Supplementary material

Figure Legends

Figure S1: Phostag gel analysis reveals ECD is phosphorylated on six residues. (A) *in vitro* phosphorylated WT ECD, 6S/A mutant, or WT phosphatase treated samples were analyzed on phostag PAGE or normal SDS PAGE.(B) FLAG-tagged ECD and the mutant 3′S/A were transfected in to T98G cells, subjected to IP, followed by western blotting with anti-p-Ser or anti-FLAG antibodies.

Figure S2: DS_PDD/E motifs of ECD are dispensable for cell cycle function. (A) Cell cycle rescue experiment was performed by transfecting WT type or indicated phospho mutants of ECD in to $Ecd^{fl/fl}$ MEFs, followed by removal of endogenous Ecd using Cre adenovirus. Graph indicates Cre/Ctrl ratio of cell numbers at different time intervals. The outcome Cre/Ctrl ratio was calculated per sample at each time point (mean ± standard deviations). Each time point indicates the average cell number of biological triplicates. (B) Shown are Δ499-527 (Lacking two DS_PDD motifs) expression levels at different time points after Cre adenovirus infection. Note that reconstituted cells express both mouse (m; upper band) Ecd and human (lower band) Δ499-527 ECD mutant.

Figure S3: Phospho-defective mutants retain the ability to interact with PIH1D1 and other components of R2TP complex in cells. (A) HEK293T cells were transfected with GFP tagged vector, WT, 3S/A or 6S/A. Cell lysates were subjected to immunoprecipitaion with PIH1D1 antibody and complex was eluted with 2x sample lysis buffer followed by western blotting with indicated antibodies. (B & C) Phosphatase treatment did not disrupt the interaction between ECD and PIH1DI. HEK293T cells were transfected with WT ECD, lysates were collected in lysis buffer without phosphatase inhibitor and then subjected to phosphatase treatment and subsequent immunoprecipitation and western blotting with indicated antibodies. (D) Mimicking of phosphoserines in to aspartic acid did not mimic binding to PIH1D1. *In vitro* interaction of GST-PIH1D1 with ECD and its phospho mutants. GST-PIH1D1 was immobilized on GST beads and incubated with HEK-293T cells lysate expressing FLAG-WT, 3S/A, 3′S/A, 6S/A and 6S/D proteins. (E) Interaction between ECD and PRP8 was confirmed by coimmunoprecipitation..

Figure S4: RUVBL1 interacts with only full length ECD. (A) Schematic representation of various GFP-tagged C-terminal deletion constructs of ECD. (B) HEK293T cells were transfected with indicated GFP tagged constructs. Cell lysates were subjected to immunoprecipitaion with RUVBL1 antibody, the complex was eluted with 2x sample lysis buffer followed by western blotting with indicated antibodies. Arrow indicates the size of protein of interest. (C) Endogenous RUVBL1 interacts with GST-ECD. Indicated GST-tagged ECD proteins were immobilized on GST beads and incubated with 293T cell lysate, followed by GST pull down washed and western blotting with RUVBL1 antibody. (D) Interaction of R2TP complex components with GST-PIH1D1 from the lysates expressing deletion mutants of ECD. (E)

Expression of various pMSCV- Flag-tagged constructs of ECD. (F) Phosphodefective mutants retain the ability to interact with retinoblastoma (RB) protein. HEK293T cells were transfected with FLAG tagged vector, WT, 3S/A or 6S/A, immunoprecipitated with anti-RB antibody followed by western blotting with indicated antibodies.

Figure S5: Full blots of various Figures. Boxed region shows the cropped region used in the main manuscript. Subnumbering refer to the no in main Figures.

Fig.S1

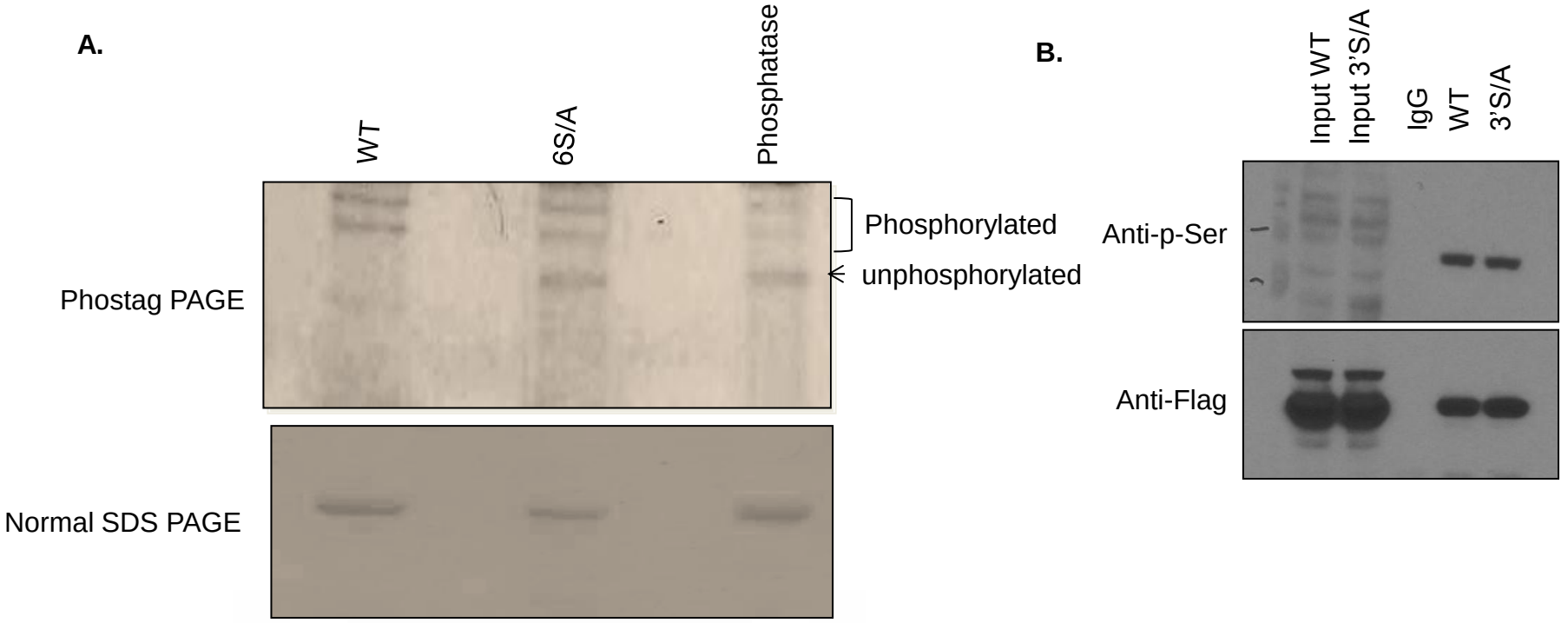
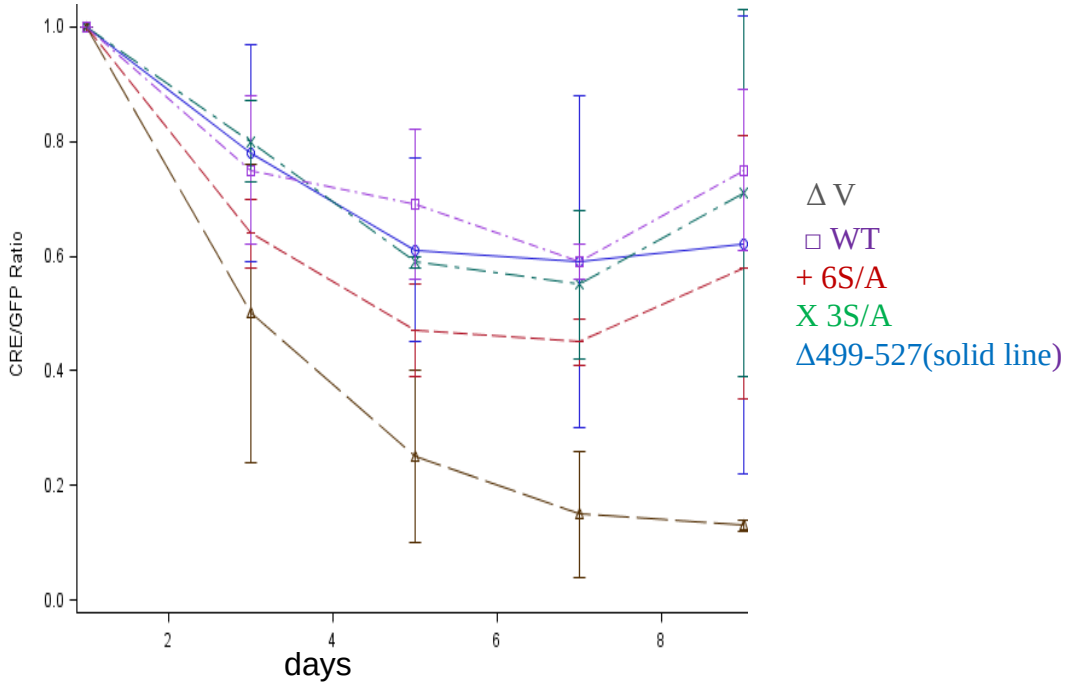

A.
WT
6S/A
Phosphatase
Phosphorylated
unphosphorylated
Phostag PAGE
Normal SDS PAGE
B.
Input WT
Input 3'S/A
IgG
WT
3'S/A
Anti-p-Ser
Anti-Flag

Figure S2

A.


CRE/GFP Ratio
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
days
Δ V
□ WT
+ 6S/A
X 3S/A
Δ499-527(solid line)

B.

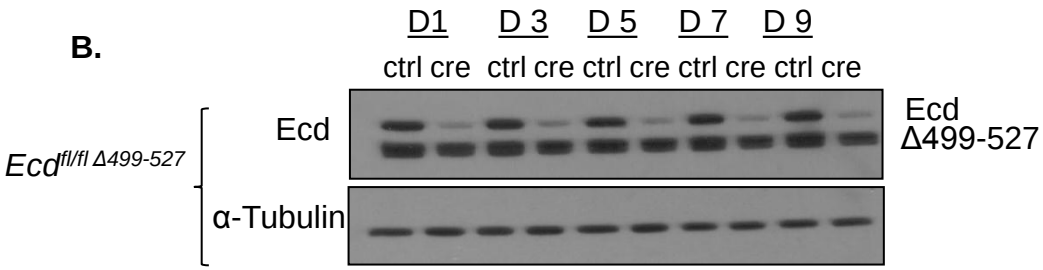

D1 D 3 D 5 D 7 D 9
ctrl cre ctrl cre ctrl cre ctrl cre ctrl cre
Ecd
α-Tubulin
Ecd Δ499-527
Ecdfl/fl Δ499-527

Figure S3

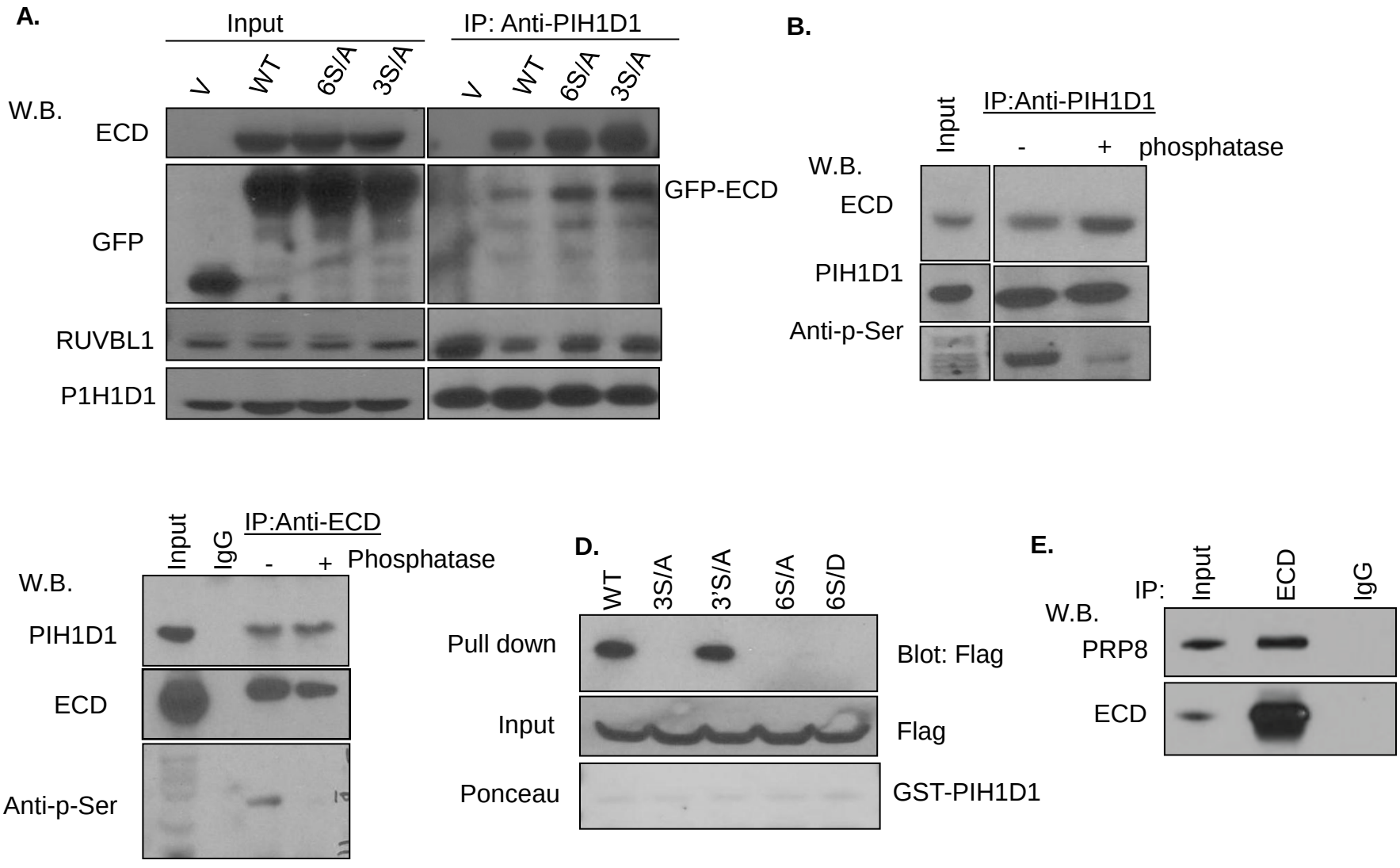

A.
Input
IP: Anti-PIH1D1
V
WT
6S/A
3S/A
W.B.
ECD
GFP
GFP-ECD
RUVBL1
P1H1D1
B.
Input
IP:Anti-PIH1D1
-
+
phosphatase
W.B.
ECD
PIH1D1
Anti-p-Ser
C.
Input
IgG
IP:Anti-ECD
-
+
Phosphatase
W.B.
PIH1D1
ECD
Anti-p-Ser
D.
WT
3S/A
3'S/A
6S/A
6S/D
Pull down
Blot: Flag
Input
Flag
Ponceau
GST-PIH1D1
E.
IP:
Input
ECD
IgG
W.B.
PRP8
ECD

Figure S4

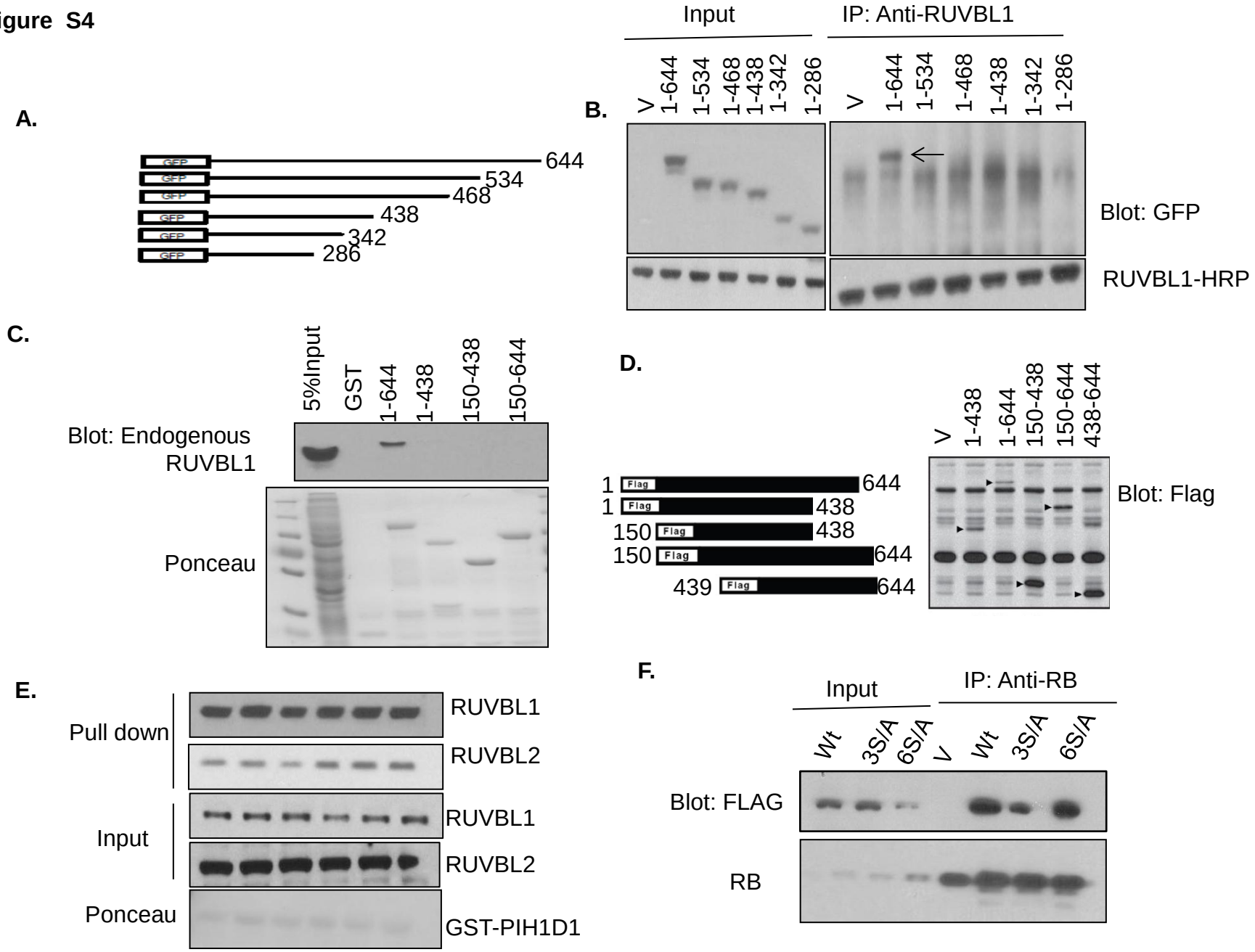

A.
GFP
644
534
468
438
342
286
B.
Input
IP: Anti-RUVBL1
V
1-644
1-534
1-468
1-438
1-342
1-286
Blot: GFP
RUVBL1-HRP
C.
5%Input
GST
1-644
1-438
150-438
150-644
Blot: Endogenous RUVBL1
Ponceau
D.
V
1-438
1-644
150-438
150-644
438-644
1 Flag 644
1 Flag 438
150 Flag 438
150 Flag 644
439 Flag 644
Blot: Flag
E.
Pull down
RUVBL1
RUVBL2
Input
RUVBL1
RUVBL2
Ponceau
GST-PIH1D1
F.
Input
IP: Anti-RB
Wt
3S/A
6S/A
V
Wt
3S/A
6S/A
Blot: FLAG
RB

Figure S5

Fig 1B

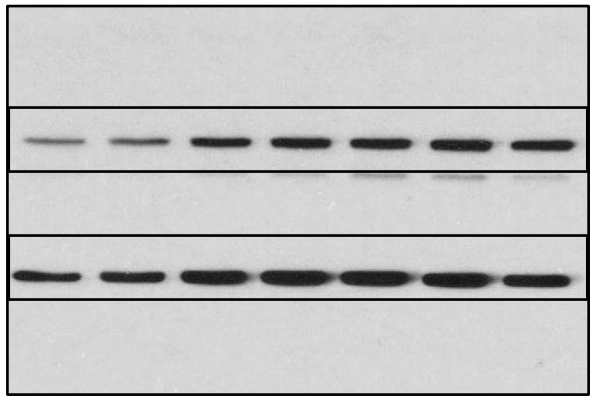


Fig 1C

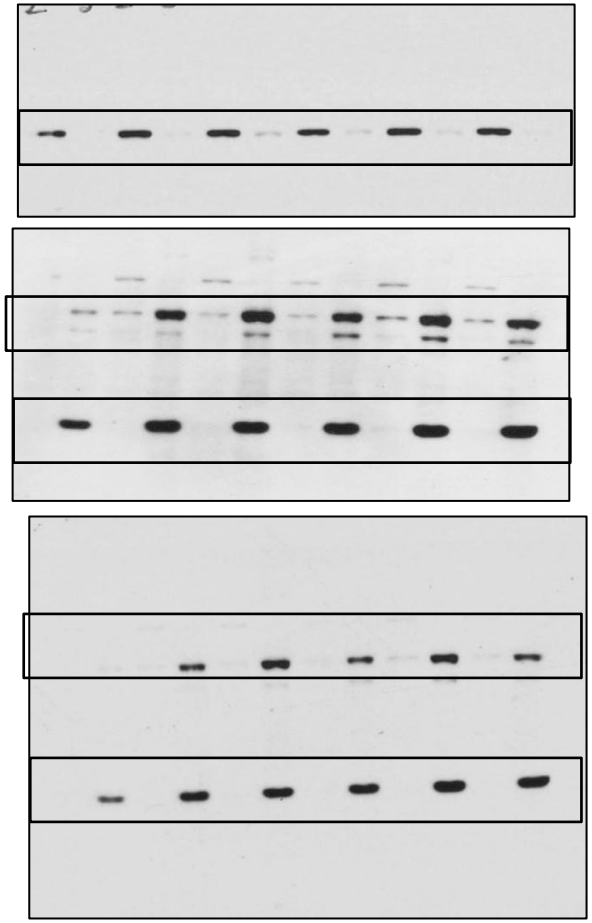


Fig 2C

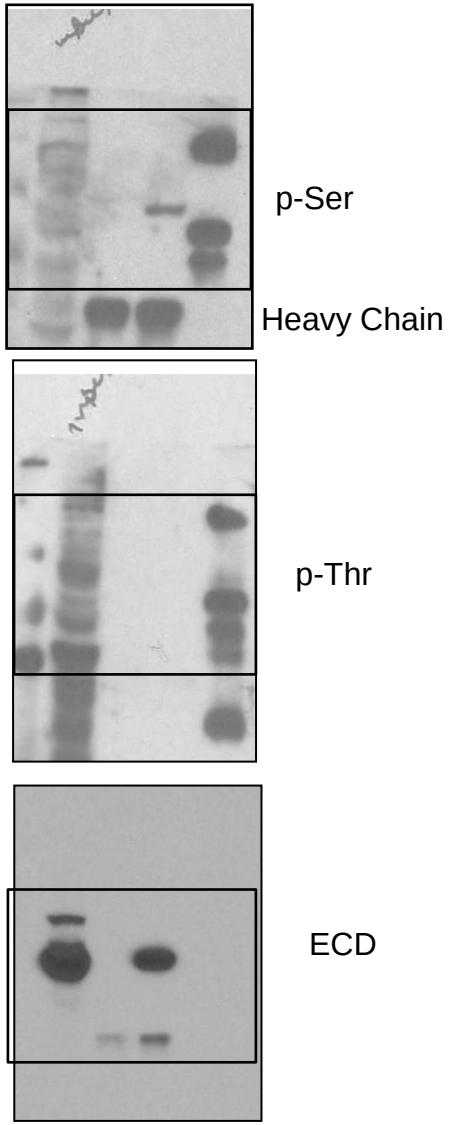


Figure S5 continue

Fig 3B.

Autorad.

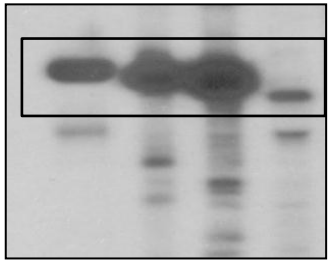
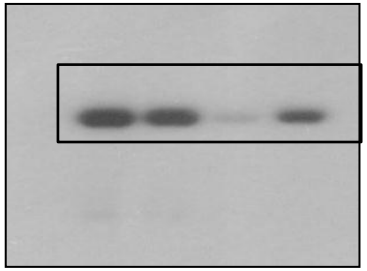
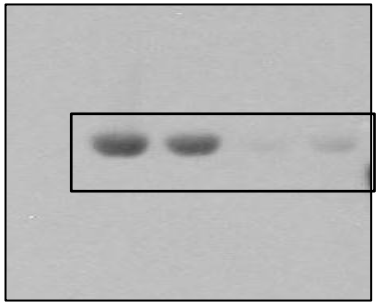


Fig 3D.

Autorad.



p-Ser



ECD

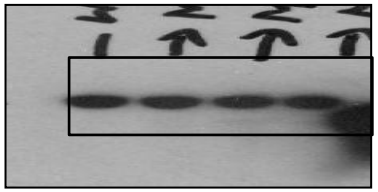
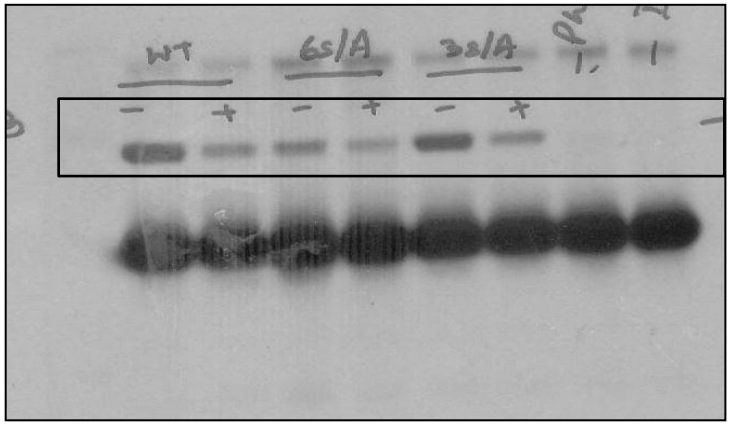


Fig 3F.



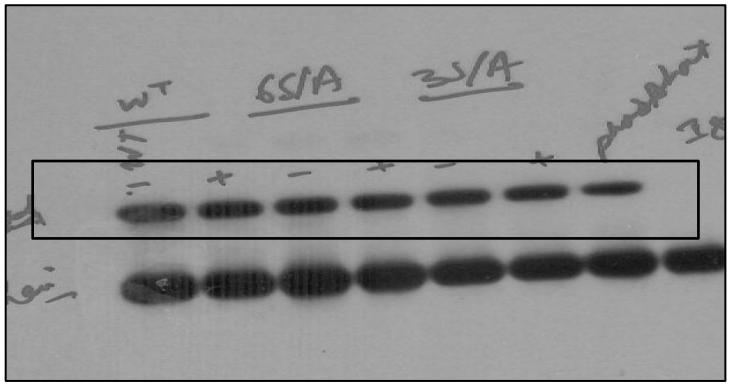


Figure S5 continue

Fig.4B

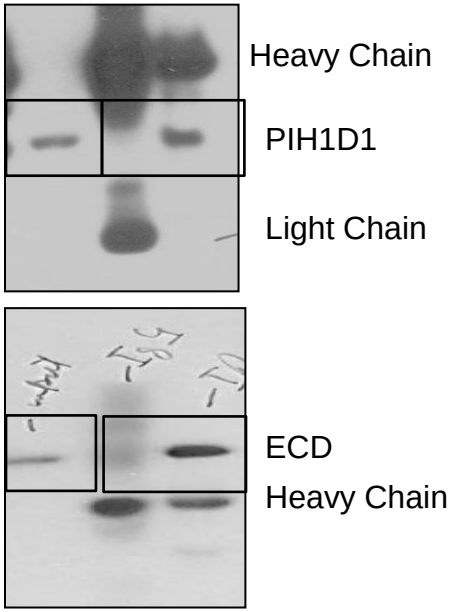


Fig.4C

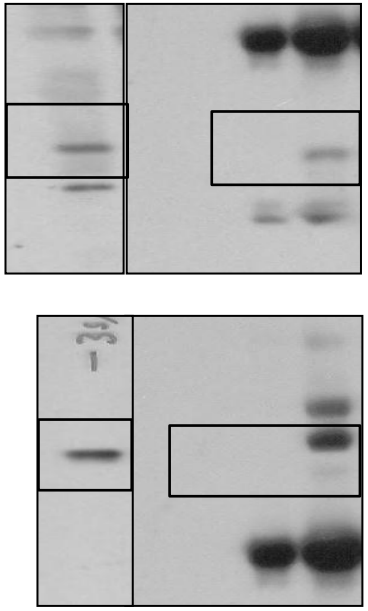


Fig.4D

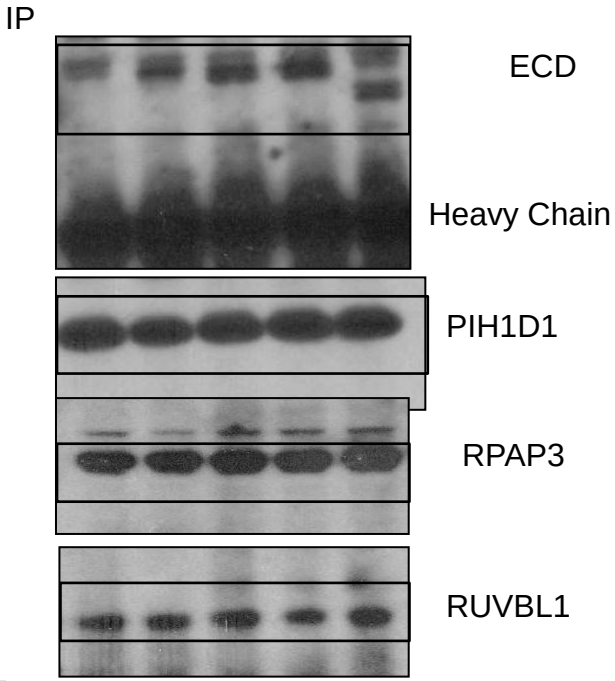


Fig.5B

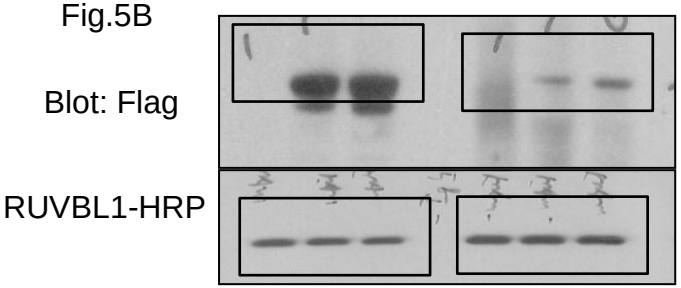


Fig.5C

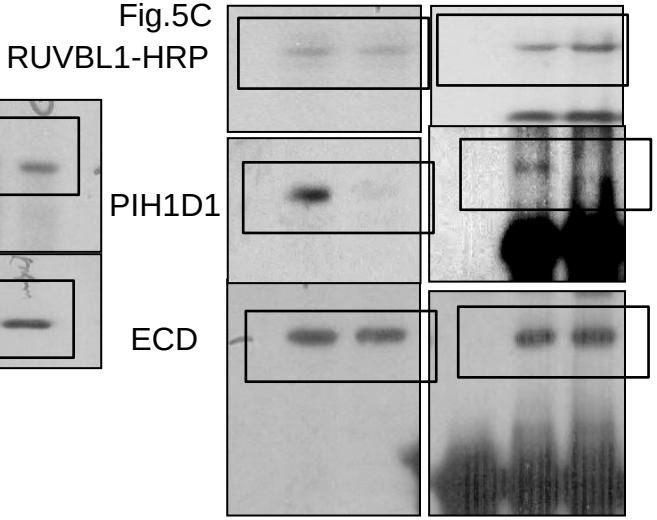


Table S1. Cre/Ctrl ratio and 'p' Values for all groups of cells

Day	cell type	N	Mean	Std Dev	overall p-value	p-value of other type vs. Wt comparison with simulation's correction
1	6S/A	9	1	0	0.99	0.99
	3S/A	9	1	0		0.99
	V	9	1	0		0.99
	WT	9	1	0		--
3	6S/A	9	0.68	0.04	<.0001	<.0001
	3S/A	9	0.77	0.08		0.29
	V	9	0.64	0.14		0.0007
	WT	9	0.83	0.07		--
5	6S/A	9	0.51	0.04	<.0001	<.0001
	3S/A	9	0.71	0.08		0.98
	V	9	0.25	0.06		<.0001
	WT	9	0.72	0.05		--
7	6S/A	9	0.49	0.04	<.0001	<.0001
	3S/A	9	0.65	0.08		0.05
	V	9	0.12	0.05		<.0001
	WT	9	0.75	0.1		--
9	6S/A	9	0.5	0.1	<.0001	<.0001
	3S/A	9	0.62	0.12		0.11
	V	9	0.09	0.03		<.0001
	WT	9	0.71	0.06		--

Table S1 . Table lists statistically significant differences among the four groups in Cre/Ctrl ratio at day 3, 5, 7 or 9 (all p values are <0.0001). Further analysis with Simulation's correction to control for multiple testing revealed that the mean ratio of WT group was significantly higher than that of V group and 6S/A group at day 3, 5, 7, and 9 ($p<0.001$); and there was no evidence of a difference in Cre/Ctrlratio between WT group and 3S/A group at day 1, 3, 5, and 9, and at day 7 there was a marginally significant difference between WT group and 3S/A group ($p=0.05$).

Name of construct	Primers for Cloning	Forward	Reverse
pMSCVpuro ECD Δ499-527	Primers for cloning of Δ499-527 by three fragment ligation.	**Ecd BglII** I 5′CGCCGGAATTAGATCTATGG AAGAAACCATGAA-3′ **Ecd SalI F 5′** TTTAGGGCCAGTCGACCCT GGCGAAGAGGCT -3′	**Ecd HpaIR15'-**CCGGTAGAATTCGTTAACGTC GACTGGCCCTAAAATC-3′ **Ecd HpaI R 5′** – CCGGTAGAATTCGTTAACT TAATTTTTTGTTGG-3′
pMSCVpuro ECD	Primers used for cloning of ECD in to pMSCV puro vector.	**Ecd BglII** F 5′-AGATCTATGGAAGAAA CCATGAA-3′	**Ecd HpaI R 5′-** GAATTCTTAAT TTTTTGTTGGCTT-3′
ECD pXLG Nterm TCM his Strep Dest 1-534 pXLG Nterm TCM 1-468pXLGNterm TCM 1-432pXLGNterm TCM 1-342pXLGNterm TCM 1-286pXLGNterm TCM	Primers used for cloning of ECD in ST6GAL1-XLG-NtermTCMhisStrep-DEST	**ECD F EcoRI 5′** –CACCCACGGCGAATTCATG GAAGAAACCATGAAG 3′	**ECD HindIII R5'-** GATTGGATCCAAGCTT TTAATTTTTTGTTGGCTT-3′ **HindIII 534 R** 5′ – GATTGGATCCAAGCTTTCA CCCTAAAATCTTATCAAAAT3′ **HindIII 468R 5′** – GATTGGATCCAAGCTTTCATCC CTTGTGGGTTGAGACTT-3′ **HindIII 432R 5′** – GATTGGATCCAAGCTTTCAGCCA ACAGCTTCCTGCAGCA-3′ **Hind III 342R 5′** – GATTGGATCCAAGCTTTCAATTC TTTTTCAGACTTTCA-3′ **HindIII 286R-5′ –** GATTGGATCCAAGCTTTCACAGC CTGTATCCACT-3′
PIH1D1pGEX6p1	Primers for subcloning of PIH1D1 in to pGEX-6p-1	**PIH1BglII F** 5′ –GGGAGATCTATGGCGA ACCCGAAGCTGC-3′	**PIH1XhoI R** 5′- GGGCTCGAGTCAAGAA GGCACCGGCAG-3′
ECDpET28B+ 1-534pET28B+ 1-432pET28B+	Primers for cloning of ECD in to Pet28b+	**ECD F XbaI 5**-′TCTAGAAATAATTTTGTTT AACTTTAAGAAGGAGATATACCAATGGAAG AAACCATGAAGC-3′	**ECD R XhoI** 5′-CTCGAGTTAGTGGTGGT GGTGGTGGTGATTTTTTGTTGGCTT-3′ **R XhoI 534-5'**-CTCGAGTTAGTGGTGGT GGTGGTGGTCTCGAG TTAGTGGTGGTGGTGGTGGT-3′ **R xhoI 432** -5′-CTCGAGTTAGTGGTGGTG GTGGTGGTGCCAA CAGCTTCCTGCAGCA-3′
pMSCV3S/A,6S/A pMSCV 3'S/A	Primers used for site directed mutagenesis 3S/A & 6S/A . (for 6S/A 3S/A was used template)	**ECD S503,505,518F** 5-′CCAAGGCCTAATG AGGCAGATGCTGATGATCTGGATGATGAAGA CTTTGAATGTTTAGATGCTGAT GATGACTTGGA-3′ **ECDS572,579,584AF** 5-′CAAGTGGAACCTGT AGCCCAGACTACCGATAACAATGCAGATGAGGA AGATGCTGGTACGGGAGAAT -3′	**ECD**503,505,518R 5′TCCAAGTCATCATCAG CATCTAAACATTCAAAGT CTTCATCATCC AGATCATCAGCATCT GCCTCATTAGGCCTTGG-3′ **ECD**S572, 579,584AR 5′- ATTCTCCCGTACCAGCA TCTTCCTCATCTGCATTG TTATCGGTAGTCTGGGCTA CAGGTT CCACTTG-3′
Flag - RUVBL1pCDNA3.1	Primers for cloning of RUVBL1 in to pCDNA3.1	**BamHI F** 5-AAGTCGACAAGGATCCATGAAG ATTGAGGAGGTGAAGAG-3'	**NotI 5**'- ACCGCGGCCGCTCACTTCATGTACTT ATCCTGCT-3'

Table S2: Primer Sequences Used for Cloning of Various Constructs