Scaffold function of long noncoding RNA *HOTAIR* in protein ubiquitination

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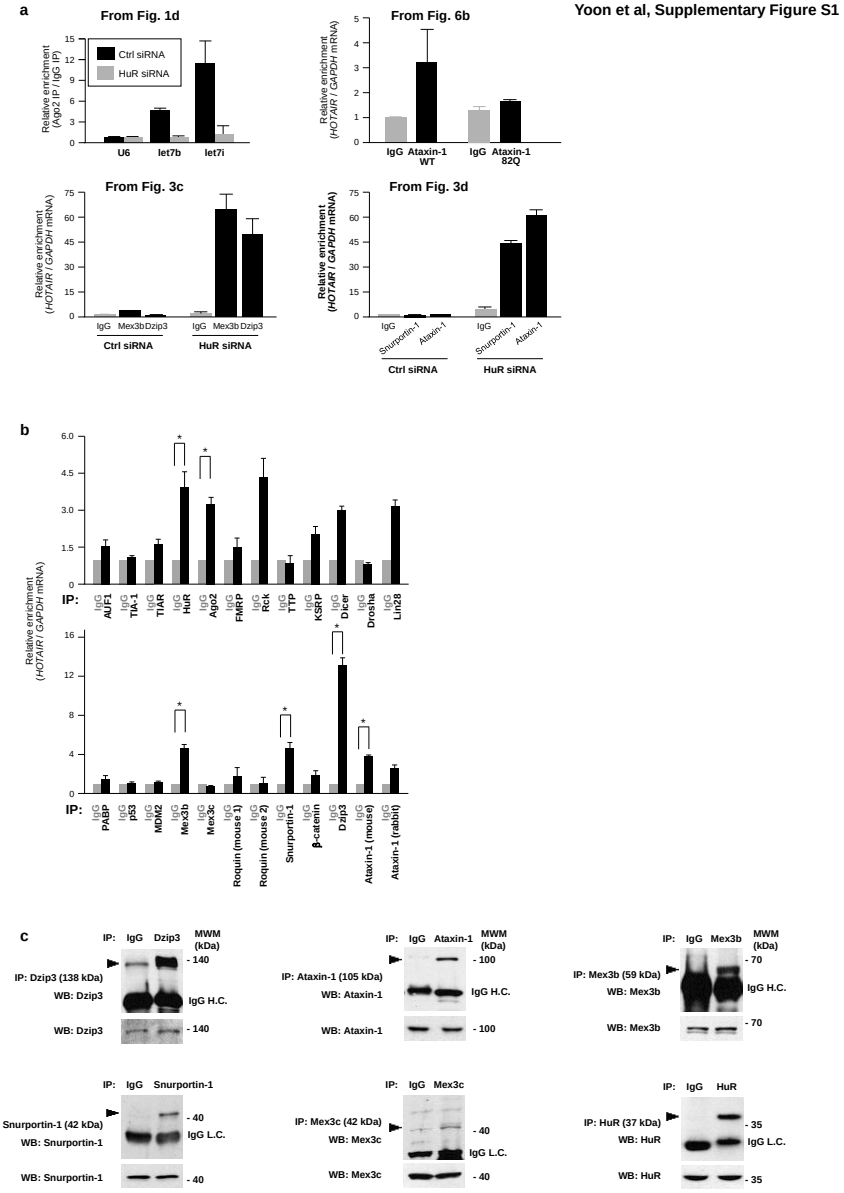
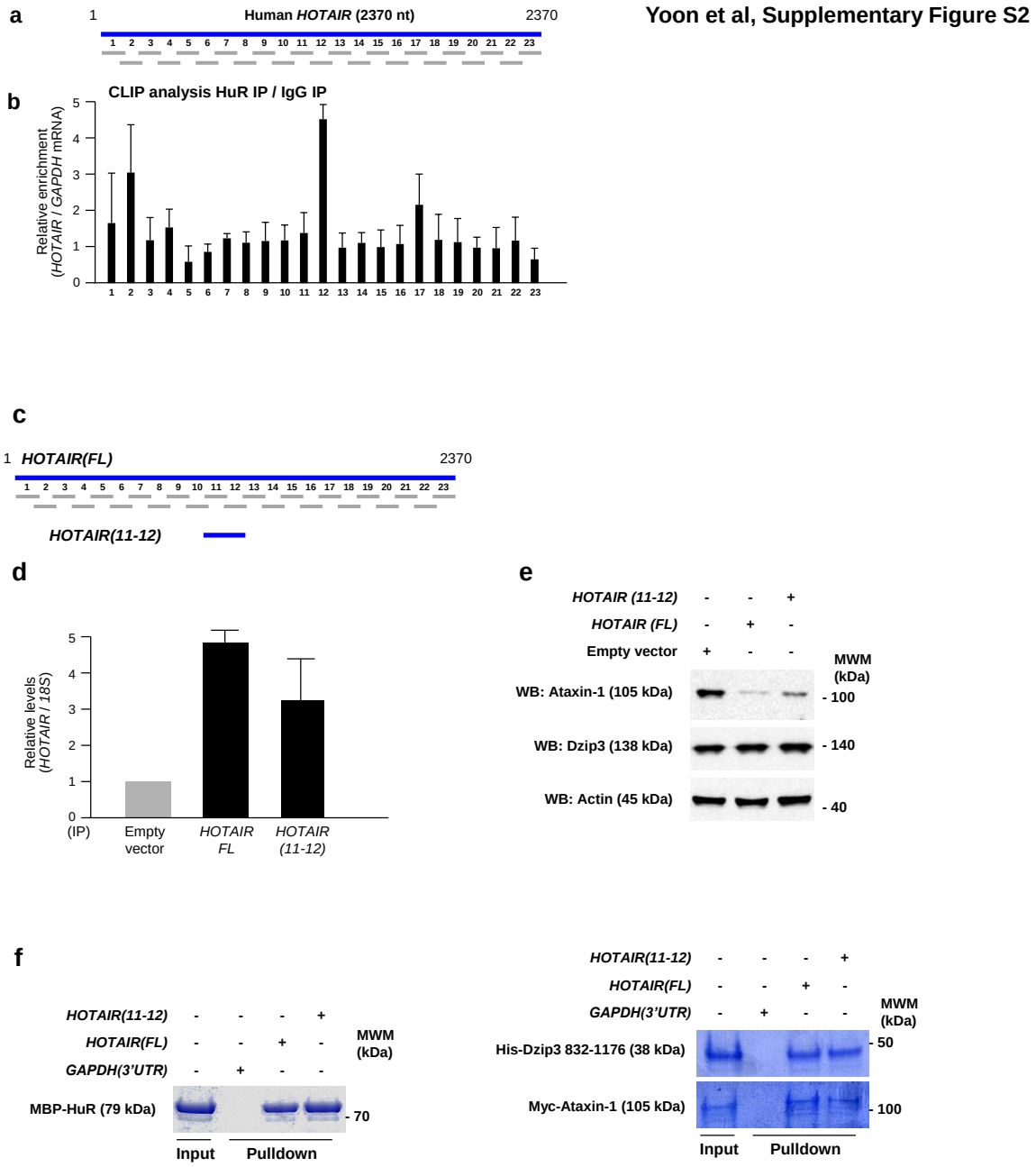
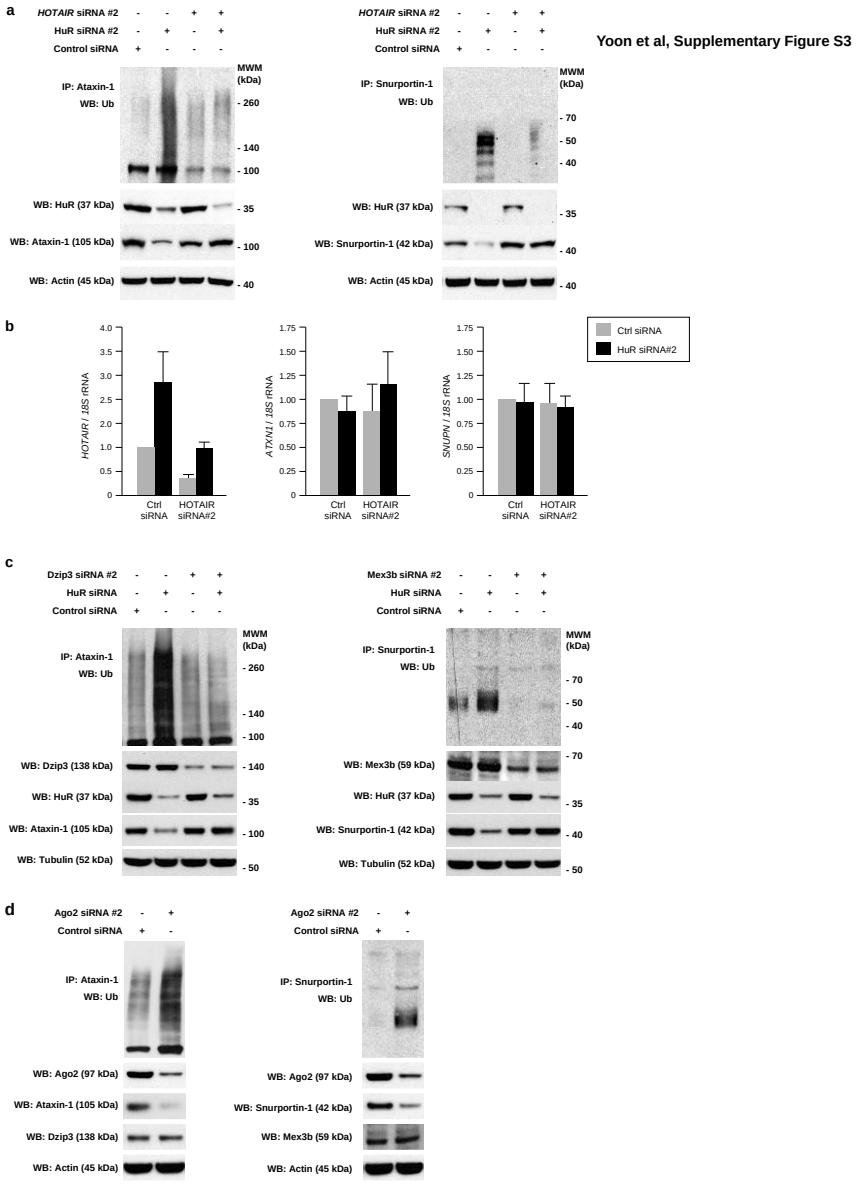
SUPPLEMENTARY FIGURES



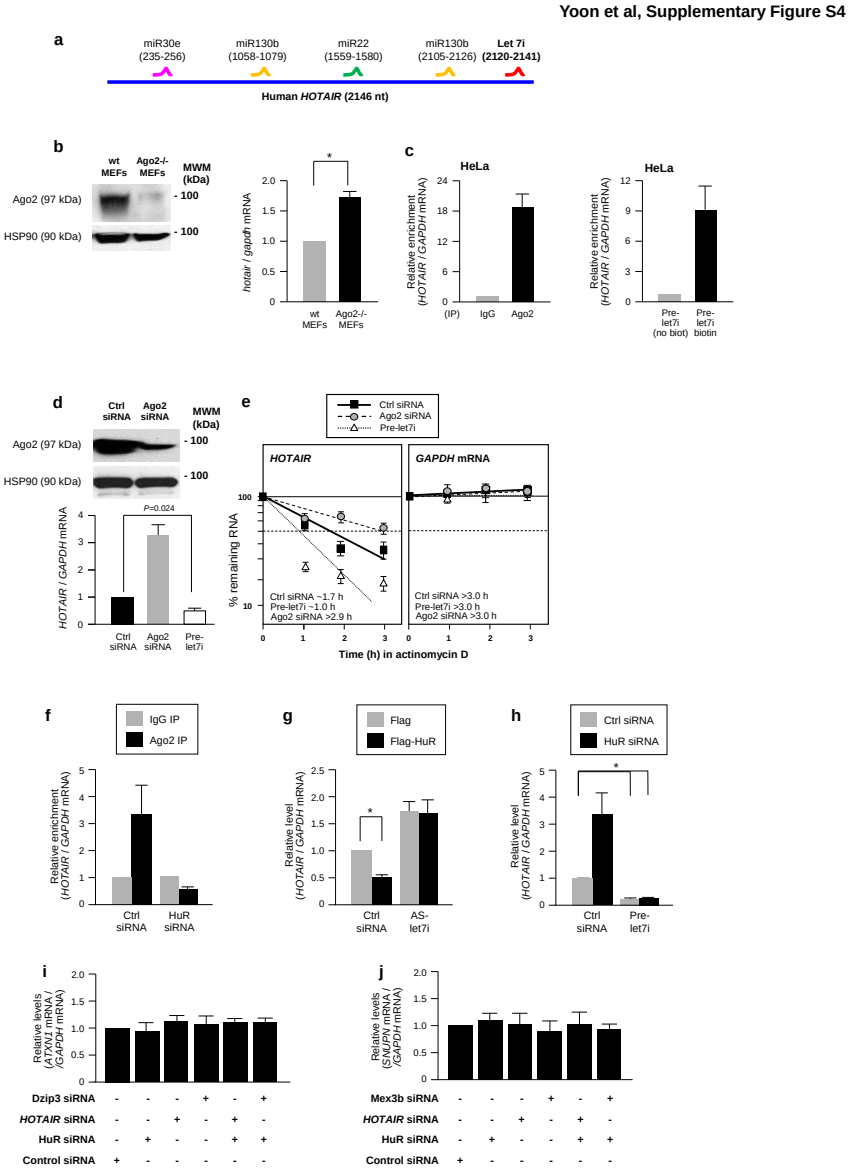
Supplementary Figure S1. Survey of RNA-binding proteins associated with *HOTAI* and IP control assays. (a) Alternative manner of representing RIP assays used in the main article, where the RNA enrichments in the IgG samples are not set as 1.0 but are set relative to the IgG in the respective control group. The graphs represent the means and S.D. from 3 independent experiments. **(b)** RIP analysis of the interaction of *HOTAIR* with a panel of RBPs in HeLa cell lysates. Antibodies recognizing the RBPs shown were used for IP in each case; control IP reactions were carried out using a corresponding IgG. *HOTAIR* levels were measured by RT-qPCR and normalized to the levels of *GAPDH* mRNA levels in the same IP samples measured by RT-qPCR analysis. Data were quantified as enrichment of *HOTAIR* in the RBP IP relative to the IgG IP. The graphs represent the means and S.D. from 3 independent experiments. *, $P < 0.05$ using Student's *t*-test. **(c)** Using HeLa cell lysates, the quality of the IP reactions was assayed by Western blot (WB) analysis of Dzip3, Ataxin-1, Mex3b, Mex3c, HuR, and Snurportin-1. Lysates were tested by WB analysis before (lower images for each protein tested) and after IP (top panels for each protein tested). IgG H.C., immunoglobulin heavy chain, IgG L.C., immunoglobulin light chain. Larger fields of blots are shown in Supplementary Figure S7.



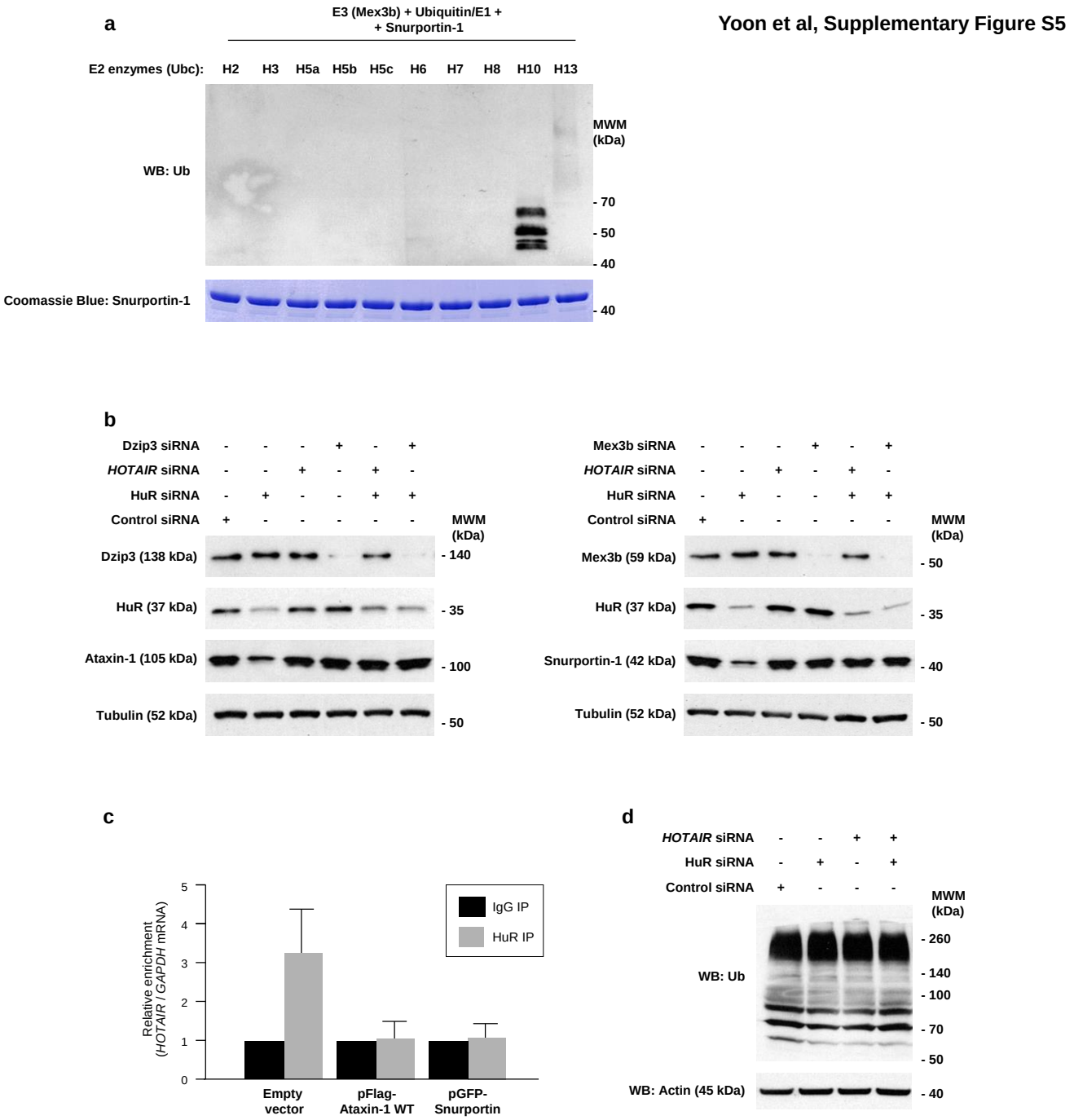
Supplementary Figure S2. Additional characterization of the interaction of *HOTAIR* with HuR, Dzip3, and Ataxin-1. (a,b) Schematic of *HOTAIR* (a) and the segments tested by CLIP analysis (b) following the procedure described in the Methods section, using 10 μg antibody and 15 mg of crosslinked lysate. **(c)** A plasmid expressing only segments 11 and 12 of *HOTAIR* (spanning nucleotide positions ~1028-1272, short blue line) was constructed and expressed in HeLa cells. **(d,e)** Forty-eight hours after transfecting HeLa cells with plasmids to express full-length [*HOTAIR(FL)*] or partial *HOTAIR* [*HOTAIR(11-12*)], as assessed by RT-qPCR (d), the levels of endogenous Ataxin-1, Dzip3, and Actin were determined by Western blot analysis (e). **(f)** Biotin pulldown analysis to determine the interaction of MBP-HuR (*left*) or His-Dzip3 (RNA binding domain) or Myc-Ataxin-1 (*right*) with biotinylated *HOTAIR(FL)*, *HOTAIR(11-12)* or control *GAPDH(3'UTR)*. In (b,d), the graphs reflect the means and S.D. from 3 independent experiments. Larger fields of blots are shown in Supplementary Figure S7.



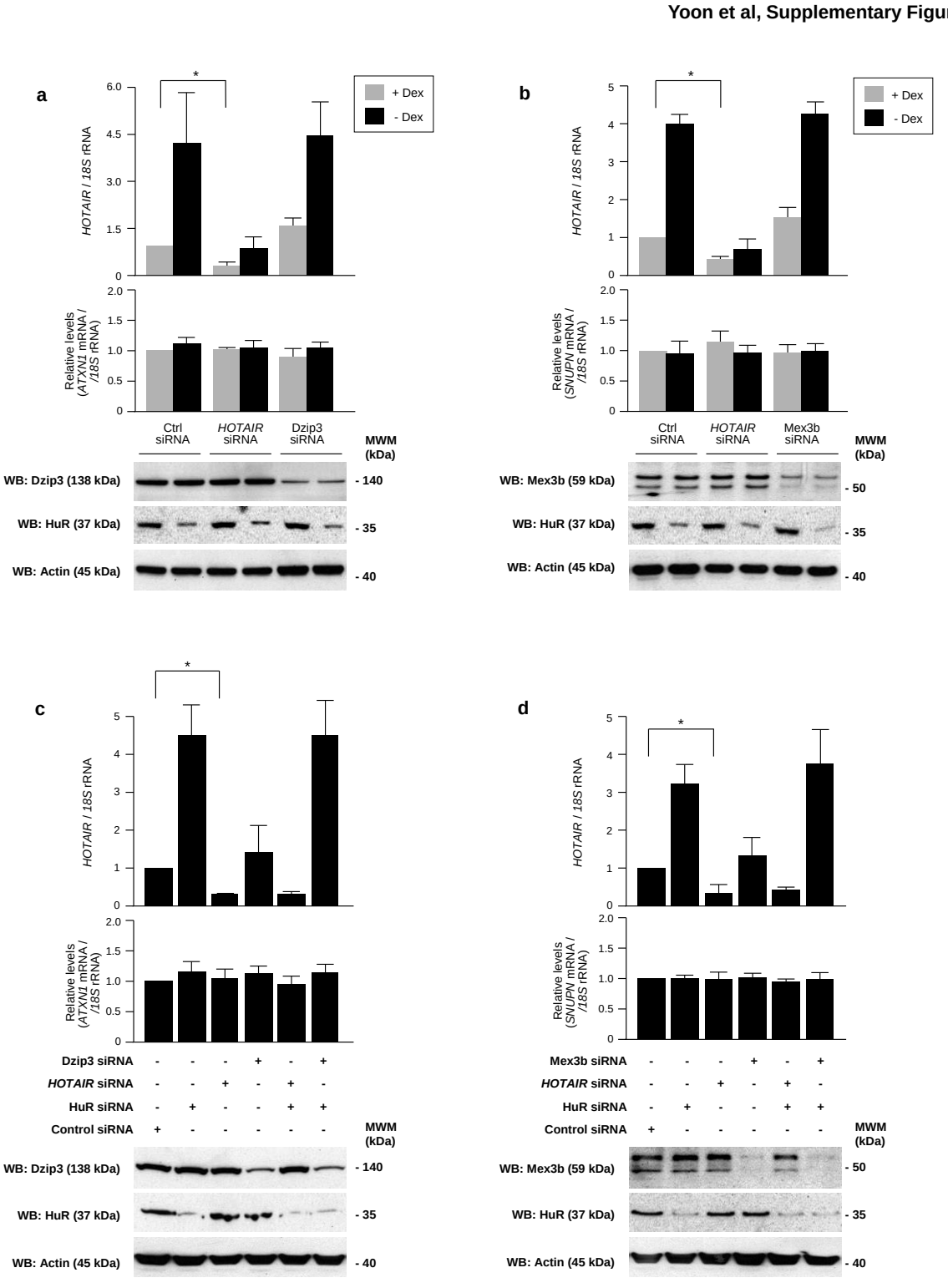
Supplementary Figure S3. Effects of additional *HOTAIR*- or HuR-directed siRNAs. (a) Forty-eight h after silencing HuR with siRNA#2 (Methods), the levels of HuR, Ataxin-1, Snurportin-1, and loading control Actin were assessed by Western blot analysis; the levels of ubiquitinated Ataxin-1, and ubiquitinated Snurportin-1, were assessed by IP of Ataxin-1 or Snurportin followed by ubiquitin Western blot analysis. **(b)** Forty-eight h after silencing *HOTAIR* with siRNA#2 (Methods) in the presence of either Ctrl siRNA or HuR siRNA#2, the levels of *HOTAIR*, *ATXN1* mRNA, and *SNUPN* mRNA relative to *18S* rRNA were assessed by RT-qPCR analysis. Data are the means and S.D. from 3 independent experiments. **(c)** Forty-eight h after silencing Dzip3 or Mex3b with siRNA#2 (Methods), the levels of HuR, Dzip3, Mex3b, Ataxin-1, Snurportin-1, and loading control Tubulin were assessed by Western blot analysis; the levels of ubiquitinated Ataxin-1, and ubiquitinated Snurportin-1, were assessed by IP of Ataxin-1 or Snurportin-1 followed by ubiquitin Western blot analysis. **(d)** Forty-eight h after silencing Ago2 with siRNA#2, the levels of Ago2, Dzip3, Mex3b, Ataxin-1, Snurportin-1, and loading control Actin were assessed by Western blot analysis; the levels of ubiquitinated Ataxin-1, and ubiquitinated Snurportin-1, were assessed by IP of Ataxin-1 or Snurportin followed by ubiquitin Western blot analysis. Larger fields of blots are shown in Supplementary Figure S7.



Supplementary Figure S4. HuR and let7/Ago2 cooperate in promoting *HOTAIR* decay. (a) *HOTAIR* sequence with predicted miRNA target sites. **(b)** In mouse embryonic fibroblasts (MEFs) isolated from Ago2 knockout mice (Ago2-/-) or wild type mice (wt), Ago2 and control HSP90 levels were assessed by Western blot analysis (*left*), and *HOTAIR* levels associated with Ago2 were assessed by RIP analysis followed by RT-qPCR (*right*). **(c)** *Left*, RIP analysis of Ago2-bound *HOTAIR* in HeLa cells. *Right*, 48 h after transfection of HeLa cells with let7i or biotin let7i, the relative enrichment of endogenous *HOTAIR* in biotin pulldown samples (isolated using streptavidin beads) was assessed by RT-qPCR quantitation of *HOTAIR*. **(d,e)** Forty-eight hours after silencing Ago2 or overexpressing pre-let7i in HeLa cells (d), the steady state levels and half-life of *HOTAIR* and control *GAPDH* mRNA were assessed as explained in Figure 2f (e). **(f)** Forty-eight hours after transfecting HeLa cells with HuR siRNA, the association of *HOTAIR* with Ago2 was assessed by RIP and RT-qPCR analysis. **(g,h)** Forty-eight hours after overexpressing Flag-HuR, silencing HuR, overexpressing let7i or expressing a let7i antagomir (AS-let7i), *HOTAIR* abundance was assessed by RT-qPCR. **(i,j)** The relative abundance of *HOTAIR*, *ATXN1*, *SNUPN* mRNA, and *GAPDH* mRNA in cells processed as shown in Fig. 4e,f was assessed by RT-qPCR analysis. In (b-j), the graphs reflect the means and S.D. from 3 independent experiments.*, P <0.05 using Student's t-test. Larger fields of blots are shown in Supplementary Figure S7.

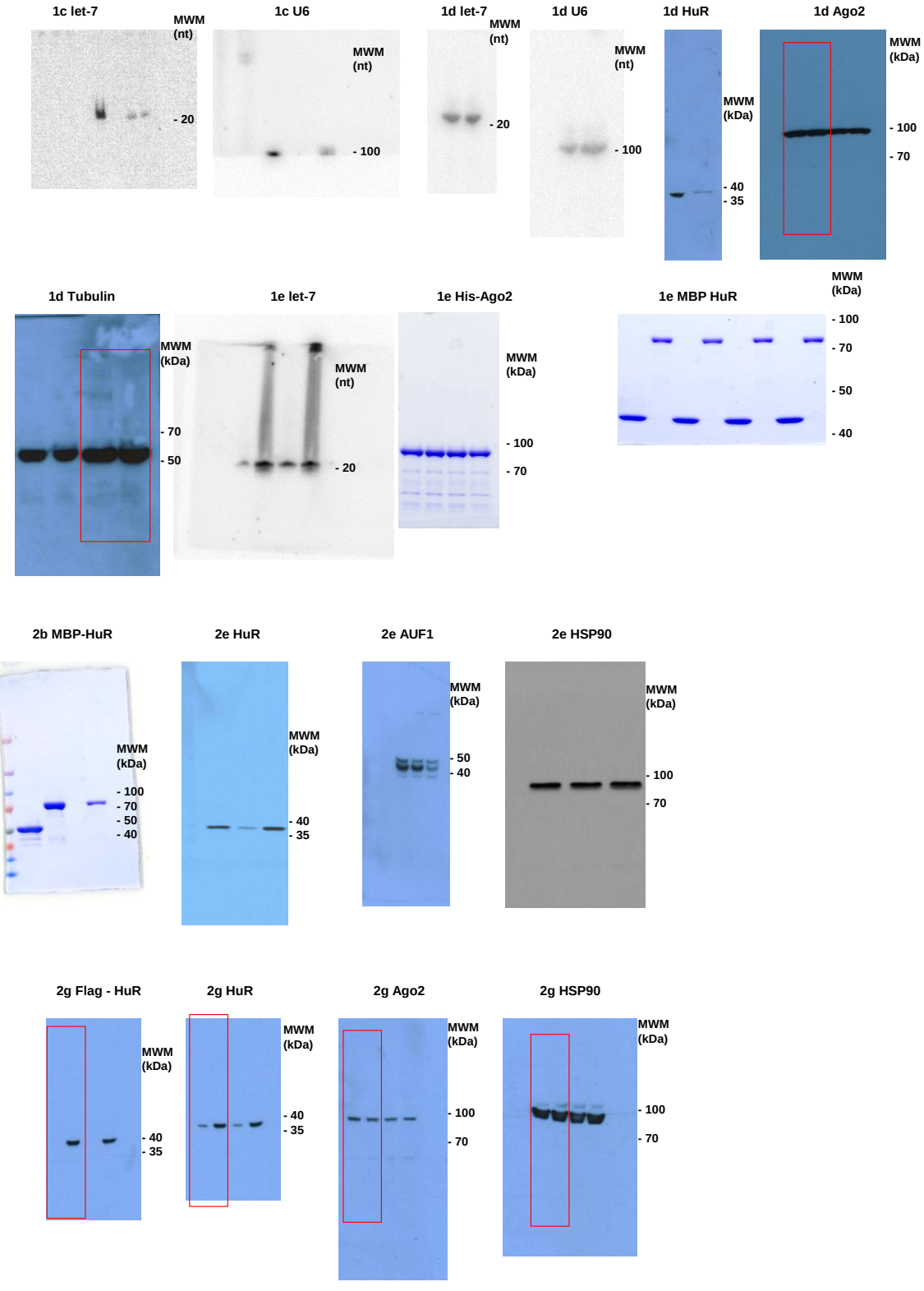


Supplementary Figure S5. Survey of Ubiquitin conjugating (Ubc) E2 enzymes for Snurportin-1, and analysis of silencing results. (a) Various E2 Ubc enzymes were assayed for their effect on ubiquitination of Snurportin-1 *in vitro* following the same procedure as that described in the main Fig. 5b. (**b)** Western blot analysis of the efficiency of silencing HuR, Dzip3, and Mex3b in HeLa cells used to measure the half-lives of Ataxin-1 and Snurportin-1. **(c)** Relative enrichment of *HOTAIR* compared to *GAPDH* mRNA from HuR IP after overexpression of empty vector, Flag-Ataxin-1 WT, or GFP-Snurportin. Data are the means and S.D. from 3 independent experiments. **(d)** Western blot analysis to detect ubiquitinated proteins in cell lysates after silencing HuR and/or *HOTAIR*. Larger fields of blots are shown in Supplementary Figure S7.



Supplementary Figure S6. RNA expression in IDH4 cells. (a,b) IDH4 cells [proliferating (+Dex) or senescent (-Dex)] were transfected as described in Fig. 6e; 5 days later, the levels of *HOTAIR*, *18S* rRNA, *ATXN1* mRNA (a) and *SNUPN* mRNA (b) were quantified and plotted. **(c,d)** Proliferating IDH4 cells were transfected as in Fig. 8; 48 h later, the levels of *HOTAIR*, *18S* rRNA, *ATXN1* mRNA (c) and *SNUPN* mRNA (d). In (a-d) the graphs reflect the means and S.D. from 3 independent experiments. .*, $P < 0.05$ using Student's *t*-test. Larger fields of blots are shown in Supplementary Figure S7.

Yoon et al, Supplementary Figure S7



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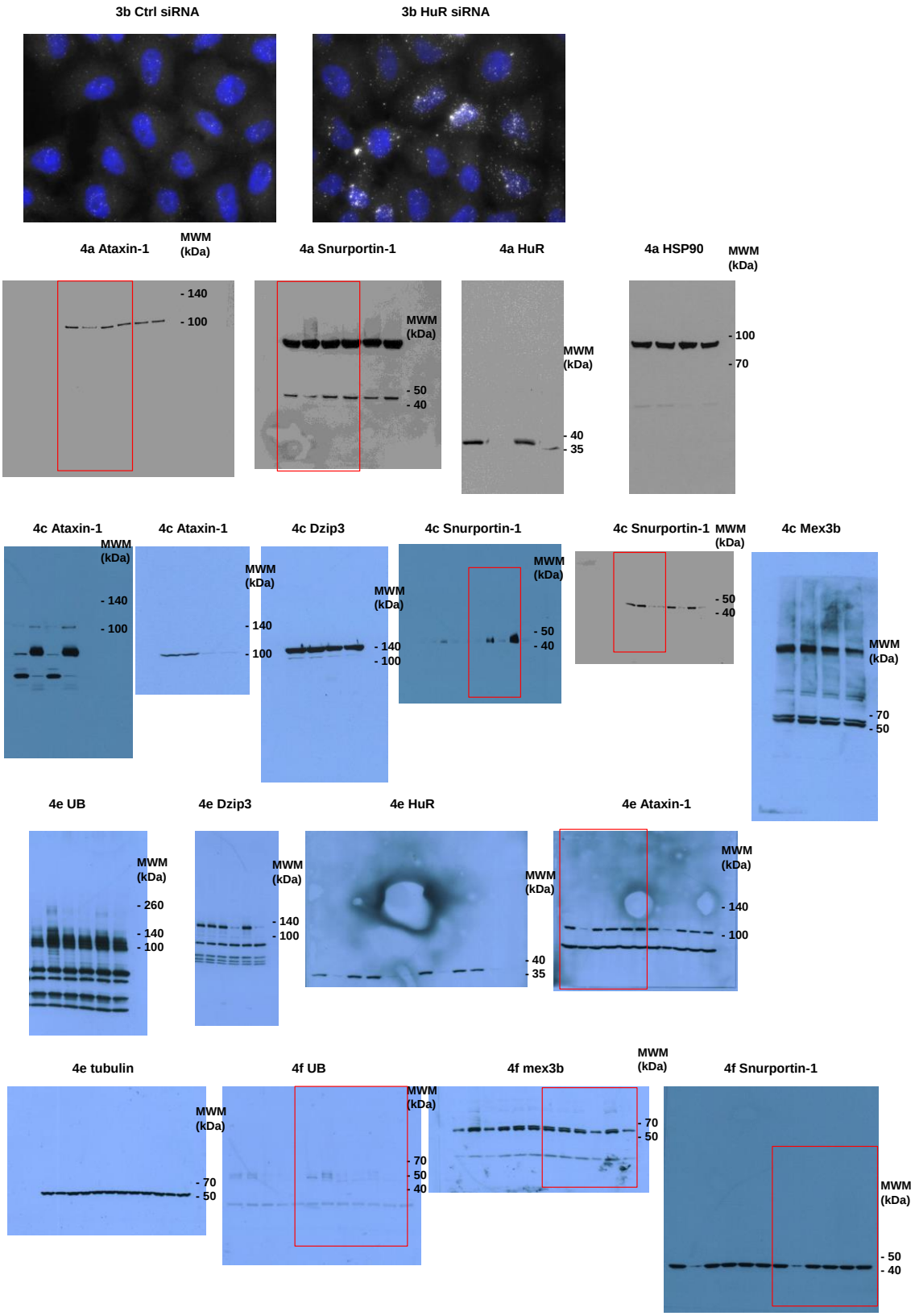

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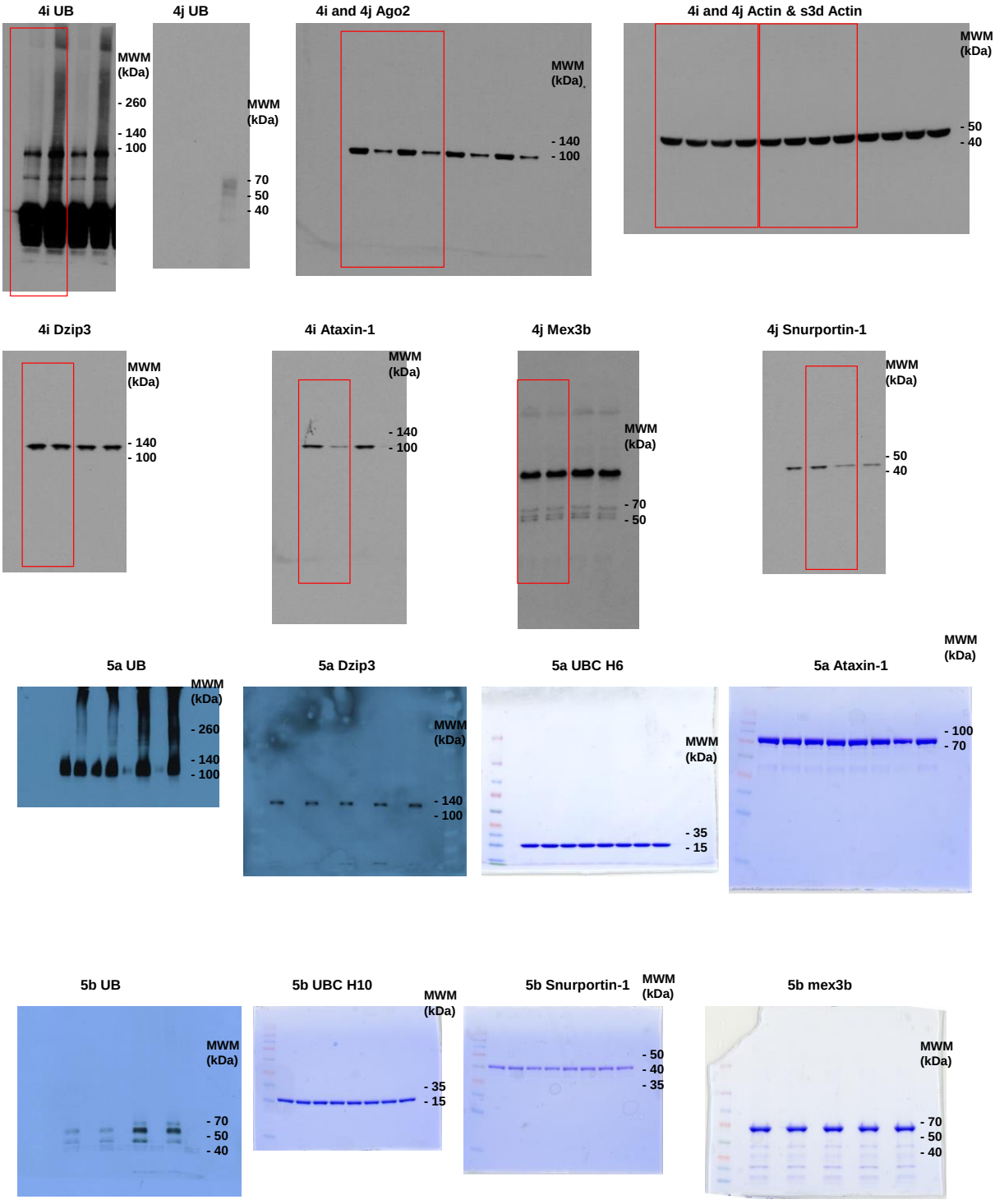

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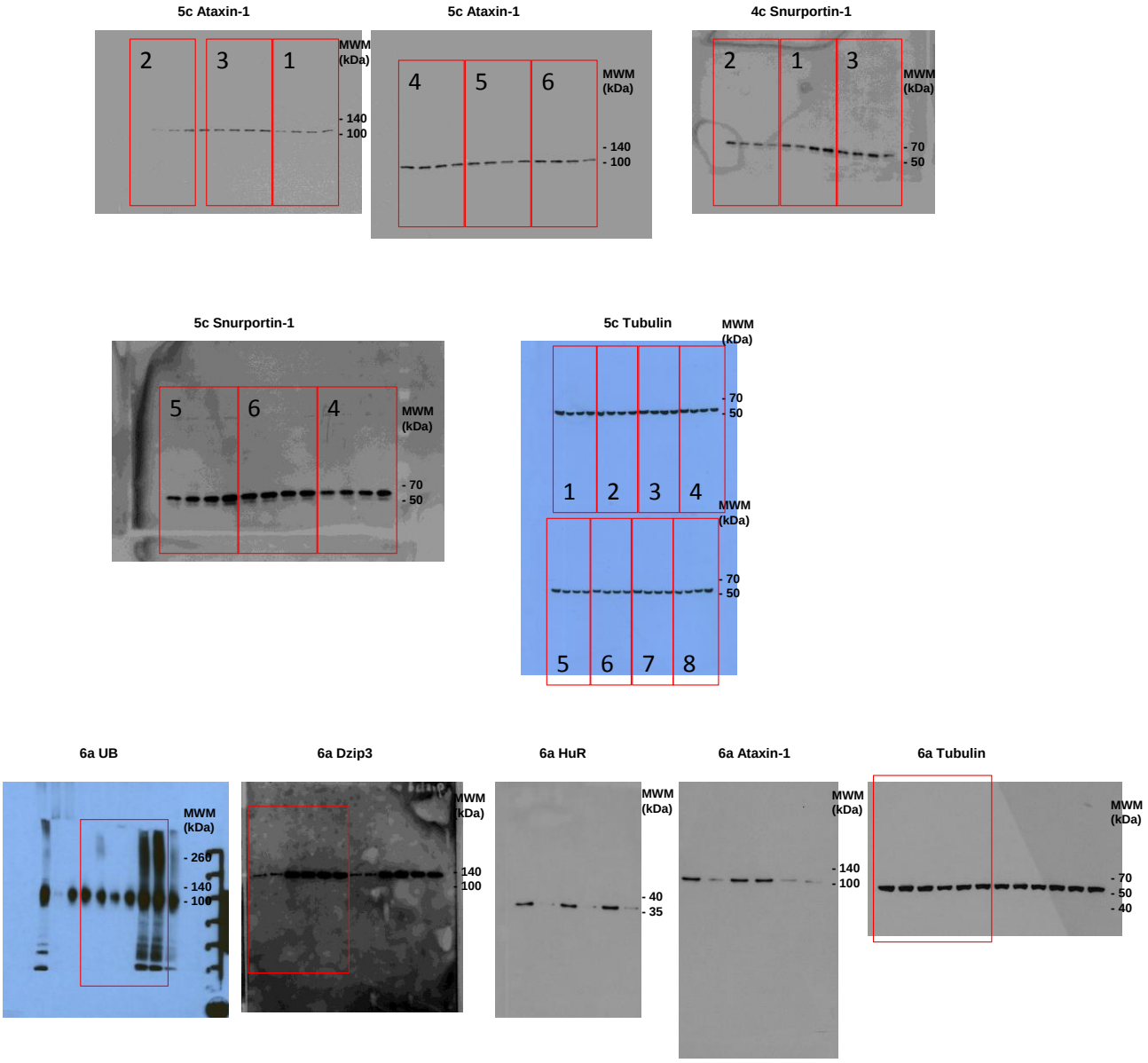

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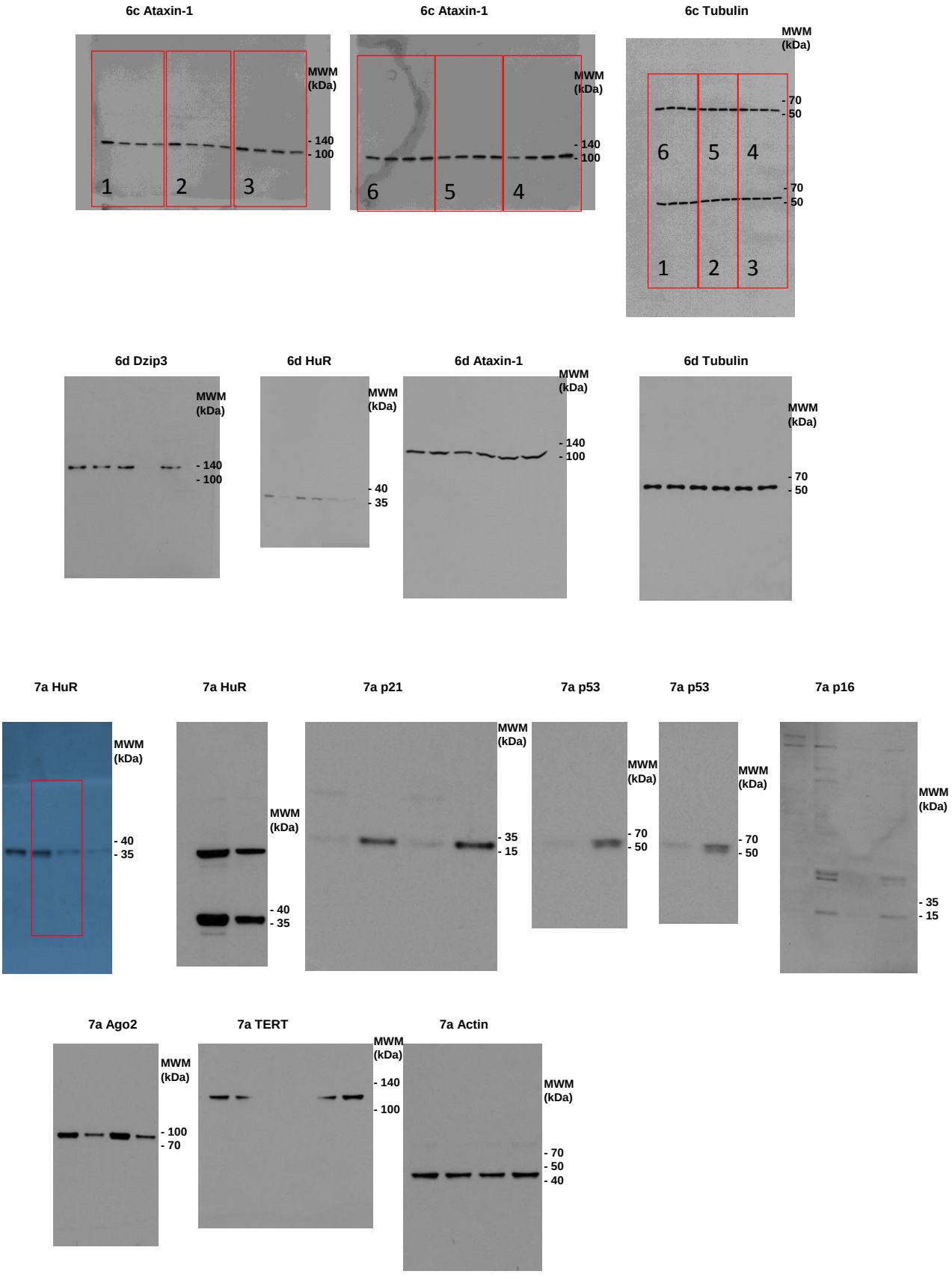

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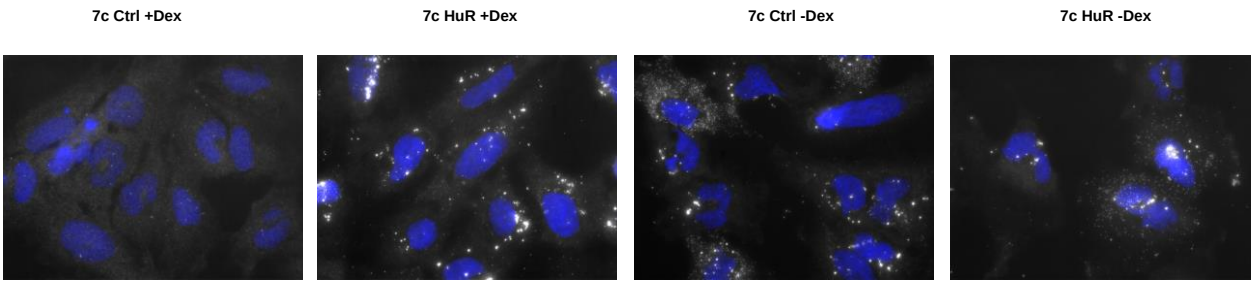

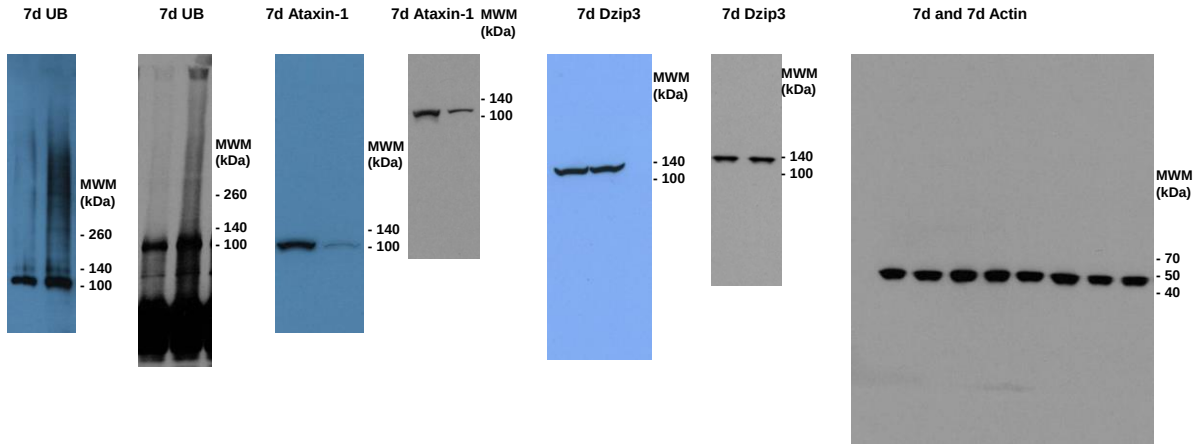

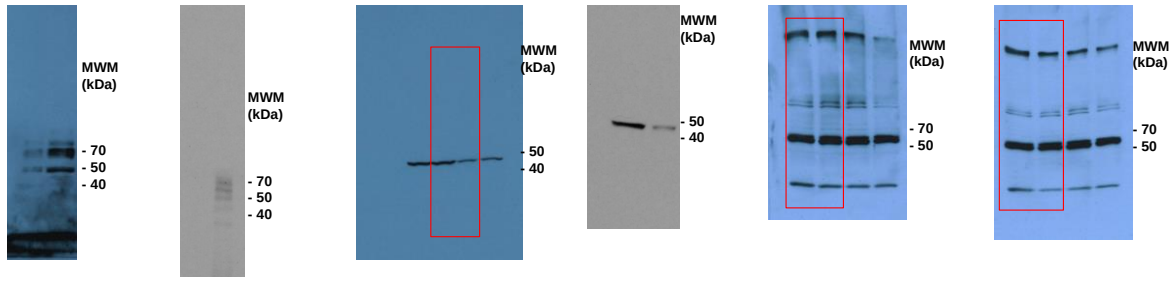

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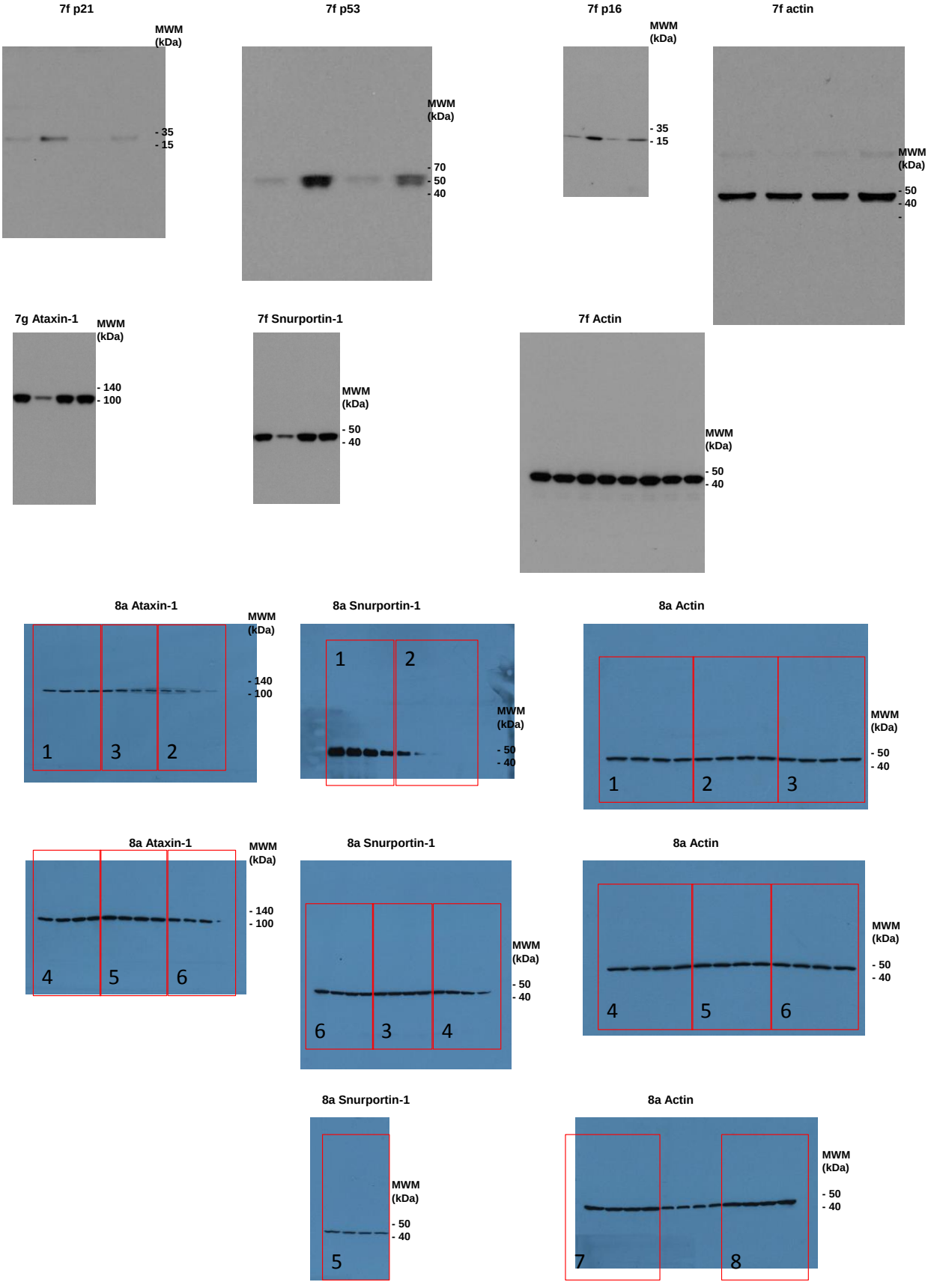

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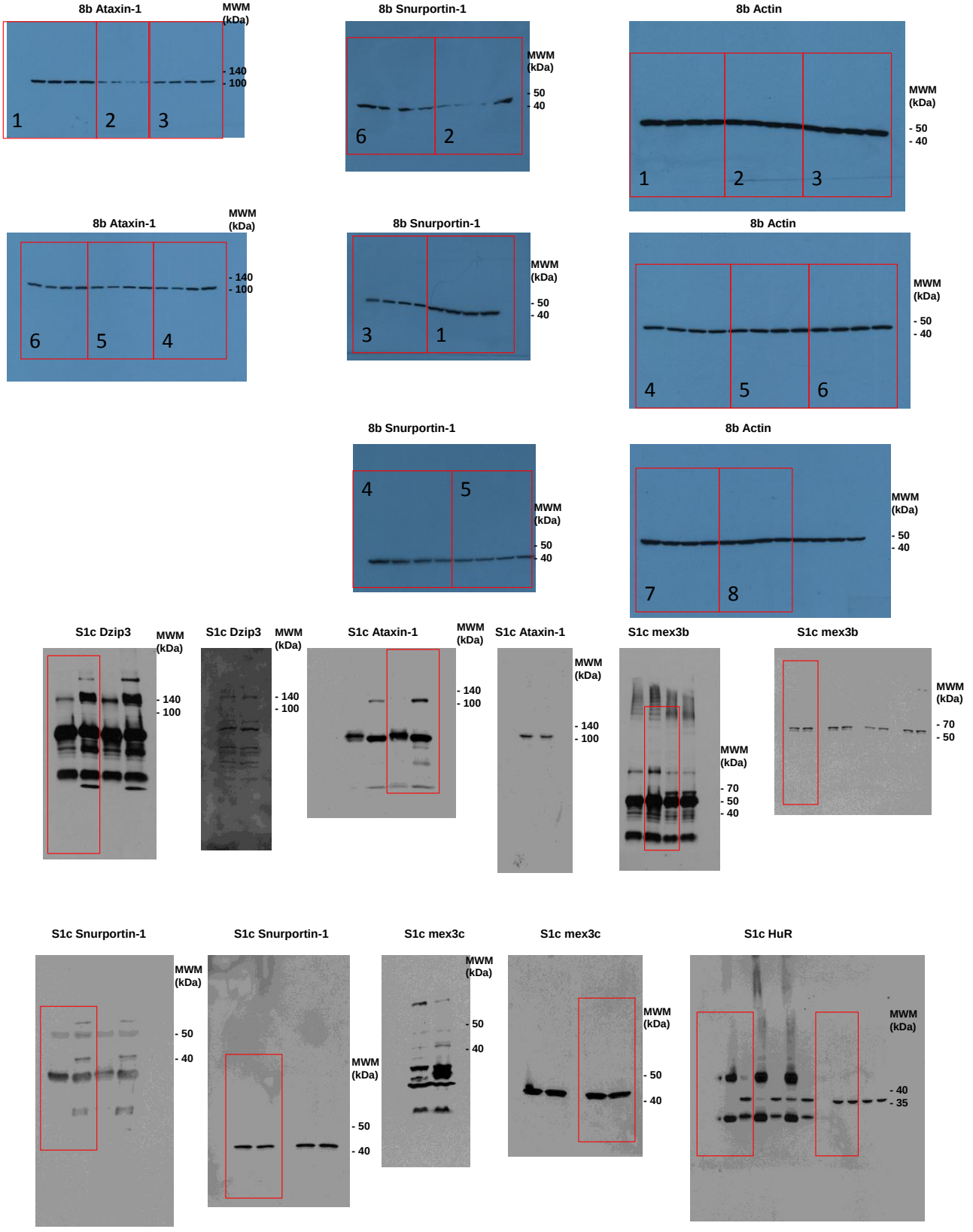

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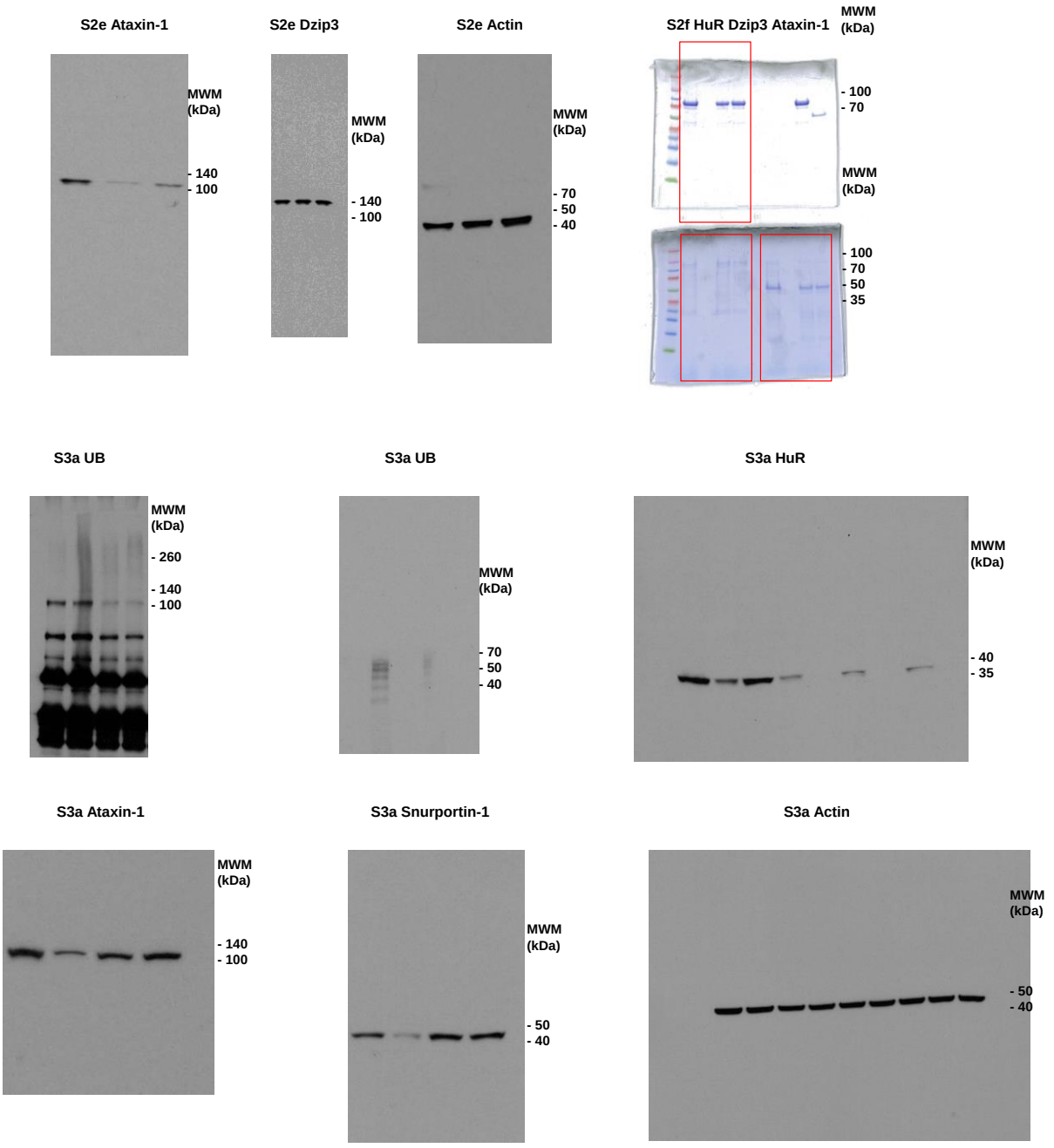

Supplementary Figure S7. Larger fields of blots shown in the Figures 1-9 and Supplementary Figures S1-S6. Larger fields of the blots depited in the main figures are indiated. The corresponding figure and panel, as well as the molecules (proteins or nucleic acids) detected are indicated above each blot. MWM, molecular weight markers [kDa (kilodaltons) or nt (nucleotides)] are indicated. In blots in which only select lanes were used, red rectangles highlight such lanes.

Name	Species	Sequence	Notes
HOTAIR F	human	GGGGCTTCCTTGCTCTTCTTATC	
HOTAIR R	human	GGTAGAAAAAGCAACCACGAAGC	
GAPDH F	human	AGCCACATCGCTCAGACAC	
GAPDH R	human	GCCCAATACGACCAAATCC	
mouse *hotair* F	mouse	TGATATGGCTGCACTGAACA	
mouse *hotair* R	mouse	TCCTGTTCTTGGCATCTCTG	
mouse *gapdh* F	mouse	GGGAAATTCAACGGCACAGT	
mouse *gapdh* R	mouse	AGATGGTGATGGGCTTCCC	
let7b	human	TGAGGTAGTAGGTTGTGTGGTT	
let7i	human	TGAGGTAGTAGTTTGTGCTGTT	
U6 F	human	CGCTTCGGCAGCACATATAC	
U6 R	human	AAAATATGGAACGCTTCACGA	
ATXN1 F	human	TCGGTGGAGCTTGGTTTACAA	
ATXN1 R	human	GGGAGGACCCAATGAACTGG	
SNUPN F	human	CAAGCGGCTGGATTATGTGAA	
SNUPN R	human	AGGAACGTCAATTAACCACTCAG	
18S rRNA F	human	CGAACGTCTGCCCTATCAACTT	
18S rRNA R	human	ACCCGTGGTCACCATGGTA	
let7 Northern blot	human	AACCACACAACCTACTACCTCA	
U6 Northern blot	human	AAAATATGGAACGCTTCACGA	
GAPDH 3'UTR F	human	**CCAAGCTTCTAATACGACTCACTATAGGGAGA** CCTCAACGACCACTTTGTCA	
GAPDH 3'UTR R	human	GGTTGAGCACAGGGTACTTTATT	
HOTAIR 1001 F	human	AAAAGGATCC TGAGGCTTGTTAACAAGACCAG	
HOTAIR 1200 R	human	AAAAGCGGCCGCTTGAGAGACAGTGCACTCACGC	
			HOTAIR **fragments amplified**
HOTAIR segment 1F	human	GACAGGGTCTGGGACAGAAG	27-144
HOTAIR segment 1R	human	GAGTCAGAGTTCCCCACTGC	
HOTAIR segment 2F	human	GCAGTGGGGAACTCTGACTC	125-250
HOTAIR segment 2R	human	GGGTGTTGGTCTGTGGAACT	
HOTAIR segment 3F	human	AGAGAGCACCAGGCACTGAG	223-380
HOTAIR segment 3R	human	TCCCCTACTGCAGGCTTCTA	
HOTAIR segment 4F	human	CAGTGGAATGGAACGGATTT	342-471
HOTAIR segment 4R	human	TCAGACTCTTTGGGGCCTTA	
HOTAIR segment 5F	human	AAGGCCCCAAAGAGTCTGAT	453-581
HOTAIR segment 5R	human	CAGGTCGGTACTGGCTTAGG	
HOTAIR segment 6F	human	CTGGCAGAGAAAAGGCTGAA	505-628
HOTAIR segment 6R	human	CTTCCCTCCTCTGGCTCTCT	
HOTAIR segment 7F	human	AGCCAGAGGAGGGAAGAGAG	613-800
HOTAIR segment 7R	human	TTTTCCCTTTTCCTCATGGA	
HOTAIR segment 8F	human	GGGCACTCACAGACAGAGGT	725-881
HOTAIR segment 8R	human	TCAGGTTTTTCCAGCGTTCT	
HOTAIR segment 9F	human	AGAACGCTGGAAAAACCTGA	862-991
HOTAIR segment 9R	human	TGGAGATGATAAGAAGAGCAAGG	
HOTAIR segment 10F	human	GTCAGCCACTGCCCCACAC	910-1031
HOTAIR segment 10R	human	GCCAGCTCTCTGGTCTTGTT	
HOTAIR segment 11F	human	TGGCCAAGCACCTCTATCTC	1028-1143
HOTAIR segment 11R	human	GTGTAGACGCCGCCATATTT	
HOTAIR segment 12F	human	ACGGAACCCATGGACTCATA	1142-1272
HOTAIR segment 12R	human	TGGTCCCATTTGGATCTTTC	
HOTAIR segment 13F	human	CAAATGTCAGAGGGTTCTGGA	1217-1324
HOTAIR segment 13R	human	TTGGGGAAGCATTTTCTGAC	
HOTAIR segment 14F	human	TGGGAGTGTGTTTTGTTGGA	1331-1441
HOTAIR segment 14R	human	CTACACAACCCCTTCGCTTC	
HOTAIR segment 15F	human	GAAGCGAAGGGGTTGTGTAG	1422-1596
HOTAIR segment 15R	human	AGGCTAGGGCTGGTTTCACT	
HOTAIR segment 16F	human	CCCTAGCCTTTGGAAGCTCT	1588-1700
HOTAIR segment 16R	human	TGCTCTGTGCTGCCAGTTAG	
HOTAIR segment 17F	human	AGGAATCCACCTGCCTGTTA	1628-1755
HOTAIR segment 17R	human	ACCCATGTGTCTCAAGATGC	
HOTAIR segment 18F	human	AATGCATCTTGAGACACATGG	1733-1833
HOTAIR segment 18R	human	AGAGTGCAAAGTCCCGTTTG	
HOTAIR segment 19F	human	CAAACGGGACTTTGCACTCT	1814-1925
HOTAIR segment 19R	human	CCCCTTCTGTGTCTACATGC	
HOTAIR segment 20F	human	GCATGTAGACACAGAAGGGGTA	1906-2005
HOTAIR segment 20R	human	CAGGCATTGGGAATGGTAAT	
HOTAIR segment 21F	human	GCCTGAACTTCCTCCTGCTA	2001-2157
HOTAIR segment 21R	human	TGCATACCTACCCAATGTATGG	
HOTAIR segment 22F	human	TTCCATACATTGGGTAGGTATGC	2134-2236
HOTAIR segment 22R	human	GCACAGAAAATGCATCCAGA	
HOTAIR segment 23F	human	TCTGGATGCATTTTCTGTGC	2217-2331
HOTAIR segment 23R	human	ACCACCACACACACACAACC	

Supplementary Table S1. DNA primers used in this study.