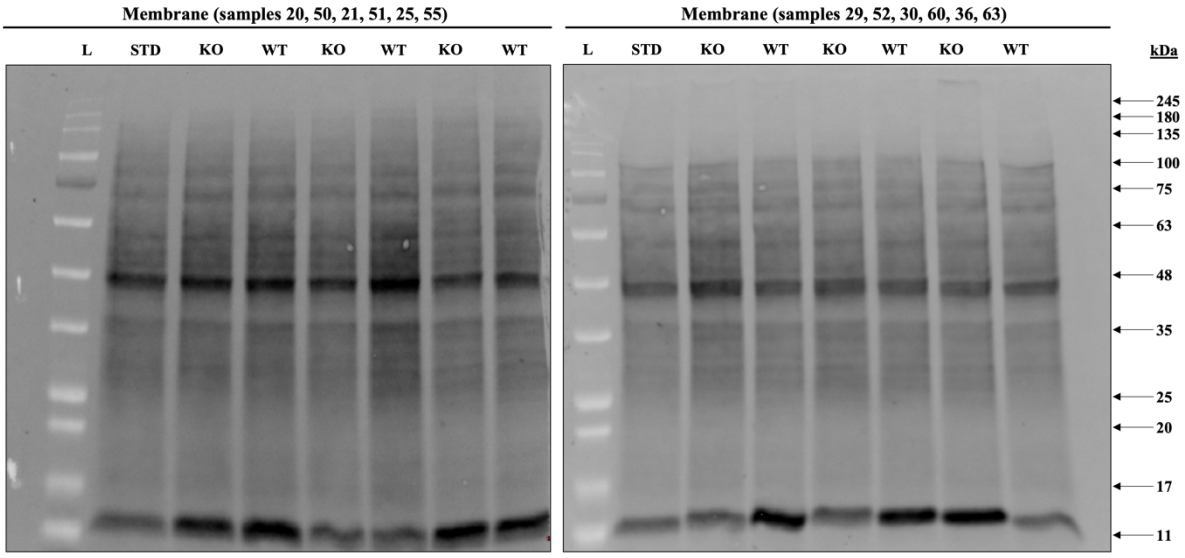

Fig. D.9 Representative total protein (stain-free) images for tumor lysates of WT and SAADKO mice. No cropping of gels to show the molecular weight markers. Sample numbers indicated above each membrane correspond to the order of loading. WT, Wild-type; KO, SAA1/2 double-knockout; L, molecular weight marker; STD, standard.