SUPPLEMENTAL MATERIAL
MS ID#: JBC/2009/055004
MS TITLE: High-temperature requirement A3 (HtrA3) promotes etoposide- and cisplatin-induced cytotoxicity in lung cancer cell lines

SUPPLEMENTAL FIGURE LEGENDS

SUPPLEMENTAL FIGURE 1. **HtrA3 modulates half maximal inhibitory concentrations (IC_{50}) for etoposide and cisplatin treatment in lung cancer cell lines.** *A-B*, Mean IC_{50} and 95% confidence intervals for etoposide (*A*) and cisplatin (*B*) with exogenous expression of HtrA3 variants and vector control. *C-D*, Mean IC_{50} and 95% confidence intervals for etoposide (*C*) or cisplatin (*D*) with endogenous HtrA3 expression or with HtrA3 downregulation. *E-F*, Mean IC_{50} and 95% confidence intervals for etoposide (*E*) or cisplatin (*F*) with re-expression of HtrA3 variants and vector control.

SUPPLEMENTAL FIGURE 2. **HtrA3 expression is induced by etoposide and cisplatin.** *A*, Immunoblots showing induction of HtrA3 expression with 20 μM etoposide for 24 hours in the human bronchial cell line BEAS-2B and in lung cancer cell lines Hop62 and HCC827. β-actin shows equal loading. Densitometric analysis of fold changes in HtrA3 intensity normalized by β-actin loading controls is given for each cell line. *B-C*, Immunoblots showing gradual induction of HtrA3 expression with 5 μM etoposide (*B*) or cisplatin (*C*) treatment in Hop62 and HCC827 over 72 hours. Densitometric analysis of fold changes in HtrA3 intensity normalized by β-actin loading controls is represented graphically for each cell line.

SUPPLEMENTAL FIGURE 3. **HtrA3 is upregulated and cleaved with etoposide and cisplatin treatment in H526.** *A-B*, Immunoblots showing induction of HtrA3 expression and with 5 μM etoposide (*A*) or cisplatin (*B*) treatment in lung cancer cell line H526. HtrA3 is cleaved following 24 hours of treatment, resulting in the appearance of a 35 kDa cleavage product. β-actin shows equal loading. *C*, Immunoblots showing HtrA3 induction and cleavage with etoposide treatment. Blots showing HtrA3 expression with polyclonal and monoclonal antibodies are provided for comparison. Results show that the monoclonal antibody, which was raised against the PDZ domain, is able to detect the 35 kDa cleavage product. This result implies that the cleavage product contains the PDZ domain. β-actin shows equal loading. *D*, Immunoblot showing induction of HtrA3 expression and HtrA3 cleavage following treatment with 5 μM etoposide and 10 μM of the broad-spectrum caspase inhibitor z-VAD-FMK. *E*, Immunoblot showing induction of HtrA3 expression but inhibition of cleavage following treatment with 5 μM etoposide and 50 μM of the serine protease inhibitor AEBSF. *F*, Analysis of apoptotic activity following HtrA3 cleavage. H526 transiently transduced with either a non-targeted vector control (normal HtrA3 expression) or an shRNA targeting the 3' UTR (HtrA3 downregulation) were treated with 5 μM etoposide. Immunoblot shows HtrA3 expression. β-actin shows equal loading. Apoptotic activity was measured by annexin V labeling followed by flow microfluorimetry. Results show greater cell death following 48 hours of etoposide treatment, when the HtrA3 cleavage product was more greatly expressed than WT HtrA3, than with HtrA3 downregulation.

SUPPLEMENTAL FIGURE 4. **Bcl-2 overexpression attenuates HtrA3 translocation from mitochondria with etoposide-induced cytotoxicity.** *A*, Immunoblots showing expression of HtrA3, endogenous and S-tagged exogenous Bcl-2 and endogenous mitochondrial marker MTC02 in human bronchial cell line BEAS-2B and lung cancer cell line Hop62. HtrA3 expression was determined using an HtrA3 polyclonal antibody. Bands specific for HtrA3 expression are indicated with an asterisk (*). Either S-tagged Bcl-2 or vector control was expressed in BEAS-2B and Hop62, which each express endogenous HtrA3. β-actin shows equal loading. *B*, Immunocytochemistry using an HtrA3 polyclonal antibody showing endogenous HtrA3 and MTC02 co-localization in BEAS-2B and Hop62 before and after etoposide-induced cytotoxic stress with Bcl-2 overexpression. Hoechst stain binds nuclear material.

Original magnification, x100. *C*, Immunoblots showing expression of GFP, exogenous HtrA3 variants, endogenous and S-tagged exogenous Bcl-2 and endogenous mitochondrial marker MTC02 in lung cancer cell line H157. S-tagged Bcl-2 and either pEGFP-N1-tagged wild-type or protease inactive full length HtrA3 or vector control were expressed in H157, which lacks detectable HtrA3 expression. HtrA3 expression was determined using an HtrA3 monoclonal antibody. β-actin shows equal loading. *D*, Immunocytochemistry showing co-localization of MTC02 and pEGFP-N1 tagged wild-type and protease inactive full length HtrA3 variants in H157 before and after etoposide treatment with Bcl-2 overexpression. Hoechst stain binds nuclear material. Original magnification, x100.

Supplemental Table 1

Oligonucleotides used for the production of plasmid constructs and site-directed mutagenesis

Plasmid	Oligonucleotide	Orientation	Sequence (5' to 3')
pcDNA3.1(+)myc-His A			
Full Length HtrA3	BsuF	Forward	TGAGGTGGTCATGGGGC
	NotIR	Reverse	GGCCGCCCCATGACCACC
PDZ-deleted HtrA3	KpnIF	Forward	CTTGGTACCGGTCCGGAATTCCCGGGATGGGAGC
	PDZ NotIR	Reverse	GGGGCGGCCGCTTTGATCTGCTTGTCTTGGAAC
pEGFP-N1			
Full Length HtrA3	KpnIF	Forward	CTTGGTACCGGTCCGGAATTCCCGGGATGGGAGC
	AgeIR	Reverse	GGGACCGGTCCCATGACCACCTCAGGTGCGATGC
PDZ-deleted HtrA3	KpnIF	Forward	CTTGGTACCGGTCCGGAATTCCCGGGATGGGAGC
	PDZ AgeIR	Reverse	GGGACCGGTCCTTTGATCTGCTTGTCTTGGAAC
S305A HtrA3 mutants	S305A F	Forward	CATCAACTACGGGAACGCCGGGGGACCACTGG
	S305A R	Reverse	CCAGTGGTCCCCCGGCGTTCCCGTAGTTGATG

Supplemental Table 2

Antibodies used for immunoblotting and immunocytochemistry

Antibody	Manufacturer	Application	Conditions
Primary			
Bcl-2	Dako (Carpinteria, CA, USA)	IB	1:500 in 3% BSA/.1% TBST
β-actin	Sigma-Aldrich (St. Louis, MO, USA)	IB	1:5000 in 3% BSA/.1% TBST
Caspase 7	BD Pharmingen (San Jose, CA, USA)	IB	1:1000 in 3% BSA/.1% TBST
Cleaved Caspase 3	Cell Signaling Technology (Beverly, MA, USA)	IB	1:500 in 3% BSA/.1% TBST
Cytochrome c	BD Pharmingen (San Jose, CA, USA)	IB	1:200 in 3% BSA/.1% TBST
HtrA2 (V-12)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	IB	1:200 in 3% BSA/ .1% TBST
HtrA3 monoclonal	Mayo Antibody Core Facility Rochester (MACFR)	IB	1:200 in 3% BSA/.1% TBST
HtrA3 polyclonal	Abcam (Cambridge, MA, USA)	IB	1:200 in 3% BSA/.1% TBST
		ICC	1:1000 in 3% BSA/.1% TBST
Frataxin*	Pacific Immunology Corp. (Ramona, CA, USA)	IB	1:5000 in 5% NFDM/.1% PBST
GFP (FL)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	IB	1:200 in 3% BSA/.1% TBST
Mitochondrial Marker MTC02	Abcam (Cambridge, MA, USA)	IB	1:200 in 3% BSA/.1% TBST
		ICC	1:1000 in 3% BSA/.1% TBST
MMP7	Abcam (Cambridge, MA, USA)	IB	1:200 in 3% BSA/.1% TBST
S-tag**	Mayo Antibody Core Facility Rochester (MACFR)	IB	1:1000 in 3% BSA/.1% TBST
Secondary			
Anti-mouse IgG (whole molecule)-TRITC antibody produced in goat	Sigma-Aldrich (St. Louis, MO, USA)	ICC	1:1000 in 3% BSA/.1% TBST
Anti-rabbit IgG (whole molecule)-FITC antibody produced in goat	Sigma-Aldrich (St. Louis, MO, USA)	ICC	1:1000 in 3% BSA/.1% TBST
Donkey anti-rabbit IgG, HRP-linked (whole antibody)	GE Healthcare UK (Little Chalfont, Buckinghamshire, UK)	IB	1:7500 in 5% NFDM/.1% TBST
Rabbit anti-goat IgG (H&L) F(ab')2, HRP-linked	Millipore (Billerica, MA, USA)	IB	1:7500 in 5% NFDM/.1% TBST
Sheep anti-mouse IgG, HRP-linked (whole antibody)	GE Healthcare UK (Little Chalfont, Buckinghamshire, UK)	IB	1:7500 in 5% NFDM/.1% TBST

Abbreviations: IB, immunoblotting; ICC, immunocytochemistry; HRP, horse radish peroxidase; NFDM, non-fat dry milk

*The frataxin antibody was provided by Grazia Isaya.

**The S-tag antibody was provided by Scott Kaufmann (Hackbarth, Lee et al. 2004).

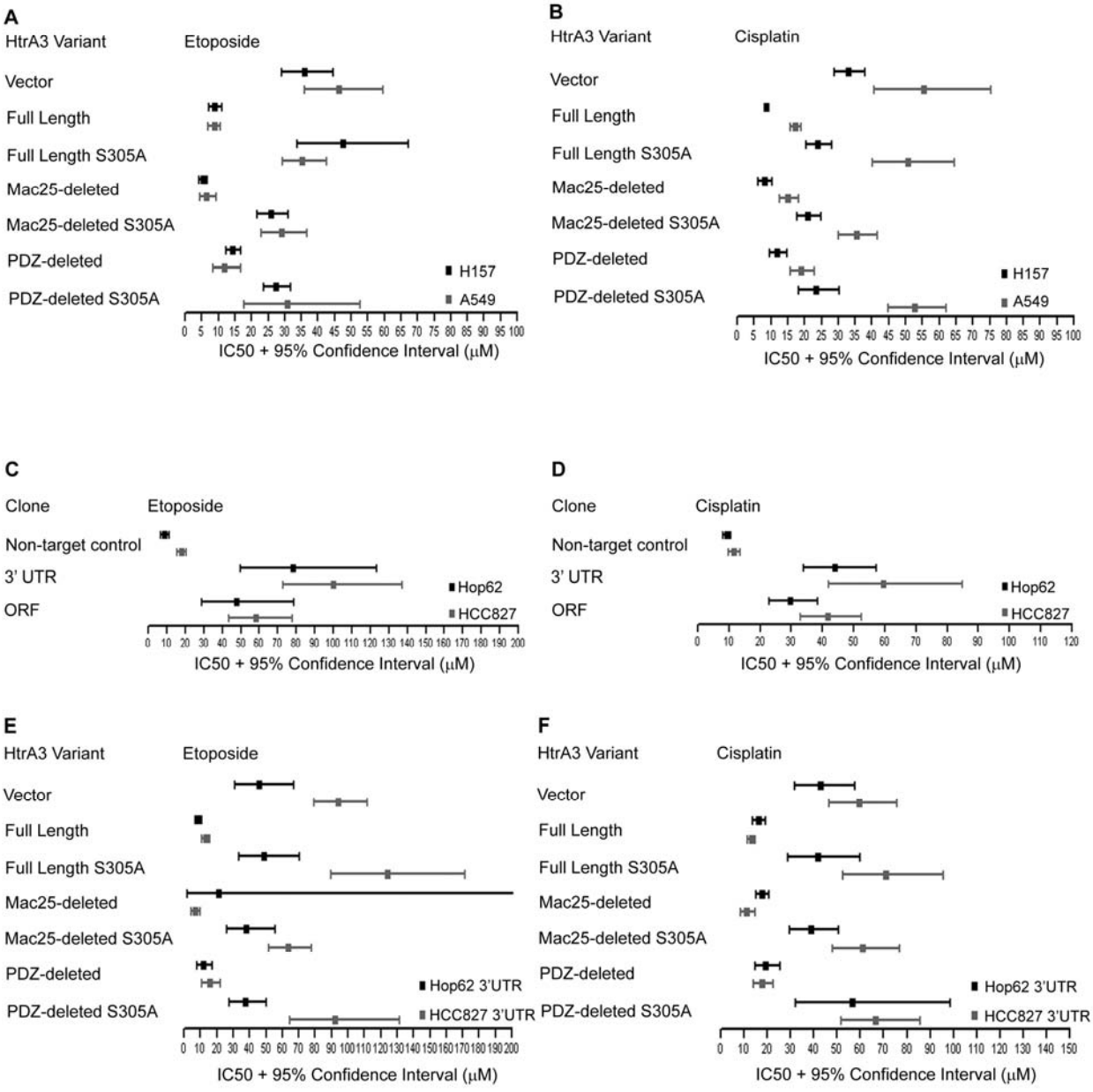
Supplemental Table 3

Half maximal inhibitory concentrations (IC_{50}) and 95% confidence intervals for etoposide and cisplatin with varying HtrA3 expression in lung cancer cell lines

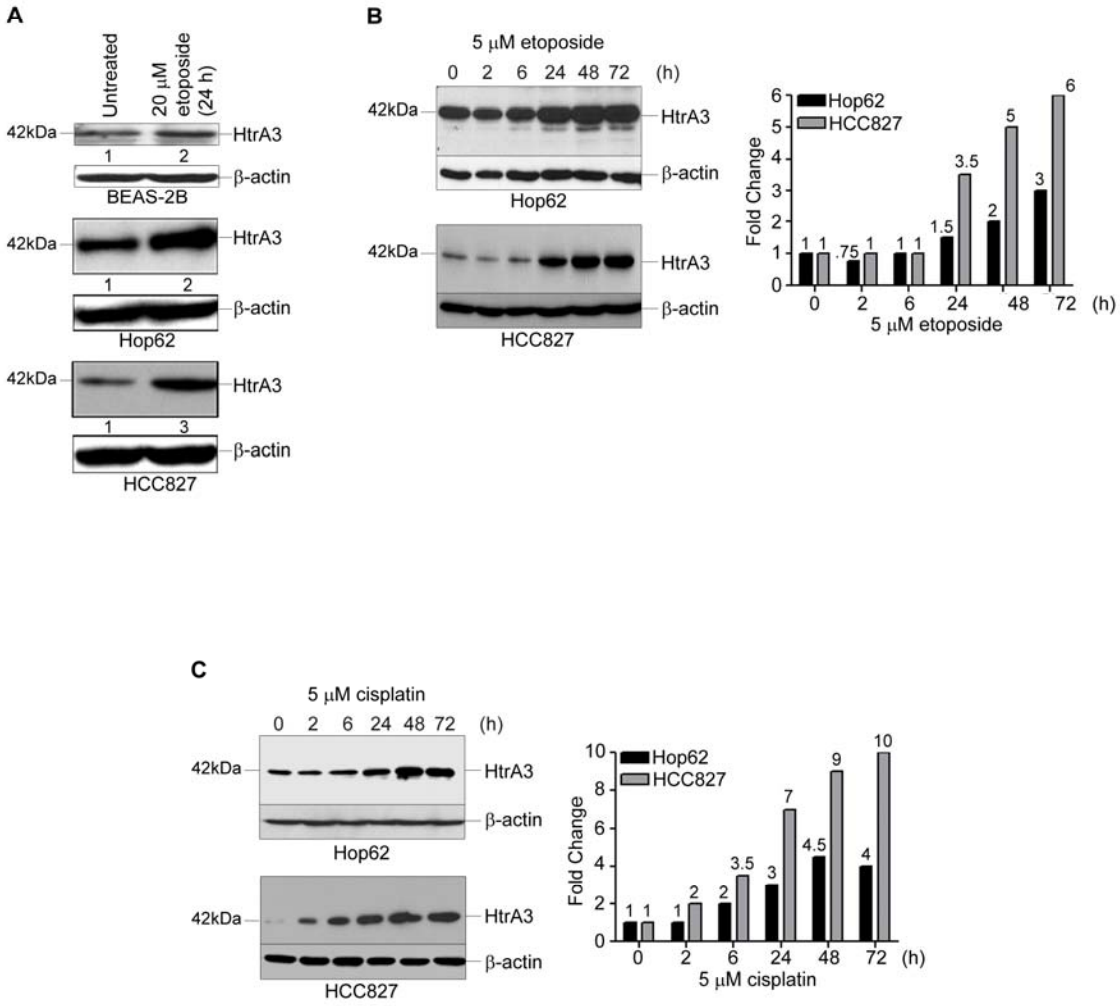
Sample	Etoposide		Cisplatin	
	IC_{50} ± SEM* (μM)	95% Confidence Interval	IC_{50} ± SEM* (μM)	95% Confidence Interval
(A) Half maximal inhibitory concentrations of etoposide and cisplatin with HtrA3 overexpression in H157 and A549				
i. **H157**				
Vector	35.94 ± 1.108	28.99 - 44.57	32.96 ± 1.068	28.71 - 37.85
Full Length	8.898 ± 1.111	7.136 - 11.09	8.721 ± 1.040	8.036 - 9.464
Full Length S305A	47.59 ± 1.180	33.72 - 67.15	23.91 ± 1.080	20.37 - 28.07
Mac25-deleted	5.301 ± 1.120	4.180 - 6.723	8.018 ± 1.132	6.183 - 10.40
Mac25-deleted S305A	25.89 ± 1.089	21.64 - 30.97	20.93 ± 1.086	17.63 - 24.85
PDZ-deleted	14.38 ± 1.078	12.29 - 16.82	11.94 ± 1.107	9.657 - 14.76
PDZ-deleted S305A	27.38 ± 1.072	23.67 - 31.67	23.46 ± 1.129	18.19 - 30.26
ii. **A549**				
Vector	46.34 ± 1.127	36.07 - 59.53	55.42 ± 1.200	40.72 - 75.42
Full Length	8.383 ± 1.200	5.766 - 12.19	17.20 ± 1.078	14.72 - 20.11
Full Length S305A	35.33 ± 1.094	29.26 - 42.67	50.85 ± 1.120	40.11 - 64.48
Mac25-deleted	6.486 ± 1.185	4.544 - 9.259	15.06 ± 1.093	12.51 - 18.12
Mac25-deleted S305A	28.97 ± 1.1185	22.92 - 36.62	35.35 ± 1.082	29.99 - 41.67
PDZ-deleted	11.93 ± 1.176	8.490 - 16.76	18.97 ± 1.092	15.78 - 22.81
PDZ-deleted S305A	30.71 ± 1.300	17.85 - 52.82	52.74 ± 1.080	44.89 - 61.96
(B) Half maximal inhibitory concentrations of etoposide and cisplatin with HtrA3 downregulation in Hop62 and HCC827				
i. **Hop62**				
Non-target control	8.714 ± 1.138	6.654 - 11.41	9.187 ± 1.066	8.035 - 10.50
3'UTR shRNA	78.38 ± 1.242	49.84 - 123.3	44.03 ± 1.133	33.88 - 57.22
ORF shRNA	47.63 ± 1.271	28.82 - 78.72	29.59 ± 1.132	22.84 - 38.34
ii. **HCC827**				
Non-target control	17.85 ± 1.070	15.62 - 20.39	11.61 ± 1.080	9.904 - 13.62
3'UTR shRNA	100.1 ± 1.163	73.00 - 137.3	59.60 ± 1.190	41.80 - 84.96
ORF shRNA	58.28 ± 1.147	43.71 - 77.70	41.55 ± 1.120	32.87 - 52.54
(C) Half maximal inhibitory concentrations of etoposide and cisplatin with HtrA3 re-expression in Hop62 3'UTR and HCC827 3'UTR				
i. **Hop62 3'UTR shRNA**				
Vector	45.70 ± 1.203	31.05 - 67.24	42.85 ± 1.151	31.94 - 57.50
Full Length	8.703 ± 1.106	7.049 - 10.75	16.24 ± 1.084	13.71 - 19.23
Full Length S305A	48.67 ± 1.195	33.52 - 70.66	41.65 ± 1.190	28.93 - 59.96
Mac25-deleted	21.20 ± 3.300	1.777 - 252.9	17.74 ± 1.078	15.15 - 20.76
Mac25-deleted S305A	37.97 ± 1.200	25.95 - 55.57	38.68 ± 1.139	29.45 - 50.80
PDZ-deleted	11.73 ± 1.200	8.071 - 17.06	19.30 ± 1.141	14.66 - 25.42
PDZ-deleted S305A	37.28 ± 1.153	27.67 - 50.23	56.43 ± 1.306	32.28 - 98.63
ii. **HCC827 3'UTR shRNA**				
Vector	94.19 ± 1.085	79.40 - 111.7	59.42 ± 1.123	46.61 - 75.75
Full Length	12.77 ± 1.183	8.986 - 18.14	13.06 ± 1.109	10.52 - 16.22
Full Length S305A	124.0 ± 1.166	89.86 - 171.2	70.83 ± 1.156	52.33 - 95.86
Mac25-deleted	6.743 ± 1.209	4.537 - 10.02	11.11 ± 1.143	8.406 - 14.68
Mac25-deleted S305A	63.48 ± 1.101	51.92 - 77.62	60.81 ± 1.120	48.01 - 77.03
PDZ-deleted	15.53 ± 1.182	10.94 - 22.05	17.74 ± 1.125	13.88 - 22.68
PDZ-deleted S305A	92.18 ± 1.185	64.60 - 131.5	66.65 ± 1.128	51.84 - 85.69

* Half maximal inhibitory concentrations are expressed as mean IC_{50} ± standard error of mean (SEM)

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3

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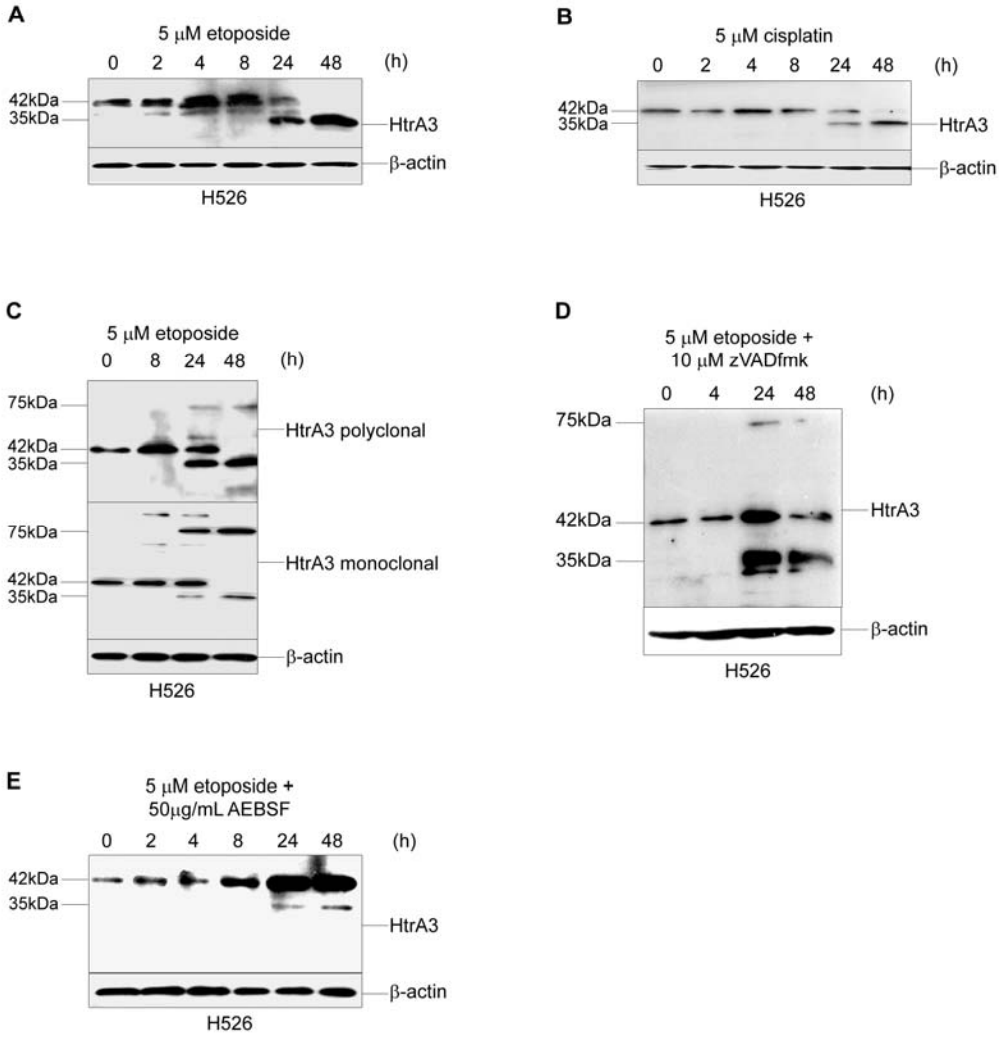

Supplemental Figure 3
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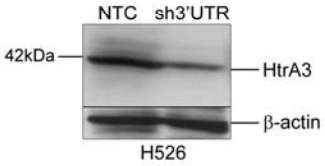
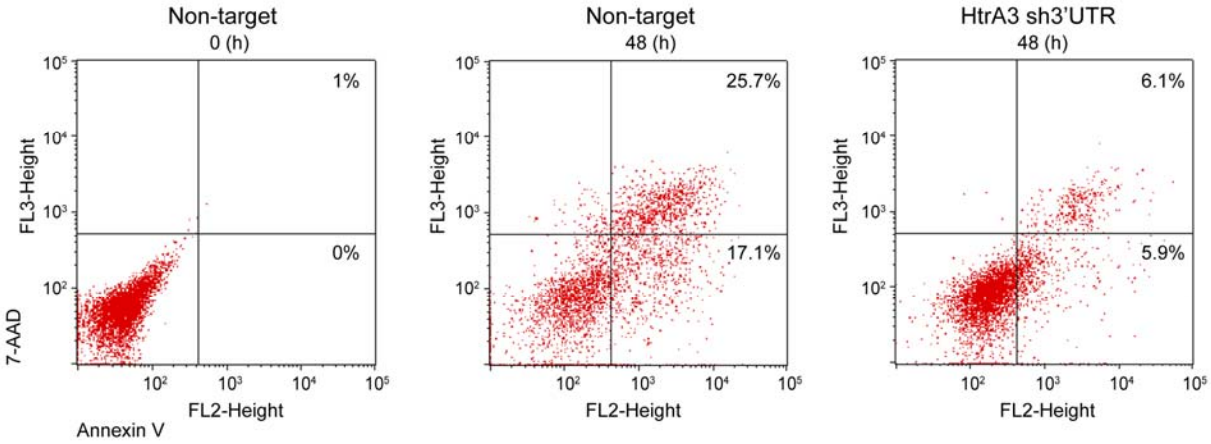
F
H526
5μM etoposide





Supplemental Figure 4
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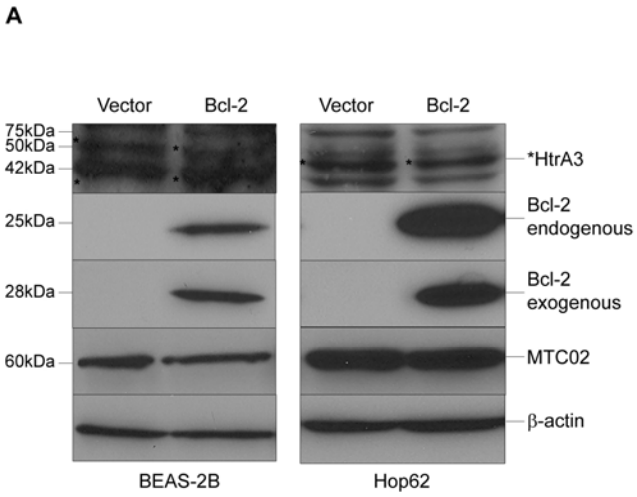

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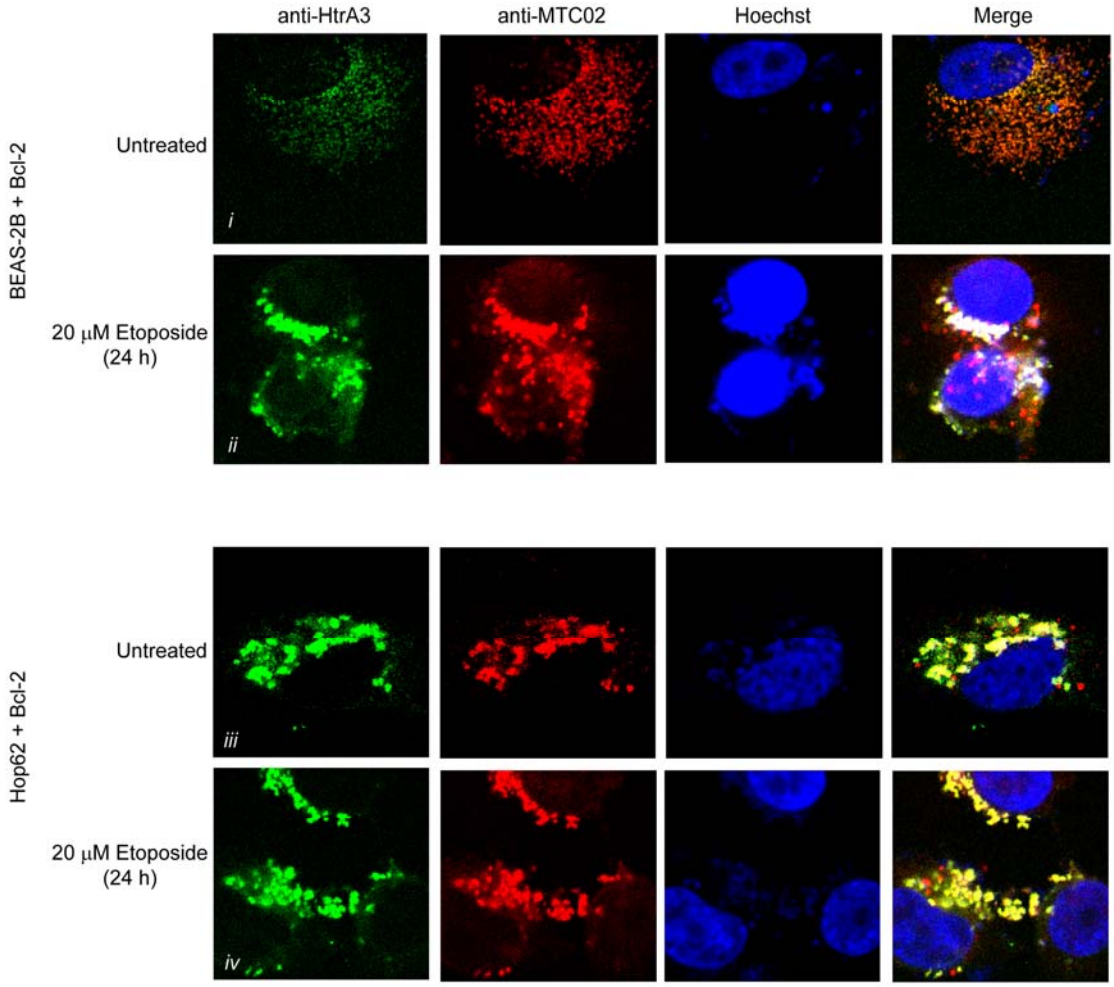

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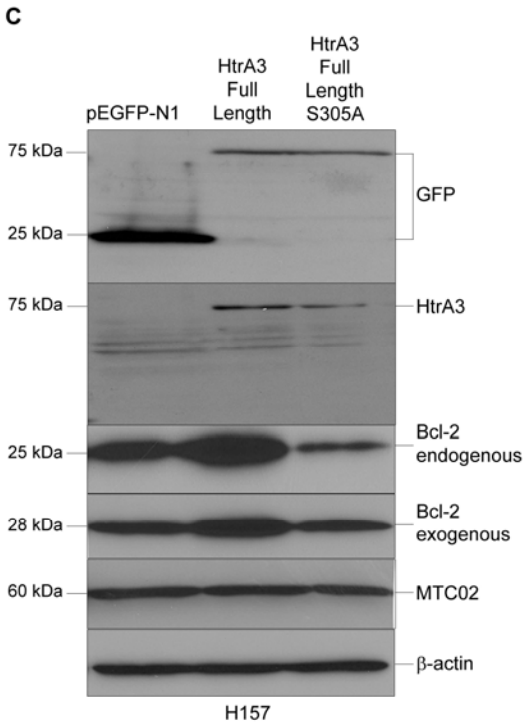

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