Supplementary Data

Selective targeting of activating and inhibitory Smads by distinct WWP2 ubiquitin ligase isoforms differentially modulates TGFβ signalling and EMT

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Quantifications using RT-PCR
The following forward (F) and reverse (R) primer pairs were used to quantify WWP2-isoform and human EMT marker transcripts using RT-PCR at an annealing temperature of 59^{o}C and 58^{o}C, respectively.

Target	Primer Sequences	Product (bp)
WWP2-FL (human)	TGAATGATGAACCCACAACAG (F) CTGGTAGAGGAATCTTTGGCTGA (R)	476
WWP2-FL (mouse)	TGAATGAGGAACCTACTCCAG (F) CTGGTAGAGGAATCTTTGGCTGA (R)	476
WWP2-N (human)	TGAATGATGAACCCACAACAG (F) CAGAGTCTCAGGTCTTCTCAGTTG (R)	334
WWP2-N (mouse)	TGAATGAGGAACCTACTCCAG (F) CCATTCTAGAGTCTCAAGTC (R)	327
WWP2-C (human)	GTGTGGTTCTTGGAGAAAG (F) GGGCGAGGATCCTTAAAGGT (R)	682
WWP2-C (mouse)	GTGTGGTTCTTGGAGAAAG (F) GGGCAAGGATCCTTAAAGGT (R)	684
Vimentin	TGGCACGTCTTGACCTTGAA (F) GGTCATCGTGATGCTGAGAA (R)	749
Fibronectin	CCGTGGGCAACTCTGTC (F) TGCGGCAGTTGTCACAG (R)	427
Snail	AATCGGAAGCCTAACTACAGCGAG (F) CCTTCCCACTGTCCTCATCTGACA (R)	240
p21	CAGCAGAGGAAGACCATGTG (F) GGCGTTTGGAGTGGTAGAAA (R)	439
GAPDH	TGTGAACCATGAGAAGTATGACAACAG (F) ACACGGAAGGCCATGCCAGT (R)	303

PCR primers for generating WWP2-isoform mammalian expression plasmids
The following primer pairs were used to PCR WWP2 coding sequences using pHM6-WWP2-HA expression plasmid (a gift from Fiona McDonald, University of Otago, NZ) as a template. PCR products were cloned downstream of the FLAG epitope-tag in plasmid pRK5 as *Eco*RI/*Xho*I restriction fragments:
WWP2-FL;
GATATCCCGAATTCATATGGCATCTGCCAGCTCTAGCCGGGGG (F)
CTCGAGTTACTCCTGTCCAAAGCCCTCGG (R)
WWP2-N:
GATATCCCGAATTCATATGGCATCTGCCAGCTCTAGCCGGGGG (F)
Primer 2, CTCGAGCTACCCTGGAGGAAGGGGCCGC (R)

WWP2-C;
GATATCCCGAATTCATATCCAGGAACCAGCTCTGCCCCGG (F)
Primer 2, CTCGAGTTACTCCTGTCCAAAGCCCTCGG (R)

PCR primers for generating bacterial WWP2-isoform expression plasmids
The following WWP2-isoforms were PCR cloned into pET28A as *Nde*I/*Hind*III digested restriction fragments:
WWP2-FL;
CTCGAGCATATGCTGCCTTTTGAGAAGTCTCAGCTC (F)
Primer 2, AAGCTTTTACTCGGTCTCCTCAATGGC (R)
WWP2-N;
CTCGAGCATATGCTGCCTTTTGAGAAGTCTCAGCTC (F)
AAGCTTTTAGCCTGGAGGAAGGGGCCG (R)
WWP2-C;
CTCGAGCATATGCGCAGTTTTCGGTGGAAGTATCAC (F)
AAGCTTTTACTCGGTCTCCTCAATGGC (R)

PCR primers for generating mammalian and bacterial WW-domain expression plasmids
Mammalian expression plasmids corresponding to Flag epitope-tagged WW domains of WWP2 (in pRK5) were PCR cloned (via PCR Blunt; Invitrogen) as restriction fragments digested with *Xho*I/*Hind*III:
WW1;
GAATTCCATATGGACGCTCTGCCTGCTGGATGG (F)
AAGCTTGTCGACTTAAGGAAGGGGCCGCTCCCAGGT (R)
WW2;
GAATTCCATATGCGGCCCCTTCCTCCAGGCTGG (F)
AAGCTTGTCGACTTACGCGGTCGGACGCTGCCAGGT (R)
WW3;
GAATTCCATATGGGCCCCCTCCCTCCTGGCTGG (F)
AAGCTTGTCGACTTAGGTCCGGGGATCCTCCCACTG (R)
WW4;
GAATTCCATATGCCAGCTCTGCCCCCAGGATGG (F)
AAGCTTGTCGACTTACGGGCGAGGATCCTTAAAGGT (R)
Extended-WW4;
GAATTCCATATGATGATCCAGGAACCAGCTCTGCCCCC (F)
AAGCTTGTCGACTTACACGTGGCTAGGTAGGGCATTTGA (R)

PCR primers for generating WWP2 ligase-null mutants (Stratagene QuikChange mutagenesis kit)
CCCAGAAGCCACACCGCCTTCAACCGTCTGGA (F)
TCCAGACGGTTGAAGGCGGTGTGGCTTCTGGG (R)

PCR primers for generating shRNA-pTER plasmids specific for WWP2-FL
GATCTCCGGAGTACGTGCGCAACTATGATCAAGAGTCATAGTTGCGCACGTACTCCTTT
TTA (sense)
GATCTAAAAAGGAGTACGTGCGCAACTATGACTCTTGATCATAGTTGCGCACGTACTC
CGGA (antisense)

Supplementary Figure Legends

Supplementary Figure S1: WWP2 binds to the PY motif in Smad7
(A) HEK293 cells expressing tagged-WWP2-FL were lysed and whole cell lysates (WCL) incubated with approximately 1μg of the indicated GST-Smad7 derived fusion proteins in a GST pull down assay (as described in Wicks *et al*, 2005). Protein complexes were washed and analysed using an anti-Flag antibody and Western blot analysis. (B) GST pulldowns were carried out as described above using the isolated GST-WW4 domain (extended version) and HEK-293 lysates from cells expressing Smad7-Flag.

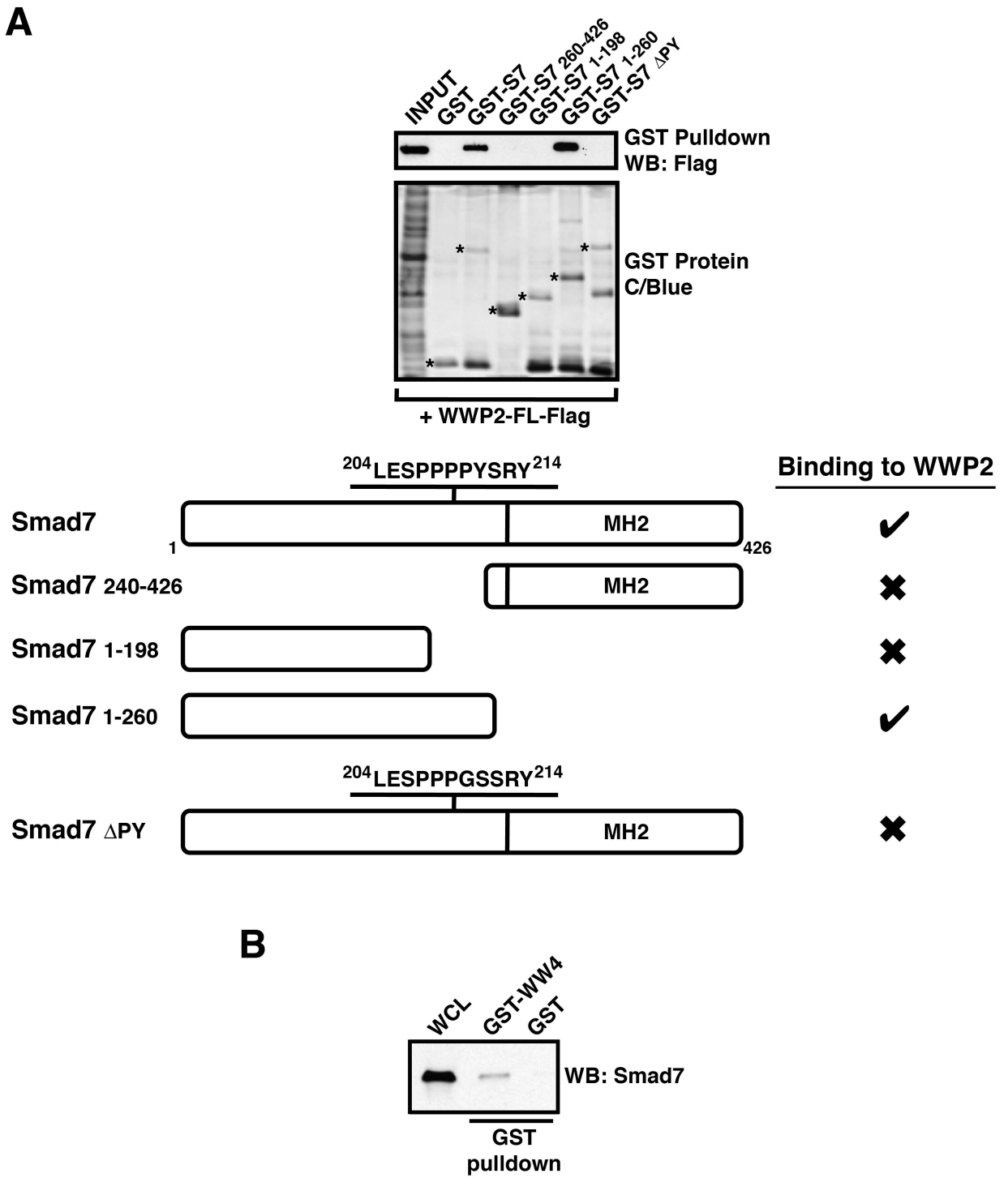
Supplementary Figure S2: WWP2 mediates Smad2 and Smad3 polyubiquitination
HEK293 cells were transfected with tagged-Smad2 (left panel) or Smad3 (right panel) 100ng HA-Ubiquitin and 100ng WWP2-FL as indicated. Cells were were pre-treated with MG132 for 5hrs and then stimulated with TGFβ for 16hrs, followed by anti-HA immunoprecipitation and analysis of ubiquitination species by anti-Smad Western blot analysis.

Supplementary Figure S3: WWP2N promotes WWP2-FL-dependent Smad2/3 turnover
Cos1 cells co-transfected with 1μg Smad2 (A) or Smad3 (B), with or without WWP-FL and WWP2-N plasmids, were pre-treated with MG132 for 18 hrs. Cells washed in fresh growth medium and incubated in the absence of MG132 and in the presence of cycloheximide for 2hrs, and TGFβ stimulated for the time points indicated. Whole cell lysates were assessed for Smad expression levels (middle), β-actin expression (left panel) and WWP2-FL/WWP2-N isoform expression using anti-Flag Western blotting (right panel). Smad-specific Western blots for Smad2 (C) and Smad3 (D) were quantified by densitometric scanning, and values were standardized for β-actin expression. Values were then standardized for WWP expression and Time=0 hr (which was set to 1), and graphs were plotted using Excel and optimum best-fit trendlines determined.

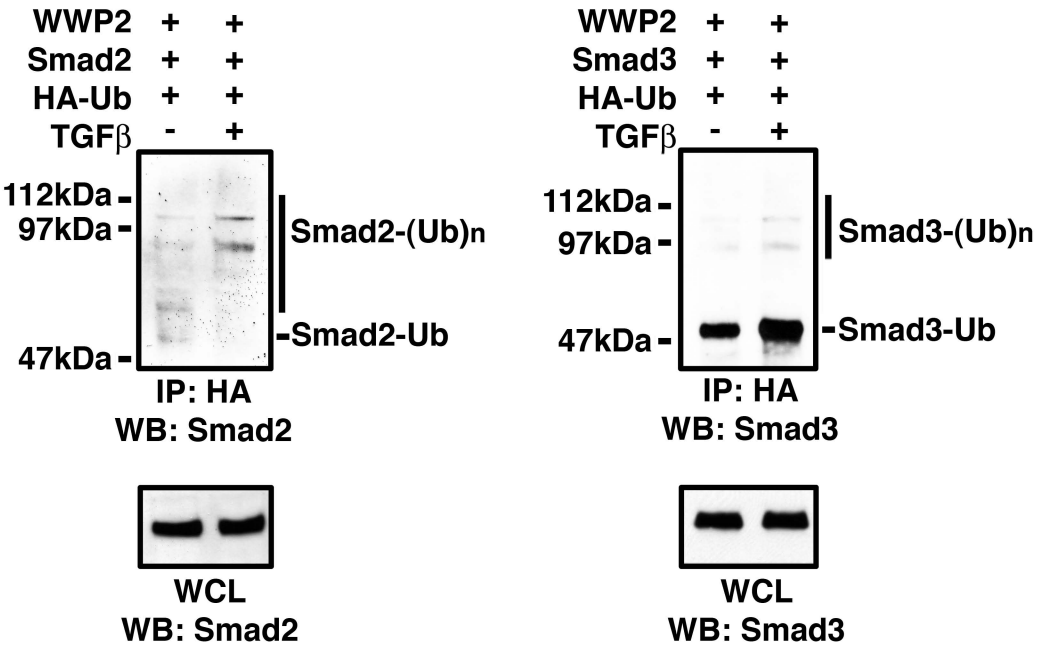
Supplementary Figure S4: WWP2-N promotes Smad ubiquitination by endogenous WWP2
HEK293 cells were transfected with tagged-Smads, 100ng HA-Ubiquitin and 100ng WWP2-FL or 100ng WWP2-N as indicated. Cells were were pre-treated with MG132 for 5hrs and then stimulated with TGFβ for 3hrs, followed by anti-HA immunoprecipitation and analysis of ubiquitination species by anti-Smad Western blot analysis.

Supplementary Figure S5: WWP2-N promotes WWP2-FL turnover
(A) Cos1 cells co-transfected with 1μg WWP2-FL (with or without WWP2-N) plasmids, were pre-treated with MG132 for 18hrs. Cells washed in fresh growth medium and incubated in the absence of MG132 and in the presence of cycloheximide for 2hrs, and TGFβ stimulated for the time points indicated. Whole cell lysates were assessed for WWP2-FL expression levels (middle), β-actin expression (left panel) and WWP2-N isoform expression (right panel). (B) Western blots were quantified by densitometric scanning and values were standardized for β-actin expression. Values were then standardized for WWP expression and Time=0 hr (which was set to 1), and graphs were plotted using Excel and optimum best-fit trendlines determined.

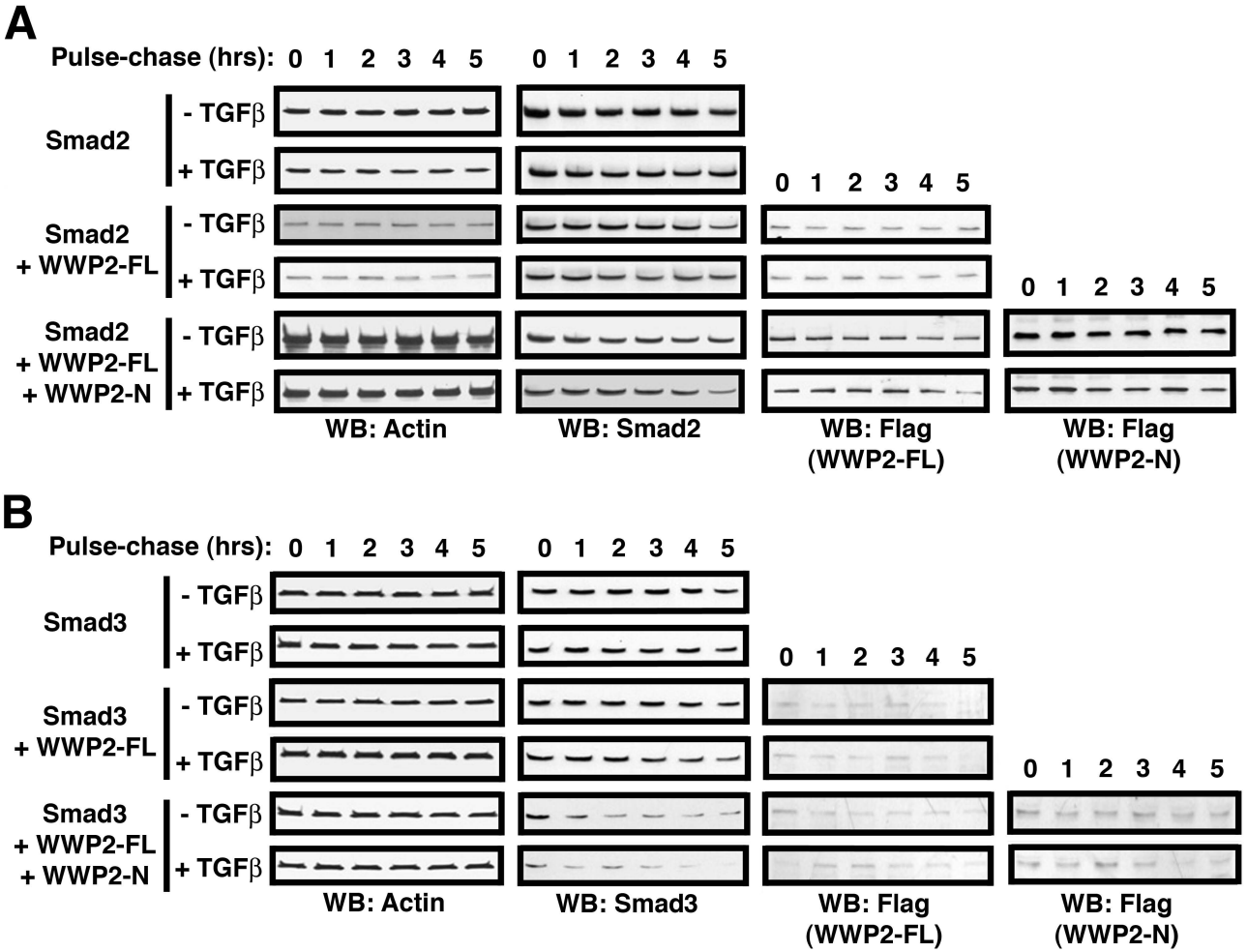
Supplementary Figure S6: Induction of classical EMT markers by TGFβ in Colo-357 cells
RNA isolated from Colo-357 cells stimulated with TGFβ for the indicated number of days, was isolated and reverse transcribed. 1.25ng cDNA were utilised as template DNA in RT-PCR using primers specific for vimentin, Snail, fibronectin, p21, fibronectin and GAPDH. Equal volumes of the PCR products were analysed by agarose gel electrophoresis.

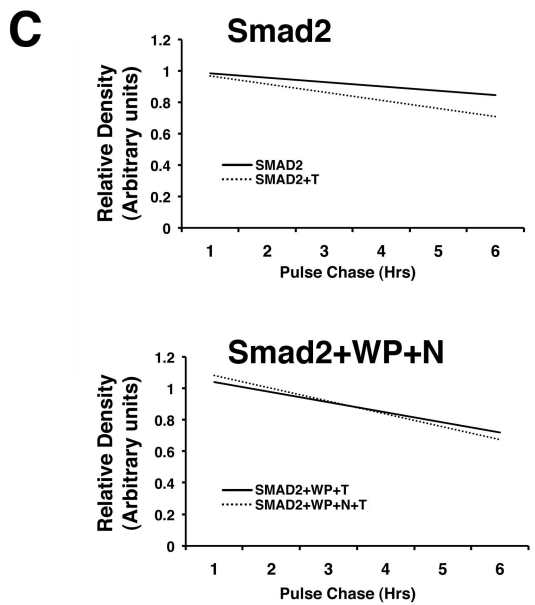


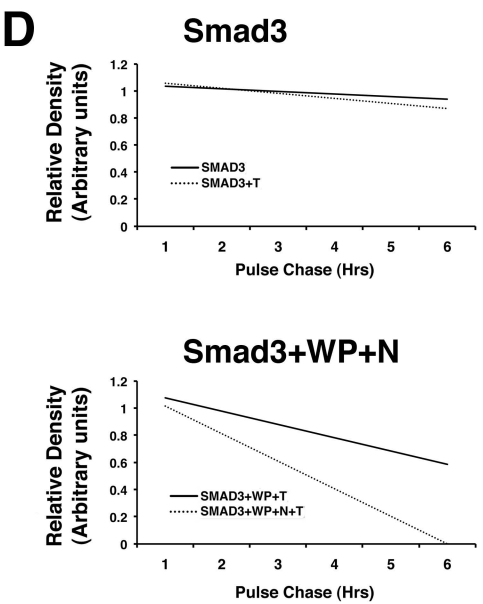
Supplementary Figure S1



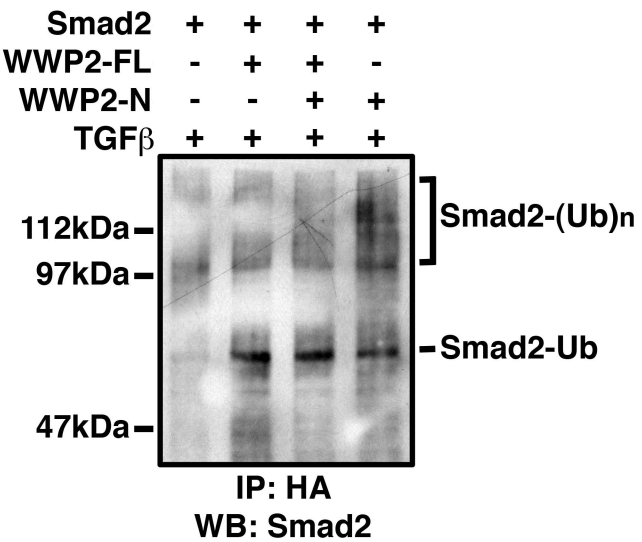
Supplementary Figure S2

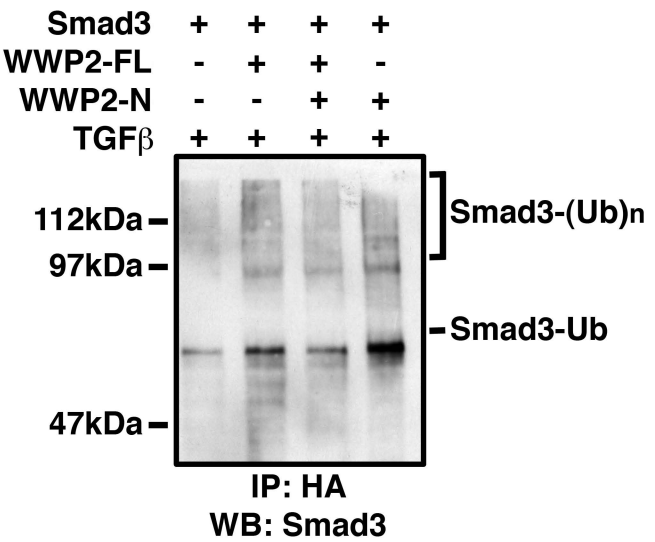




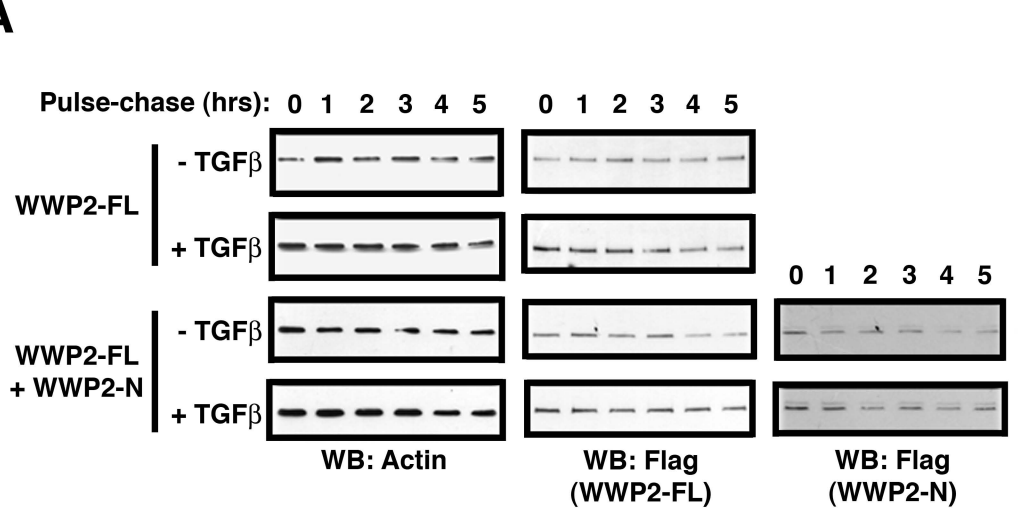


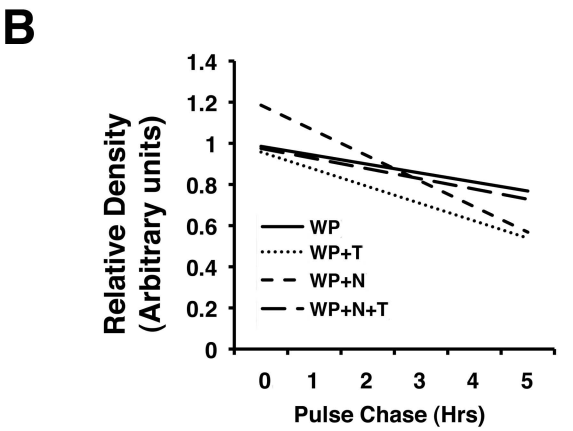
Supplementary Figure S3



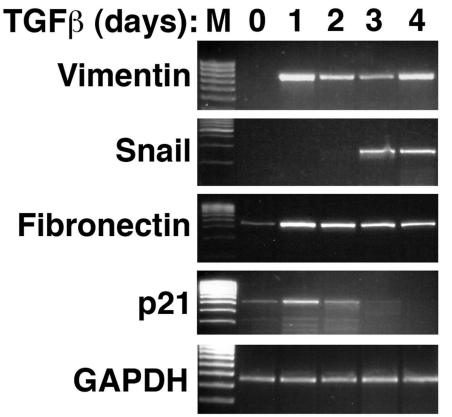


Supplementary Figure S4





Supplementary Figure S5



Supplementary Figure S6