An Integrated Stress Response via PKR Suppresses HER2+ cancers and Improves Trastuzumab Therapy.

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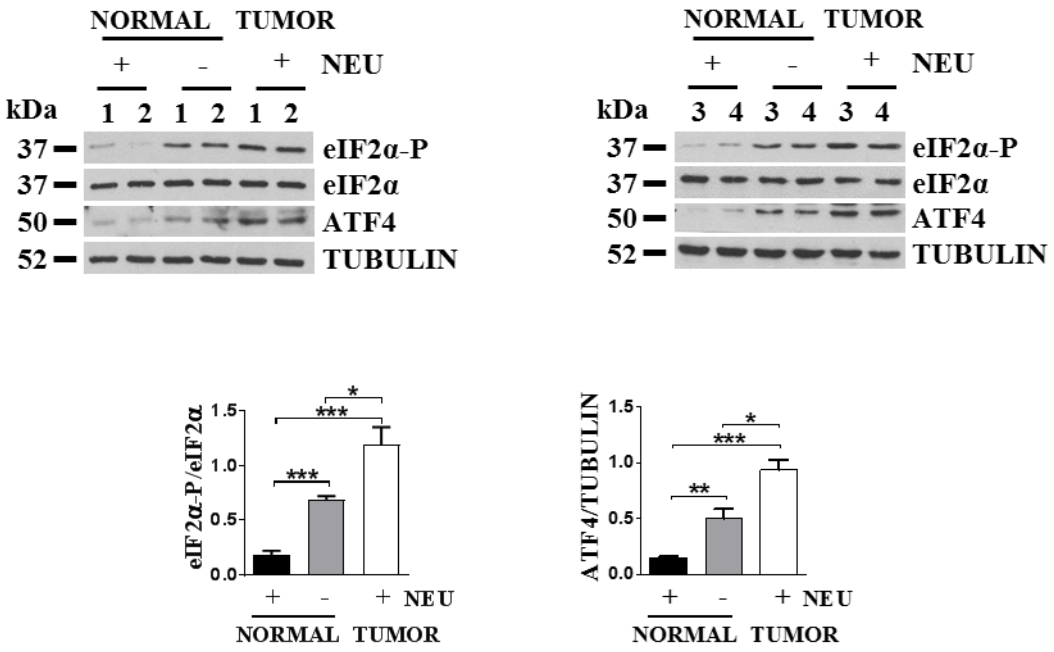
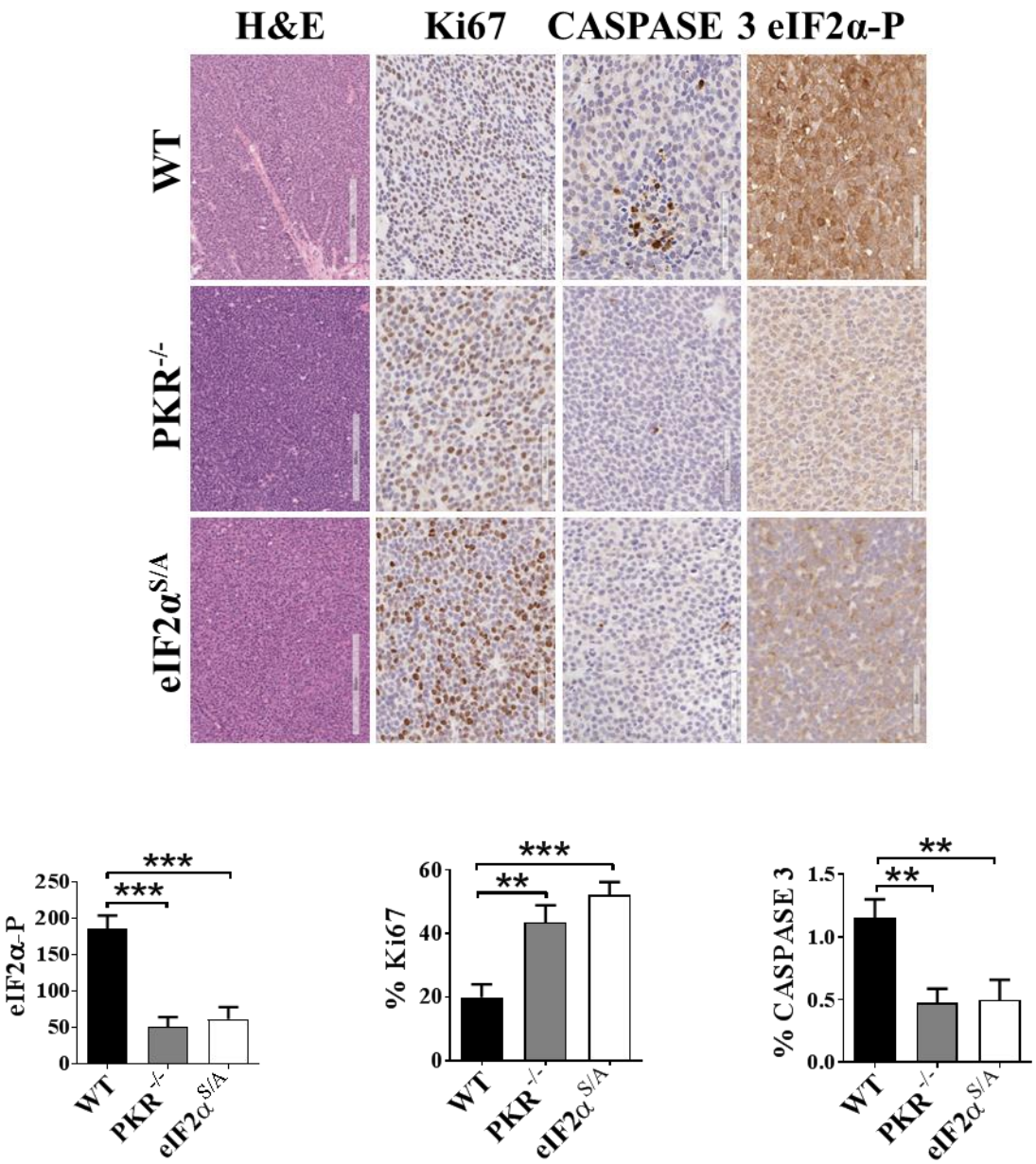
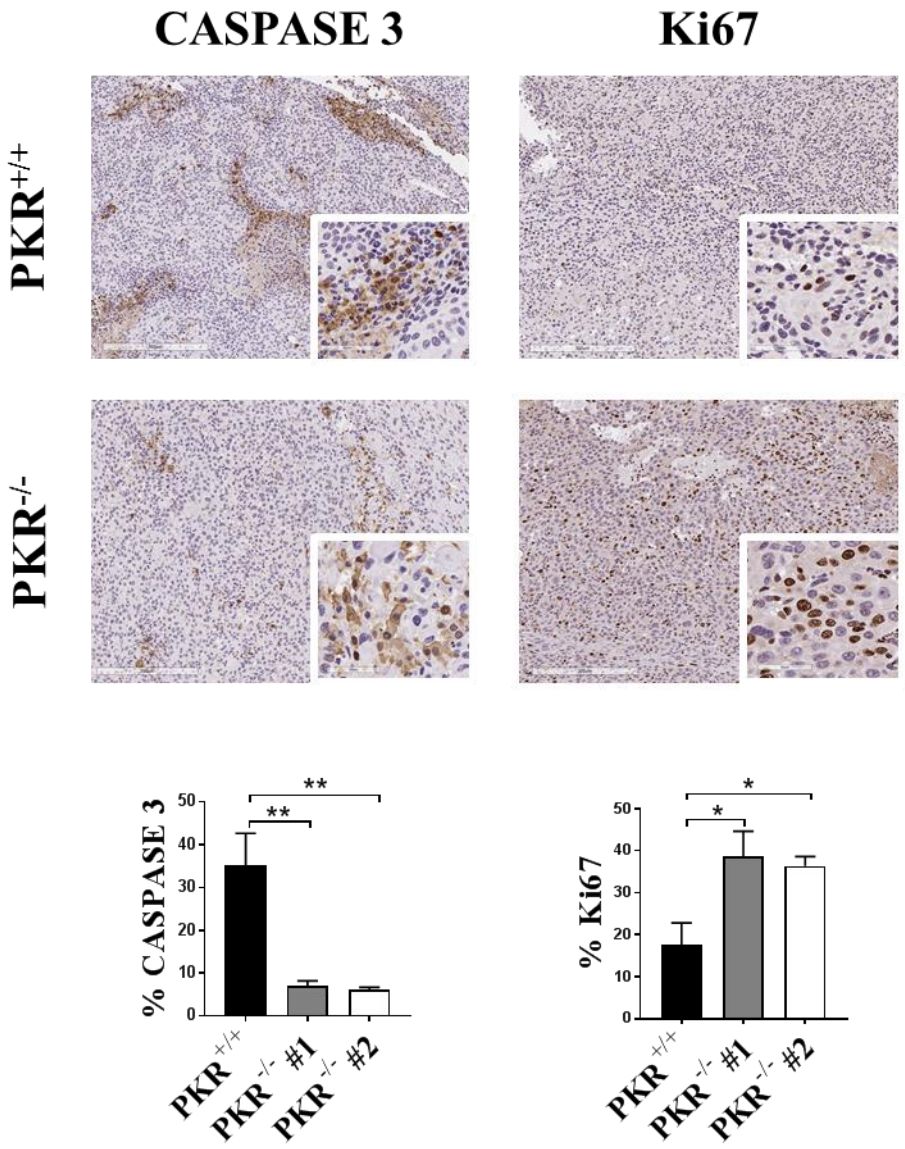
SUPPLEMENTARY INFORMATION



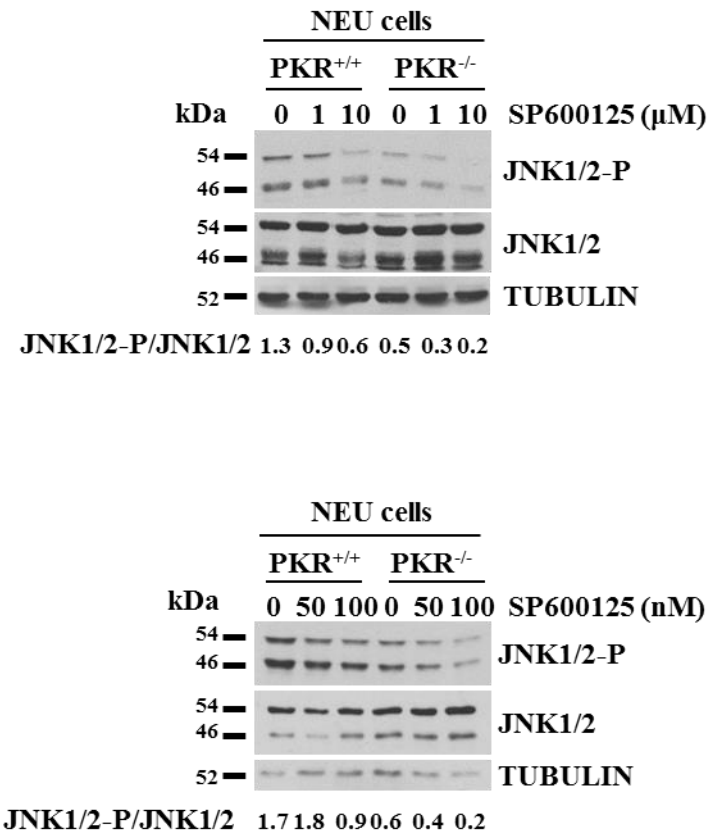
Supplementary Figure 1. eIF2α-P and ATF4 levels are low in mouse NEU mammary gland prior to tumor formation and high in formed tumors. Immunoblotting of protein extracts of mammary tissue from FVB/N mice lacking (Normal, -NEU), or expressing NEU prior to (Normal, +NEU) or after tumor formation (Tumor, +NEU). Quantification of data from the analysis of 4 mice of each group. ±SEM *p<0.05;** p<0.01; *** p<0.001.



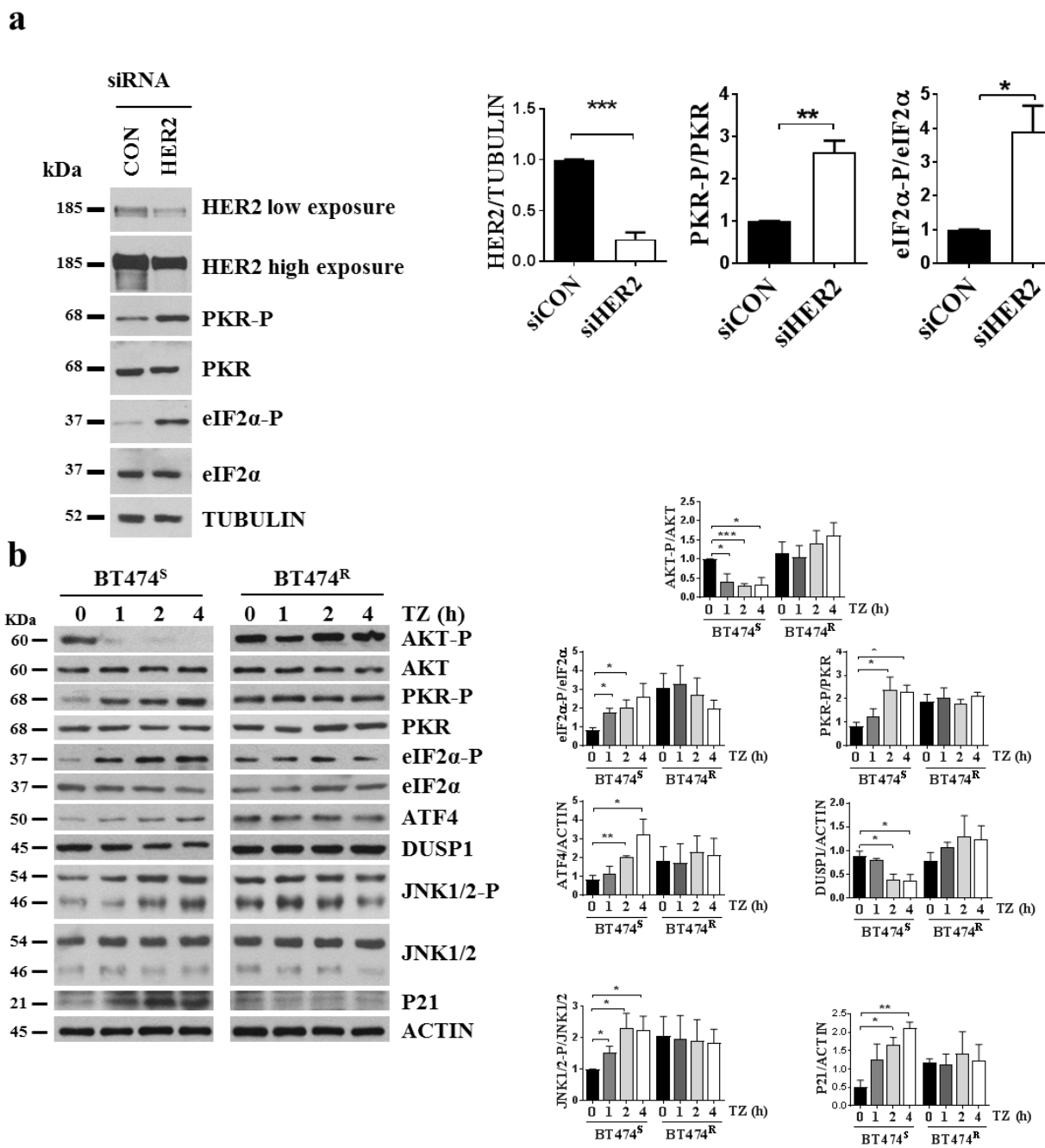
Supplementary Figure 2. PKR and eIF2α-P decrease the proliferation and increase apoptosis of mouse NEU breast tumors. H&E staining and IHC analyses of Ki67, cleaved Caspase 3 and eIF2α-P in sections from NEU WT, NEU PKR$^{-/-}$ or NEU eIF2α$^{S/A}$ tumors isolated 4 weeks after initial detection by palpation (Fig. 1a). The H-score of elF2α-P and the % of positive cells for Ki67 and Cleaved Caspase 3 were quantified by the Aperio ImageScope software from the analysis of tumors from 8 different mice per group. Data represent mean ±SEM ** $p<0.01$; *** $p<0.001$.



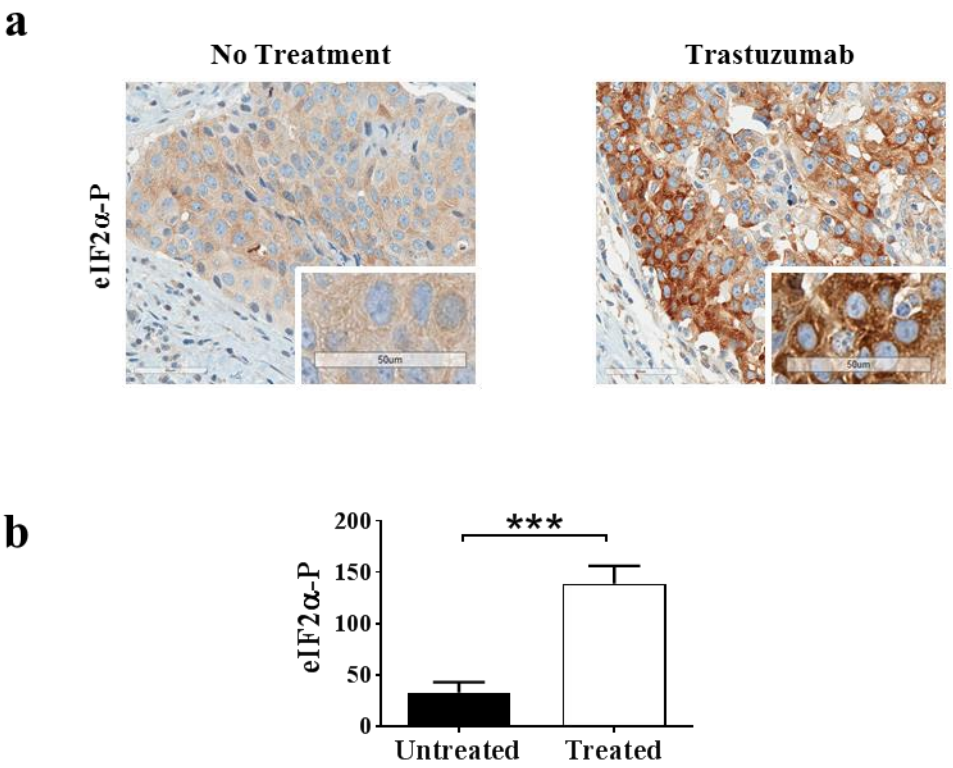
Supplementary Figure 3. PKR inhibits proliferation and increases apoptosis of mouse NEU breast tumors in SCID mice. IHC analyses of Ki67 and cleaved Caspase 3 in sections from NEU WT and NEU PKR$^{-/-}$ tumors isolated from SCID mice (Fig. 2a). The % of positive cells for Ki67 and Cleaved Caspase 3 were quantified by the Aperio ImageScope software from the analysis of tumors from 3 different mice per group. Data represent mean ±SEM *$p < 0.05$; ** $p<0.01$.



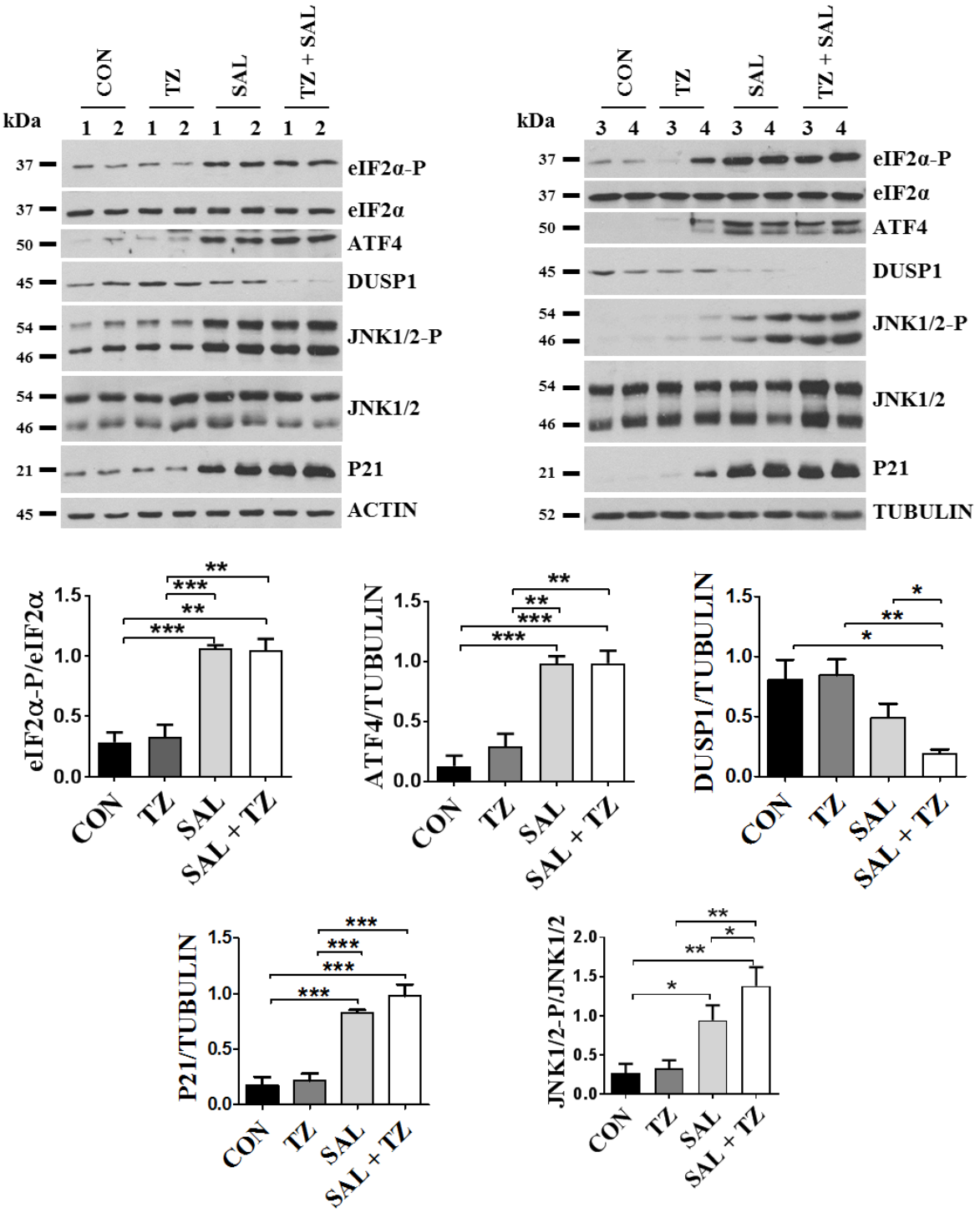
Supplementary Figure 4. Inhibition of JNK1/2-phowphorylation by SP600125. Mouse NEU PKR$^{+/+}$ and PKR$^{-/-}$ tumor cells were treated with indicated concentration of SP600125 and immunoblotted for phosphorylated and total JNK1/2 levels. Quantifications of the phosphorylated JNK1/2 normalized to total JNK1/2 for each lane are indicated.



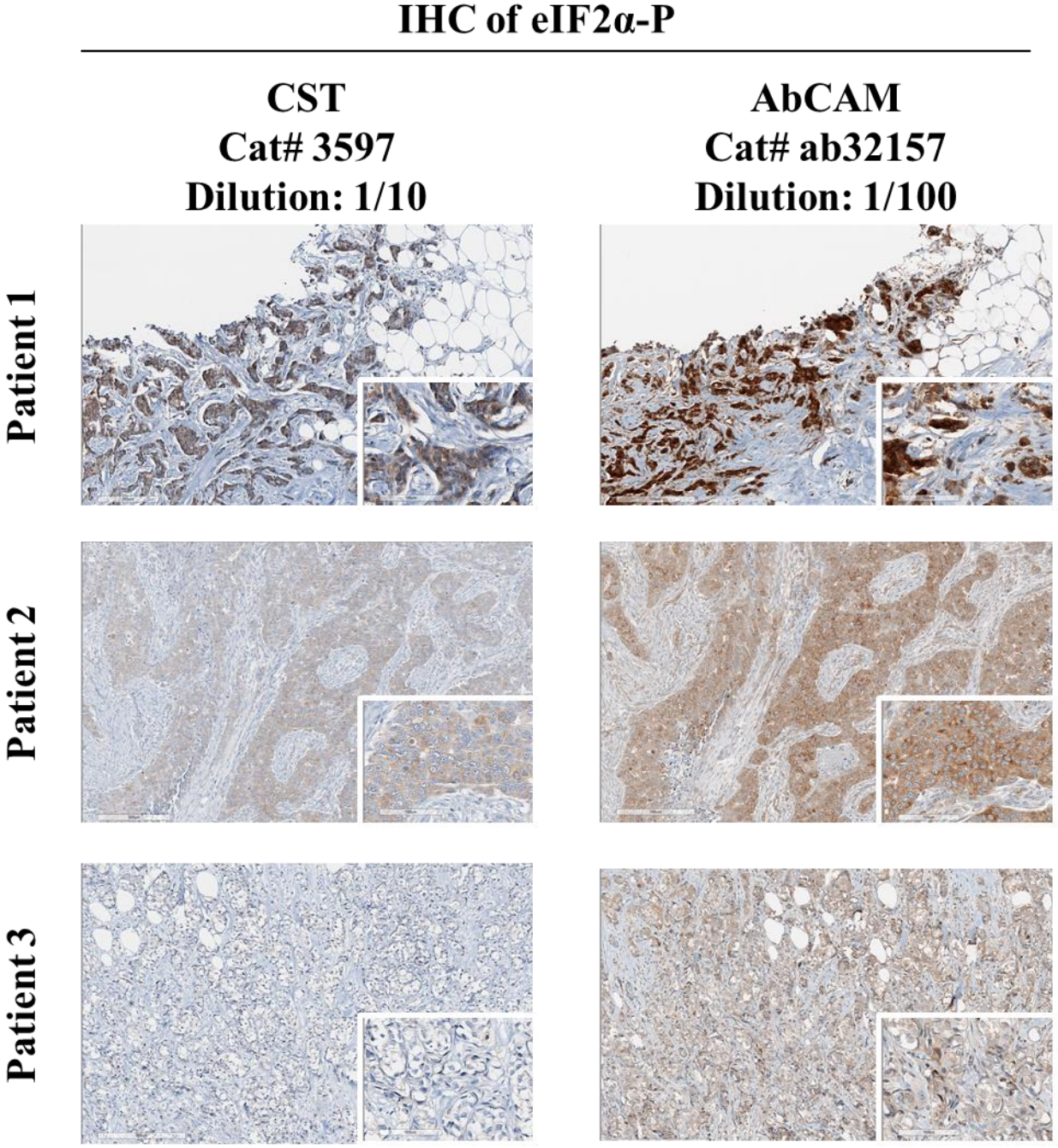
Supplementary Figure 5. PKR is inhibited by HER2 and activated by Trastuzumab in breast tumor cells. (**a**) BT474 cells were treated with scrambled siRNAs (CON) or HER2 siRNAs and subjected to immunoblotting for the indicated proteins. Quantification of blots from 3 biological replicates are indicated. (**b**) Immunoblot analyses of the indicated proteins in protein extracts from Trastuzumab sensitive (S) or resistant (R) BT474 cells treated with 21µg/ml Trastuzumab for the indicated time. Quantification of data represent from 3 biological replicates. (**a, b**) Data represent mean ±SEM *$p < 0.05$; **$p<0.01$; *** $p<0.001$



Supplementary Figure 6. Trastuzumab increases eIF2α-P in tumors from HER2+ breast cancer patients. (**a**) Representative IHC staining of eIF2α-P in HER2+ breast tumors before and after Trastuzumab treatment of the same patient, who developed resistance to therapy. (**b**) The intensity of eIF2α-P was evaluated by the Aperio ImageScope software. The graph shows the histoscore (H-score) of the intensity of eIF2α-P in tumors before and after Trastuzumab therapy of 6 patients of HER2+ breast cancer with resistance to Trastuzumab. Data represent mean ±SEM *** $p<0.001$



Supplementary Figure 7. SAL003 induces the anti-tumor pathways downstream of eIF2α-P in HER2+ gastric PDXs co-treated with Trastuzumab. Protein extracts of HER2+ gastric tumors grown in NOG mice treated with SAL003 and/or Trastuzumab in Fig. 6 were subjected to immunoblotting for the indicated proteins. Quantifications were performed with data from the analyses of 4 different tumors per type of treatment. Data represent mean ±SEM *$p < 0.05$; **$p<0.01$; *** $p<0.001$.



Supplementary Figure 8. Quality control analysis of the anti-eIF2α-P antibody. IHC analyses of identical tumor sections from 3 Trastuzumab-resistant HER2+ breast cancer patients, which were included in Supplementary Fig. 6, with an anti-eIF2α-P antibody from Cell Signaling Technology (CST Cat# 3597) or Abcam (Cat# ab32157) used in this study.

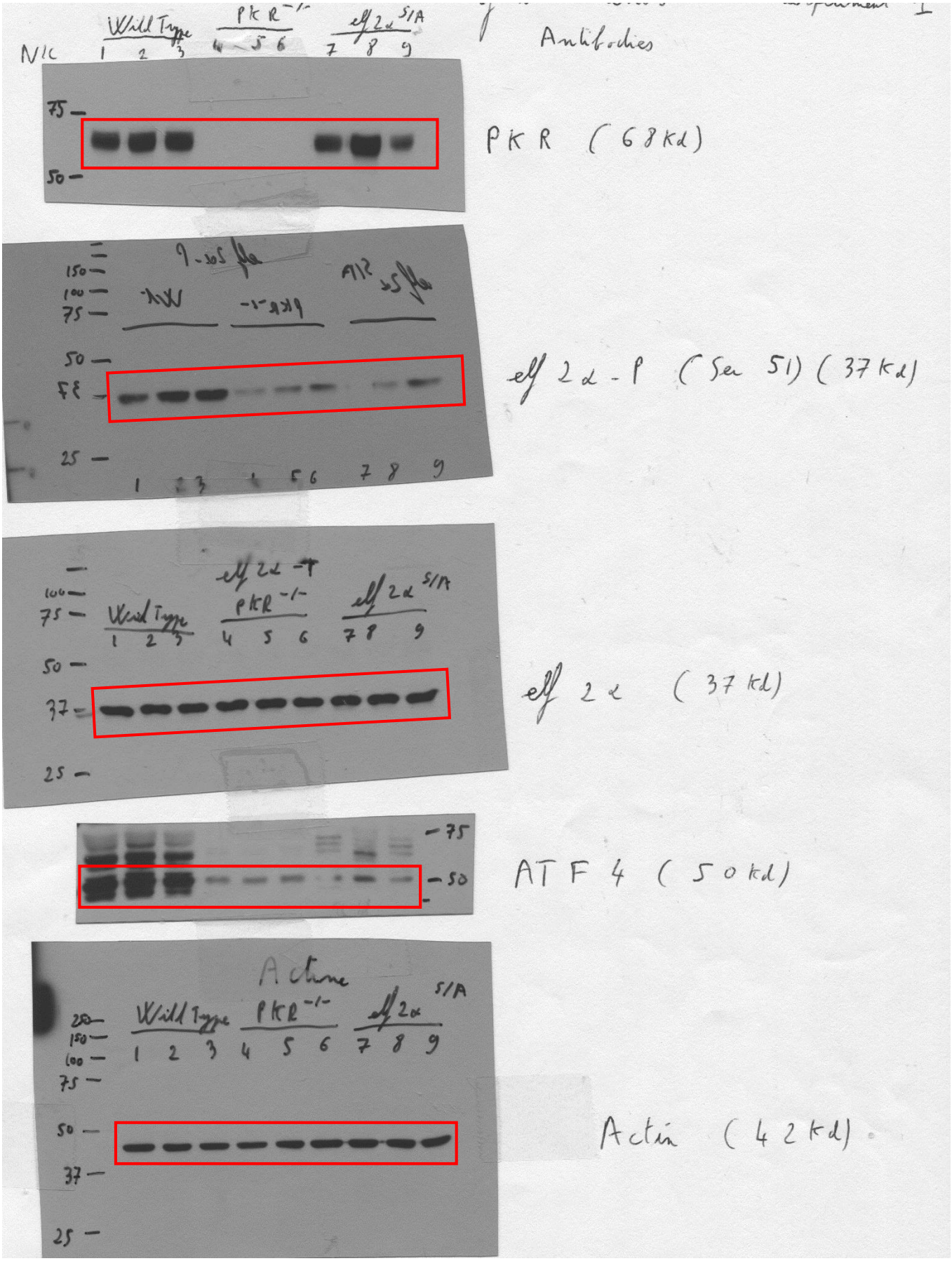
Application	Target gene	Sequence
gRNA	PKR	5'-TAATGGCTACTCCGTGCATC-3'
shRNA	DUSP1	#1: 5'-CCGGCATGAGGACTAACCGGGTAAACTCGAGTTTACCCGGTTAGTCCTCATGTTTTT-3'
		#2: 5'-CCGGGTTGTTGGATTGTCGCTCCTTCTCGAGAAGGAGCGACAATCCAACAACTTTTT-3'
	ATF4	#1: 5'-CCGGCCAGAGCATTCCTTTAGTTTACTCGAGTAAACTAAAGGAATGCTCTGGTTTTTG-3'
		#2: 5'-CCGGCGGACAAAGATACCTTCGAGTCTCGAGACTCGAAGGTATCTTTGTCCGTTTTTG-3'
RT-PCR	DUSP1	Fw 5'-GTTGTTGGATTGTCGCTCCTTC-3'
		Rv 5'-TTGGGCACGATATGCTCCAG-3'
	P21	Fw 5'-GCAGATCCACAGCGATATCC-3'
		Rv 5'-CAACTGCTCACTGTCCACGG-3'
	ACTIN	Fw 5'-CAGCAGATGTGGATCAGCAAG-3'
		Rv 5'-GCATTTGCGGTGGACGAT-3'
	GAPDH	Fw 5'-GAGAGTGTTTCCTCGTCCCG-3'
		Rv 5'-CAATCTCCACTTTGCCACTGC-3'

Supplementary Table 1. Sequences of DNA primers used in the study.

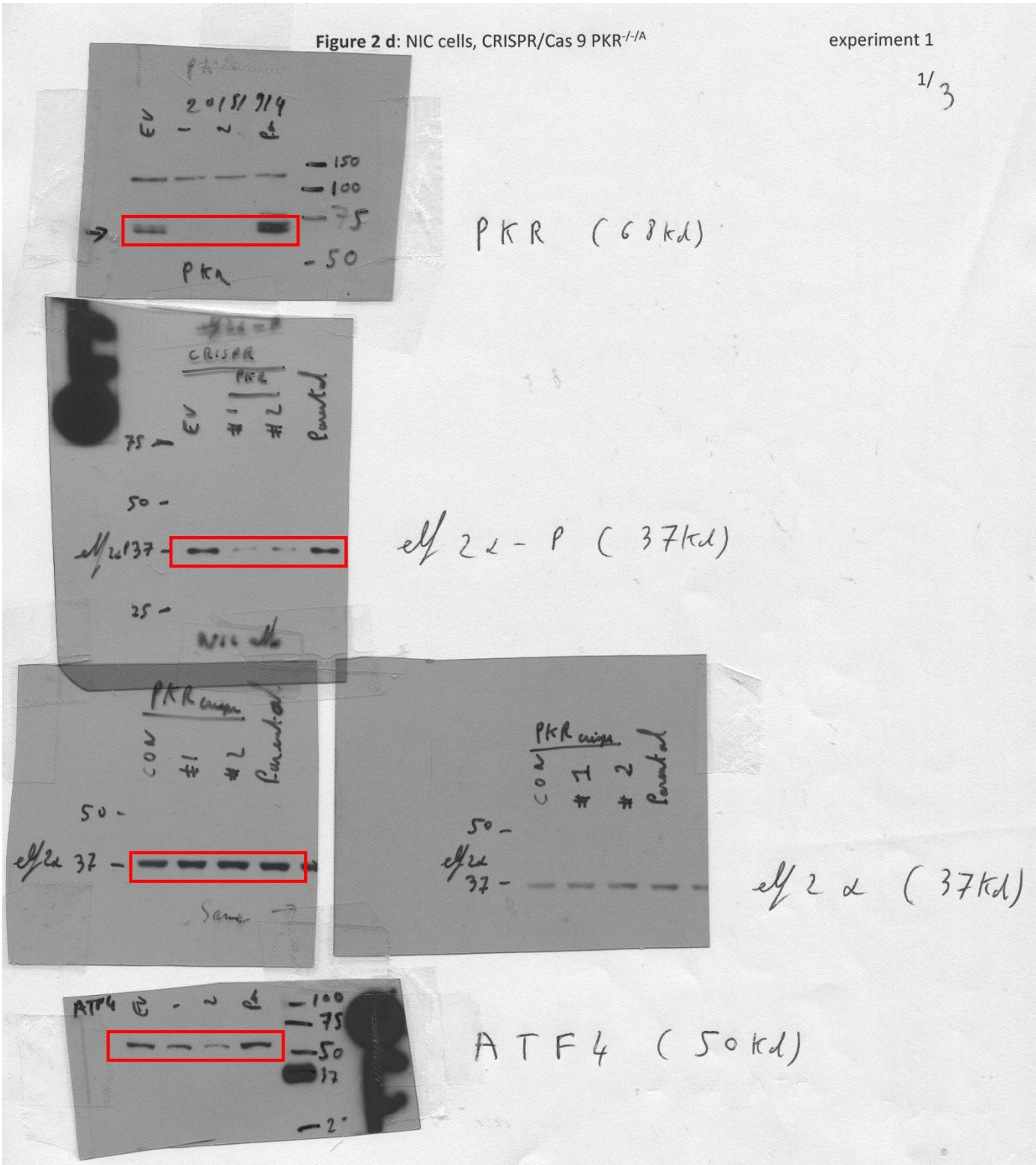
Antibody	Species	Company	Cat #	Dilution	
				WB	IHC
phosphoserine 51-eIF2α	rabbit monoclonal	Novus Biologicals	NB 110-56949	1/1000	1/100
phosphoserine 51-eIF2α	rabbit monoclonal	Cell Signaling	3597		1/10
phosphoserine 51-eIF2α	rabbit monoclonal	AbCAM	ab32157		1/100
phosphothreonine 446-PKR	rabbit monoclonal	AbCAM	ab32036	1/1000	
PKR	mouse monoclonal	Santa Cruz	sc-6282	1/1000	
ATF4	rabbit polyclonal	Proteintech	10835-1-AP	1/1000	
P21 Waf/Cip1 (12D1)	rabbit monoclonal	Cell Signaling	2947	1/1000	
Actin	mouse monoclonal	mpbio	C4	1/5000	
phospho-Akt (Ser473)	rabbit monoclonal	Cell Signaling	4060	1/1000	
Akt	rabbit polyclonal	Cell Signaling	9272	1/1000	
DUSP1	rabbit polyclonal	Millipore	7535	1/1000	
HER2	rabbit polyclonal	Cell Signaling	2165	1/1000	
eIF2α	mouse monoclonal	Cell Signaling	2103	1/1000	
JNK1/2-P	rabbit monoclonal	Cell Signaling	4668	1/1000	
JNK1/2	rabbit polyclonal	Cell Signaling	9252	1/1000	
α-Tubulin	mouse monoclonal	Sigma-aldrich	T5168	1/1000	
Ki67	rabbit polyclonal	AbCAM	ab15580		1/500
Cleaved Caspase-3 (Asp175)	rabbit polyclonal	Cell Signaling	9661		1/150
Mouse IgG-horseradish peroxidase-conjugated	goat	KPL	474-1806	1/3000	
Rabbit IgG-horseradish peroxidase-conjugated	goat	Jackson immunoReseach	111-035-144	1/3000	
Biotinylated anti-rabbit IgG	horse	Vector	BA-1100		1/150

Supplementary Table 2. Primary and secondary antibodies used in the study.

Blots of Figure 1d



Blots of Figure 2d



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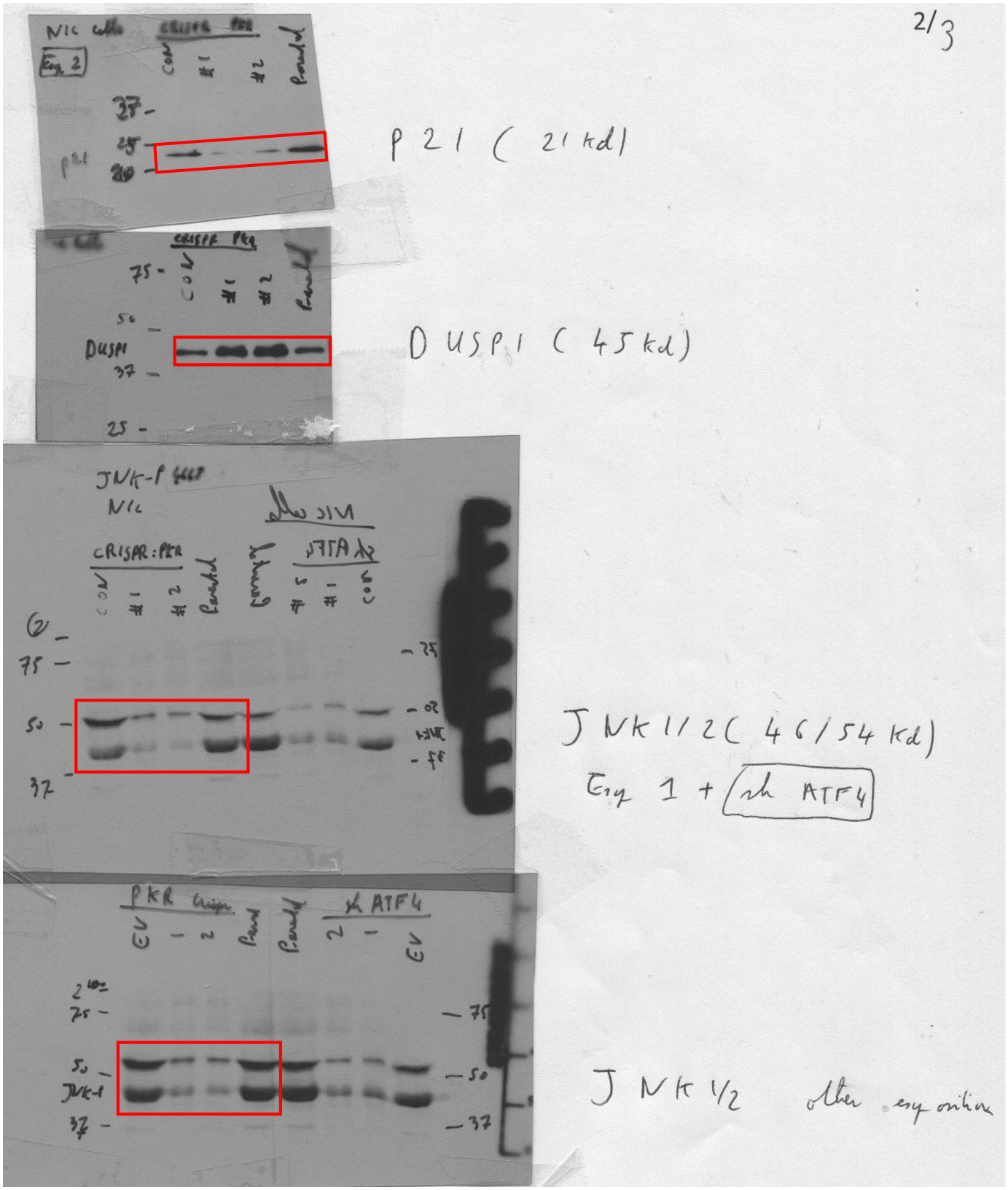

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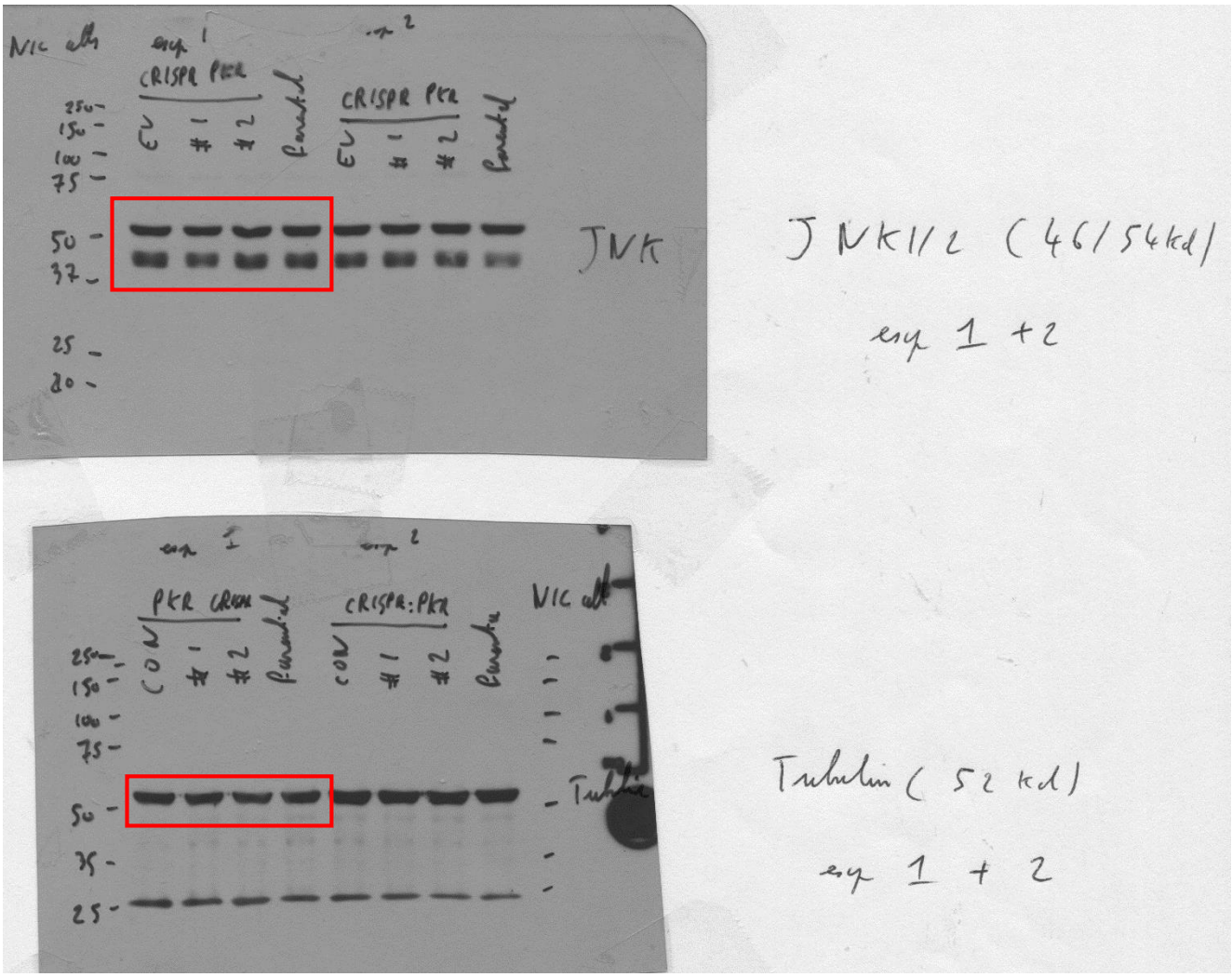
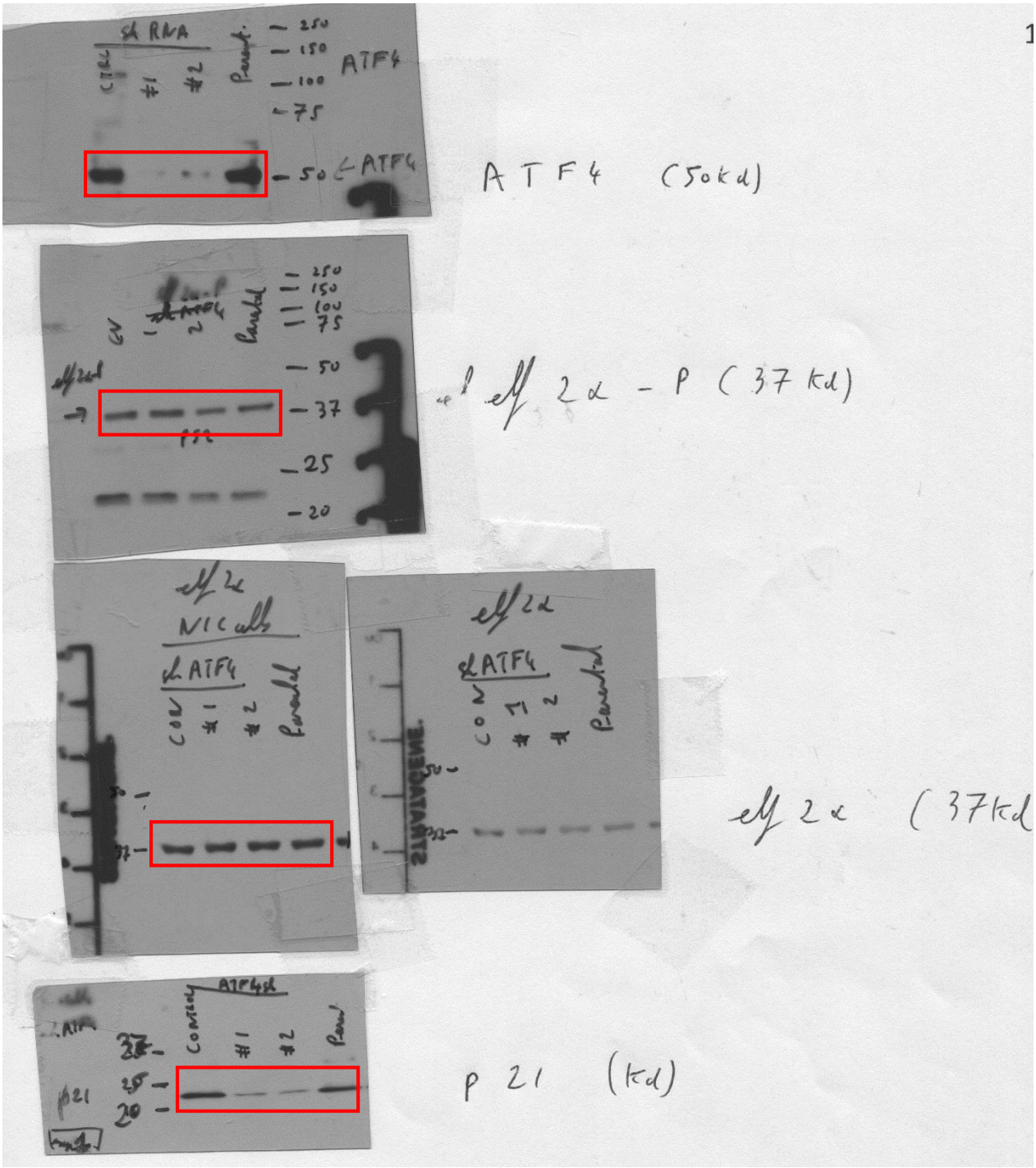


Blots of Figure 3a



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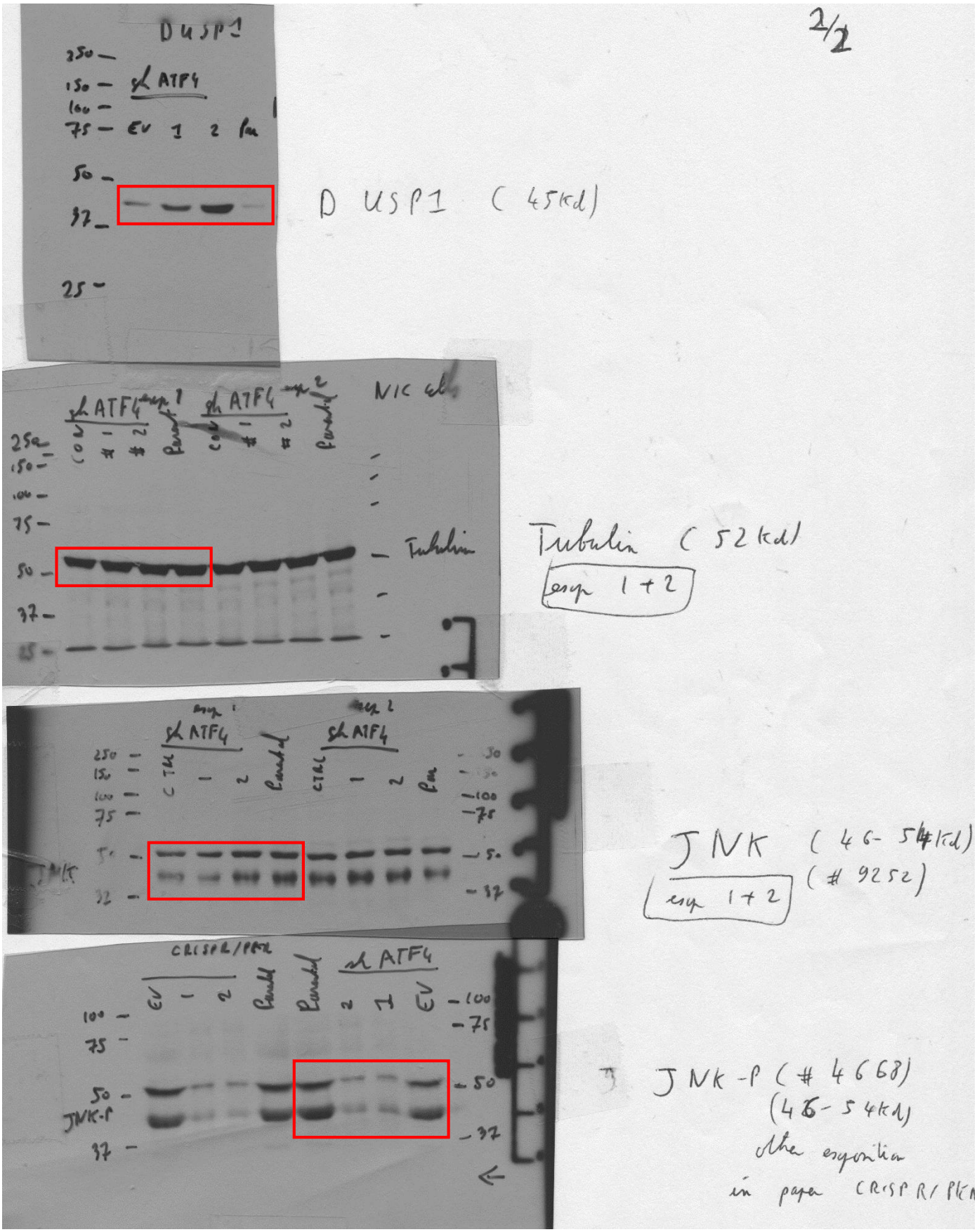
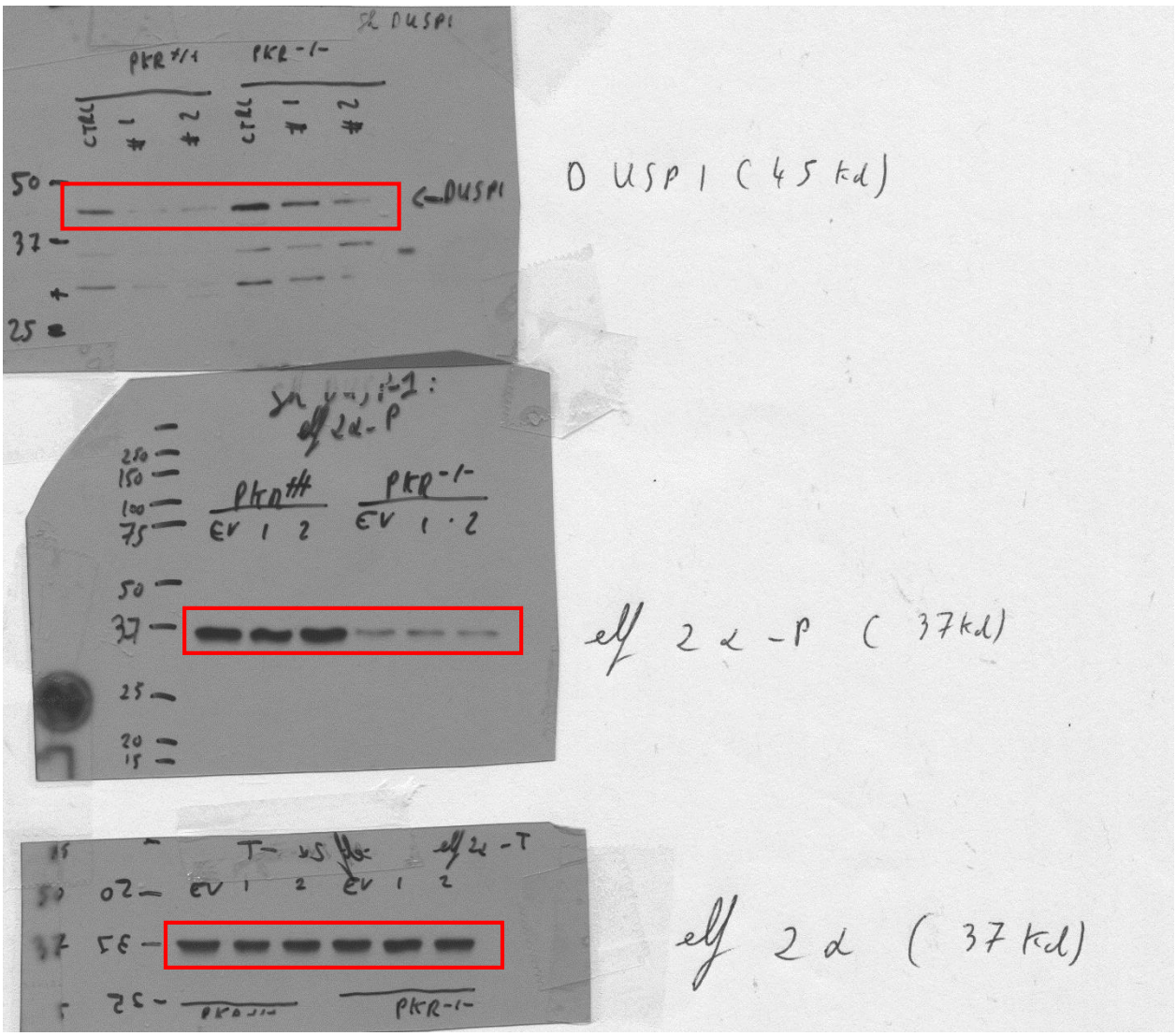


Blots of Figure 4a



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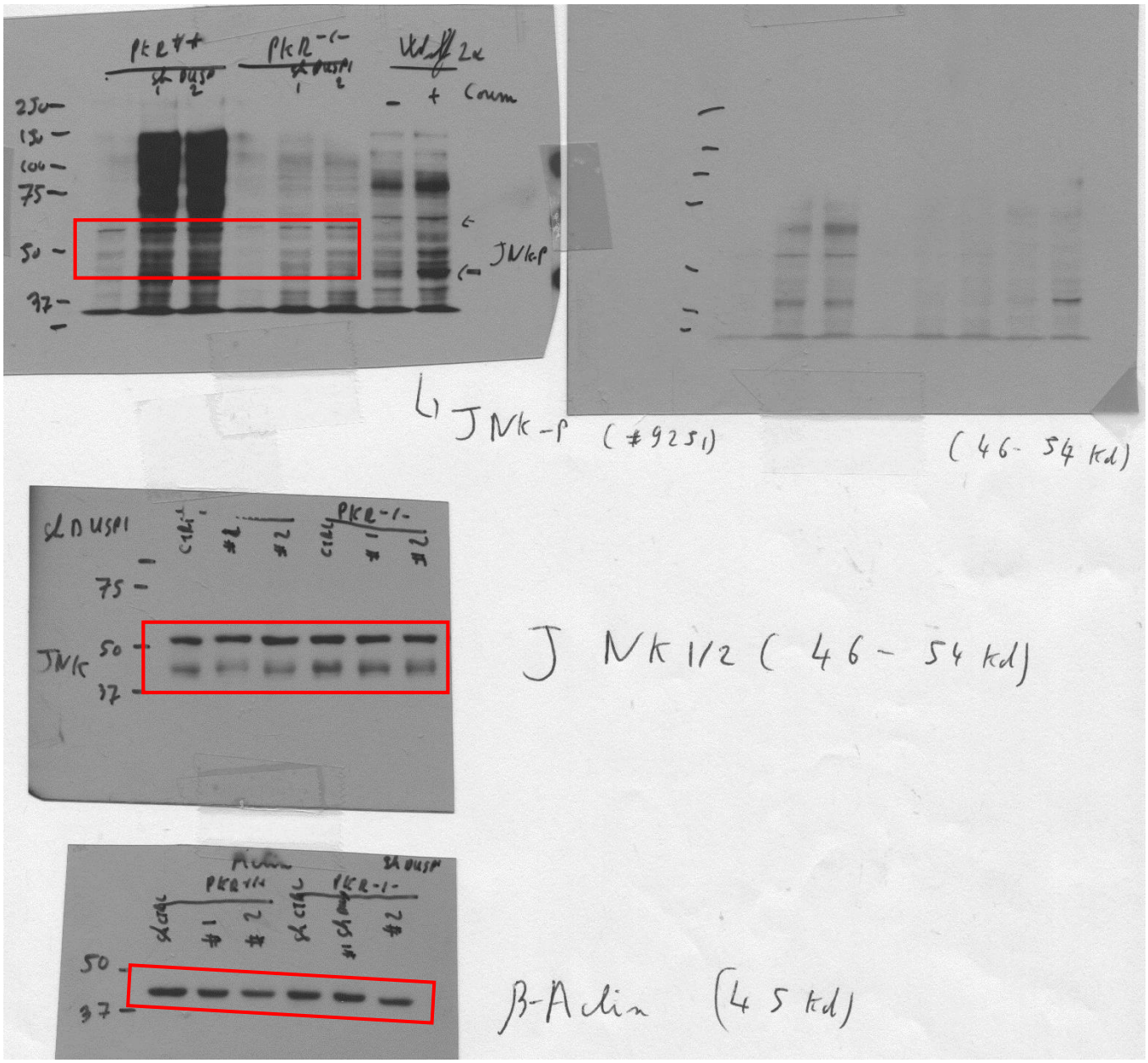
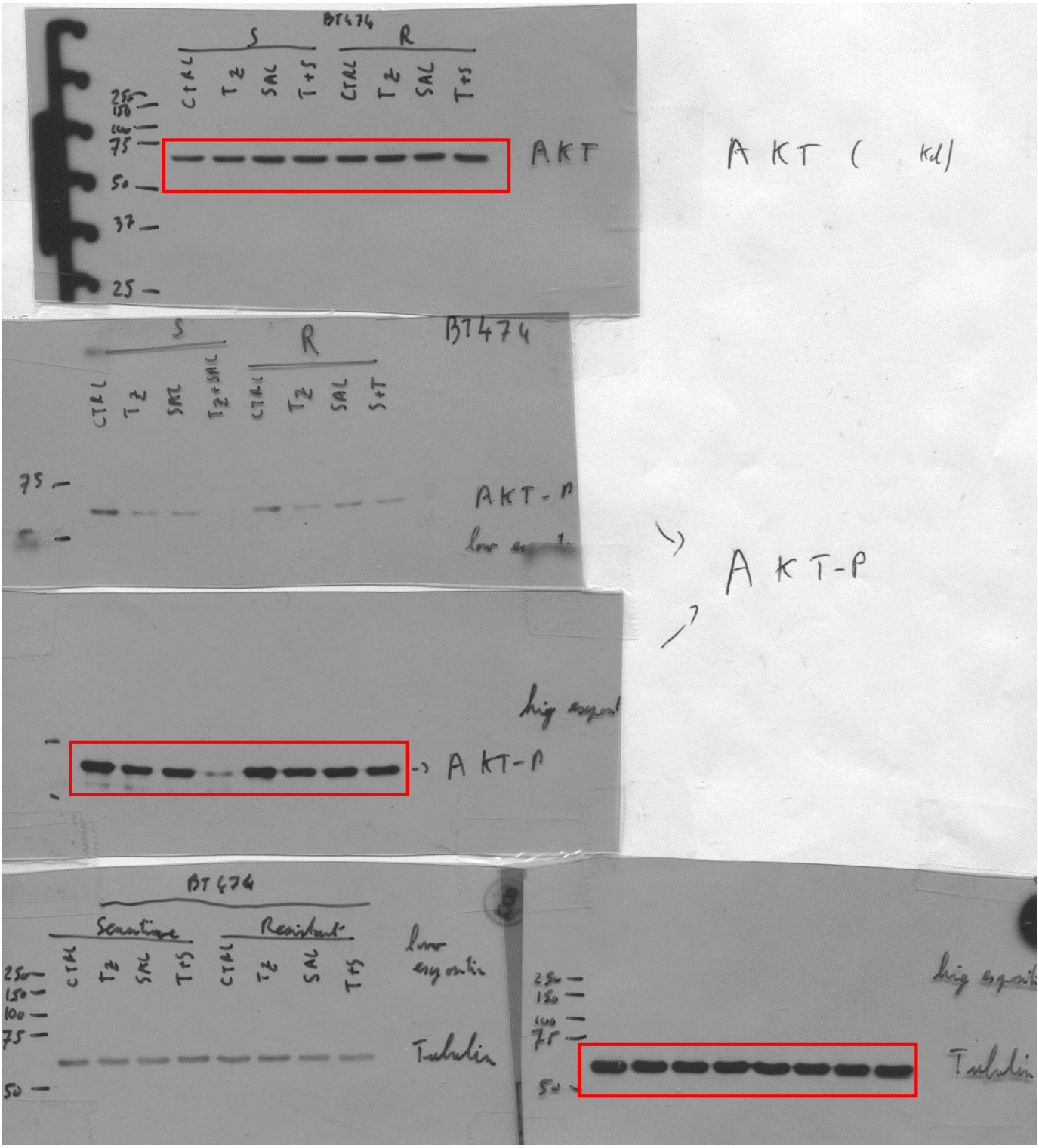


Blots of Figure 5a



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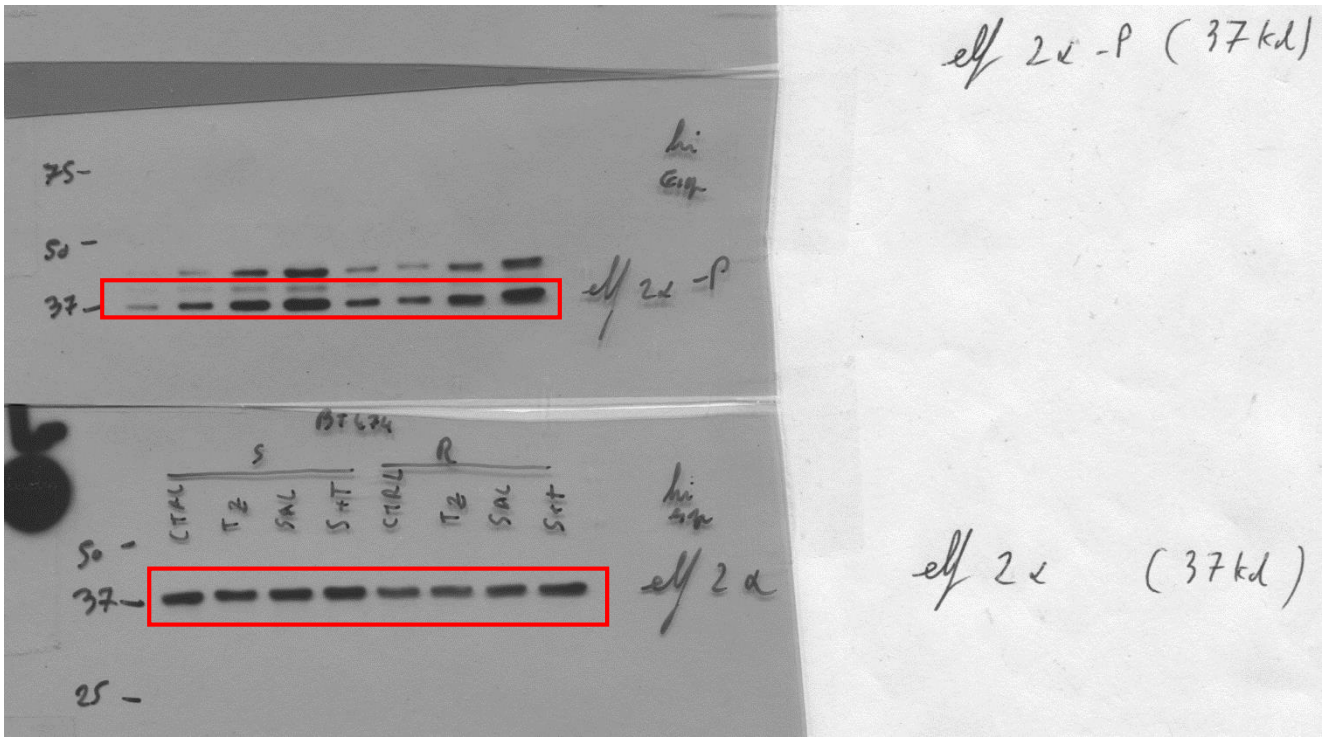

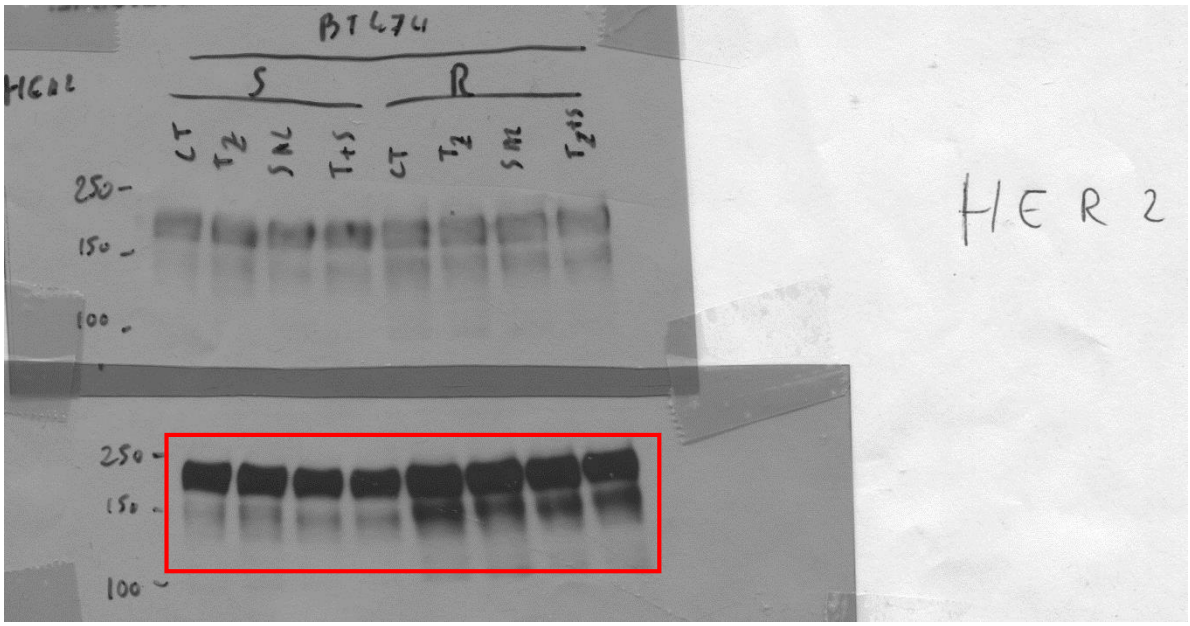

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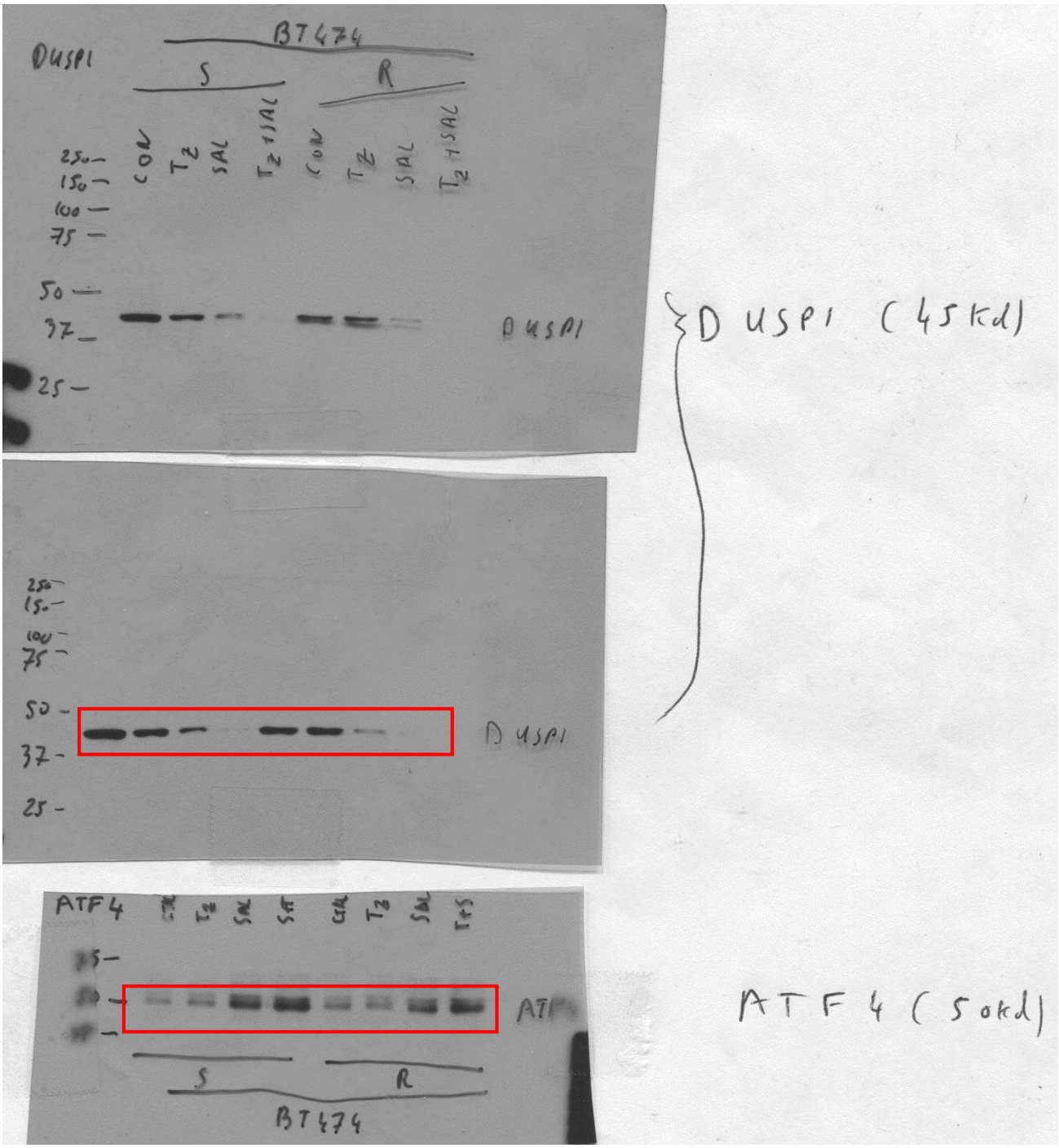

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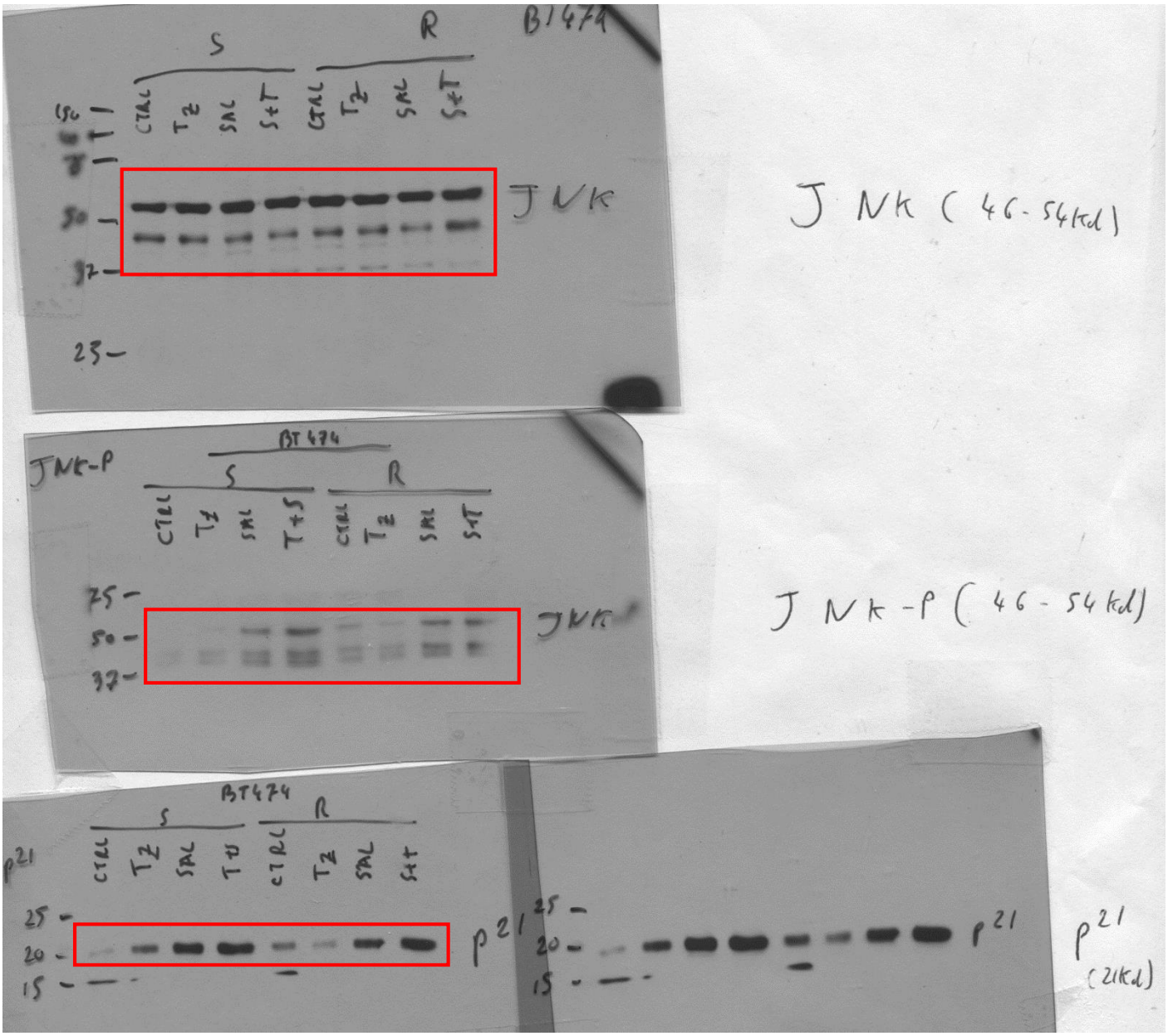
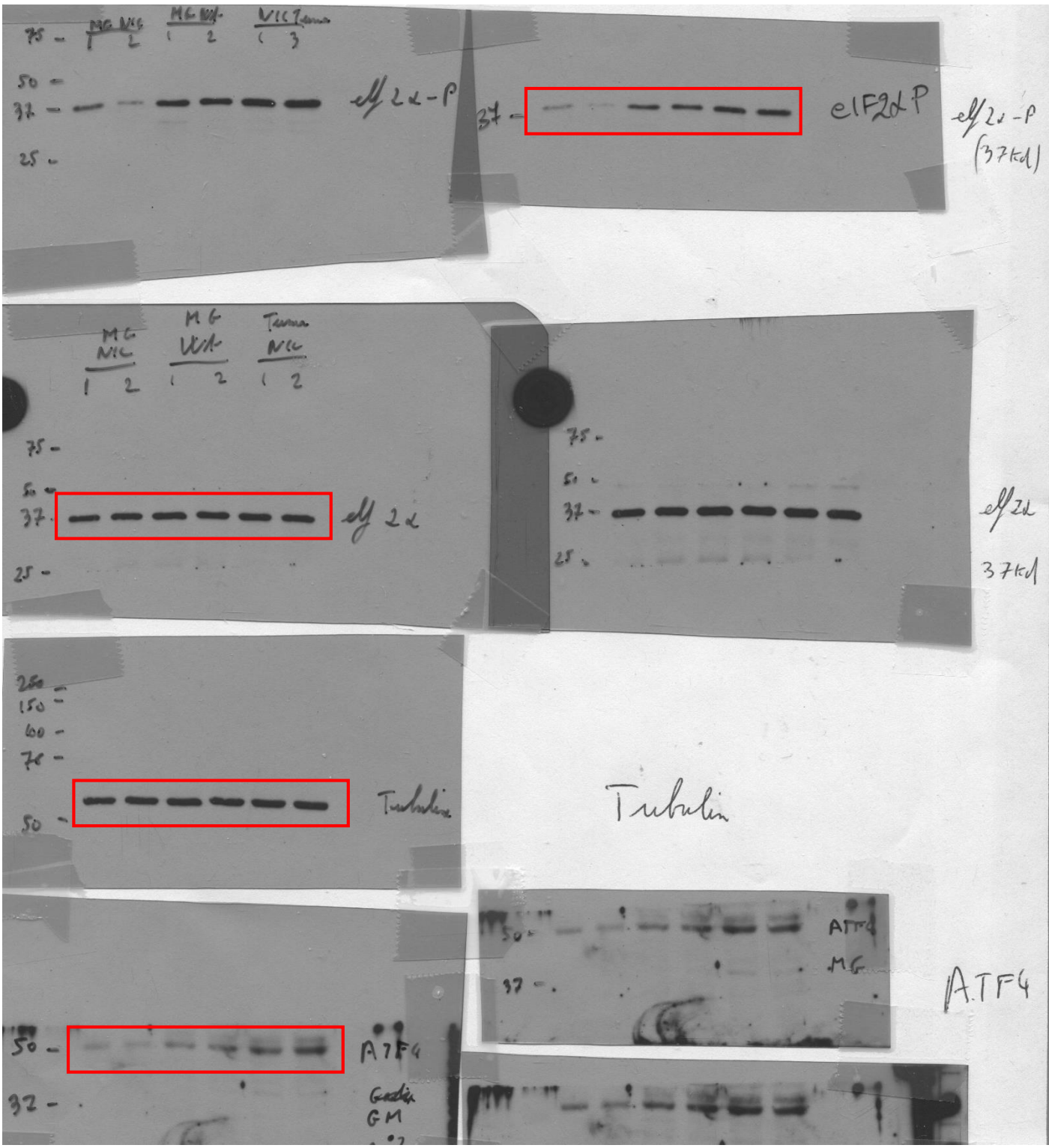


Blots of Supplementary Figure 1



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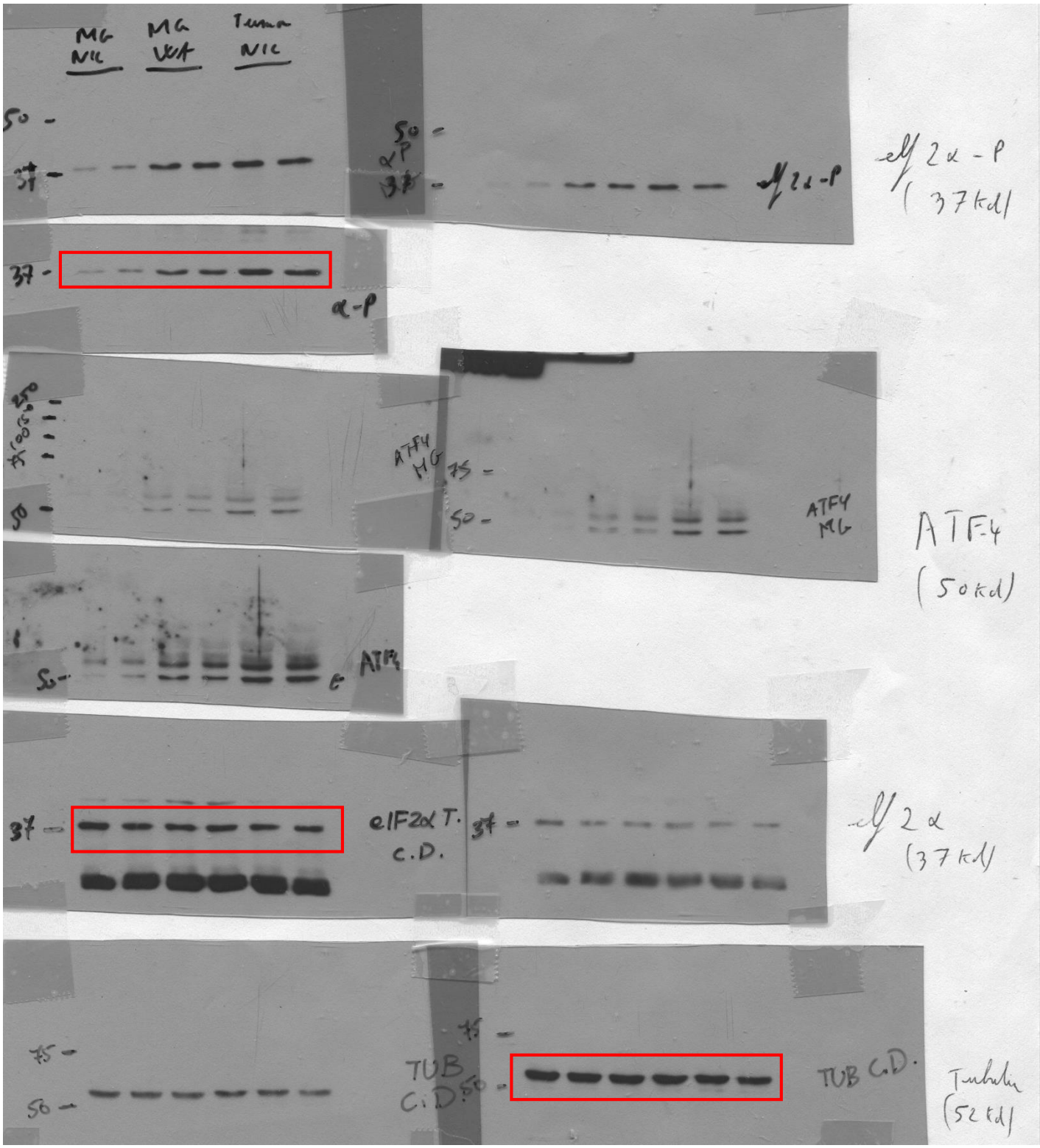
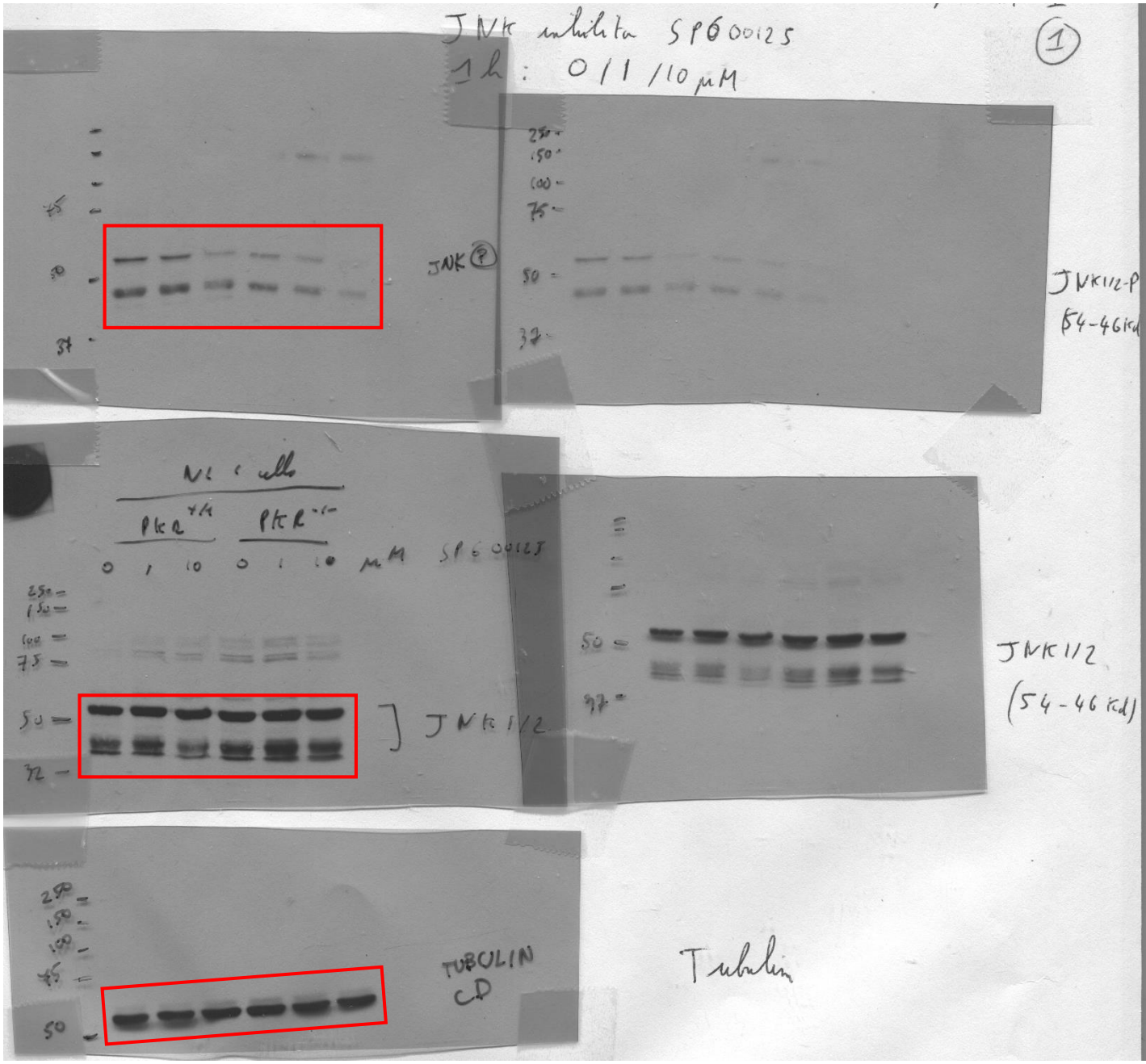


Blots of Supplementary Figure 4



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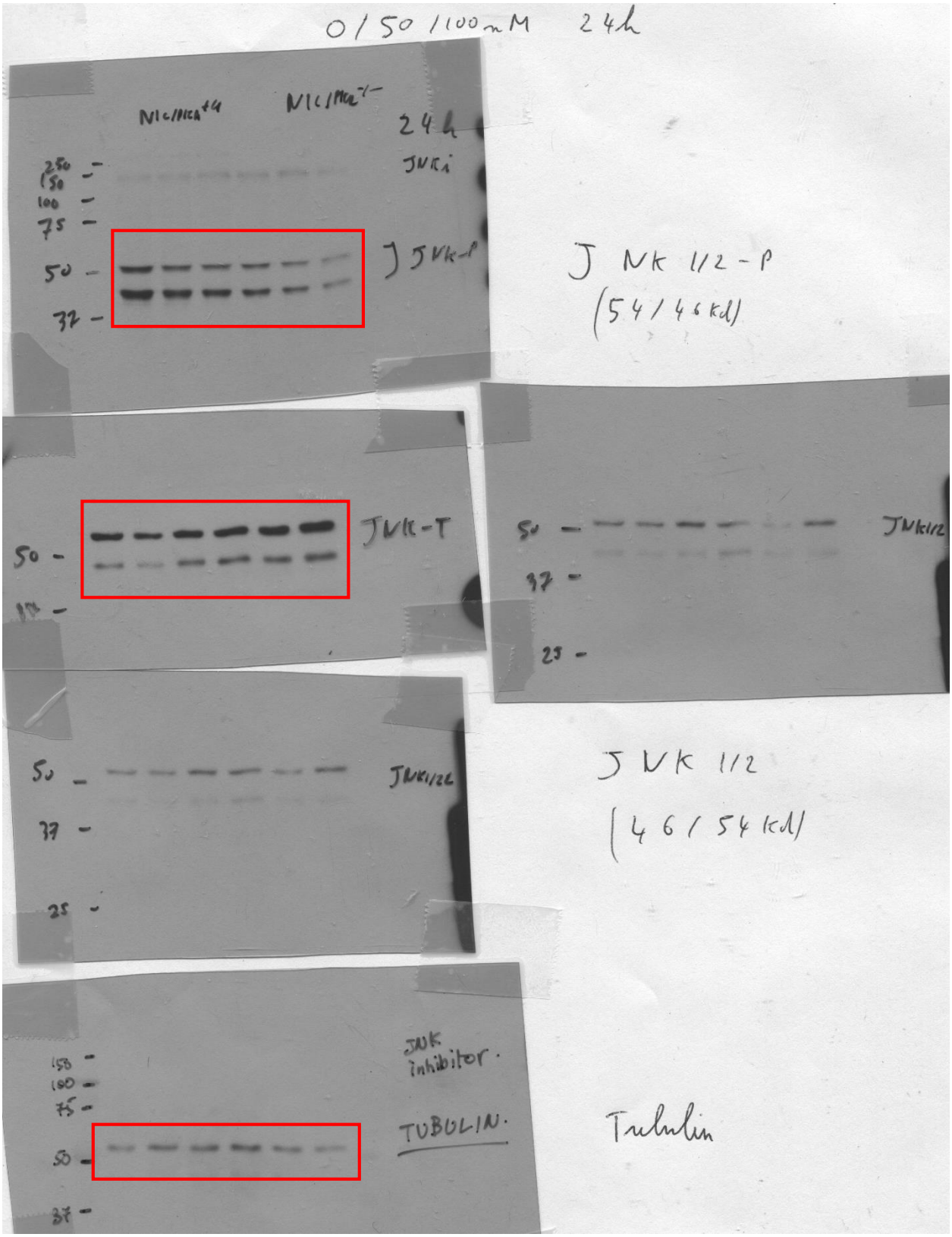

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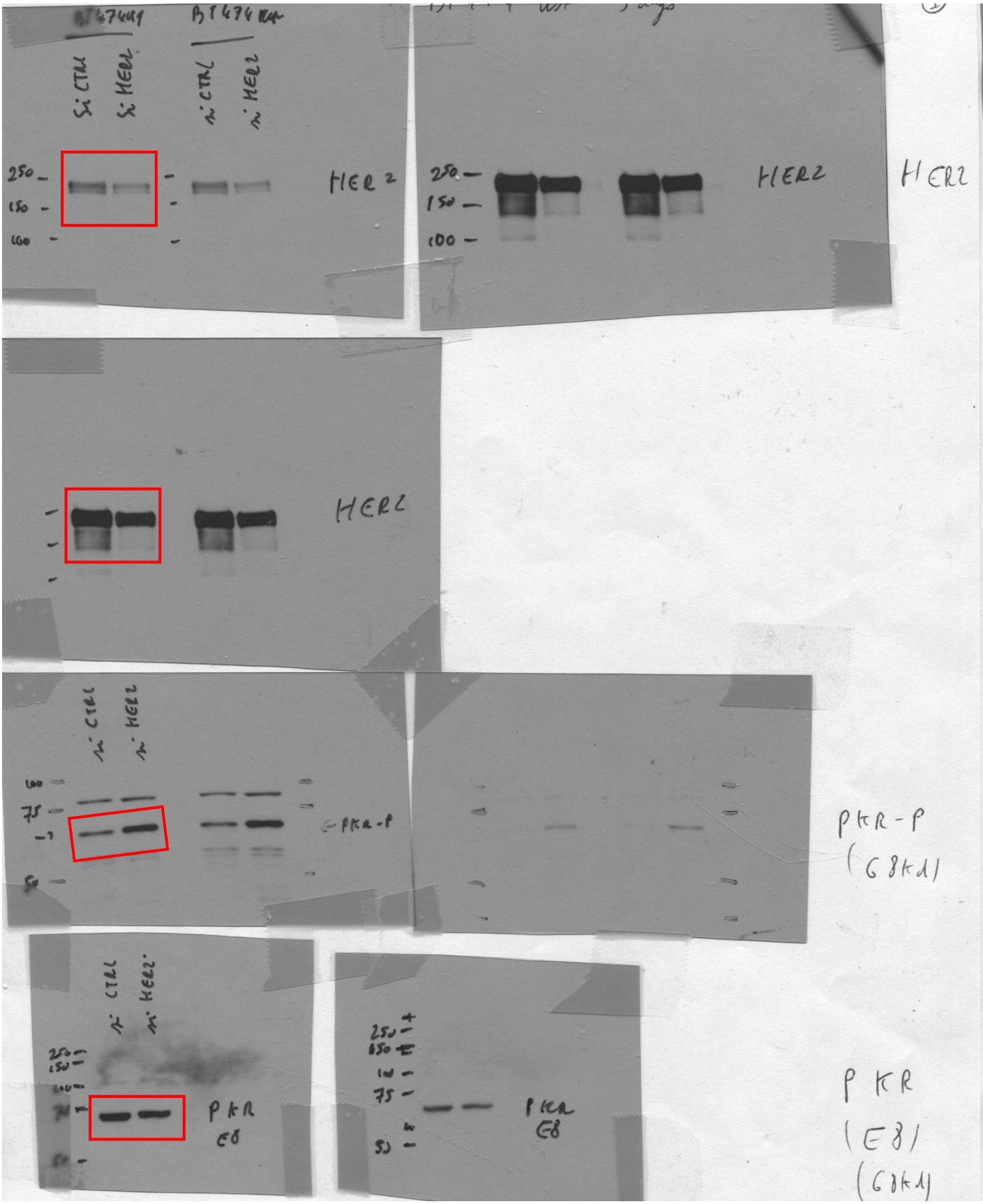

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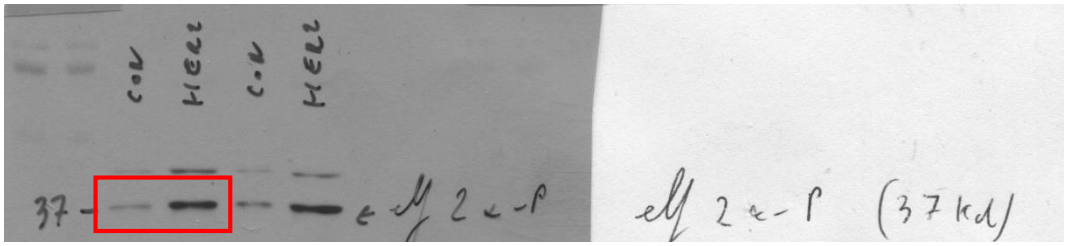
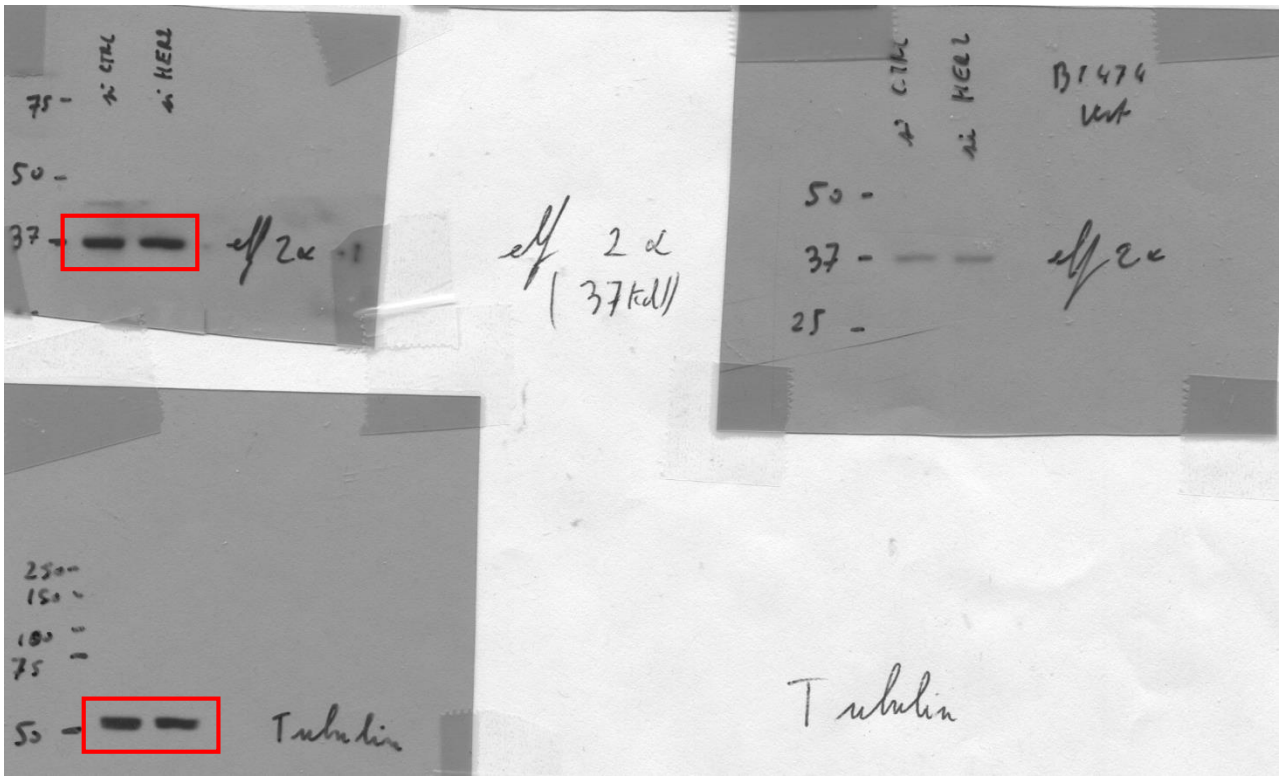
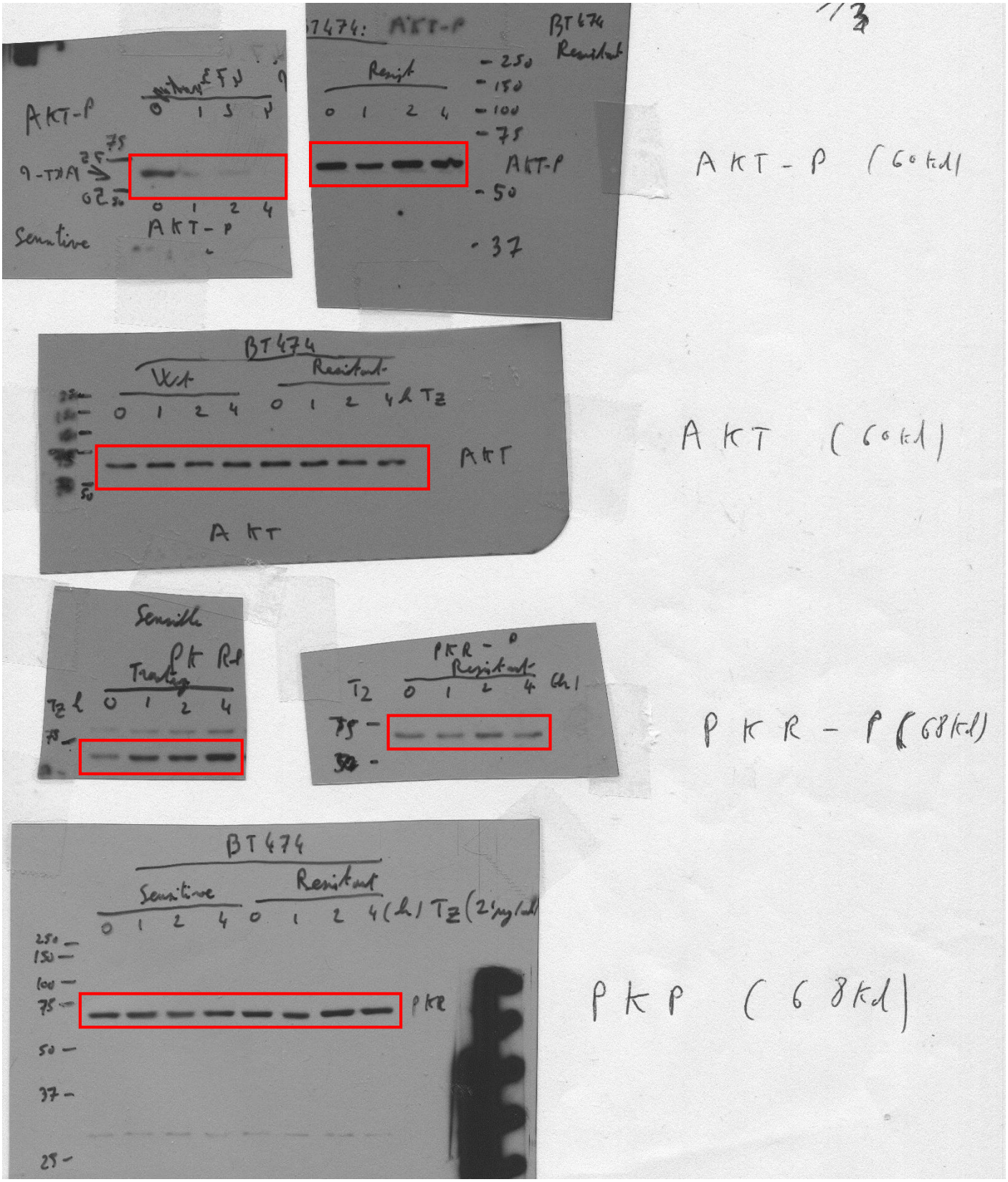




Blots of Supplementary Figure 5b



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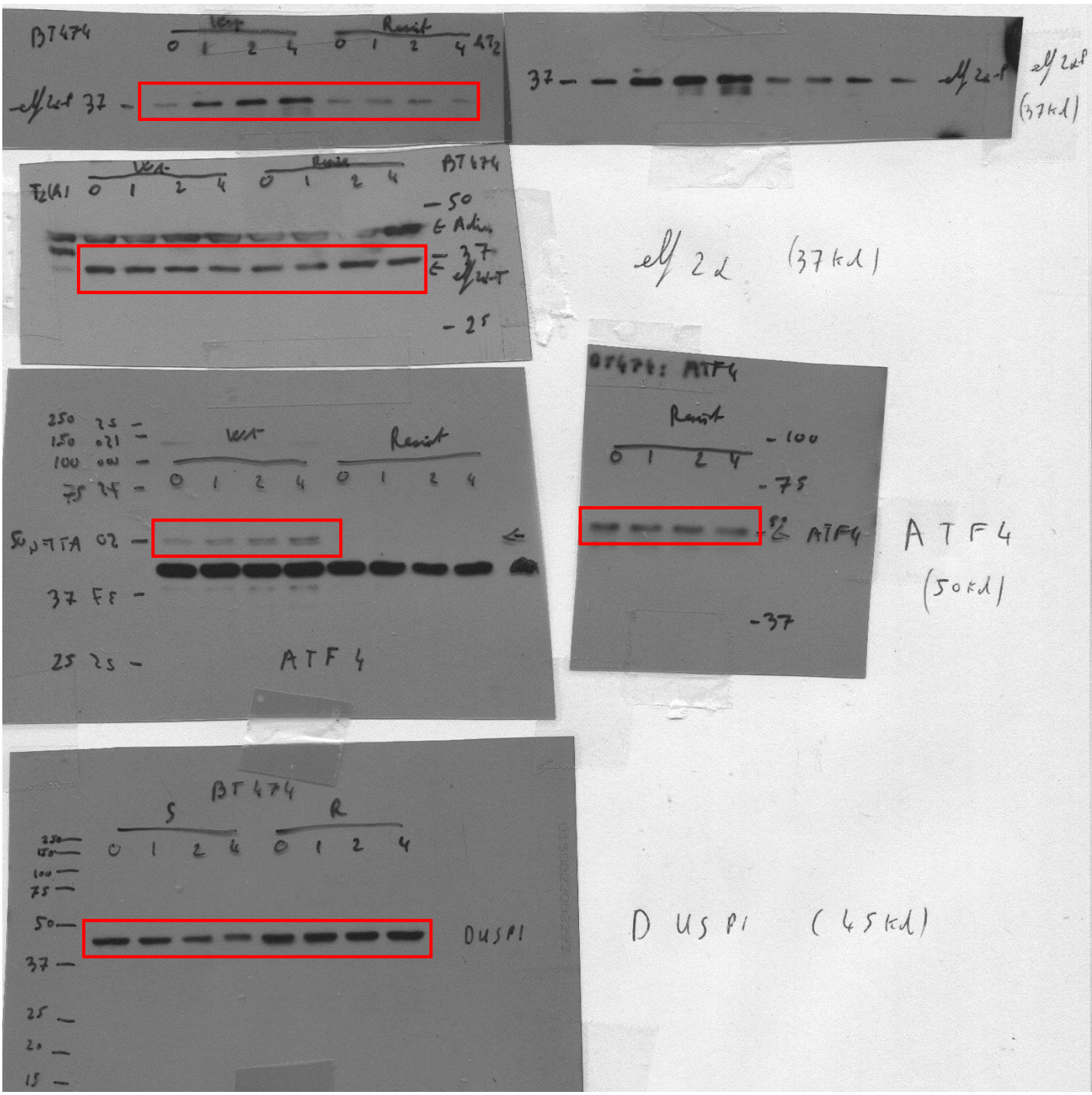

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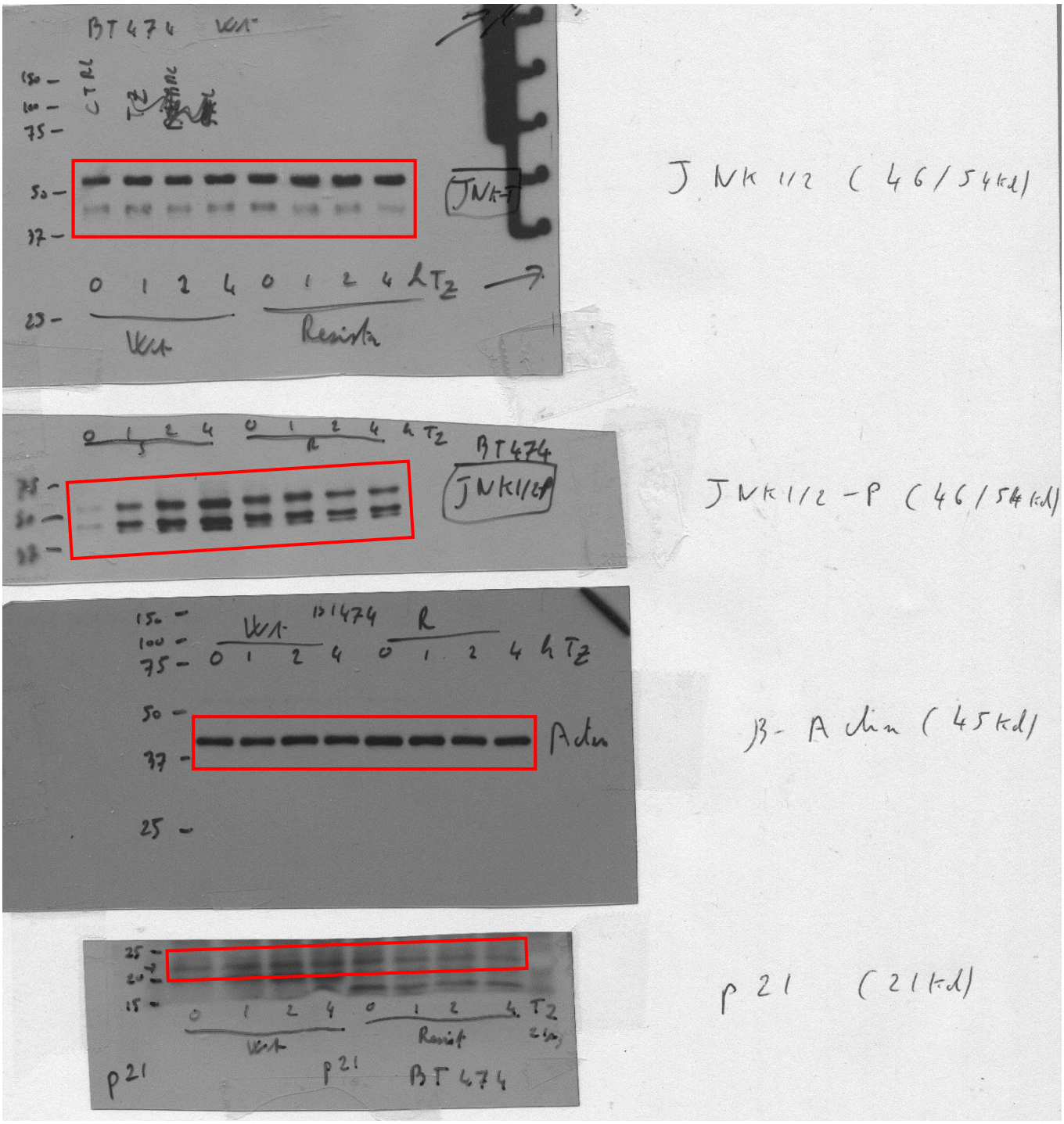
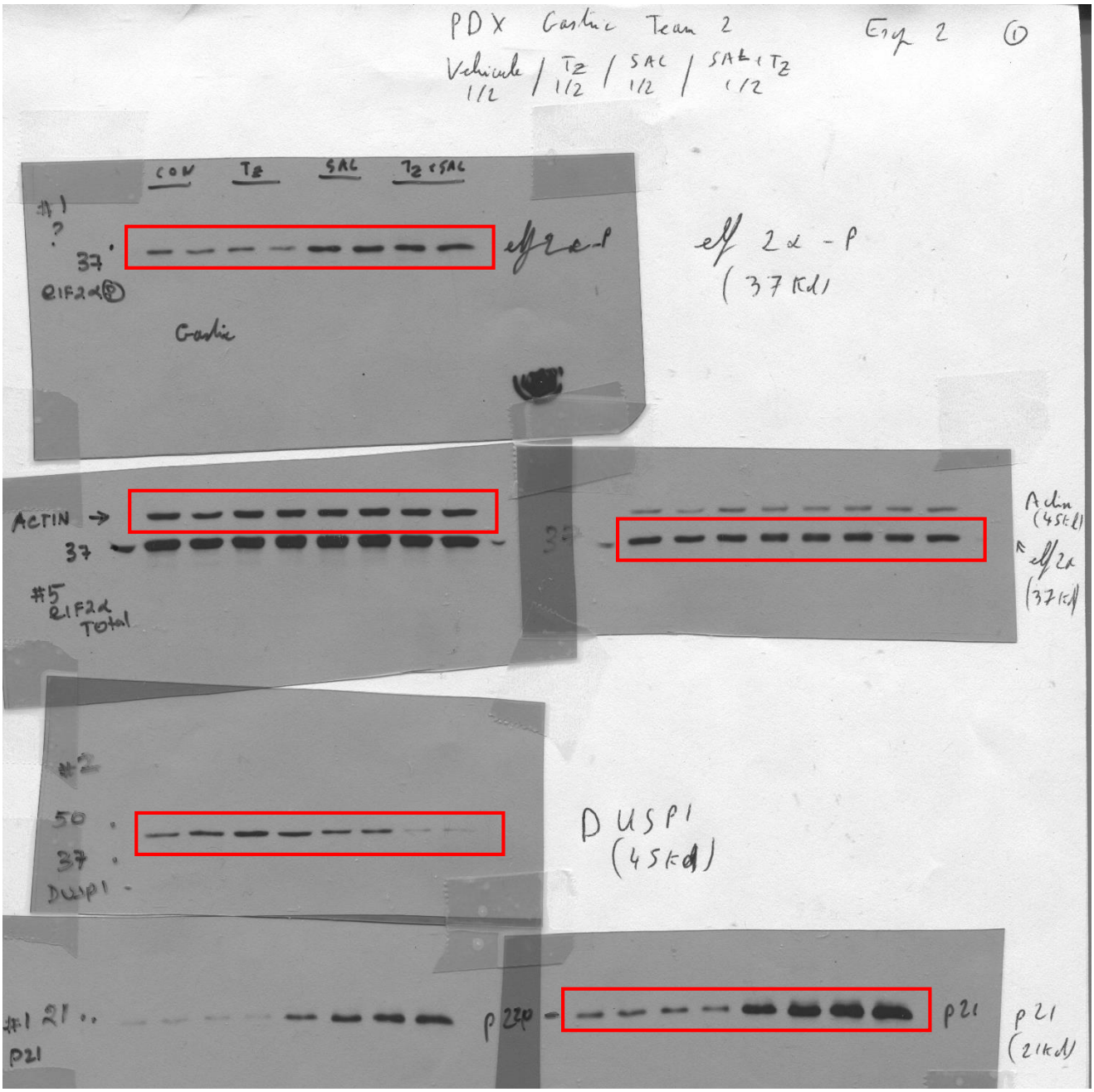


Blots of Supplementary Figure 7



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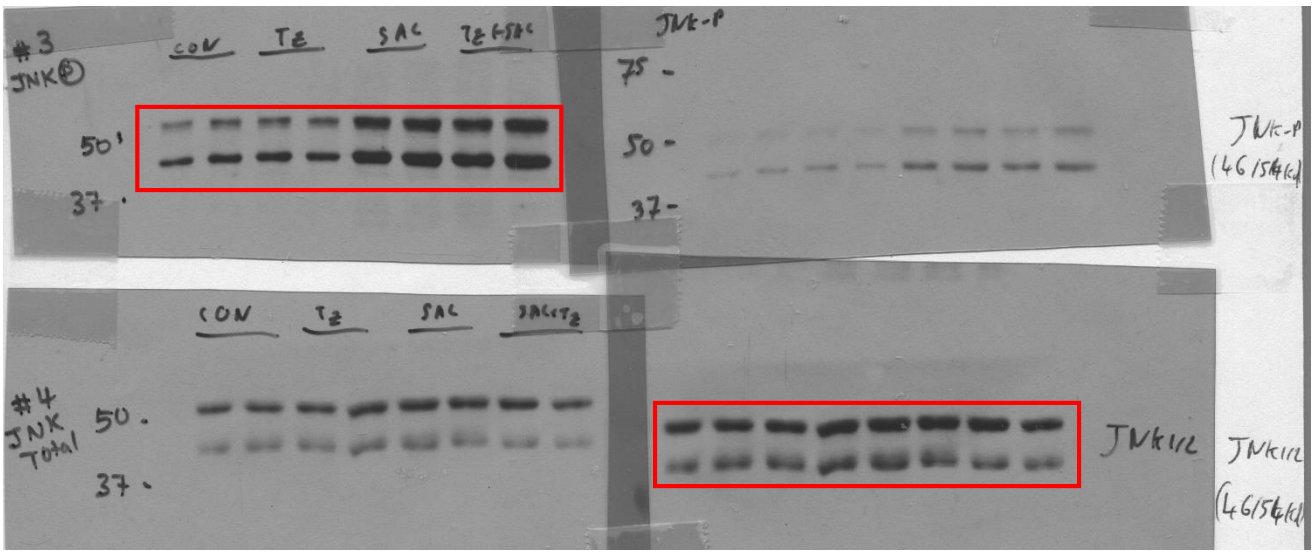

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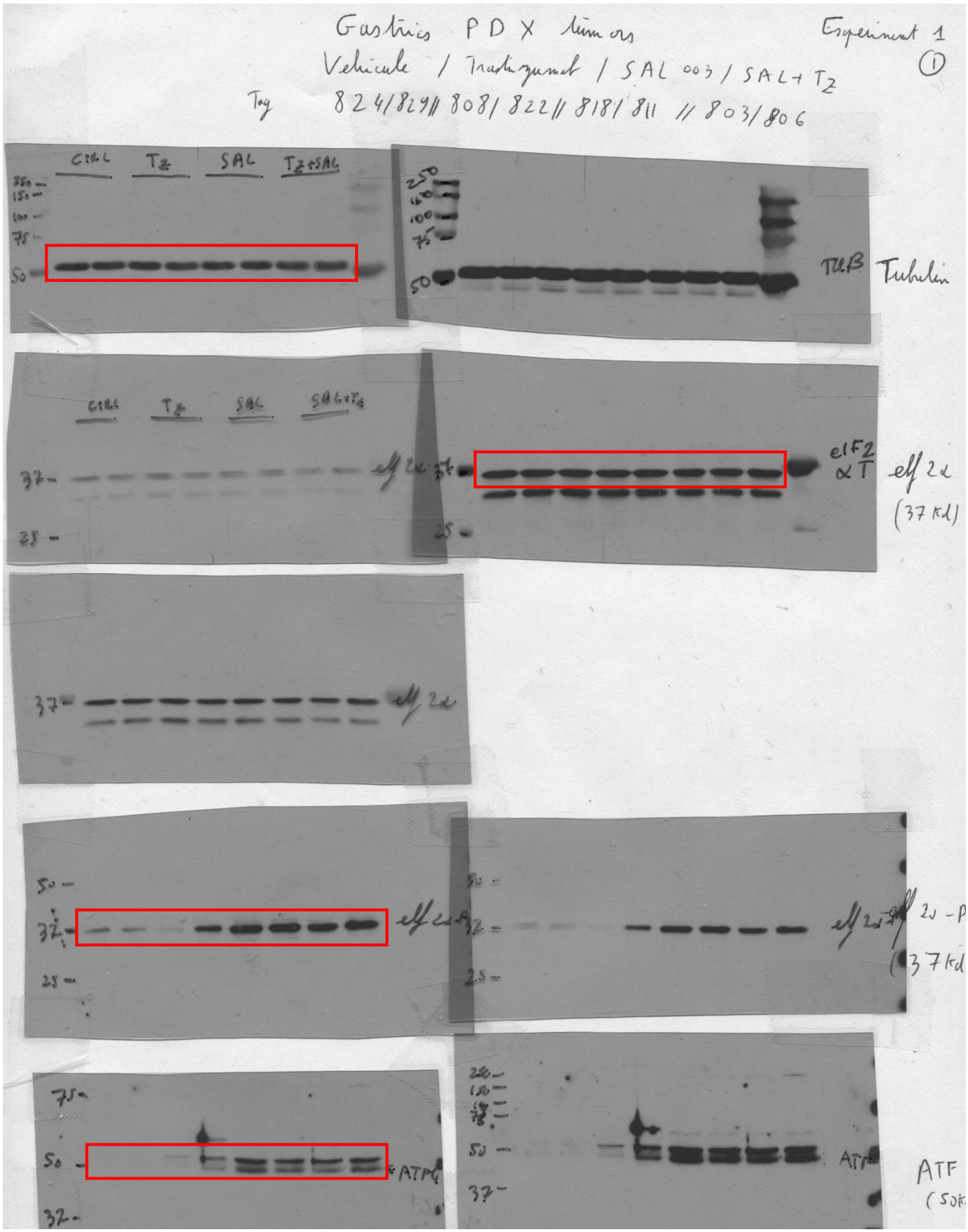

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