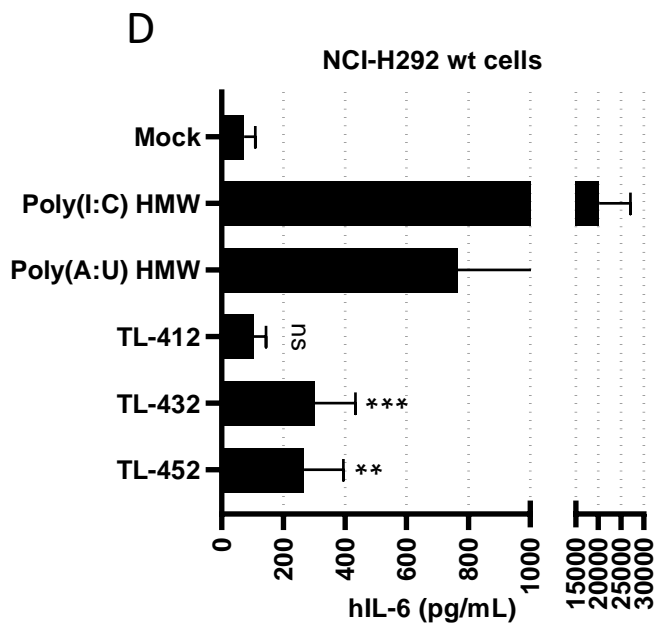
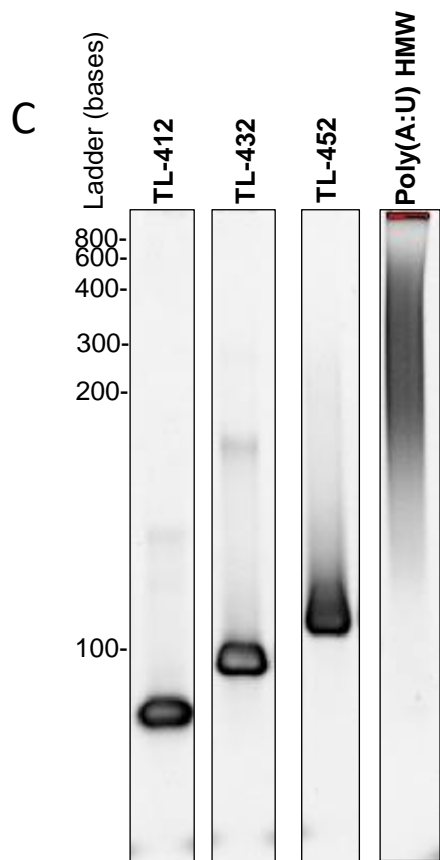
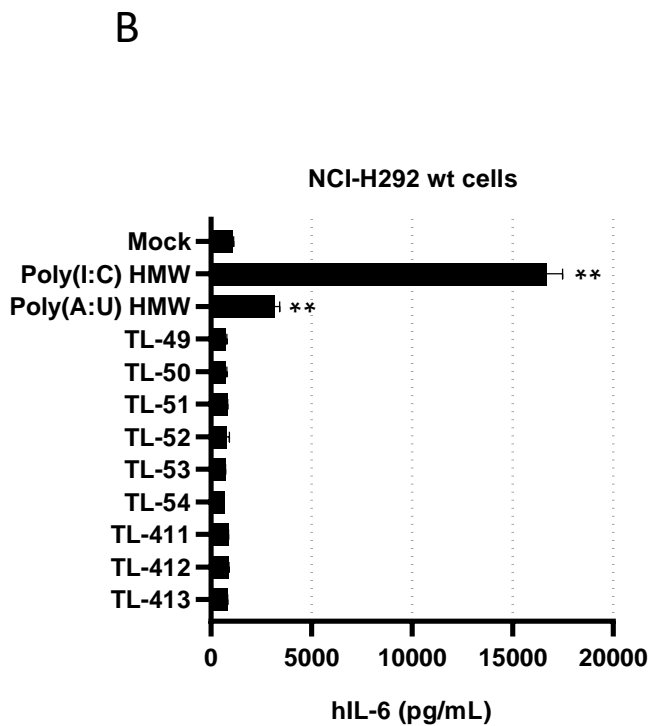
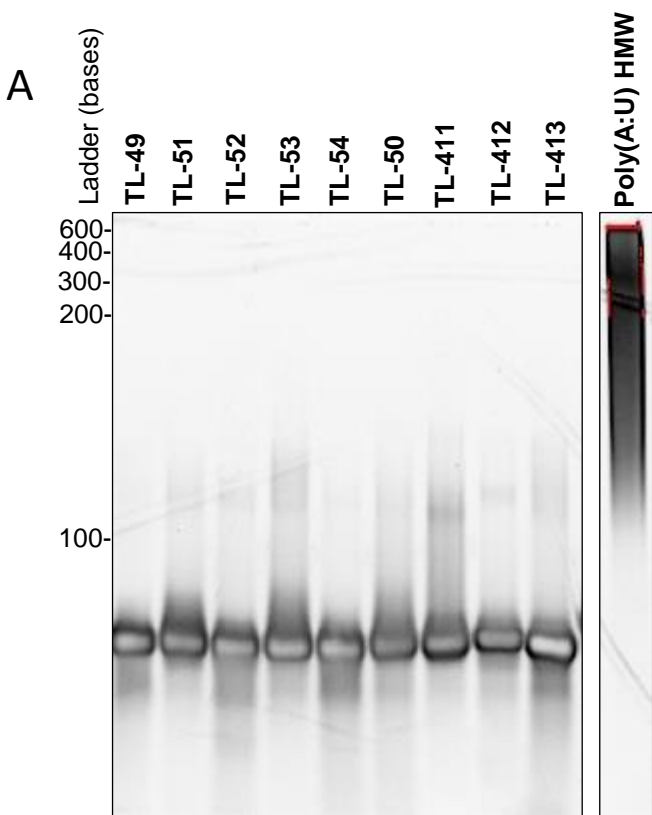
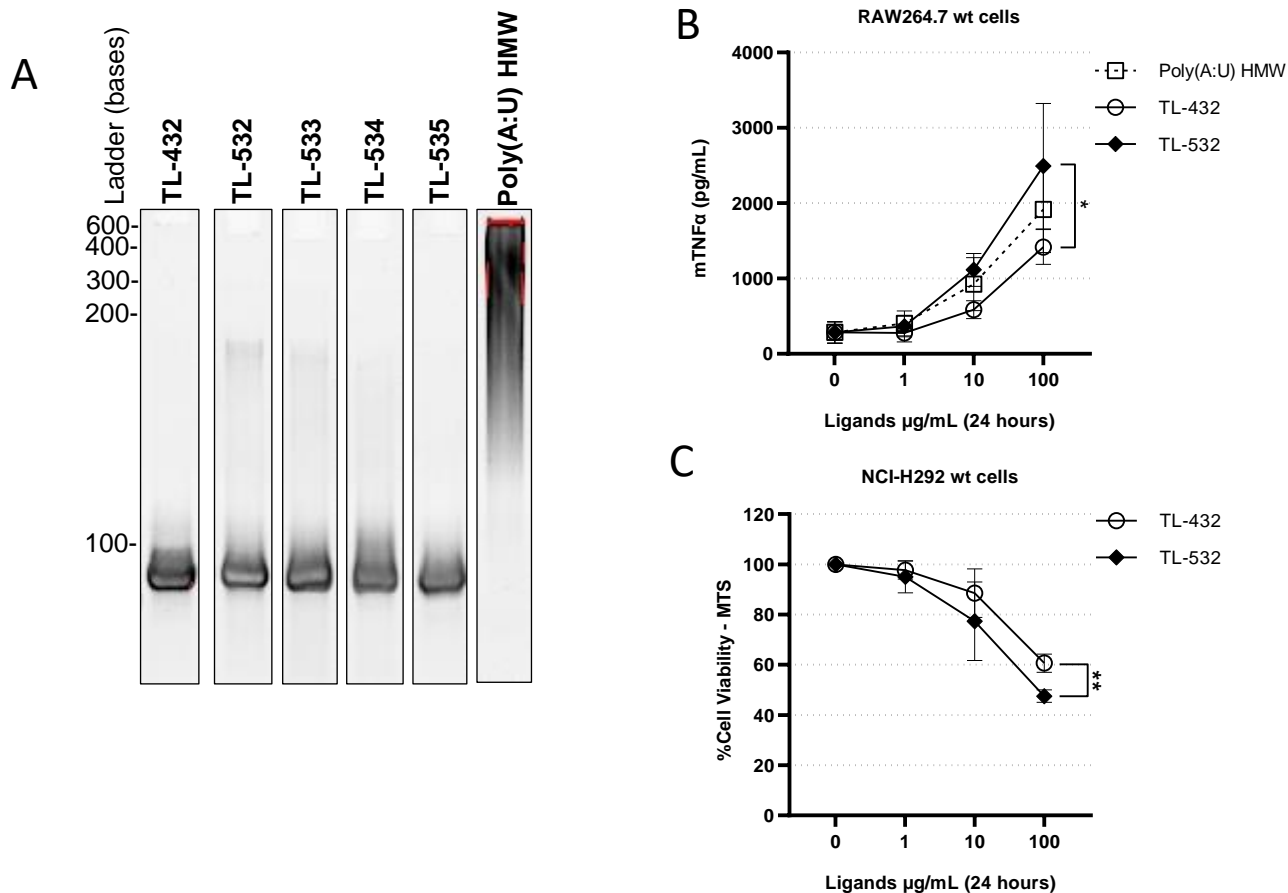


# Supplementary Figure S1



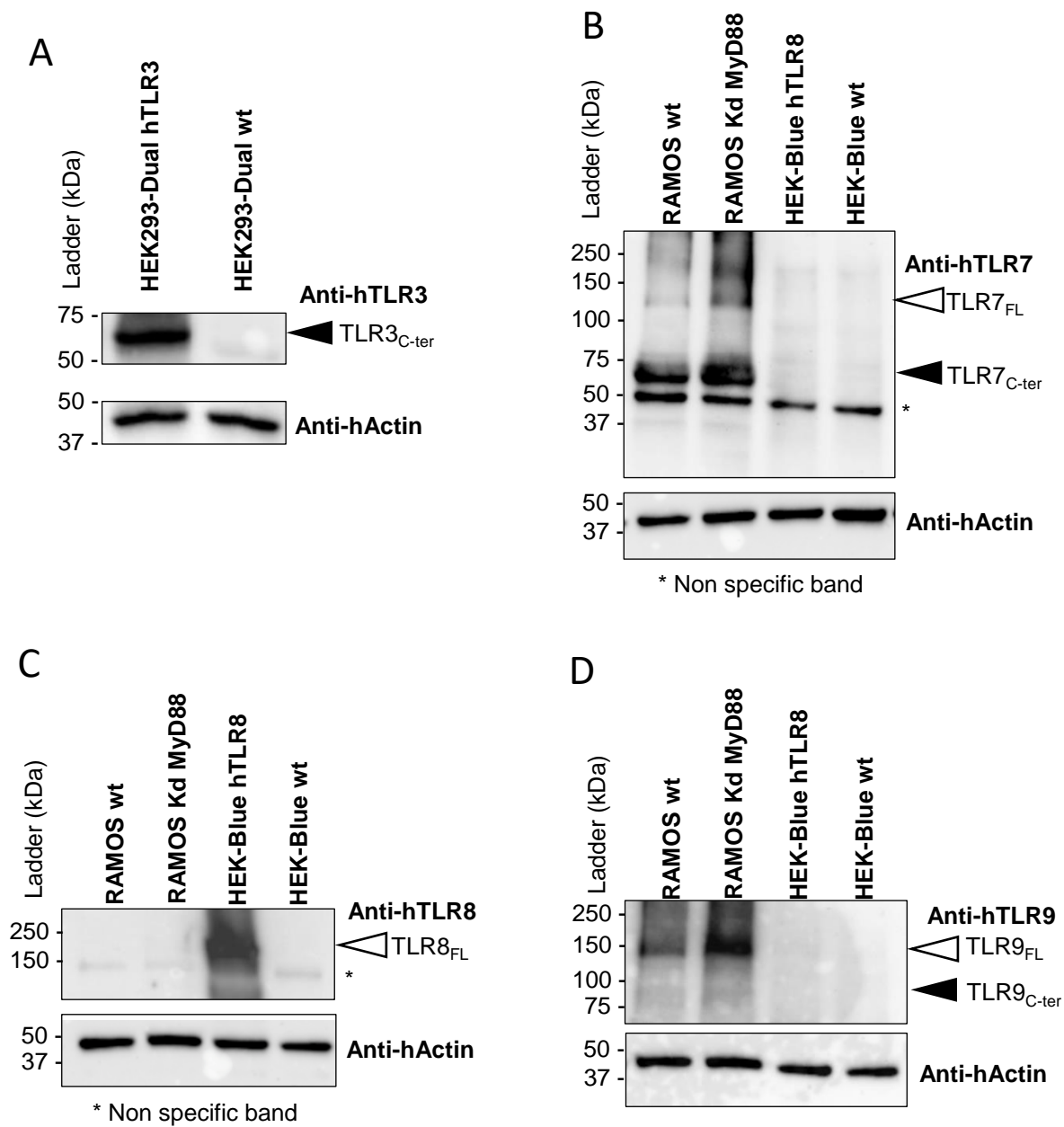
**Supplementary Figure S1: Supporting data of the discovery process.** (A) A panel of 50 bp dsRNAs were evaluated for their native gel profile in an 8% PAGE gel. 5  $\mu$ g of each dsRNAs were deposited before visualization using bromide ethidium. (C) TL-412, TL-432, and TL-452 dsRNAs of respectively 50-70-90bp were evaluated for their native gel profile in an 6% PAGE gel. 1  $\mu$ g of each dsRNA was deposited before visualization using bromide ethidium. (B-D) NCI-H292 wt cells were treated with the different molecules at 10  $\mu$ g/mL for 24 hours. The concentration of hIL-6 was measured by ELISA. Data are representative of at least two (A) or three (B-C) independent assays, or are the mean of three independent assays (D). Results are expressed as mean  $\pm$  SD. Unpaired Student's *t*-test: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns: not significant. Unpaired Student's *t*-test values are compared to Mock condition unless otherwise stated.

## Supplementary Figure S2



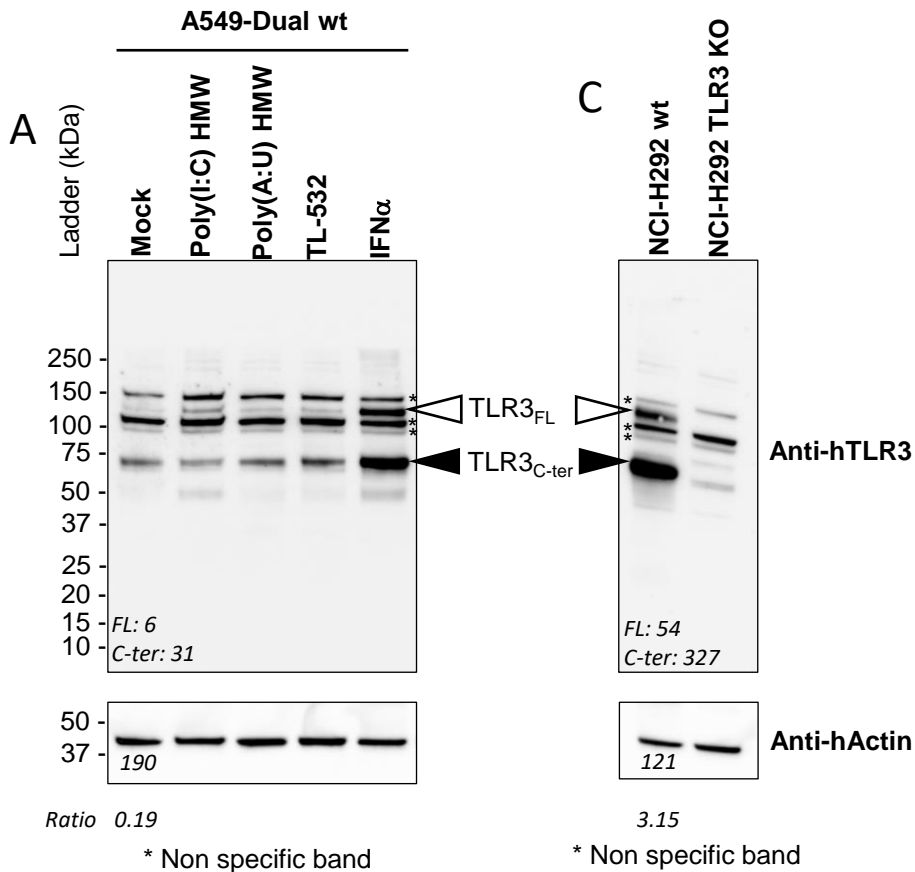
**Supplementary Figure S2: Supporting data of the discovery process.** (A) All 70bp dsRNAs TL-432, TL-532 thru -535 were evaluated for their native gel profile in an 6% PAGE gel. 1.5  $\mu\text{g}$  of each dsRNA was deposited before visualization using bromide ethidium. (B) RAW264.7 wt cells were treated with the different molecules from 1 to 100  $\mu\text{g/mL}$  for 24 hours. The concentration of mTNF $\alpha$  was measured by ELISA. (C) NCI-H292 wt cells were treated with the different molecules from 1 to 100  $\mu\text{g/mL}$  for 24 hours. The cell viability was determined by MTS assay. Data are representative of at least two independent assays (A) or are the means of at least two (B) or three (C) independent assays. Results are expressed as mean  $\pm$  SD. Unpaired Student's *t*-test: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns: not significant.

### Supplementary Figure S3

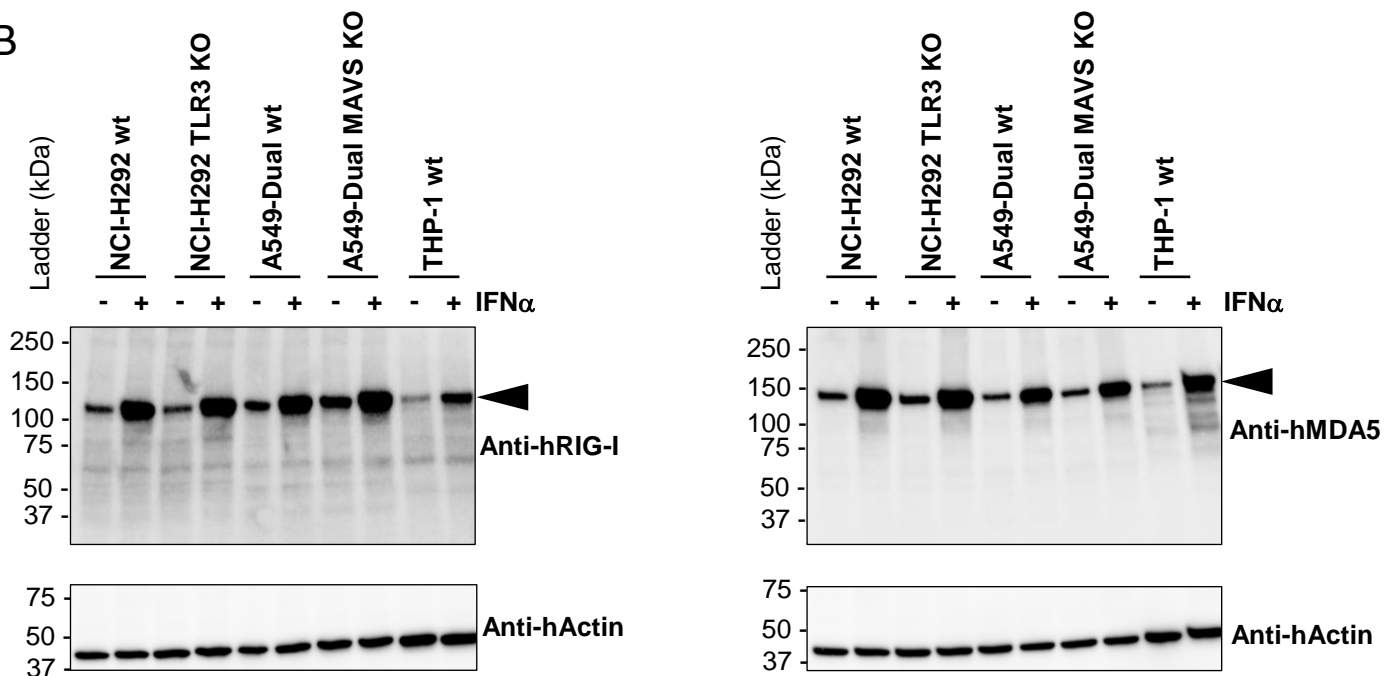


**Supplementary Figure S3: Supporting data of the TLR3 specificity experiments of TL-532.** (A) The TLR3 protein expression was evaluated by Western Blot in HEK293-Dual wt and its re-expressed human-TLR3 counterpart (HEK293-Dual hTLR3). TLR3<sub>C-ter</sub> = 72kDa cleaved C-ter band of TLR3 indicating that the receptor is localized in the endolysosomes. (B) The TLR7 protein expression was evaluated by Western Blot in RAMOS wt and Kd MyD88, compared to the TLR7 negative HEK293-Blue wt cells. TLR7<sub>FL</sub> = TLR7 Full Length; TLR7<sub>C-ter</sub> = 75kDa cleaved C-ter band of TLR7 indicating that the receptor is localized in the acidic compartments (Hipp et al., Immunity, 2013). (C) The TLR8 protein expression was evaluated by Western Blot in HEK293-Blue wt and its re-expressed human-TLR8 counterpart (HEK293-Blue hTLR8). (D) The TLR9 protein expression was evaluated by Western Blot in RAMOS wt and Kd MyD88, compared to the TLR9 negative HEK293-Blue wt cells. TLR9<sub>FL</sub> = TLR9 Full Length; TLR9<sub>C-ter</sub> = 80kDa cleaved C-ter band of TLR9 indicating that the receptor is localized in the endolysosomes (Ewald et al., Nature, 2008). Data are representative of two independent assays.

# Supplementary Figure S4



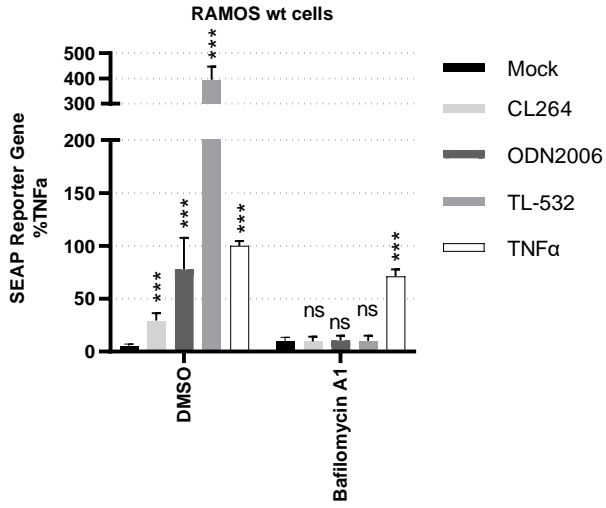
**B**



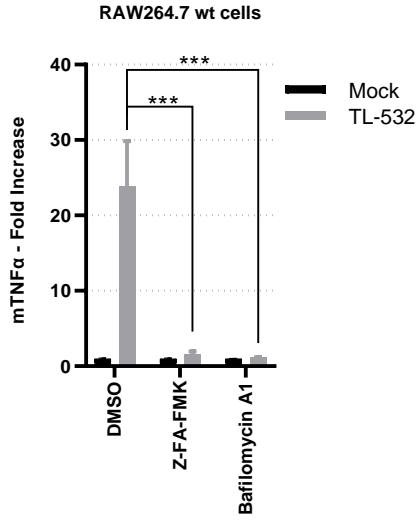
**Supplementary Figure S4: Supporting data of the TLR3 specificity experiments of TL-532.** (A-C) The TLR3 protein expression was evaluated by Western Blot in (A) A549-Dual wt cells at resting state or after 24 hours of treatment with 500  $\mu\text{g}/\text{mL}$  of the indicated dsRNA or 1000 IU/mL of IFN $\alpha$ , (C) NCI-H292 wt (express endogenous hTLR3) and TLR3 KO H292 cells, confirming the absence of TLR3 expression in the KO counterpart. Western Blot legends: TLR3<sub>FL</sub> = TLR3 Full Length; TLR3<sub>C-ter</sub> = 72kDa cleaved C-ter band of TLR3 indicating that the receptor is localized in the endolysosomes. 6 = relative band intensity. Ratio is calculated according to the following formula:  $(\text{RelativeBandIntensity}_{\text{FL}} + \text{RelativeBandIntensity}_{\text{C-ter-TLR3}}) / \text{RelativeBandIntensity}_{\text{Actin}}$ . \* = Non-specific band. (B) The RIG-I and MDA5 protein expression were evaluated by Western Blot in A549-Dual wt and MAVS KO cells at resting state or after 16 hours of treatment with 1000 IU/mL of IFN $\alpha$ . The RIG-I and MDA5 expression levels of A549 were compared to the NCI-H292 and THP-1 wt cells known to express these two cytosolic sensors (Estornes et al., CDD, 2012; Koerner et al., Nat Com, 2021). Data are representative of two independent assays.

# Supplementary Figure S5

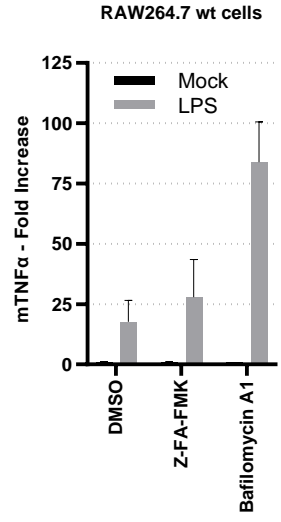
**A**



**B**



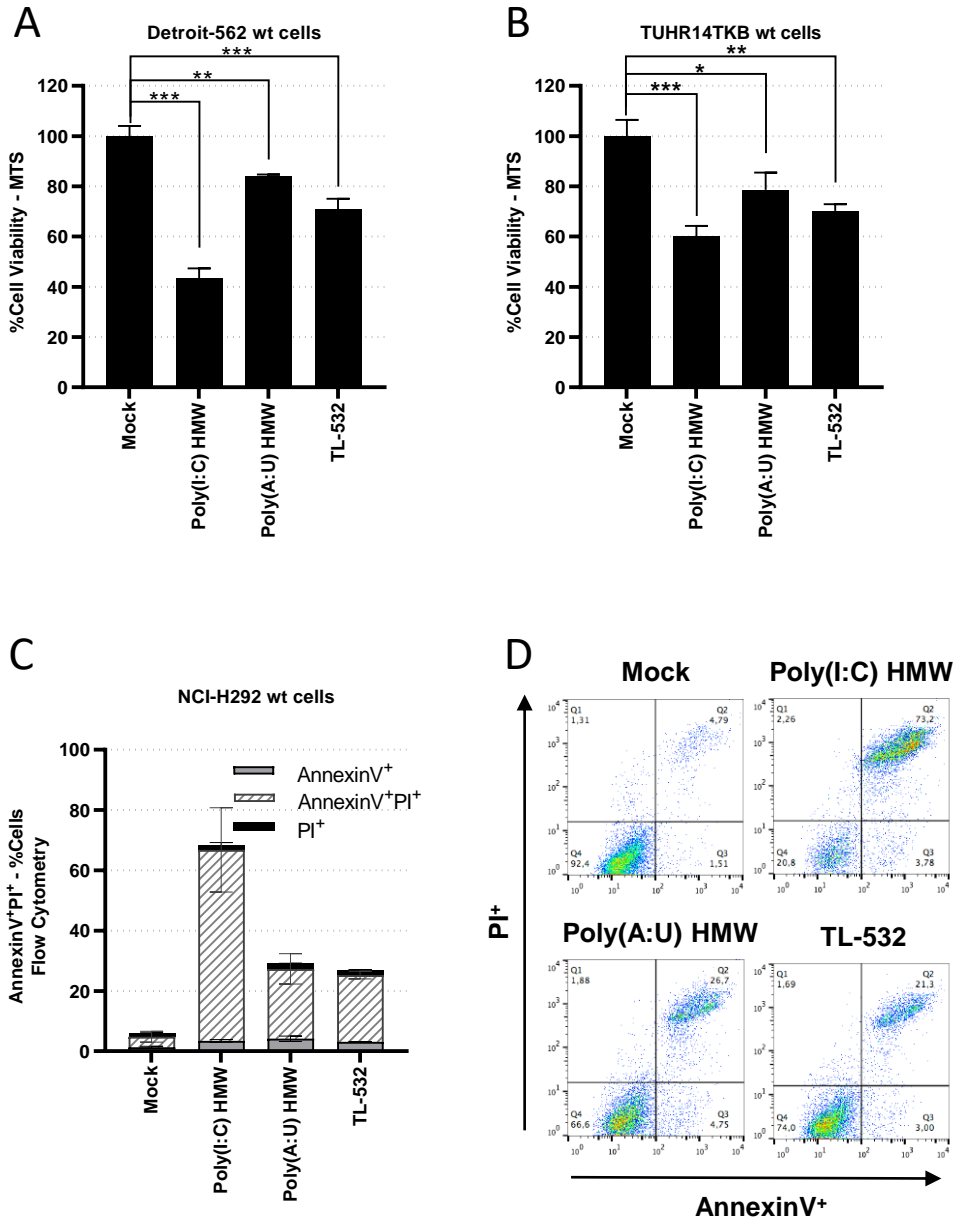
**C**





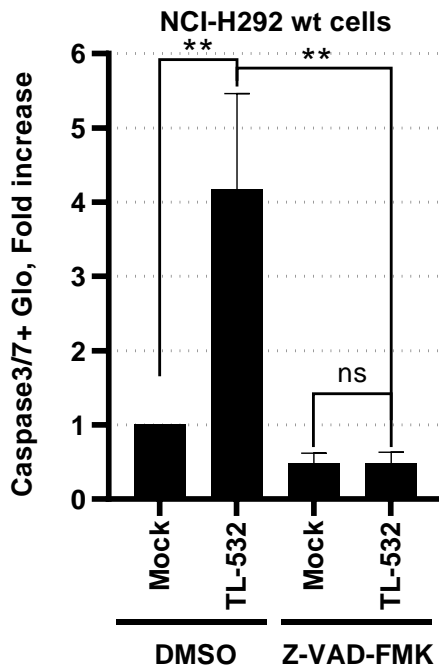
**Supplementary Figure S5: Supporting data of the TLR3 specificity experiments of TL-532.** (A) The endolysosomal transduction signaling pathway specificity of TL-532 was confirmed in RAMOS wt cells by adding the specific vacuolar H<sup>+</sup>-ATPase inhibitor Bafilomycin A1 (100 nM for 45 min) prior to treatment with 10 µg/mL of TLR7-ligand (CL264), 5 µg/mL of TLR9-ligand (ODN2006), 500 µg/mL of TL-532, and 0.1 µg/mL of TNFα for 24 hours. NF-κB activation was measured with SEAP reporter gene assay and expressed as the percentage of SEAP over the relative TNFα activation of the DMSO condition. (B-C) The endolysosomal transduction signaling pathway specificity of TL-532 was evaluated in RAW264.7 wt cells by adding the pan-cathepsin inhibitor Z-FA-FMK (20 µM for 24hr) or the specific vacuolar H<sup>+</sup>-ATPase inhibitor Bafilomycin A1 (50 nM for 45min) prior to treatment with 100 µg/mL of TL-532 (B) or 0.1 µg/mL of LPS (C) for 24 hours. The concentration of mTNFα was measured by ELISA and expressed in fold increase of mTNFα secretion over their respective Mock conditions. Data are the mean of two (A), or three independent assays (B-C). Results are expressed as mean ± SD. Unpaired Student's *t*-test: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns: not significant. Unpaired Student's *t*-test values are compared to Mock condition unless otherwise stated.

# Supplementary Figure S6



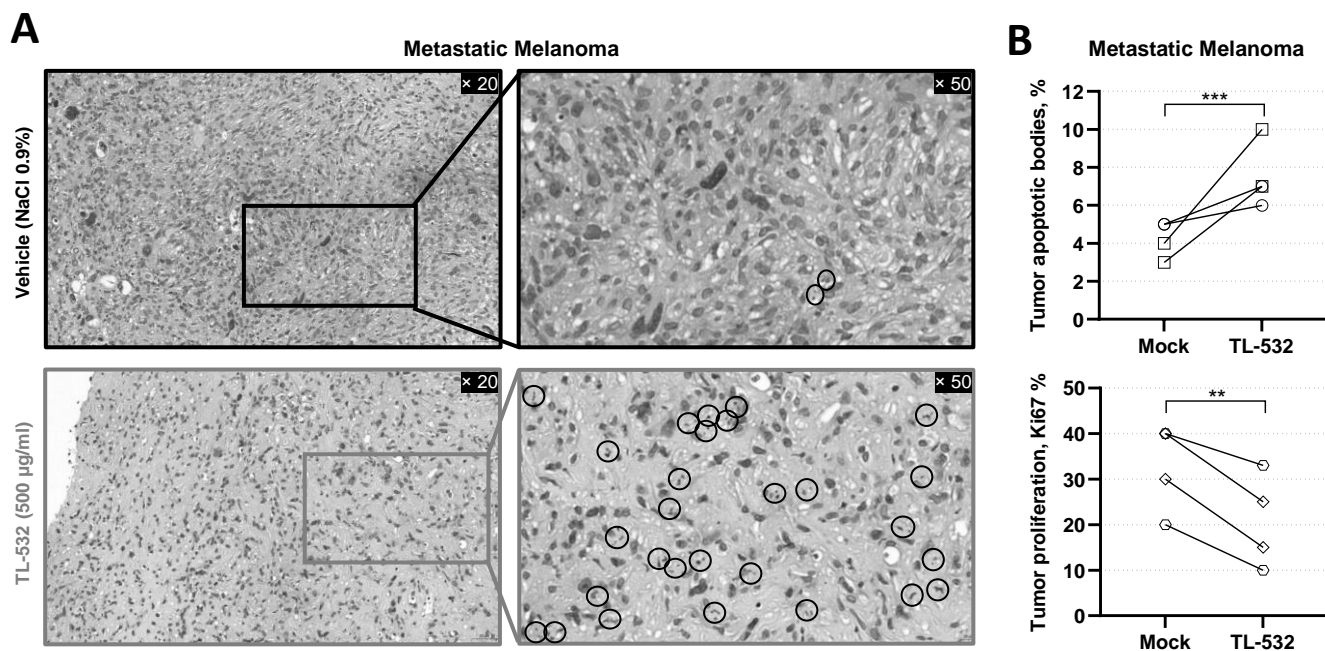
**Supplementary Figure S6: TL-532 reduces cell viability and induces apoptosis in cancer cells.** (A-B) Cell viability – expressed in percentage of untreated cells – was determined by MTS assay in head & neck Detroit-562 wt (A) and renal TUHR14TKB wt (B) cancer cell lines treated with Poly(I:C) HMW at 100  $\mu\text{g}/\text{mL}$ , Poly(A:U) HMW and TL-532 at 500  $\mu\text{g}/\text{mL}$  for 24 hours. (C-D) Apoptosis – expressed in % of positivity (C) or by FACS quadran stat (D) – was determined by AnnexinV<sup>+</sup>PI<sup>+</sup> flow cytometry staining in NCI-H292 wt cells treated with the different molecules at 5.4  $\mu\text{g}/\text{mL}$  for 24 hours. Data are representative of at least two independent assays (D), or are the mean of at least two independent assays (A-C). Results are expressed as mean  $\pm$  SD. Unpaired Student's *t*-test: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns: not significant.

## Supplementary Figure S7



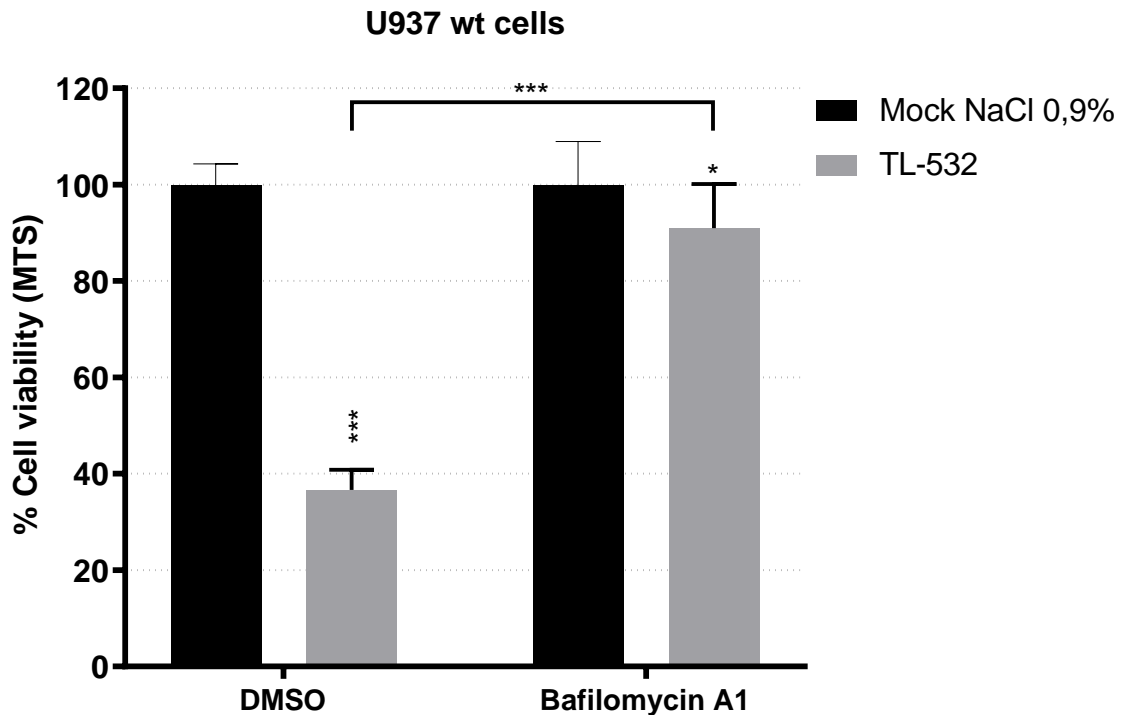
**Supplementary Figure S7: TL-532 induces activation of Caspase3/7 in cancer cells.** The caspase-dependent cell death was determined by adding or not the caspase inhibitor Z-VAD-FMK (20  $\mu$ M for 2 hours) prior to TL-532 treatment at 100  $\mu$ g/mL for 6 hours. Activation was measured with Caspase3/7-Glo assay. Data are the mean of three independent assays. Results are expressed as mean  $\pm$  SD. Unpaired Student's t-test: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , ns: not significant.

## Supplementary Figure S8



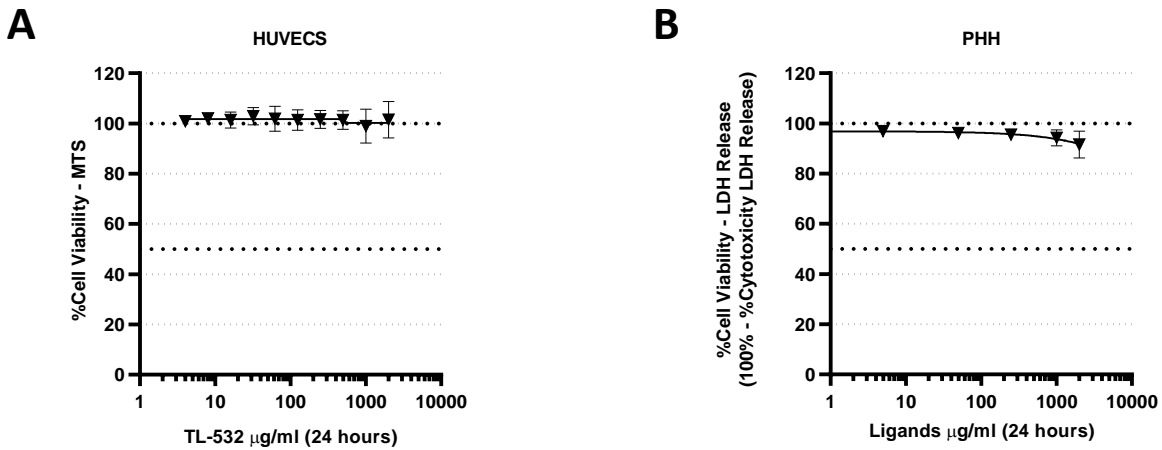
**Supplementary Figure S8 : TL-532 induces cancer cell death in two *ex vivo* samples. (A-B)** To analyze TL-532 activity in a more physiological model, two distinct tumors of vaginal metastasis with bladder invasion from melanoma belonging to the same patient were cultured and treated *ex-vivo* in presence or not with 500 µg/ml of TL-532 for 24 or 48 hours, before anatomopathological analysis. (A) Shows a representative field at 24h post-treatment. Apoptotic bodies are circled in black. (B) Shows the respective percentage of apoptotic bodies (upper panel) and proliferative cells (Ki67 positive cells - lower panel) over the untreated condition. Data are representative of two independent tumors from the same patient at two different time points post treatment as indicated above. Paired Student's t-test: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ , ns: not significant.

## Supplementary Figure S9



**Supplementary Figure S9: TL-532 reduces cell viability in U937 wt in an endolysosomal transduction signaling pathway dependent manner.** The endolysosomal transduction signaling pathway specificity of TL-532 was confirmed in U937 wt cells by adding the specific vacuolar H<sup>+</sup>-ATPase inhibitor Bafilomycin A1 (40 nM for 45 min) prior to treatment with 500 µg/mL of TL-532 for 24 hours. Cell viability was measured by MTS assay. Data are the mean of two independent assays. Results are expressed as mean ± SD. Unpaired Student's *t*-test: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ; ns: not significant. Unpaired Student's *t*-test values are compared to Mock condition unless otherwise stated.

## Supplementary Figure S10



**Supplementary Figure S10: TL-532 does not induce cytotoxicity in *in vitro* primary cell models.** (A-B) Cell viability – expressed in percentage of untreated cells – was determined by MTS assay in Human Umbilical Vein Endothelial Cells HUVECS (A) and by LDH Release in Primary Human Hepatocytes PHH (B) treated with a dose escalation of TL-532 from 4 to 2000  $\mu\text{g/mL}$  in HUVECS or from 5 to 2000  $\mu\text{g/mL}$  in PHH for 24 hours. All data are the means of three independent assays. Results are expressed as mean  $\pm$  95% confidence interval.

### Supplementary Table S1

dsRNA ID#	Sequence 5'→3'		% Inosine
TL-1	Sense	UACIUCUAUUAUUIIACCUAUIUUAUCUICIUUCCAACCUUAIUAUCAC	22
	Antisense	IUIAAUCCUAAIIUUIIACACICAIUAUAACAUIIUCCUAUAIACIUA	
TL-3	Sense	UCIUCIACICAAICIAUUAACUCCUUCACAUCAUAUUCIUUUCUUAU	23
	Antisense	AUAICAAACTAUUAUIAUUIACAI IAIUIUAUUCICUUIUCIACICIA	
TL-4	Sense	AAUIAAIUUUUICAIACAUUIAIUICCIAACAAIACCUIACCUAACIIU	23
	Antisense	ACCIUUAIUACAIUCUUIUUCIICACUCAUIUCUICCAAUACUUCUUAU	
TL-5	Sense	CICUIUUUUCIAAAUUACCCUUUAUICICIIUUAUUIAACCAICUUAUI	23
	Antisense	CAUAAICUIUIUUCAAUACCCICICAUAAAIIUAAUUUCIAAAAACIUI	
TL-6	Sense	IIAAIUIUIUCUAIUCUUAICUUAICUACUAIATIIUCCACIUUUAUI	24
	Antisense	ACUAAACIUIIACCUCUAIUIACIUAACIUAATAUCUAIACCACUUC	
TL-9	Sense	AAIATAIUCUCAUAAUACIUCCIICCCICAUICAIIIUUAUUUIIACA	25
	Antisense	UIUCCAAUUAUACCCUICICAUICICICCIACIUAUUAUIATACUCUUU	
TL-10	Sense	AUAIAAACUACAIIACUAACCUUCCUICACACIIIAIUIUIIAAUCCIU	26
	Antisense	ACIIAUUCCACCUCUCCIUIUUCIACAIIAAIIUUAUIUCCUIUAIUUUCUUAU	
TL-13	Sense	IAIIIAIUCIUCAIACCAIAUAIUCUUIAUUCCUIAUCIIAAIIAUC	26
	Antisense	IAUCCUCCIAUCAI IACUAACAAICUAUCUIIUCUAIACUCCUCCUC	
TL-14	Sense	IIAUACIAUCCIUAIUAUIUAUAAIIACACIIAAUAUCCCCI IACICA	26
	Antisense	UICIUCCIIIAUAUCCIIUUCUUUAUCAUUCUACI IAUUCUIUAUCC	
TL-16	Sense	ACIUUCUAAIAUUIIACIAAAUIUUUCICUACCUAIUAUIIUCICCC	26
	Antisense	IIICUACCUUCCUAIUUCICIAAACAUAUUCUACCAUCUUAIACTIU	
TL-17	Sense	UACIUACAAIIUACACAAICACAIUAIAUCCUICCCICIUUCCUUAUI	26
	Antisense	CAUAIIAAACICIIICAIUAUCUACUIIUCUUIUUCACCUUICUACIUA	
TL-18	Sense	CUAIIUUIUUAUUIIAUUIICCAUUCUCCIIATIUUAUACCUIIACIICCI	26
	Antisense	CIIICUACIIUAUAACACUCIIIAIAUUIICAAUCCAAUCCACAACUAI	
TL-19	Sense	CACIIUCCCAUIAAUAIUACIUCIUAICCUUACCUIACUIUACUUIIAAIU	26
	Antisense	ACUCCAAIUAACIUAUIUAUIUCUACIACUICUAUUAUAI I IACCCUIU	
TL-20	Sense	IACCI IACIACCCACAIACICUIIATAAUAUCUACUICUICUUUACAAI	26
	Antisense	CUUUIUAAAICAIUAIAUAUUCUCCAIICICUCUUIUIUUCIUCCI IUC	
TL-21	Sense	UUUCCACUICCUUAAICCIICUUIICCUUUUCUICCUUUAUAUCCAUUI	26
	Antisense	CCAUIIUAUCUACAIICAIAAAIIICAAICCIICUUAUIICAIUIIIAAA	
TL-22	Sense	ICAACUUCIATIIACCUAUIIACCIACCUAIUAUUCIICAUUIUIICAI	27
	Antisense	CUICCCACAUICCIAUCUAIUICIUACCAUUAUIUCCUCIAAIIUUC	
TL-23	Sense	IAUCUAIICUIIATACCCIIUUAUICUCCAUUACIIUCAUIUIIUCACAI	27
	Antisense	CUIUIACCCACUACCIUAAUIIACUAUAACIIUUCUCACICCAUAIUUC	
TL-24	Sense	ACUICACIUCUUAACIUIUIUCCIUACAIACICCAUCCAIUAUCACI	28
	Antisense	CIUIAUCCUIIAUIICICUUCUACI IACCAACIUUUAIAACIUCIACIU	
TL-25	Sense	ACUIIUCCAACICICAIICUAUIUUCIATIIATAAUUACCCIIIIICAAU	28
	Antisense	AUUICCCCIIIAUAUUCUCCUACUAUICCUICICIUUIICACCAUI	
TL-26	Sense	IACAACCAIUAUCUCIIUUCUICCAACCCUUCUACACICUUAUAUAC	28
	Antisense	ICUUAACAICUIUAIAACIIUUIIIICAAIACCCIIATAUICUIUUIUC	
TL-27	Sense	CAUICUAIUCIUCIIIIUACACUUCUUAACCAUUUIIACACII IACACU	28
	Antisense	AIUIUCCCIUUCCCAAUUIIUAUAICAAIUIUACCCCIACICUAICAUUI	
TL-28	Sense	AUAIACIIACAIUUIIUAUCCUAIACACAIUCIUCIUCIUAUCCUAIUC	28
	Antisense	ICUAIUAUUCI IACICICACUUIUCUAI IUAACCAAIUCUUCUUCUUAU	
TL-29	Sense	UAUCCAAUACUCCACCUUUIICAIIIIIUAUCIACUUIUCCCACTIUAACAU	28
	Antisense	AUIUUUACAIUIIIACAUICUAUCCUCCUICCAACIIUIIUAUUIIIUA	
TL-30	Sense	AUUAACAIAUUAICCUUICUAIACACCCIIICUIIIAICCUAAIUAUC	28
	Antisense	IAUACCUUAIUCUCCAIACCCICIIUUIUCUAIACAIICUAIUCUUIUACU	
TL-31	Sense	UUCAICICICAIICUUIIUCIATUAUAAAUCUCCAIUICCAAIAACCAC	28
	Antisense	IUIIUCUUIIICACUIIATAUUUUUACUUCIACCAAICCUICICICUIAA	
TL-32	Sense	ICAACIIAACIUCUUAICUCCIICAIICAAUUAIIIIAACICAAIACU	28
	Antisense	AUICUUCIUCUCCUUAAUUCUICCIICAIICAAUIIACIUUCCIIUUC	
TL-33	Sense	IUAUCAUUIUCACCUICCIUUAACACUCAACIUAUIUIIIACICCIUU	29
	Antisense	AACIICUCCCAUCUUIUAUIUIUACACCIICAIUIUCACAAUIAUC	



### Supplementary Table S1

TL-34	Sense	IUUACCCAUUUIUCCACAIACACUCIUCIUCUCCIIICUUICCUCUA	29
	Antisense	UAIATIIICAAICCCIIAATCIACIATIUUCCUUIIACCAUUIIIUAAC	
TL-36	Sense	ACICUIUCUCUIICACIUIIUIICCUAIAIAAUCACAUCCAAICCUII	30
	Antisense	CCAIICUUIIAUUIAUUCCUCUAIICCCACCACIUCCTAIAACAICTU	
TL-37	Sense	IUCIUIICAAUIUUCIUCUIIUIUIIUUCACACAUICIIICIIUCIU	30
	Antisense	ACICACCICCCICAUUIUUAITACCACCCATAIACAUIUCCACIAC	
TL-38	Sense	UIICAIACACACCIIUACCCCIUCUCUCAUIAUICCACIICIAAUIUC	31
	Antisense	IACAUCICCTUIICAUCAAUIAIAIICIIIIUCACIIUIUIUCUICCA	
TL-40	Sense	AICCCUUCUCCUUCIICCCACICCCIIUAIAUAUCACICCUUIACCCUC	33
	Antisense	IAIITUCAAIIICUIUAUCUCUACIIICUIIICCCAIIIIIATAAIIICU	
TL-41	Sense	ACICUICAIACUUIACAACIIICAIACUCIICIIICAIUCCUAIUICAI	33
	Antisense	CUICACUAIACCUICCCIIATUCUICCCIUUIICAAIUCUICAICTU	
TL-42	Sense	IICIAAIICCCUAACIIIAUAUCACIICCCACACUCIICICIAUAUCII	33
	Antisense	CCUAUUCIICCCIAUUIUIIICIIICIIUAUCUCCCIUUAIIICCUUICCC	
TL-44	Sense	ICACCAIUCUIUAATIUCCICCCACIACIACIACIICCIICIIIAIACCAC	34
	Antisense	IUIIUCUCCICCCIICCUCIUCUCIUIIICIIACCUUAACAIAUCUIIUC	
TL-45	Sense	UCCUIIAIIAIIICIIHAUAICCCUUAACCCUIICCCACCTIUIIICIIU	34
	Antisense	ACCICCAACIIUIIICACIIIUAAIAIICUAUCCICCCUCCUCAIIA	
TL-47	Sense	UICICCIUCCCCAICCCICUCUAUCUCIICACCICCAUAACCAIACCI	36
	Antisense	CIUCUIIUUAUIICIIUUCIIAICAUAIACIICIIICUIIICACCIICICA	
TL-48	Sense	UAICCICCCUUIICCCIIUCCICUACCUUICAIIAUAUCIACCIUCC	36
	Antisense	IACIICCUICUAUCCUICAAIUAACIACCIICCCAIIIICIIICUA	
TL-49	Sense	ICUCUUCIICICCCIIICACCCUICCIICIIICIIIAUCICCCI	43
	Antisense	CIIICIAUCCICCCCIICAIIIUIICICCCIIICICICCAIACIAC	
TL-50	Sense	IAUAUIIICIIICCCIIICIIICICCCIIICCCCIICIIICIIICIIU	46
	Antisense	AACICCCICCCIIICIIICIIICCCIIICCCIIICCCICCCUUAUCC	
TL-51	Sense	IUCIICCCIIICCCCIICCCCAIICIIICUCUCCIIICCCIIICCCIC	47
	Antisense	IICIIICCCIIICIIIIAICCCICUICIIICCCIIIIICCCIIICCCIAC	
TL-52	Sense	IIIIICCCACIICIIICIIICCCIIICIIICCCCIICIIICCCCIICUCI	48
	Antisense	CIACIICIIICICCCIIIIICICCIICIIICIIICCCIIICIIICCCCIIC	
TL-53	Sense	CIIICCIICIIIIICIIICIIIIICCCUUIICCCICCCIIICCCCIIC	49
	Antisense	CICIIICICCIICIIICIIICCAIIICCCCIICCCIIICCCCIICCIICCI	
TL-54	Sense	IIICCIICIIICCCIIICIIICIIICCCIIICCCIIICIIICIIICIIIC	50
	Antisense	IICICCCCIICCIICIIICIIICIIICCCIIICCCIIICIIICCCIIICCC	
TL-411	Sense	II	50
	Antisense	CC	
TL-412	Sense	AA	0
	Antisense	UU	
TL-413*	Sense	AUUUAAAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAU	0
	Antisense	AUUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAUAAU	

**Supplementary Table S1: Sequence list of the 47 tested 50bp dsRNAs.** dsRNAs TL-1 to -48 were manufactured by IDT. dsRNAs TL-49 to -54, and -411 to -413 were manufactured by Horizon Discovery. All dsRNAs have a phosphate backbone, bear 5'-OH and 3'-OH ends at the two strands, and do not have other modifications. All of the tested dsRNAs were chemically manufactured on solid-phase support and purified by chromatography. \*: 50% adenine and 50% uracil content, the same %(A:U) as TL-412.

