SUPPLEMENTAL MATERIAL

IL-6R/STAT3/miR-34a feedback controls EMT, invasion and metastasis of colorectal cancer

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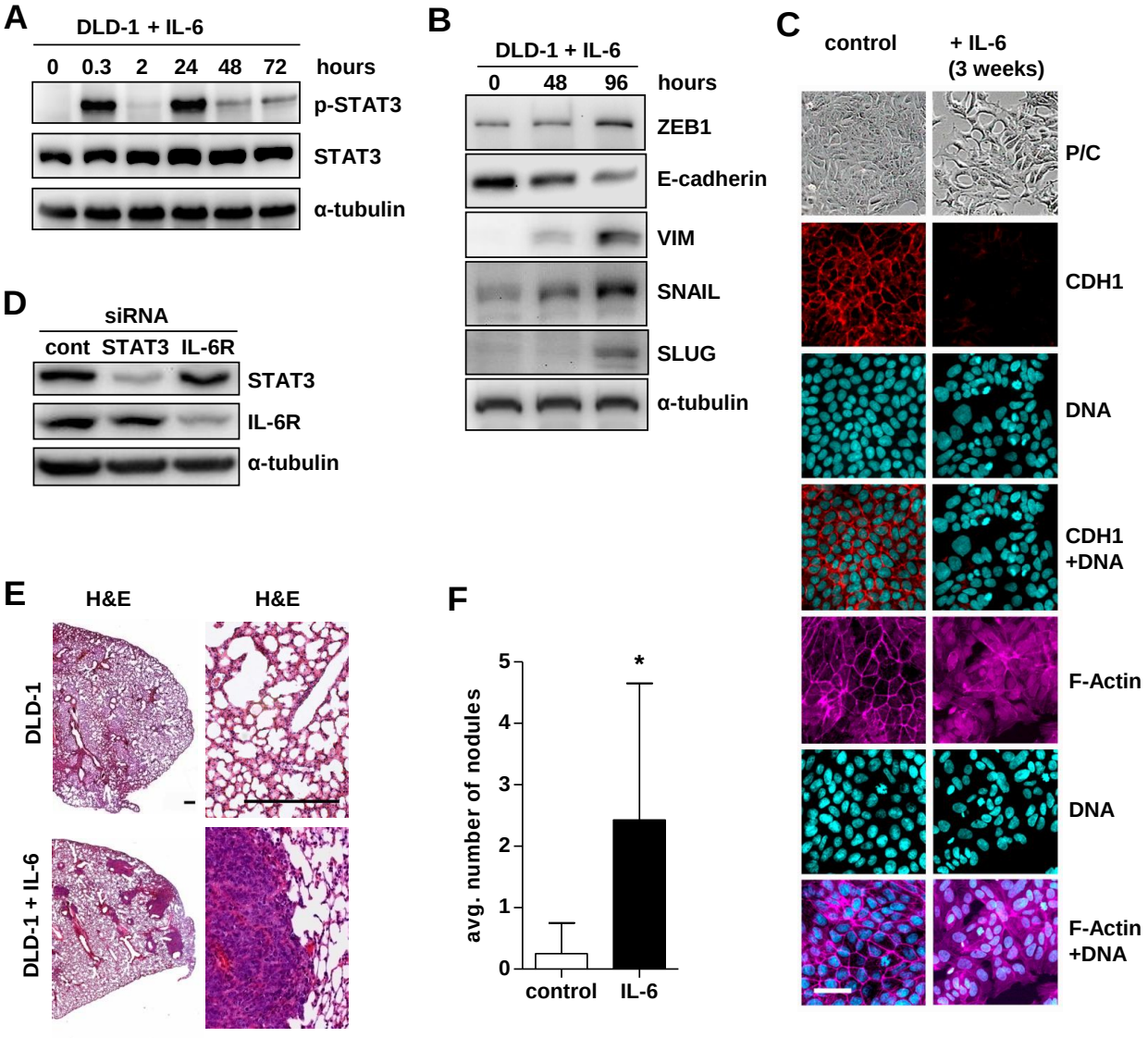
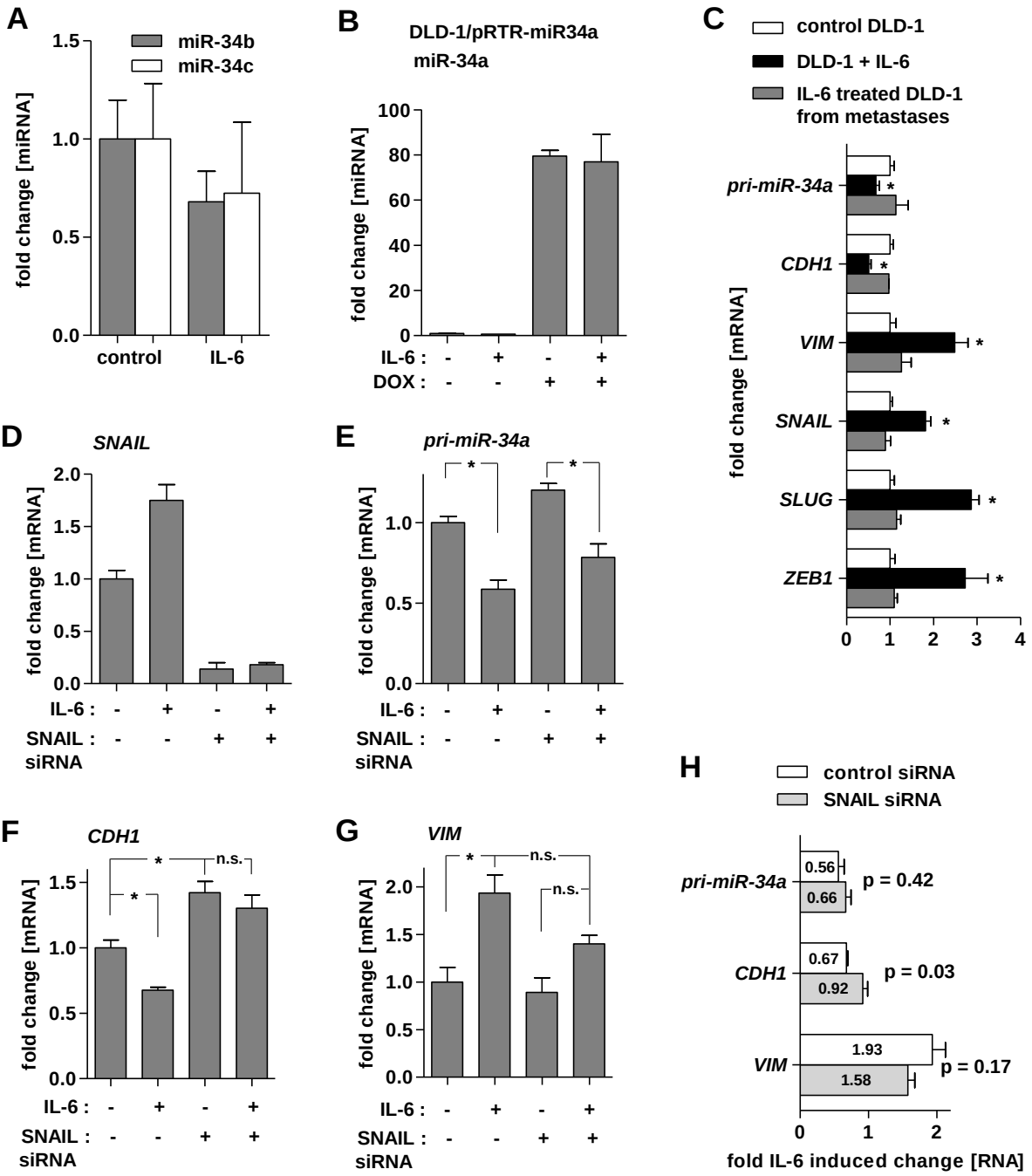
Inventory of Supplemental Information

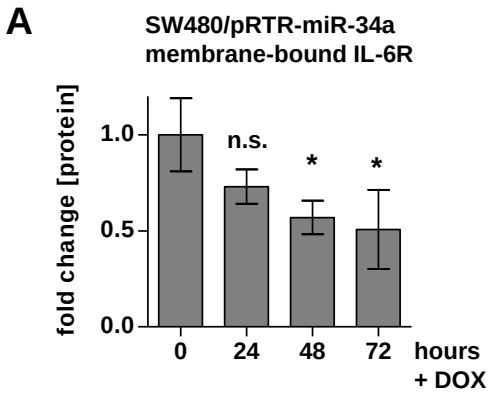


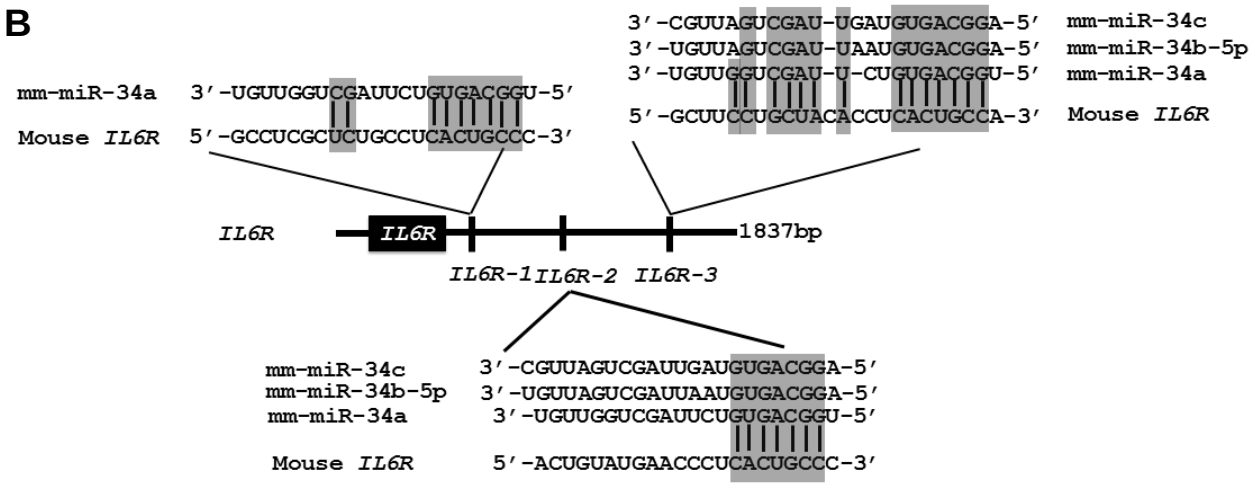
Supplemental Figure 1. IL-6 induces EMT, invasion, and metastasis of DLD-1 cells.

(A) Western blot analysis of p-STAT3-Y705 phosphorylation and STAT3 expression in DLD-1 cells after IL-6 treatment for the indicated periods. **(B)** Western blot analysis of indicated proteins in DLD-1 cells treated with IL-6 for indicated periods. **(C)** Indirect immunofluorescence detections of indicated proteins in DLD-1 cells treated with IL-6 as indicated. Pictures were taken with phase contrast (P/C) and confocal microscopy. Scale bar in the upper right panel represents 25 µm. **(D)** Western blot analysis of the indicated proteins in DLD-1 cells transfected with STAT3- or IL-6R-specific siRNAs. **(E, F)** Formation of lung metastases by DLD-1 cells in immune-compromised NOD/SCID mice. H&E staining of lungs **(E)** and quantification of metastatic tumor nodules in the lung per mouse ten weeks after tail vein injection are shown **(F)**. Scale bars represent 200 µm. In panel **F** mean values ± SD (n=3) are provided. (*) $P < 0.05$



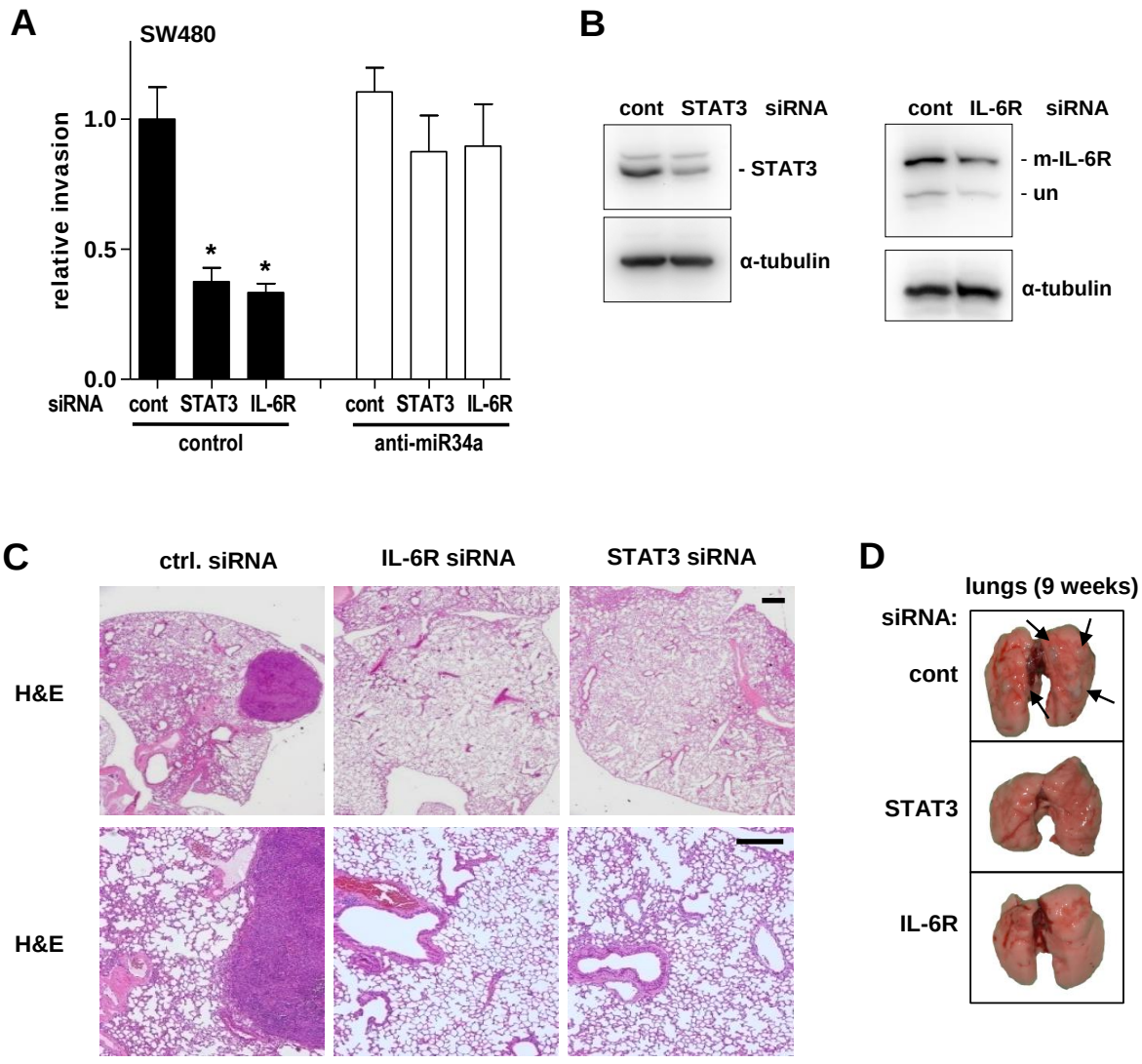
Supplemental Figure 2. IL-6-induced EMT and invasion of colorectal cancer cells are mediated by direct repression of miR-34a by STAT3. (A) qPCR detection of mature miR-34b and miR-34c expression in DLD-1 cells after treatment with IL-6 for 72 hours. **(B)** miR-34a expression in DLD-1 cells stably harboring an episomal DOX-inducible pRTR/miR-34a expression plasmid. Cells were treated with IL-6 and/or DOX for 48 hours. **(C)** qPCR analyses of the indicated mRNAs in parental (control) DLD-1 cells, DLD-1 cells treated with IL-6 for 5 days, and cells explanted from lung metastases that formed from IL-6 treated DLD-1 cells. **(D, E, F, G, H)** qPCR analysis of *SNAIL* **(D)**, *pri-miR-34a* **(E)**, *CDH1* **(F)**, and *VIM* **(G)** expression in DLD-1 cells transfected with control or SNAIL siRNAs for 24 hours and subsequently treated with IL-6 for 72 hours. **(H)** Fold IL-6 induced changes in expression of indicated genes with control or *SNAIL*–specific siRNA. In panels **A - H** mean values ± SD (n=3) are provided. (*) $P < 0.05$





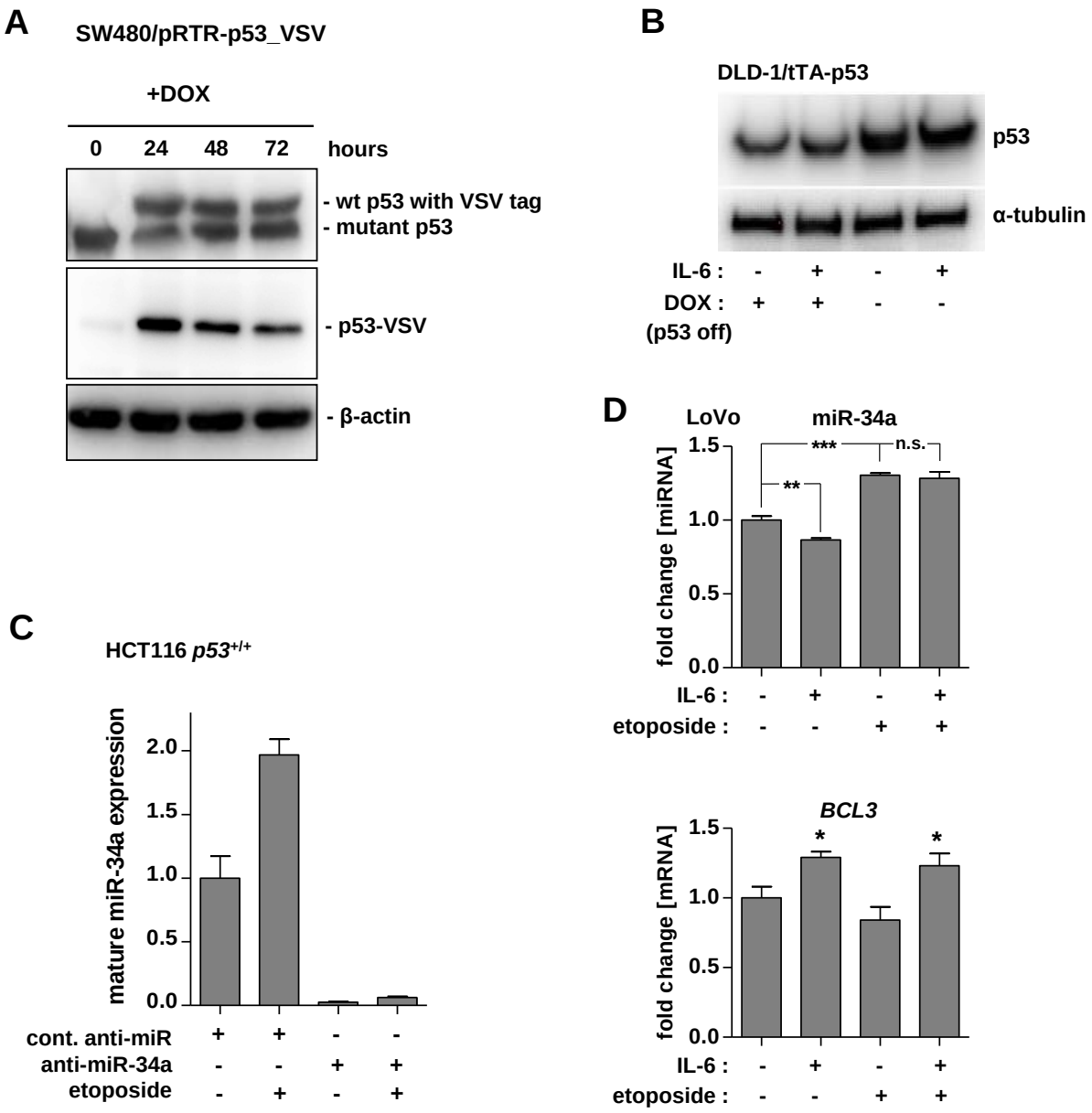
Supplemental Figure 3. *IL6R* is a direct target of miR-34.

(A) Densitometric quantification of membrane-bound IL-6R protein expression with normalization to β-actin in SW480/pRTR-miR-34a cells after treatment with DOX (see also Figure 2C). **(B)** Schematic representation of the mouse *IL6R* 3'-UTR with miR-34 seed-matching sequences. In **A** mean values ± SD (n=3) are provided. (*) $P < 0.05$



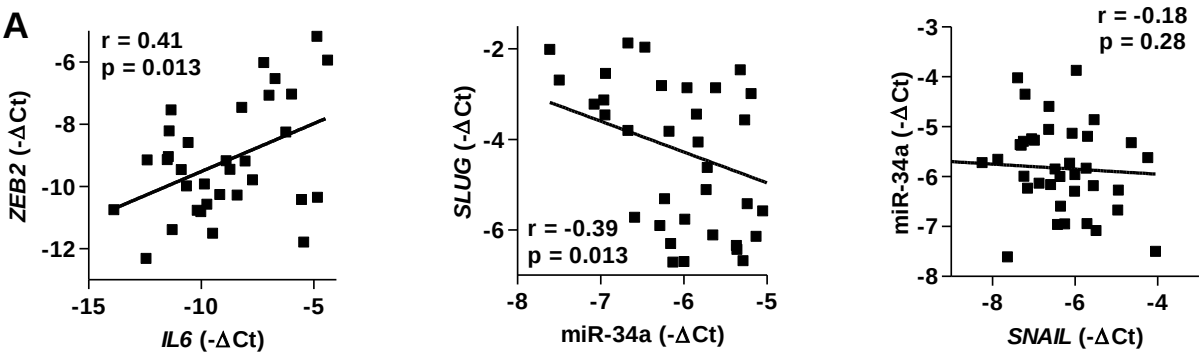
Supplemental Figure 4. The mesenchymal phenotype of cancer cell lines is associated with an active IL-6R/STAT3/miR-34a loop.

(A) Knockdown of STAT3 or IL-6R suppresses invasion in a miR-34a-dependent manner. SW480 cells were transfected with STAT3 or IL-6R siRNA and miR-34a antagomirs. Subsequently, cells were allowed to migrate through a matrigel-coated filter for 48 hours and counted using DAPI staining. **(B)** SW620-luc2 cells were transfected with control, STAT3-, or IL-6R-specific siRNAs for 48 hours. Expression of the indicated proteins was determined by Western blot analysis. **(C, D)** Representative examples of H&E stained lungs **(C)** and images of lungs **(D)** after tail vein injection of SW620 cells transfected with the respective siRNAs. Scale bars represent 200 µm. In panel **A** mean values ± SD (n=3) are provided. (*) $P < 0.05$



Supplemental Figure 5. p53 disrupts the IL-6R/STAT3/miR-34a feed-back loop by inducing miR-34a. **(A)** Western blot analysis of p53 and VSV protein expression in SW480/pRTR-p53_VSV cells after treatment with DOX for indicated periods. **(B)** Western blot analysis of p53 expression after removal of DOX and IL-6 for 72 hours (please note that in DLD-1_tTA_p53 cells the expression of ectopic wild-type p53 is induced after the removal of DOX). **(C)** Expression of mature miR-34a in HCT116 *p53*+/+ cells transfected with control or anti-miR-34a oligos followed by treatment with etoposide for 48 hours. **(D)** Expression of mature miR-34a (upper) and the IL-6/STAT3 target *BCL3* (lower) in LoVo cells treated with etoposide and IL-6 for 72h. In panels **C** and **D** mean values ± SD (n=3) are provided. (*) $P < 0.05$; (**) $P < 0.01$ and (***) $P < 0.001$.

A



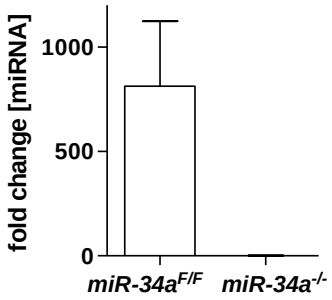
B

	miR34a	IL6	IL6R	SNAIL	SLUG	ZEB1	ZEB2	VIM	CDH1
miR34a	1.00								
IL6	-0.35	1.00							
IL6R	-0.59	0.33	1.00						
SNAIL	-0.18	0.29	0.48	1.00					
SLUG	-0.39	0.07	0.39	-0.05	1.00				
ZEB1	0.11	0.42	0.52	0.46	-0.30	1.00			
ZEB2	0.02	0.41	0.45	0.60	-0.05	0.87	1.00		
VIM	-0.11	0.39	0.58	0.70	0.16	0.44	0.67	1.00	
CDH1	0.27	0.09	-0.14	0.37	-0.63	0.30	0.21	-0.01	1.00

Approximately r > 0.3 or < -0.3 is significant

Supplemental Figure 6. **Evidence for the presence of the IL-6R/STAT3/miR-34a feed-back loop in primary CRC tumors.**

(A, B) Correlations of the indicated mRNAs and miRNAs in the TUM human colon tumor collection (n = 48). The significance was calculated using the Spearman correlation coefficient.



Supplemental Figure 7. Loss of miR-34a facilitates the formation of invasive colorectal tumors in the AOM/DSS mouse model. Expression of miR-34a in intestine epithelia of indicated mice.

Supplemental Table 1. **Oligonucleotides used for qPCR**

gene	forward	reverse
GAPDH	TGTTGCCATCAATGACCCCTT	CTCCACGACGTACTCAGCG
BCL3	CCCTATACCCCATGATGTGC	TACCCTGCACCACAGCAATA
CDH1	CCCGGGACAACGTTTATTAC	GCTGGCTCAAGTCAAAGTCC
IL6R	TTGTTTGTGAGTGGGGTCCT	TGGGACTCCTGGGAATACTG
JAK2	CCTTGTACTTCACGATGTTGTC	GTGGAGATGTGCCGCTATG
pri-miR-34a	CGTCACCTCTTAGGCTTGGA	CATTGGTGTCGTTGTGCT
SLUG	GGGGAGAAGCCTTTTTCTTG	TCCTCATGTTTGTGCAGGAG
SOCS3	GACTTCGATTCGGGACCA	GGAAACTTGCTGTGGGTGAC
STAT3	GGGAAGAATCACGCCTTCTAC	ATCTGCTGCTTCTCCGTCAC
SNAIL	GCACATCCGAAGCCACAC	GGAGAAGGTCCGAGCACAC
VIM	TACAGGAAGCTGCTGGAAGG	ACCAGAGGGAGTGAATCCAG
ZEB1	TCAAAAGGAAGTCAATGGACAA	GTGCAGGAGGGACCTCTTTA

Supplemental Table 2. Oligonucleotides used for qChIP

gene	forward	reverse
AchR	CCTTCATTGGGATCACCACG	AGGAGATGAGTACCAGCAGGTTG
miR-34a	GGAATCCTTTCTCCCCAGAG	GTAGCCTCCGTAAGGGGAAG

Supplemental Table 3. Oligonucleotides used for *IL6R*-3'UTR cloning and mutagenesis

gene	forward	reverse
human *IL6R* 3'-UTR	GTTTTCCACTGTGGGCTTGT	TACTGACCCTTTGCCCCATA
human *IL6R* 3'-UTR site1 mutant	CTCAGCAAAAGATGCTTCTCAGTCGGATGCCAGCTTATCTCAGGGG	CCCCTGAGATAAGCTGGCATCCGACTGAGAAGCATCTTTTGCTGAG
human *IL6R* 3'-UTR site2 mutant	GTTTCTGCAGCACCCCCAGTCGGTTGAGTCCCCAGCAGTG	CACTGCTGGGGACTCAACCGACTGGGGGTGCTGCAGAAAC
mouse *IL6R* 3'-UTR	CCACGAGATCAGCACACAAG	CTAGAGCGGACAAGCAGAGG

Supplemental Table 4. Oligonucleotides used for genotyping of *miR-34a*$^{-/-}$ mice

oligo	sequence
a	ACCTTGCAGGTGCTCAGAAT
b	TGGAGCTAACGGAGTGTGTG
c	CTACCCAAGCTCGACGAAGT
d	TGCAGCACTTCTAGGGCAGT

Supplemental Table 5. List of antibodies

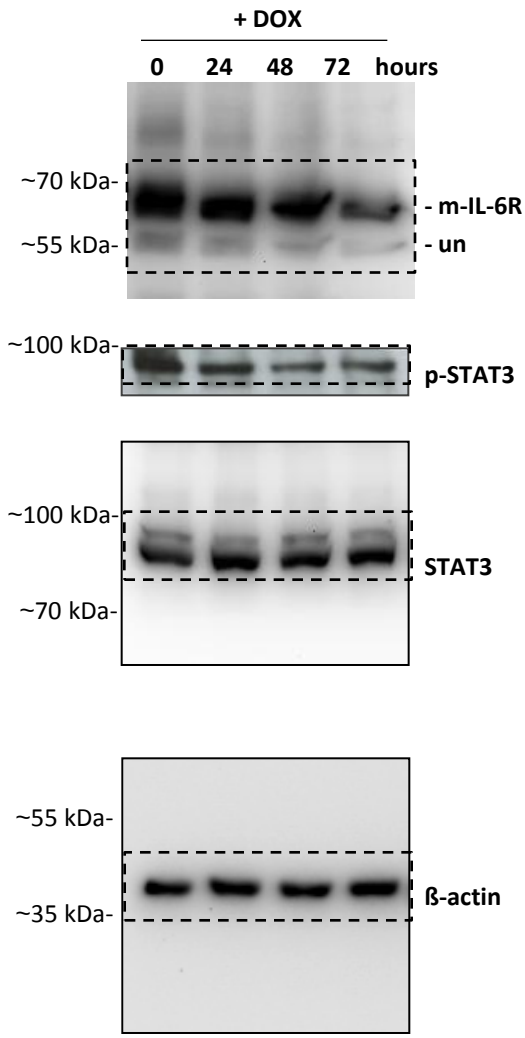
Primary antibodies

epitope	species	catalog no.	company	use	dilution	source
Vimentin	human	#2707-1	Epitomics	WB	1:5000	rabbit
E-cadherin	human	#334000	Invitrogen	WB; IF	1:1000; 1:50	mouse
p53	human	#sc-126	Santa Cruz	WB	1:1000	mouse
α-tubulin	human mouse	#T-9026	Sigma	WB	1:1000	mouse
ZEB1	human mouse	#sc-25388	Santa Cruz	WB	1:1000	rabbit
SNAIL	human	#3879S	Cell Signaling	WB	1:500	rabbit
SNAIL	mouse	#NBP1-19529	Novus Biosciences	WB	1:500	rabbit
SNAIL	mouse	AP20370PU-N	Acris	IHC	1:150	rabbit
IL-6R	human mouse	#sc-661	Santa Cruz	WB; IHC	1:1000; 1: 500	rabbit
STAT3	human	#sc-482	Santa Cruz	WB	1:1000	rabbit
p-STAT3	human mouse	#9145 XP	Cell Signaling	WB; IHC	1:1000; 1: 300	rabbit
cleaved Caspase-3	mouse	#96618	Cell Signaling	IHC	1: 300	rabbit
BrdU		#MCA2060	AbD Serotec	IHC	1: 400	rat
β-actin	human	#A2066	Sigma	WB	1:1000	rabbit

Secondary antibodies or conjugates

name	ordering no.	company	use	dilution	source
anti-mouse HRP	# W4021	Promega	WB	1:10.000	goat
anti-rabbit HRP	# A0545	Sigma	WB	1:10.000	goat
anti-rat-biotin	# E0468	Dako	IHC	1 : 500	rabbit
anti-rabbit-biotin	# E0432	Dako	IHC	1 : 600	goat
Alexa Flour 555-conjugated anti-mouse	# A21422	Invitrogen	IF	1:500	goat
Phalloidin conjugated Alexa-647	# A22287	Invitrogen	IF	1:40	

Full unedited gel for Figure 2C



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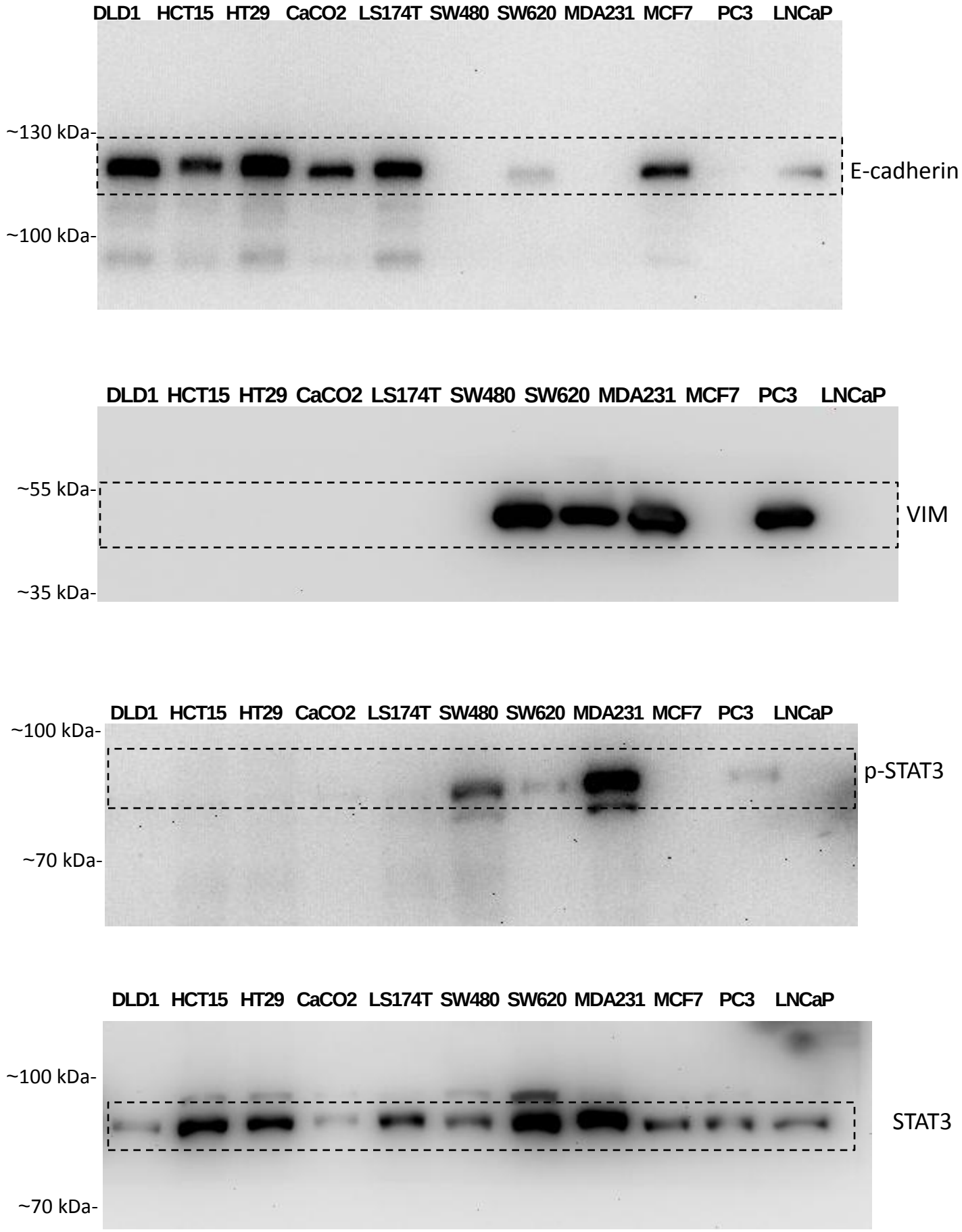

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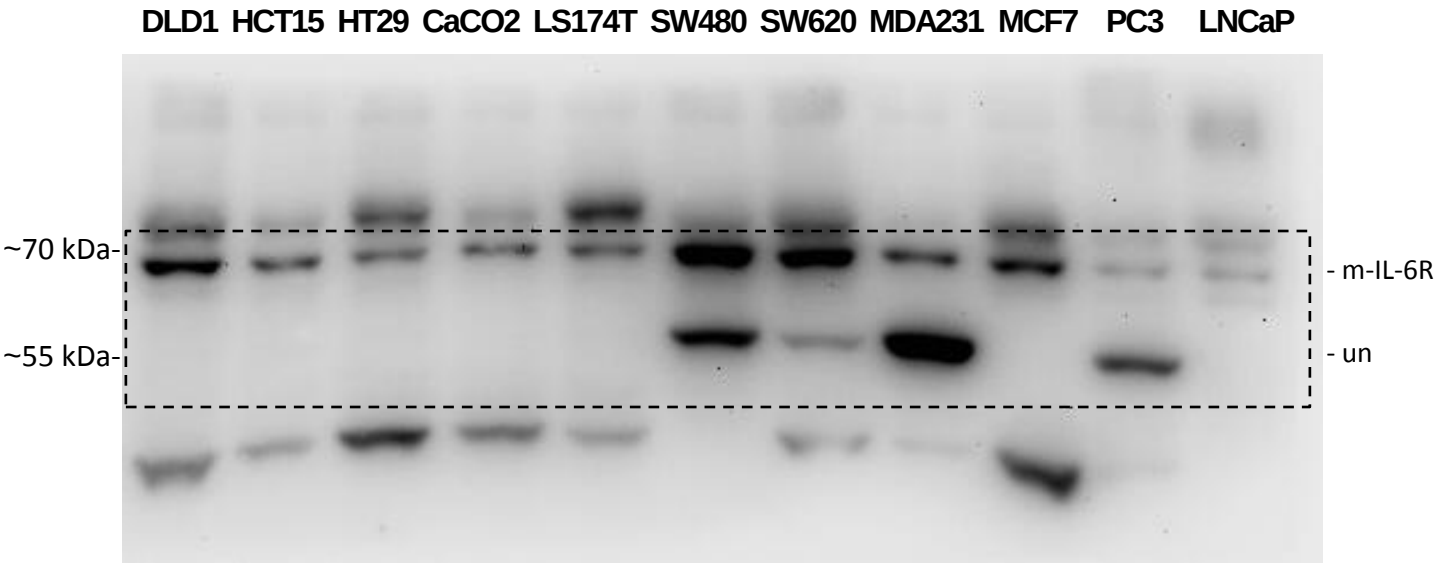

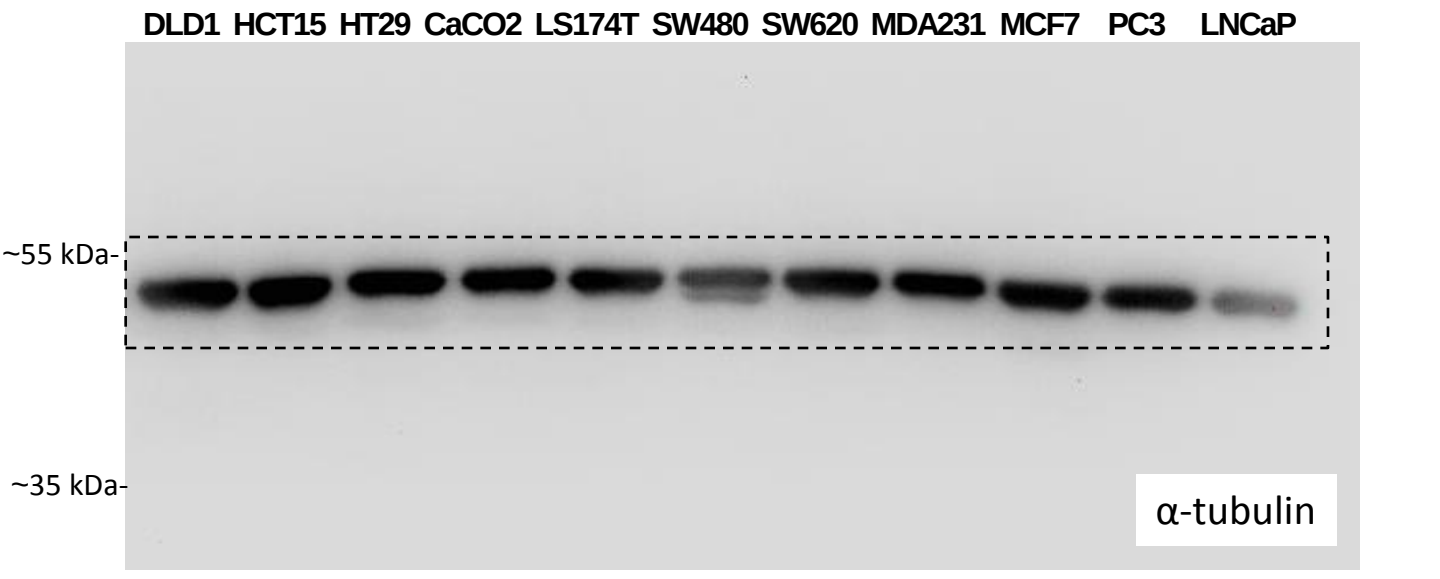

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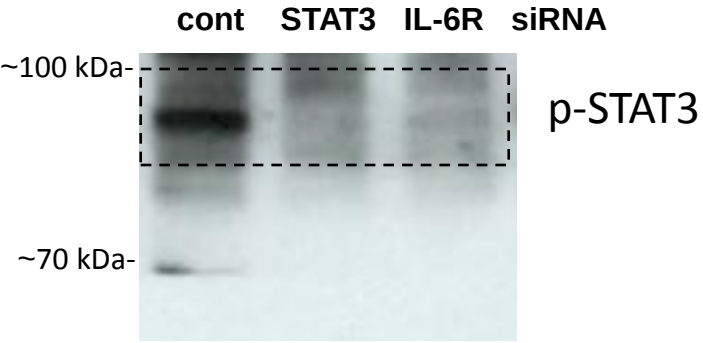

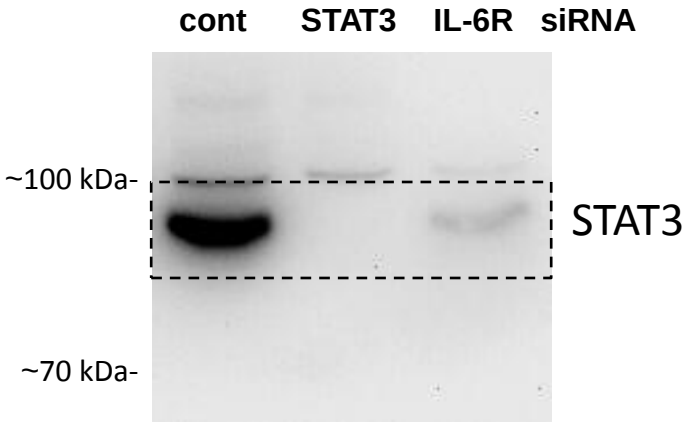

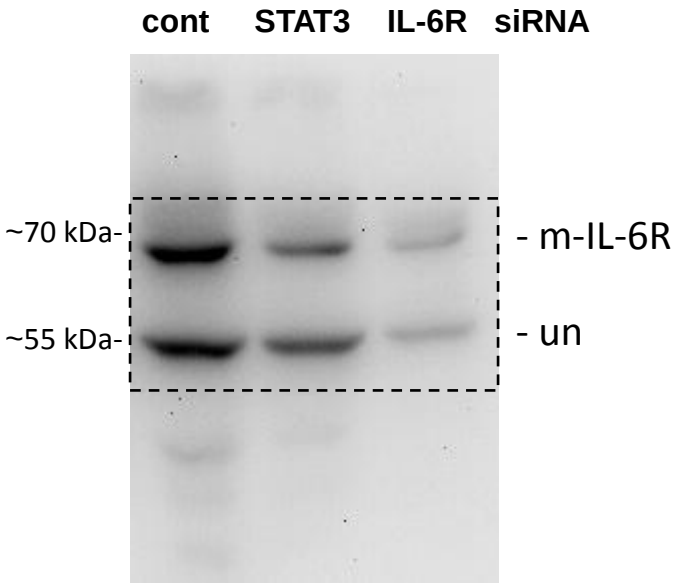

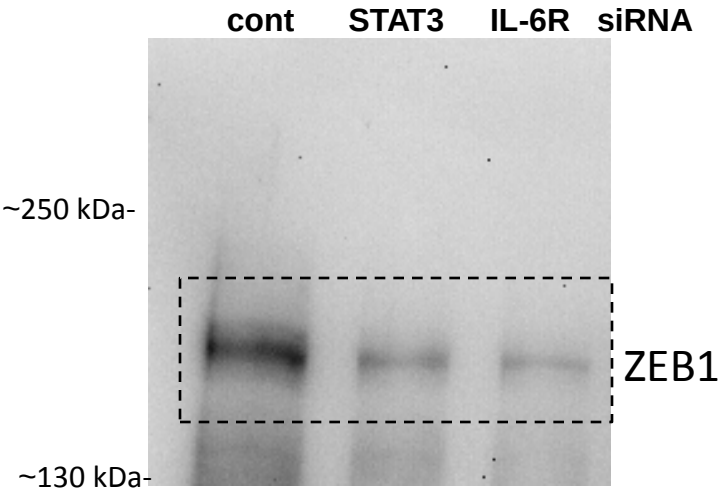

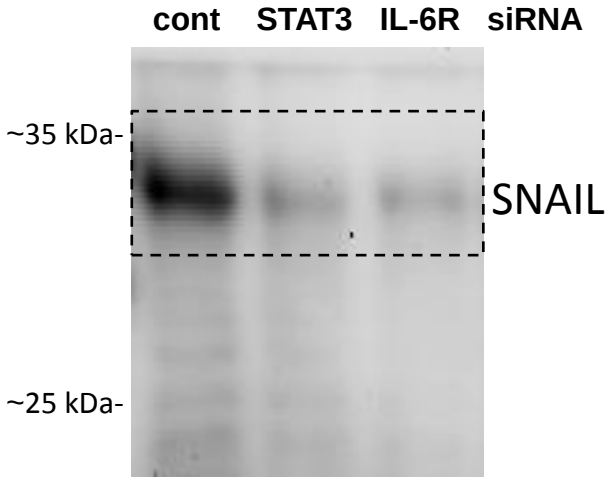

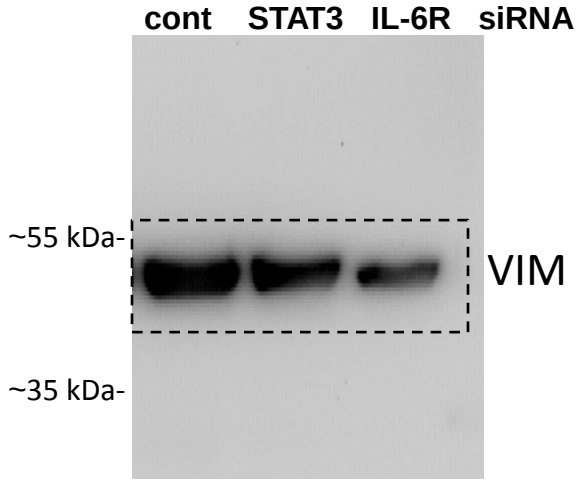

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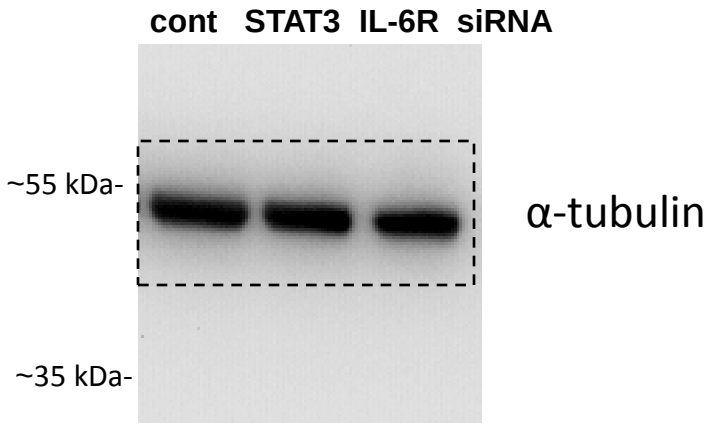

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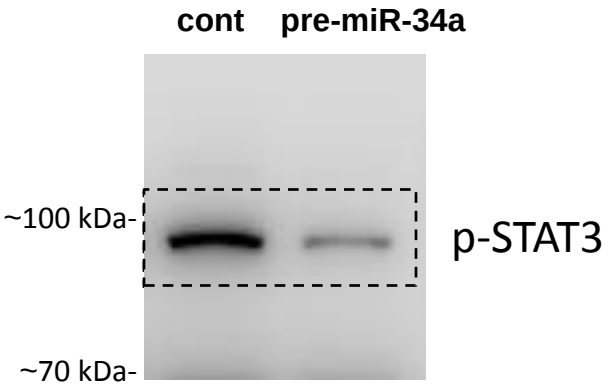

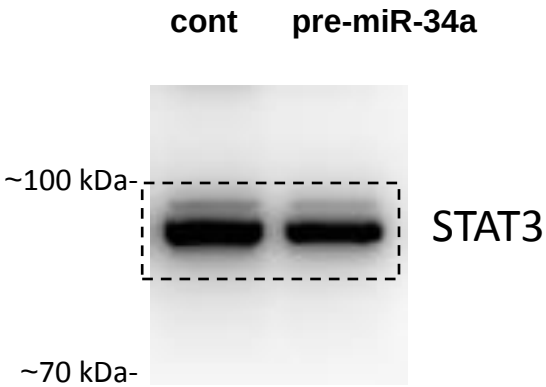

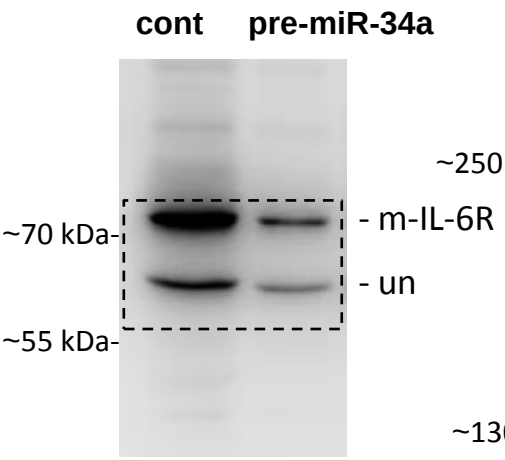

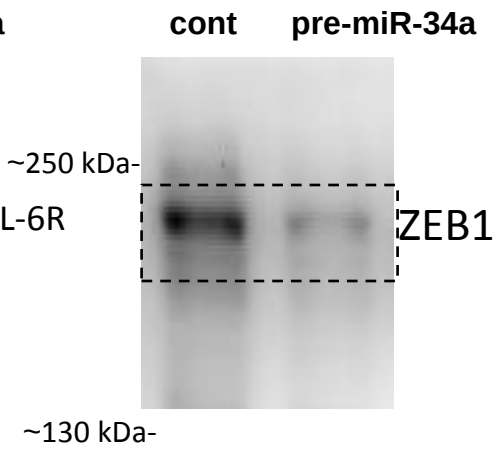

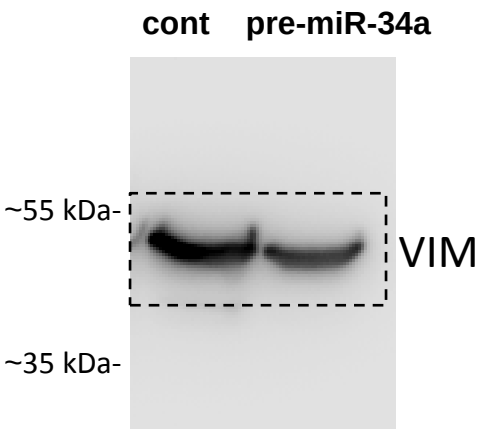

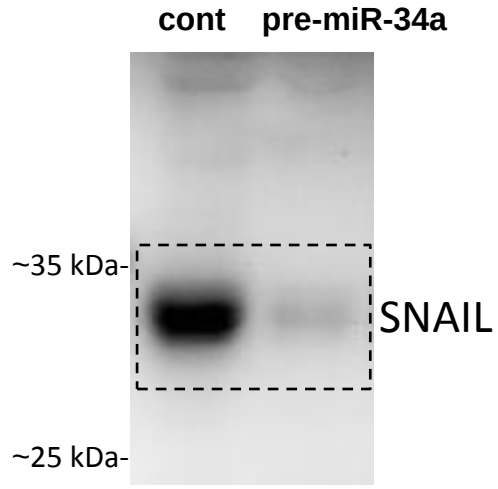

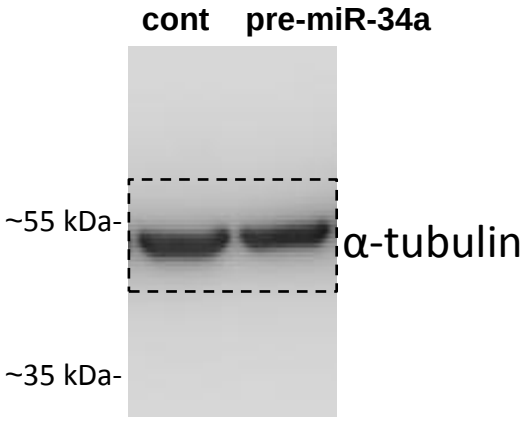

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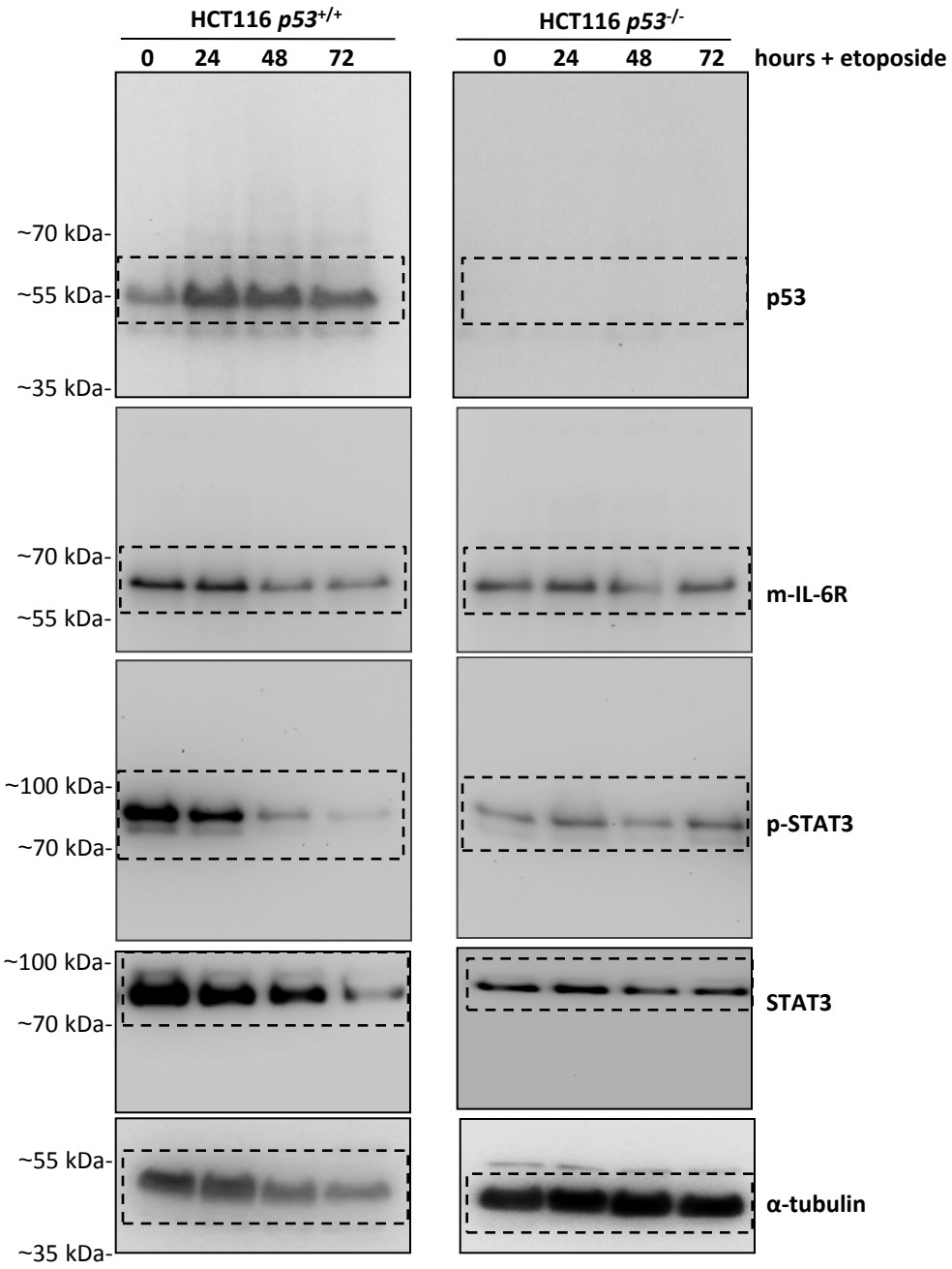

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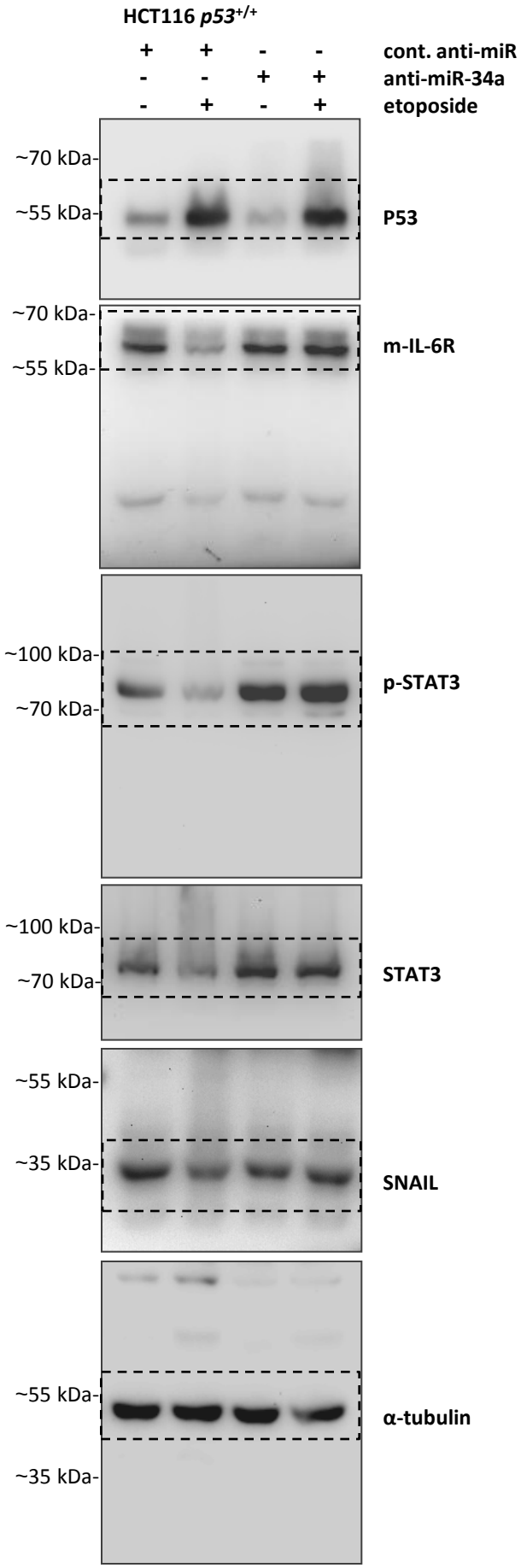

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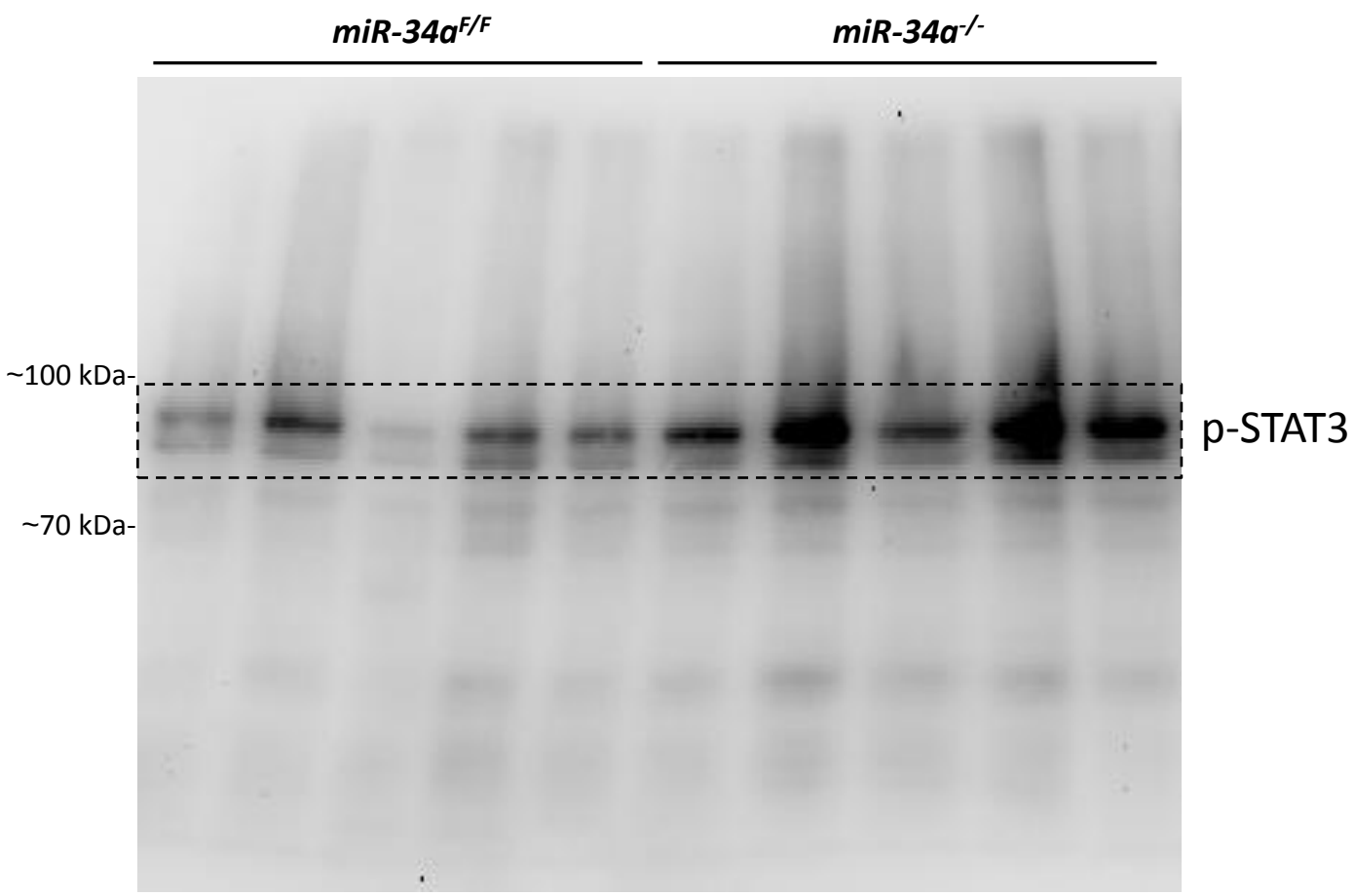

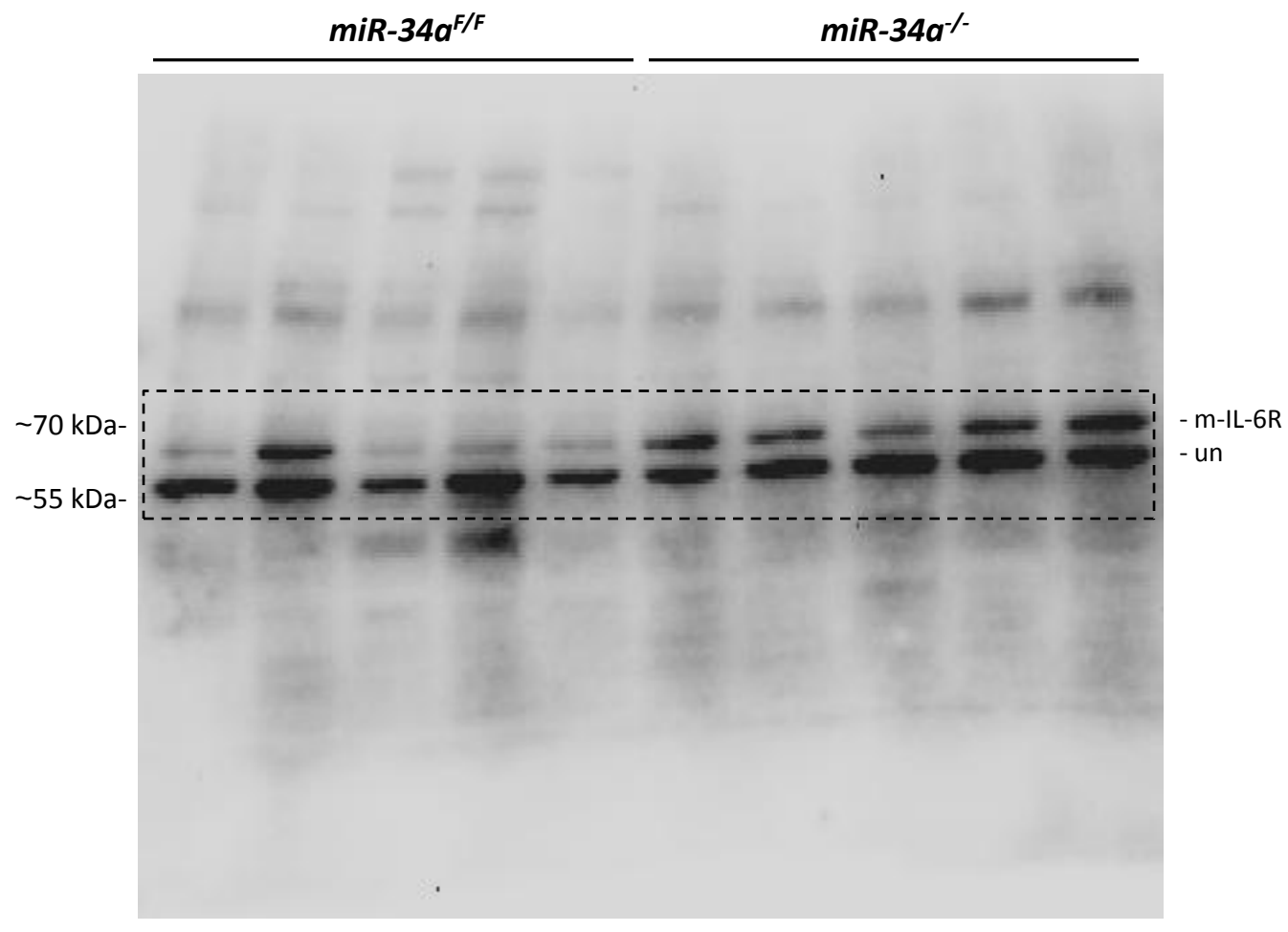

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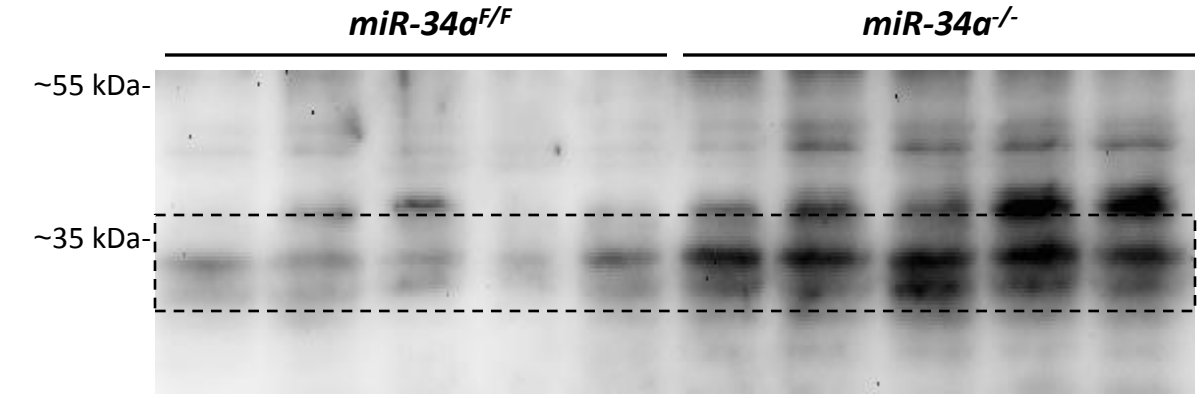

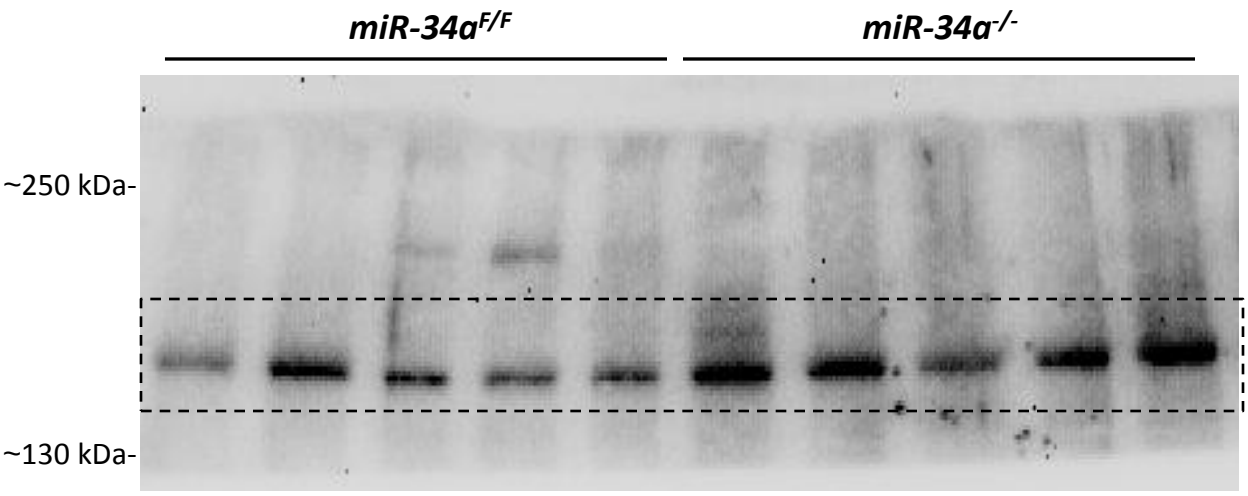

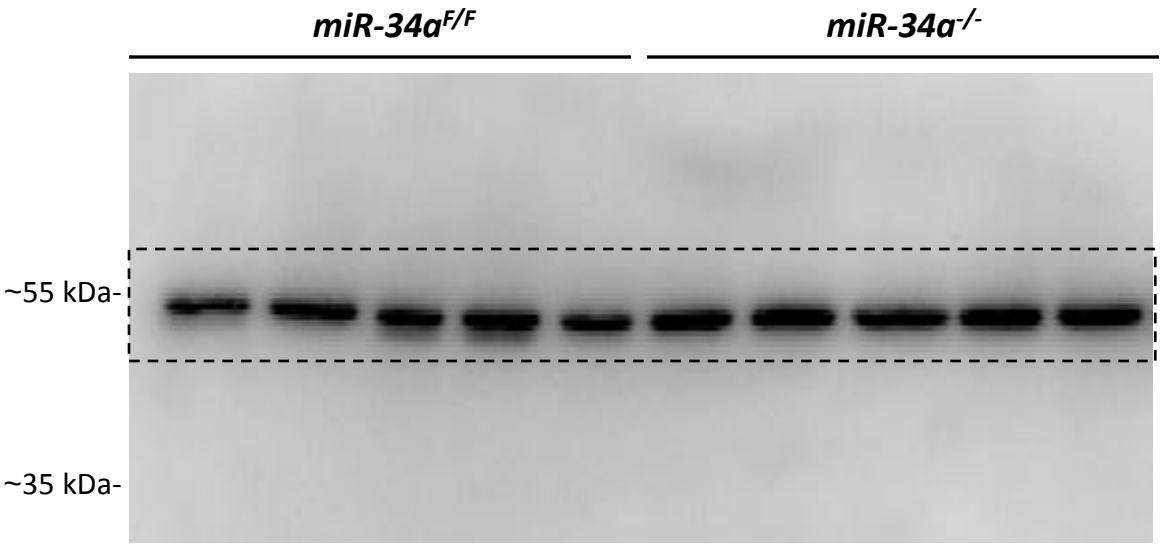

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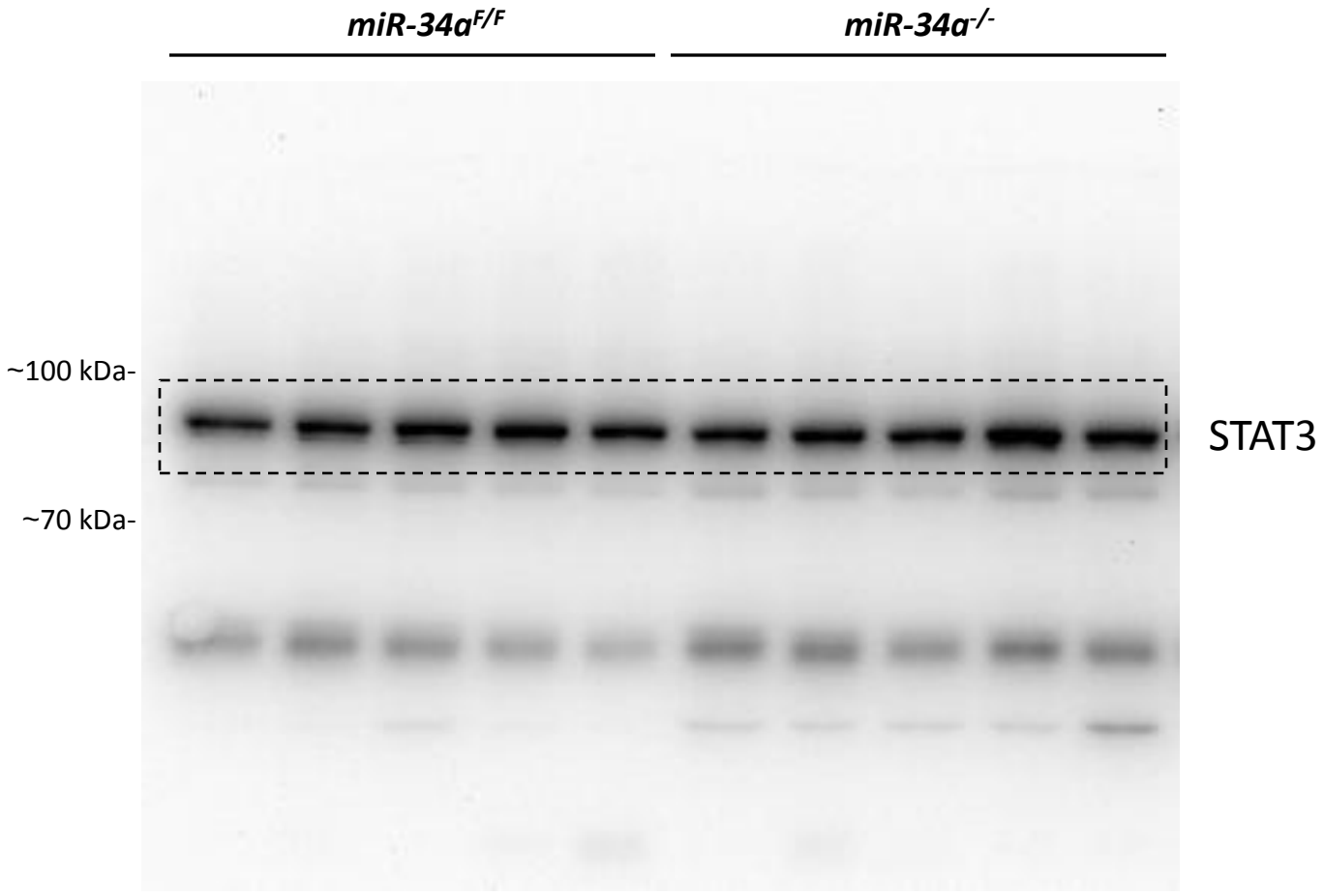

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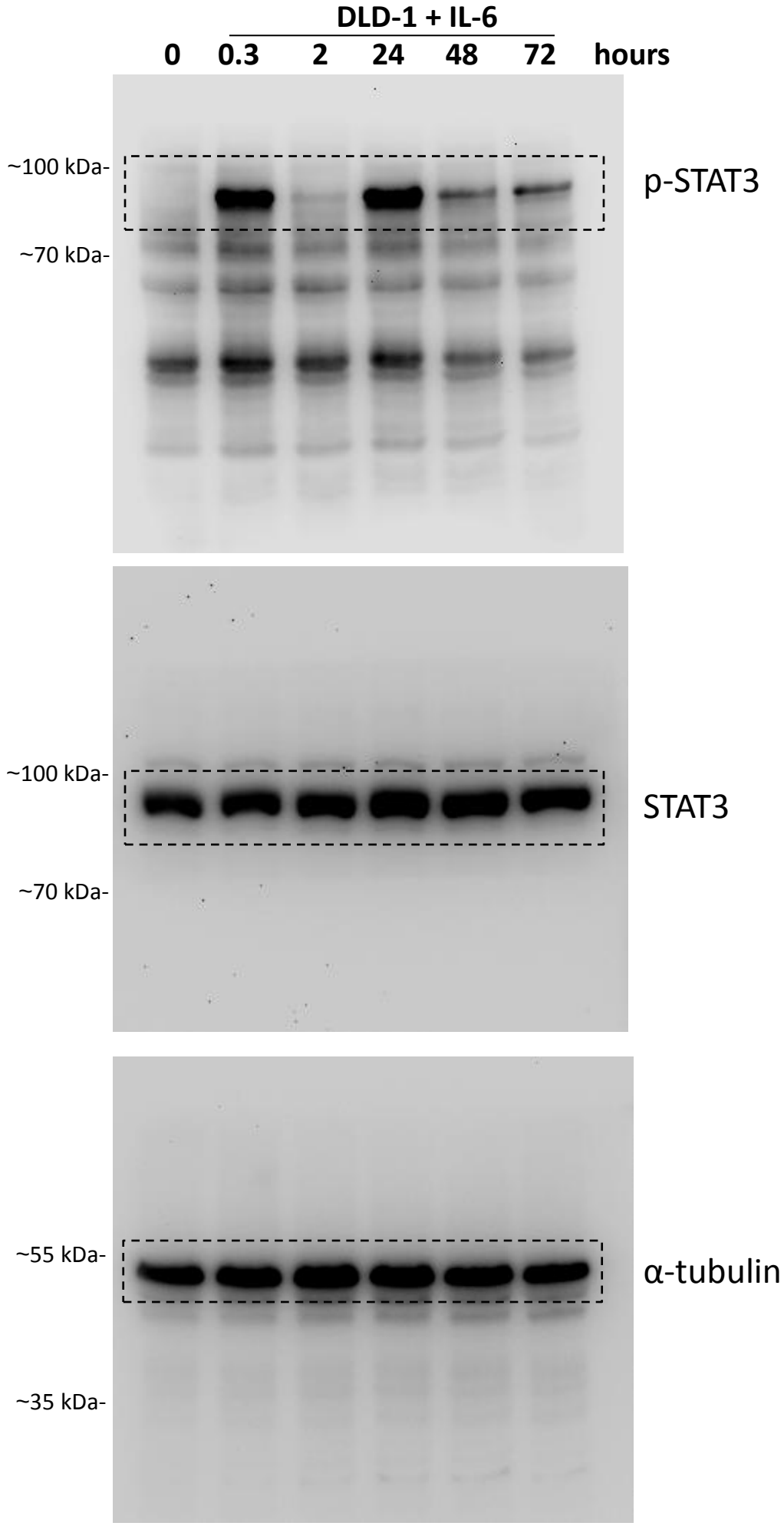

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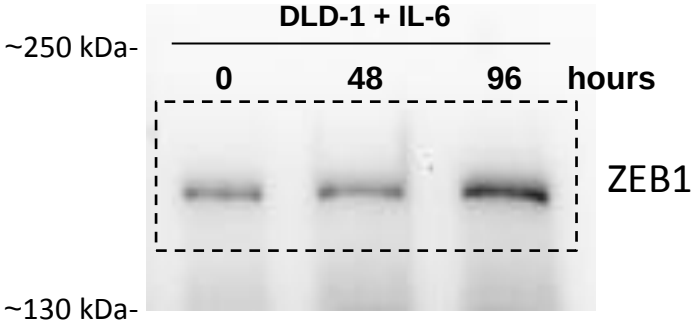
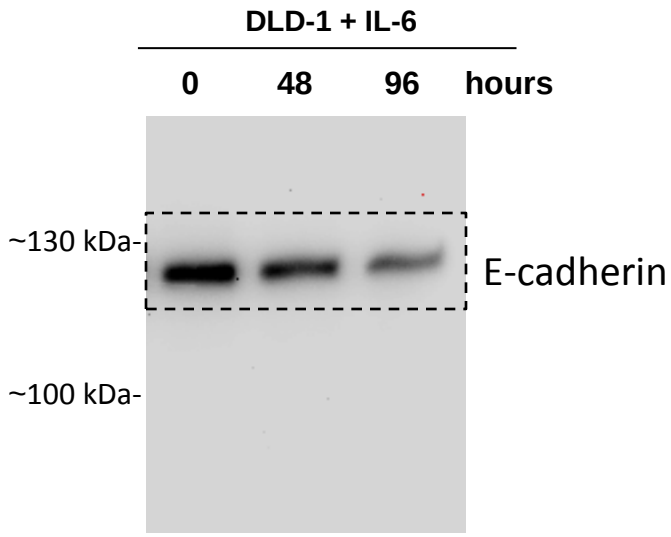
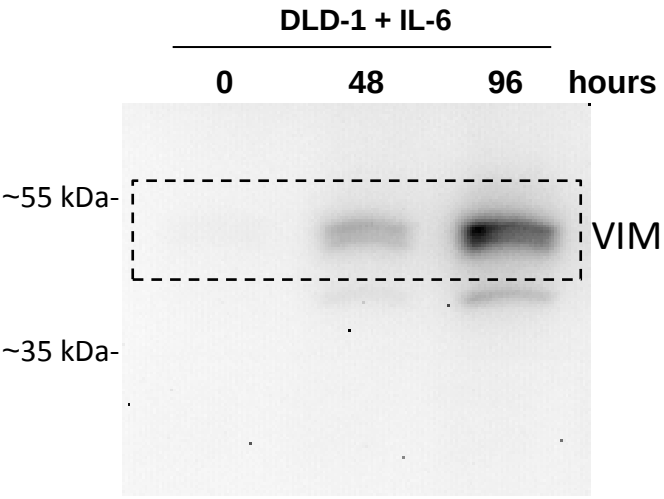
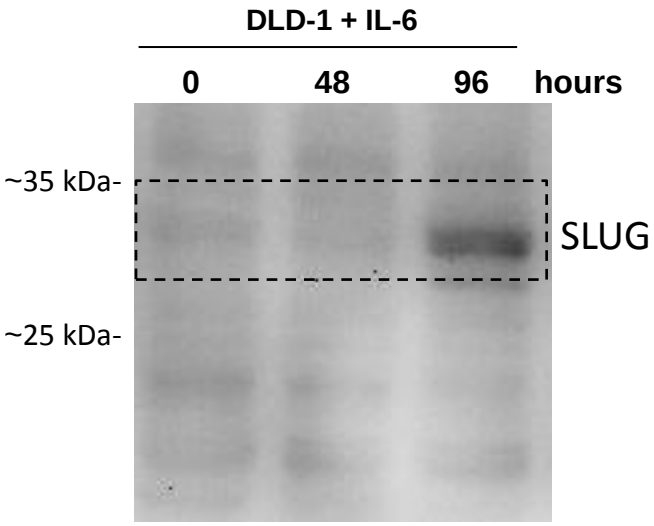
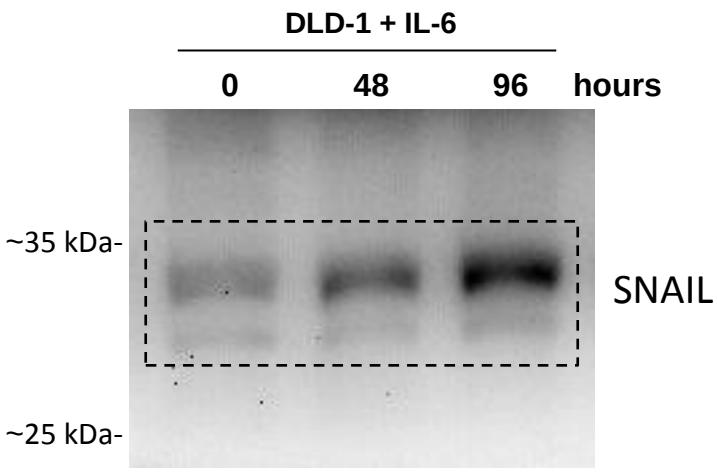
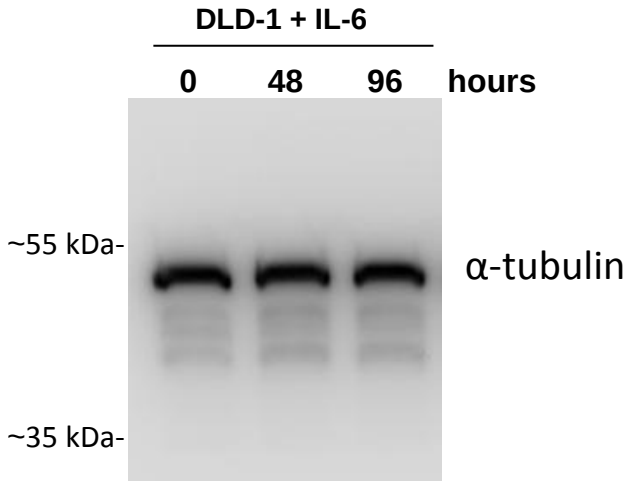




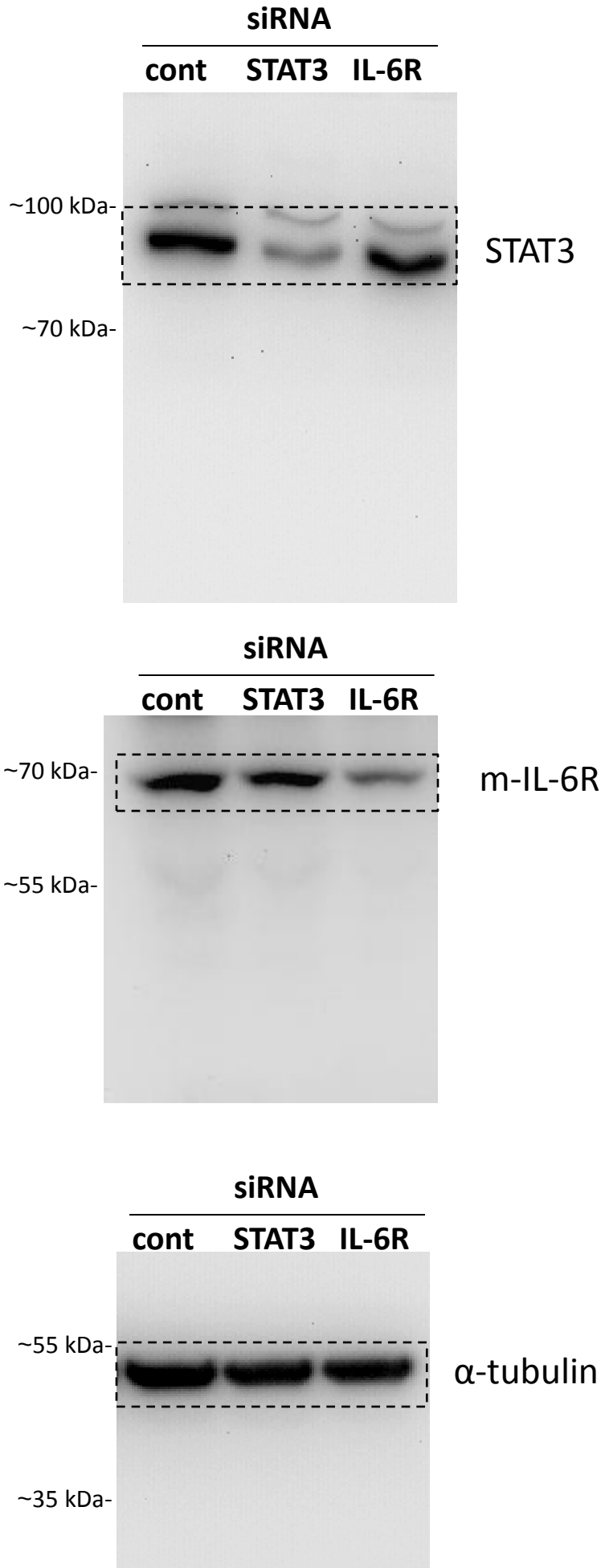




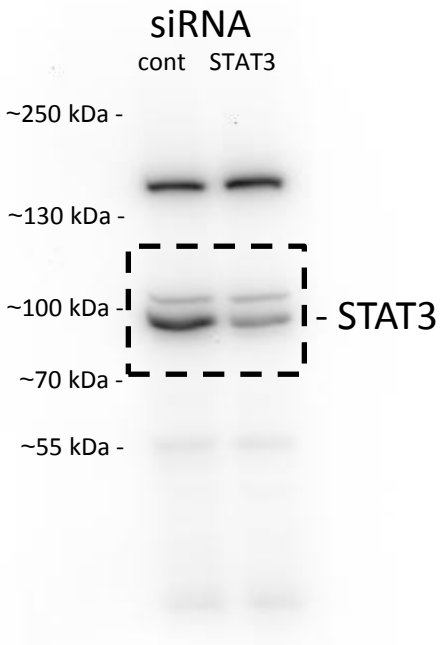


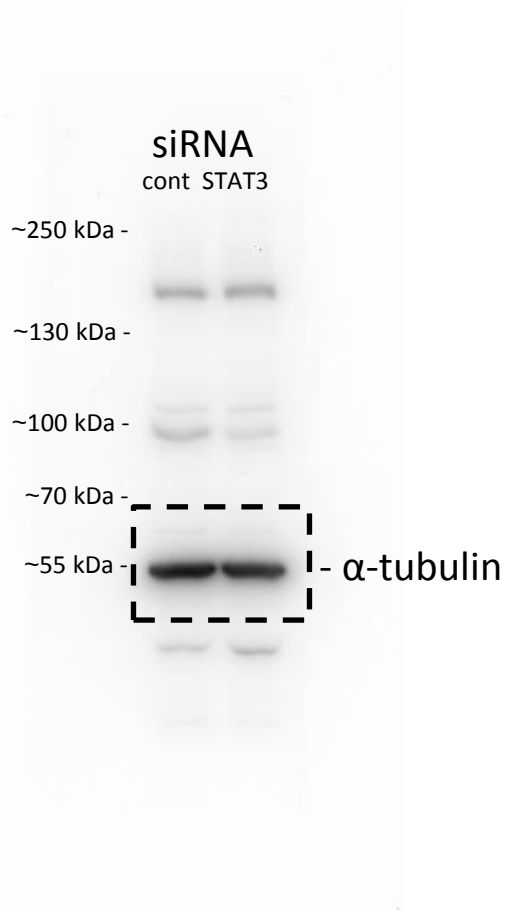


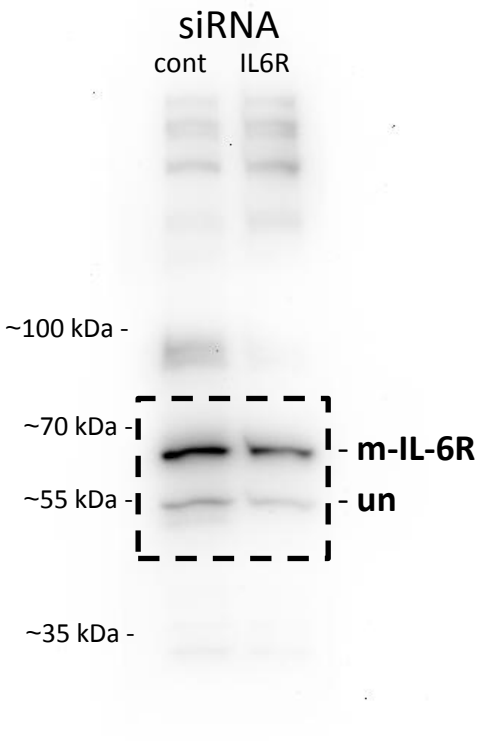
Full unedited gel for Supplemental Figure 1D

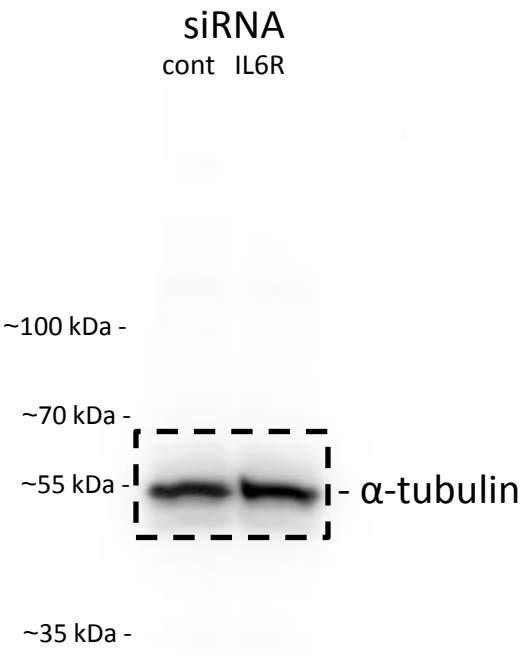


Full unedited gel for Supplemental Figure 4B

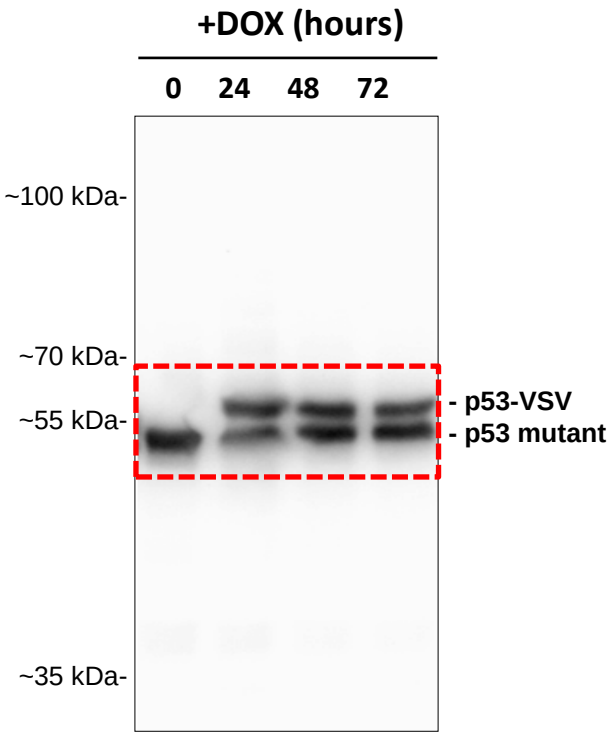


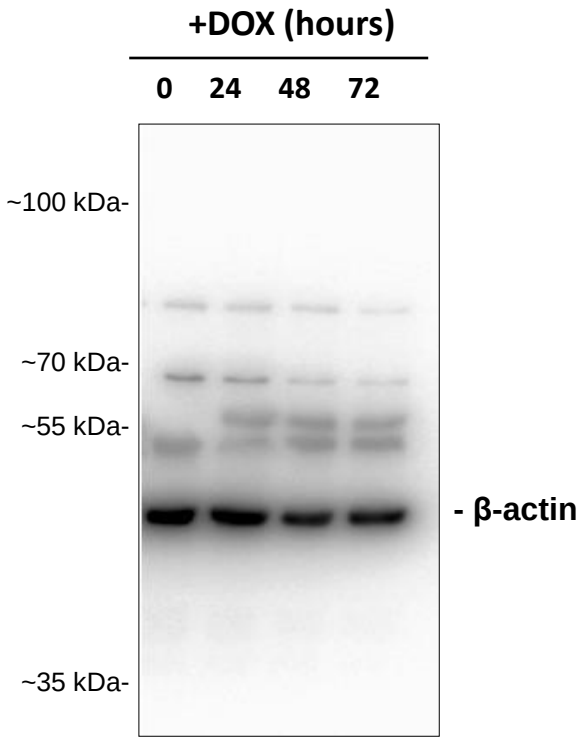


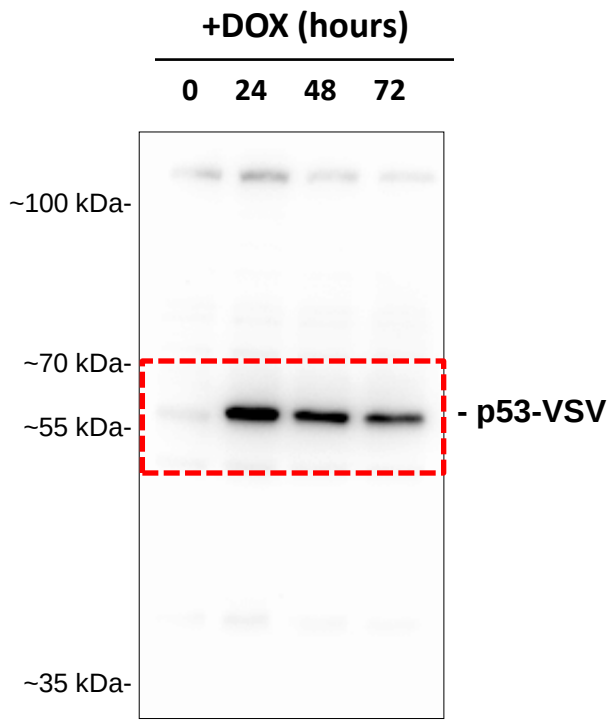


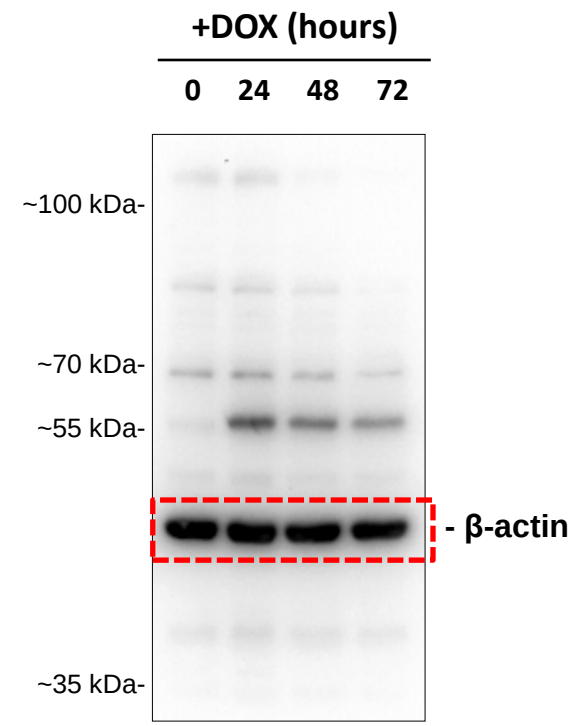


Full unedited gel for Supplemental Figure 5A









Full unedited gel for Supplemental Figure 5B