SUPPLEMENTARY DATA

IMPAIRED TRANSFORMING GROWTH FACTOR-BETA (TGF-β) TRANSCRIPTIONAL ACTIVITY AND CELL PROLIFERATION CONTROL OF A MENIN IN-FRAME DELETION MUTANT ASSOCIATED WITH MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN1)*

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SUPPLEMENTARY METHODS

Reagents–Recombinant IL-1β and TGF-β and Flag antibodies (monoclonal M-2) were from Sigma. The mouse monoclonal antibody F5 against p21 (sc-6246), the goat anti-p15 PAF antibody (sc-67922) and the rabbit anti-β-tubulin polyclonal H-235 antibody (sc-9104) were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. The rat insulinoma Rin-5F and somatolactotroph GH4C1 cell lines were from the American Type Culture Collection (Manassa, VA) and the human embryonic kidney (HEK293) cells were from NPS Pharmaceuticals. Dulbecco's modified Eagle's medium, fetal bovine serum and antibiotics were from Invitrogen.

Constructs–Human menin wild-type (WT) cDNA in pcDNA3.1 was as described previously (S1) and was used as template for the other menin constructs. The EcoRI insert from this plasmid was cloned into pCMV-tag2B (Stratagene) to create a menin WT (Flag-tagged) construct. The correctness of this and all other constructs was confirmed by DNA sequencing.

Menin Δ(1-40) was generated by PCR using the following primers: F1: 5' TCACTAAGCTTATGCTGGGCTTCGTGGAGCATTTTCTGGCT 3' (HindIII site is underlined); R1: 5' GAGTGGGTCTGGCCGGCCAGGGGTG 3' [an FseI site at position c.885 in menin cDNA (c.1 is the A of the ATG initiation codon) that is not present in the vector is underlined]. The HindIII-FseI- digested PCR fragment was cloned into HindIII-FseI- digested menin WT in pcDNA3.1.

Menin Δ(41-277) was generated by overlap PCR using the following primers: F1: 5' ATGGGGCTGAAGGCCGCCCAGAAGACG 3'; R1 5' ATCTGCCAGGTTCCCTAAGGCCATCACCAAGGAAAGGAGCACCAGGTC 3'

F2: 5' GACCTGGTGCTCCTTTCCTTGGTGATGGCCTTAGGGAACCTGGCAGAT 3'; R2 5' GAGTGGGTCTGGCCGGCCAGGGGTG 3' (FseI site at c.885 of menin cDNA is underlined). Two PCRs were done initially with primer sets, F1-R1 and F2 and R2, to generate 135-bp and 88-bp products, respectively. The conditions were: 1 cycle; 94°C 1 min, 25 cycles; 94°C 30 sec, 60°C 35 sec, and 72°C 30 sec, and final cycle; 72°C 10 min. The BmgBI-FseI digested fragment was cloned into both BmgBI-FseI digested menin WT (in pcDNA3.1) and menin WT (in pCMV-tag2B).

Menin Δ(184-218) was generated by overlap PCR using the following primers: F1: 5'ATCGAGGATCCATGGGGCTGAAGGCCGCCCAGAAGACGCTGTTC3' (BamHI site is underlined); R1: 5' GCGCATGTATGATCCTTTCAGGTACAGCCAGCTCCAGGCATGATCCTCAGACAGGGCGAGGT GGAC3'; F2: 5' GTCCACCTCGCCCTGTCTGAGGATCATGCCTGGAGCTGGCTGTACCTGAAAGGATCATACAT GCGC 3'; R2: TGTGGTAGAGGGTGAGTGGGTCTGGC 3'. Two PCRs were done initially with primer sets, F1-R1 and F2-R2, to generate 596-bp and 288-bp products, respectively. The conditions were: 1 cycle; 94°C 35 sec, 25 cycles; 94°C 35 sec, 58°C 35 sec, and 72°C 45 sec, and final cycle; 72°C 10 min. PCR products were gel purified and used as template for a final PCR using primers F1 and R2 (same PCR conditions except the elongation step was; 72°C 1 min). The BmgBI-FseI digested 817-bp final PCR product was cloned into both BmgBI-FseI digested menin WT (in pcDNA3.1) and menin WT (in pCMV-tag2B).

Menin Δ(278-477) was generated by overlap PCR using the following primers:

F1 5' GCTGAGGTCACCTGGCACGGCAAGGGCAACGAGGAC 3' (the BstEII site which which cuts at position c.585 of the menin cDNA and is not present in vectors is underlined); R1 5' CCCGCCGTGGGCCCCGCCGCCGGCCGGGGTACCTTTCCAGATGTCCCAGG 3';

F2 5' CCTGGGACATCTGGAAAGGTACCCCGGCCGGCGGCGGGGCCCACGGCGGG 3'; R2 5' TACAGGCGGCCGC**TCA**GAGGCCTTTGCGCTGCCGCTTGAGGA 3' (NotI site is underlined and

stop codon is in bold). Two PCRs were done initially with primer sets, F1-R1 and F2-R2, to generate 277-bp and 438-bp products, respectively. The conditions were: 1 cycle; 94°C 1 min, 25 cycles; 94°C 30 sec, 60°C 35 sec, and 72°C 45 sec, and final cycle; 72°C 10 min. The BstEII-NotI- digested fragment was cloned into both BstEII-NotI digested menin WT (in pcDNA3.1) and menin WT (in pCMV-tag2B).

Menin Δ(478-610) was generated by PCR using the following primers: F: 5' GAGCAGACAGCTGAGGTCACCTGGCACGGC 3' (the BstEII site at c.585 is underlined); R: 5' TACAGGCGGCCGC**TCA**CCGGGCTTCCTCGCCCCACGGCTCCT 3' (the NotI site is underlined and the stop codon is in bold). The conditions were: 1 cycle; 94°C 1 min, 25 cycles; 94°C 30 sec, 60°C 35 sec, and 72°C 45 sec, and final cycle; 72°C 10 min. The BstEII-NotI- digested fragment was cloned into both BstEII-NotI digested menin WT (in pcDNA3.1) and menin WT (in pCMV-tag2B).

Human menin missense mutants (L22R, H139D, A242V, W423R, S443Y) were prepared using the menin WT (Flag-tag) construct as template with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

EGFP-menin constructs–The vectors used were, pEGFP-C1-3, in which the GFP is encoded N-terminally, and pEGFP-N1-3, in which GFP is encoded C-terminally, to the multiple cloning site (MCS), respectively (Clontech). The numbers, C1-3 and N1-3, designate the three different reading frames. For the cloning of menin inserts, the pEGFP vectors were digested with restriction enzymes to cut appropriately within the MCS.

The EcoRI insert of meninWT (in pcDNA3.1) was cloned into both pEGF-N1 and pEGF-C1 to create pEGFP-meninWT-N and pEGFP-meninWT-C, respectively.

Menin Δ(1-40) (in pcDNA3.1) was digested with HindIII and EcoRI and the insert cloned into pEGFP-C2 to create pEGFP-menin Δ(1-40)-C.

MeninWT (in pcDNA3.1) was digested with SalI and EcoRI and the insert cloned into pEGFP-C2 to create pEGFP-menin Δ(1-100)-C.

The EcoRI insert of menin Δ(41-277) (in pcDNA3.1) was cloned into pEGFP-N1 to create pEGFP-menin Δ(41-277)-N.

Menin Δ(278-477) (in pcDNA3.1) was digested with HindIII and PmeI and the insert cloned into HindIII-SmaI-digested pEGFP-C2 to create pEGFP-menin Δ(278-477)-C.

Menin Δ(478-610) (in pcDNA3.1) was digested with EcoRI and NotI and the insert cloned into pEGFP-N1 to create pEGFP-menin Δ(478-610)-N.

GST-menin and -Smad constructs–The vectors used were pGEX-5X1-3 (GE Healthcare).

MeninWT (in pcDNA3.1) was digested with EcoRI and the insert cloned into pGEX-5X1 to create pGST-meninWT.

Menin Δ(1-40) (in pcDNA3.1) was digested with PmeI and the insert cloned into SmaI-digested pGEX-5X2 to create pGST-menin Δ (1-40).

Menin Δ(41-277) (in pcDNA3.1) was digested with EcoRI and the insert was cloned into pGEX-5X1 to create pGST-menin(D41-277).

Menin Δ(278-477) (in pcDNA3.1) was digested with EcoRI and NotI and the insert was cloned into pGEX-5X1 to create pGST-menin Δ(278-477).

Menin Δ(478-610) (in pcDNA3.1) was digested with EcoRI and NotI and the insert was cloned into pGEX-5X1 to create pGST-menin Δ(478-610).

The rat Smad3 cDNA was released from the pcDNA3.1 vector with EcoRI and cloned into pGEX-5X1 to create pGST-Smad3.

Other constructs–The human JunD cDNA in expression vector pCMV6-XL5 was from Origene (cat. # SC116768). The promoterless pGL3 luciferase reporter plasmid was from Promega. The 3TP-Lux TGF-β-responsive reporter construct was from Dr. Joan Massague (S2). The pAP-1-Luc cis-reporter plasmid with a $(TGACTAA)_7$ enhancer element sequence was from Stratagene. The $(\kappa B)_6$-luciferase reporter construct was from Dr. Mark S. Nanes (S3).

Minigene Constructs–A portion of the *MEN1* gene corresponding to part of exon II, intron 2, exon III, intron 3 and exon IV was PCR-amplified from genomic DNA from patient II-1 and from an unrelated normal individual. This was done using the Expand long template PCR system (Roche, Laval, QC, Canada) that has a mixture of Taq and Pwo DNA polymerases, and primers 2F and 4R described above. The conditions were as follows: 94°C for 1 min, 94°C 45 sec, 58°C for 45 sec, and 68°C for 9 min with an increment at cycle 10 of 20 sec each cycle for 20 cycles followed by a 10 min elongation. After gel purification, the wild-type and mutant 2505-bp fragments were cloned into the TA cloning vector, pCR2.1Topo (Invitrogen), which contains SacI and XhoI sites flanking the PCR product insertion site, and were partially sequenced to establish the orientation and confirm the correctness of the exons and the flanking intronic sequences. Vectors harboring wild-type and mutant inserts in the appropriate orientation were digested with SacI, blunt-ended with Klenow, and digested with XhoI. The released inserts were subcloned into the EcoRV and XhoI sites of the mammalian expression vector pcDNA3.1 to generate wild-type and mutant minigene constructs.
Minigene Assay–Rin-5F cells were seeded at 60% confluency in DMEM with 10% FBS and incubated for 12-24 h. Cells were transfected with minigene constructs or empty vector as control (10 μg DNA/100 mm dish) using fugene (Roche). Total RNA was isolated 48 h later from the transiently transfected cells and was analyzed by RT-PCR using primers 2F-4R.
Cellular localization of menin mutants by green fluorescence–HEK293 cells were grown on coverslips in DMEM containing 10% FCS in 6-cm plates to approximately 40% confluence. Transfections were carried out with Lipofectamine (Invitrogen) plasmid DNA (8-10 μg) expressing either WT or mutant menin as well as empty vectors, pcDNA3.1 and pEGFP-N. Twenty-four hours after transfection, cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence was visualized by fluorescent microscopy, and images were collected by a charge-coupled device camera.

SUPPLEMENTARY REFERENCES

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S2. Wrana, J.L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.F., and Massague, J. (1992) *Cell* **71**, 1003-1014
S3. Farmer, P.K., He, X., Schmitz, M.L., Rubin, J., and Nanes, M.S. (2000) *Am. J. Physiol. Endocrinol. Metab.* **279**, E213-E220

SUPPLEMENTARY LEGENDS

Fig. S1. Minigene analysis of the splicing of wild-type and mutant menin transcripts. A. Genomic PCR products (2554-bp) comprising part of exon 2 (from the ATG initiation codon), intron 2, exon 3, intron 3 and exon 4 of wild-type or mutant *MEN1* genes [the position of the mutation is shown by the arrow (▼)] were cloned into pcDNA3.1 and the constructs were transfected into Rin-5F cells. B. Predicted structures of the wild-type (WT) and mutant (MUT) mRNAs and predicted sizes of RT-PCR products (WT, 792-bp; MUT, 687-bp) using primer set #1 (see Fig. 2A). C. Agarose gel electrophoresis of RT-PCR products; M, DNA markers; WT, wild-type; MUT, mutant; pcDNA3.1, empty vector; -RT, minus reverse transcriptase.

Fig. S2. Menin Δ(184-218), like wild-type menin, localizes to the nucleus. Representative examples of, A, green fluorescence in HEK293 cells transfected with EGFP-menin fusion constructs, GFP-MeninWT, GFP-MeninΔ(184-218) or GFP-MeninΔ(478-610) and, B, DAPI staining showing the nuclei from the same cells, and C, overlay of the two signals.

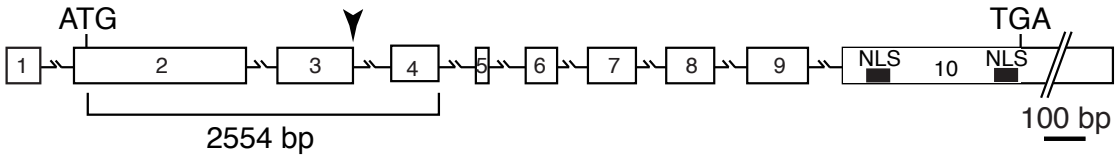
Fig. S3. Menin Δ(184-218), like wild-type menin, modulates JunD and NF-κB transcriptional activity in patient lympgoblastoid cells. A. The promoterless pGL3 or AP-1(7x) constructs were transfected without

(-) or with (+) JunD, with empty vector (V), wild-type menin (WT), menin mutants Δ(184-218), H139D or A242V into lymphoblastoid cells, either homozygous for the wild-type (WT), or heterozygous for the splice-site mutant (Mutant) *MEN1* genes. Relative luciferase activity was measured and the values shown represent the mean ± SEM. ***, $P<0.001$, JunD (+) versus (-). B. Western blot analysis of the cell extracts probed with antibodies against Flag and β-tubulin. C. The promoterless pGL3 or NF-κB(6x) constructs were transfected either with empty vector (Vector), wild-type menin (WT), menin mutants Δ(184-218) or Δ(278-477) into lymphoblastoid cells, either homozygous for the wild-type (WT), or heterozygous for the splice-site mutant (Mutant) *MEN1* genes, that were treated (+) or not (-) with IL-1β (5 ng/ml). Relative luciferase activity was measured and the values shown represent the mean ± SEM. ***, $P<0.001$; *, $P<0.05$, IL-1β (+) versus (-). D. Western blot analysis of the cell extracts probed with antibodies against Flag and β-tubulin.

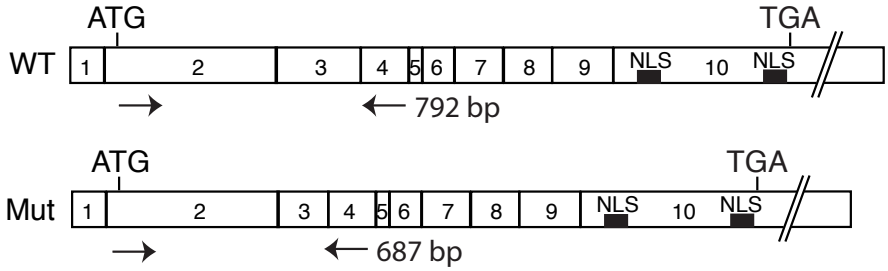
Fig. S4. Transfection with increasing amounts of the wild-type menin construct results in increasing responsiveness to TGF-β in A, Rin5F cells and B, GH4C1 cells. The 3TP-Lux construct was transfected without (-) or with 25, 50, 100, 250 or 500 ng of the WT menin construct. Cells were stimulated (+) or not (-) with 200 pM TGF-β. Relative luciferase activity was measured and the values shown represent the mean ± SEM. ***, $P<0.001$; TGF-β (+) versus (-). *, $P<0.05$; **, $P<0.01$; versus 500 ng WT menin.

Fig S1.

A


ATG
TGA
1
2
3
4
5
6
7
8
9
NLS
10
NLS
2554 bp
100 bp

B


ATG
TGA
WT
1
2
3
4
5
6
7
8
9
NLS
10
NLS
792 bp
ATG
TGA
Mut
1
2
3
4
5
6
7
8
9
NLS
10
NLS
687 bp

C

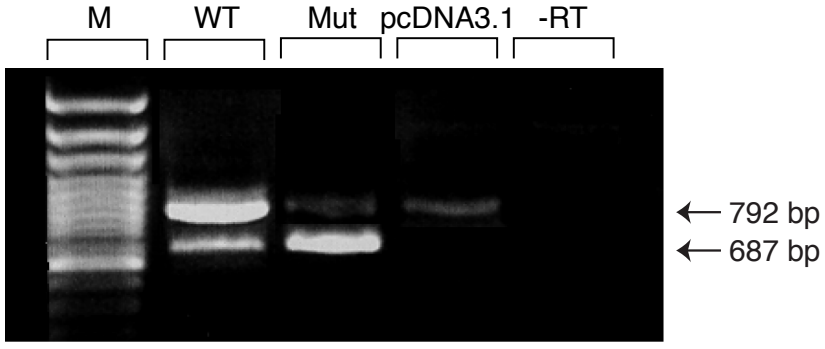

M
WT
Mut
pcDNA3.1
-RT
792 bp
687 bp

Figure S2.

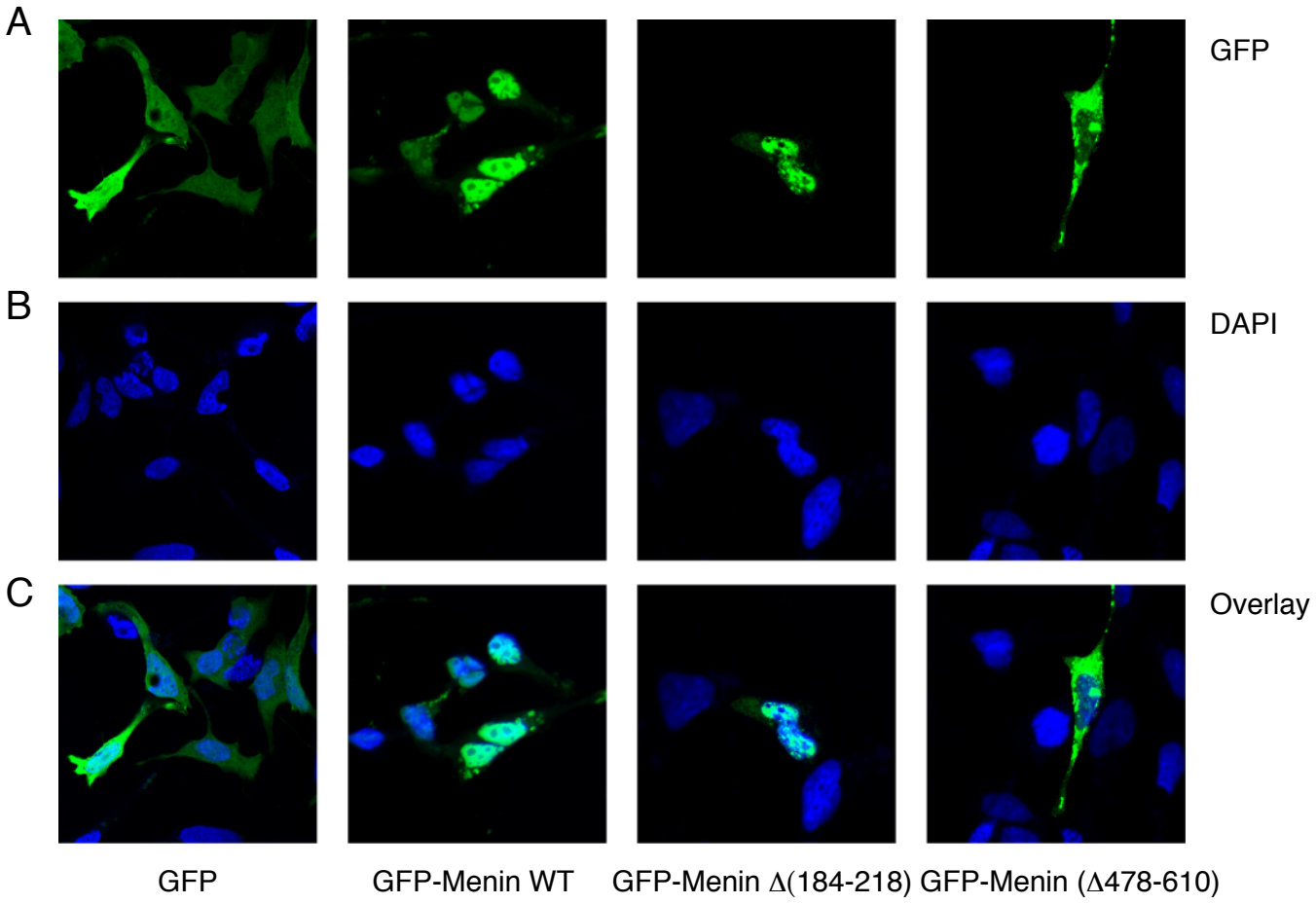

A
B
C
GFP
DAPI
Overlay
GFP
GFP-Menin WT
GFP-Menin Δ(184-218)
GFP-Menin (Δ478-610)

Figure S3.

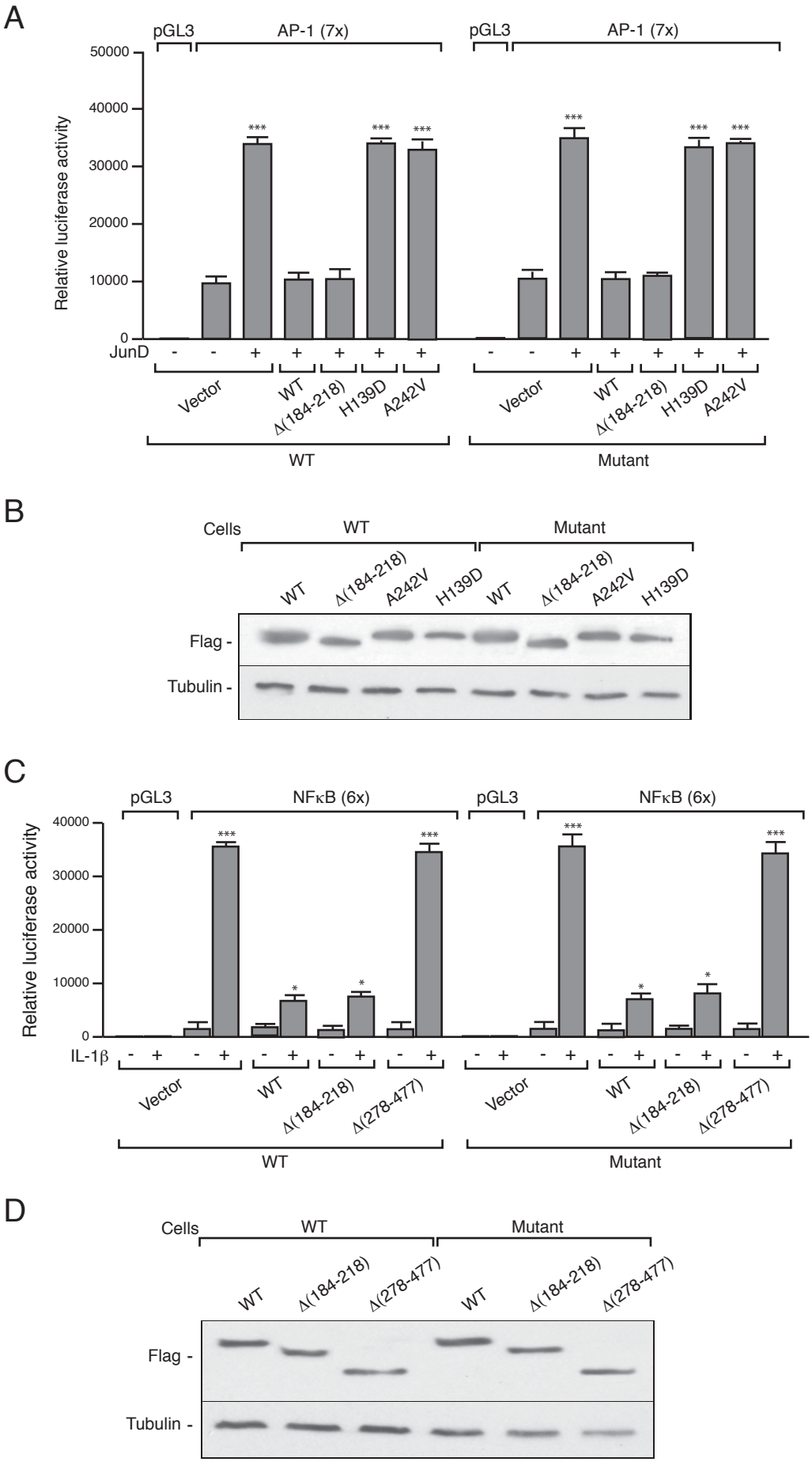
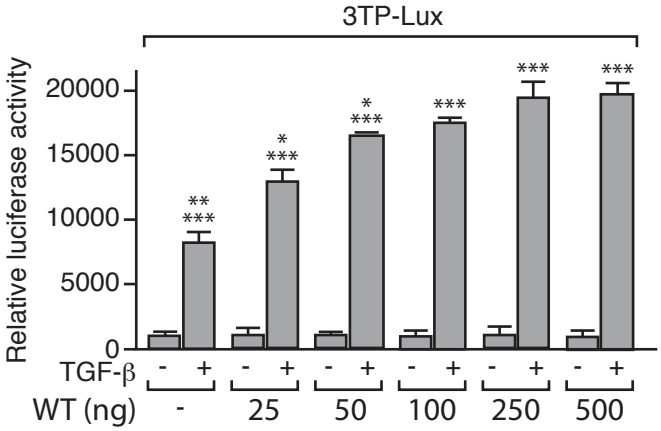

A
pGL3
AP-1 (7x)
pGL3
AP-1 (7x)
Relative luciferase activity
50000
40000
30000
20000
10000
0
***
JunD
- - + + + + +
Vector
WT
Δ(184-218)
H139D
A242V
WT
Mutant
B
Cells
WT
Mutant
WT
Δ(184-218)
A242V
H139D
Flag -
Tubulin -
C
pGL3
NFκB (6x)
pGL3
NFκB (6x)
Relative luciferase activity
40000
30000
20000
10000
0
***
*
IL-1β
- + - +
Vector
WT
Δ(184-218)
Δ(278-477)
WT
Mutant
D
Cells
WT
Mutant
WT
Δ(184-218)
Δ(278-477)
Flag -
Tubulin -

Figure S4.

A Rin-5F cells



B GH4C1 cells