Supplemental Data for

IKKβ is a β-catenin kinase that regulates mesenchymal stem cell differentiation

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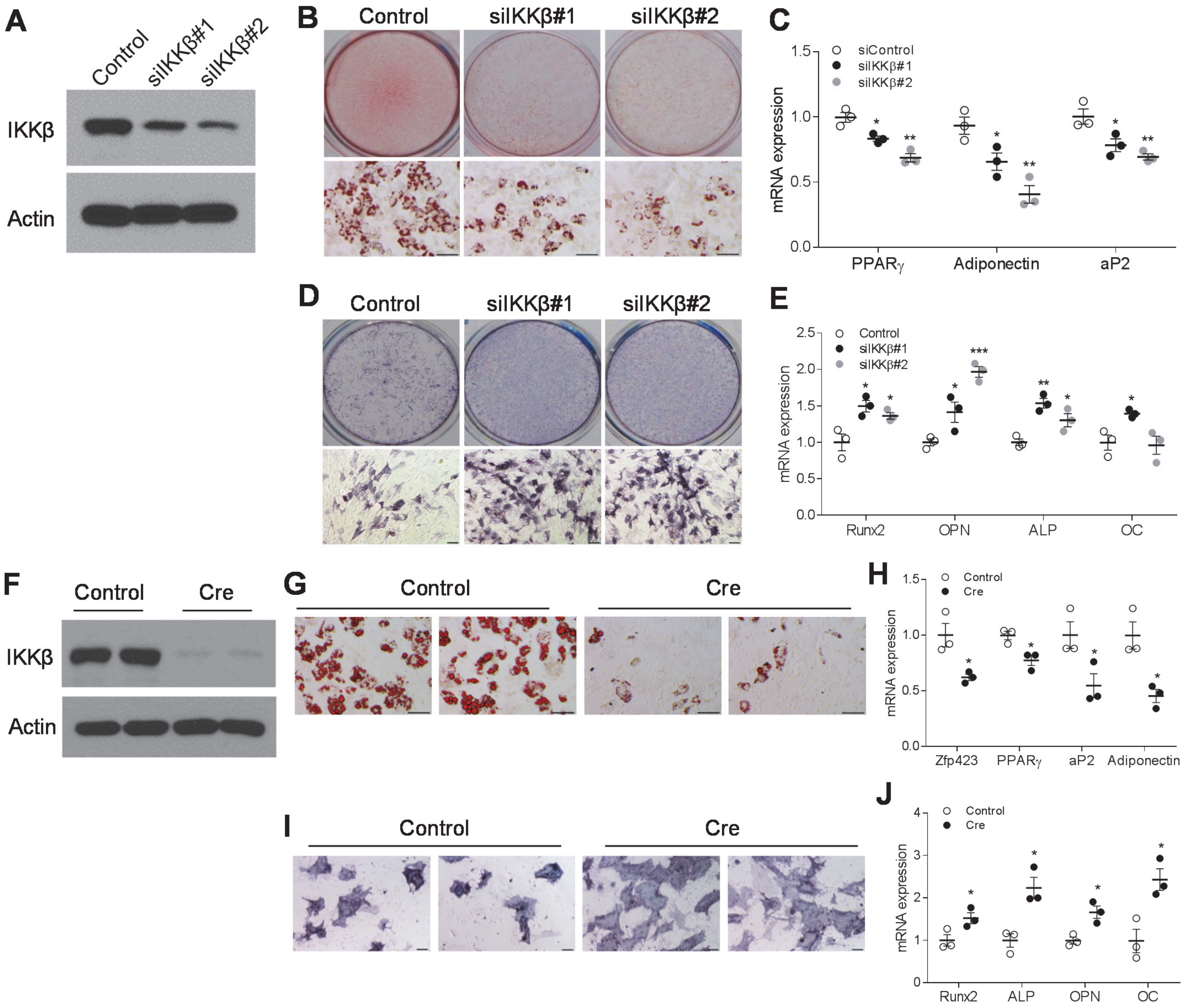
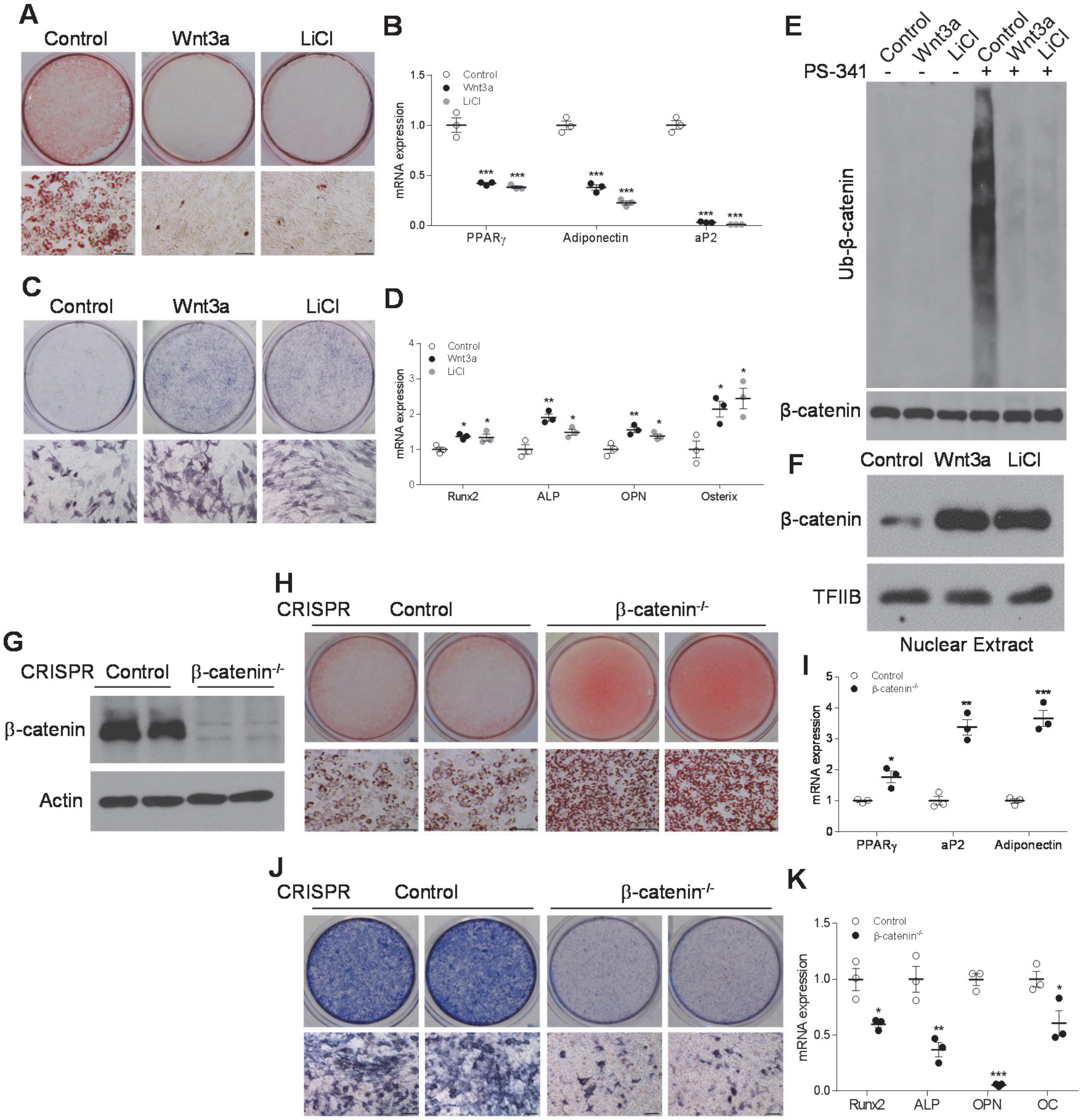


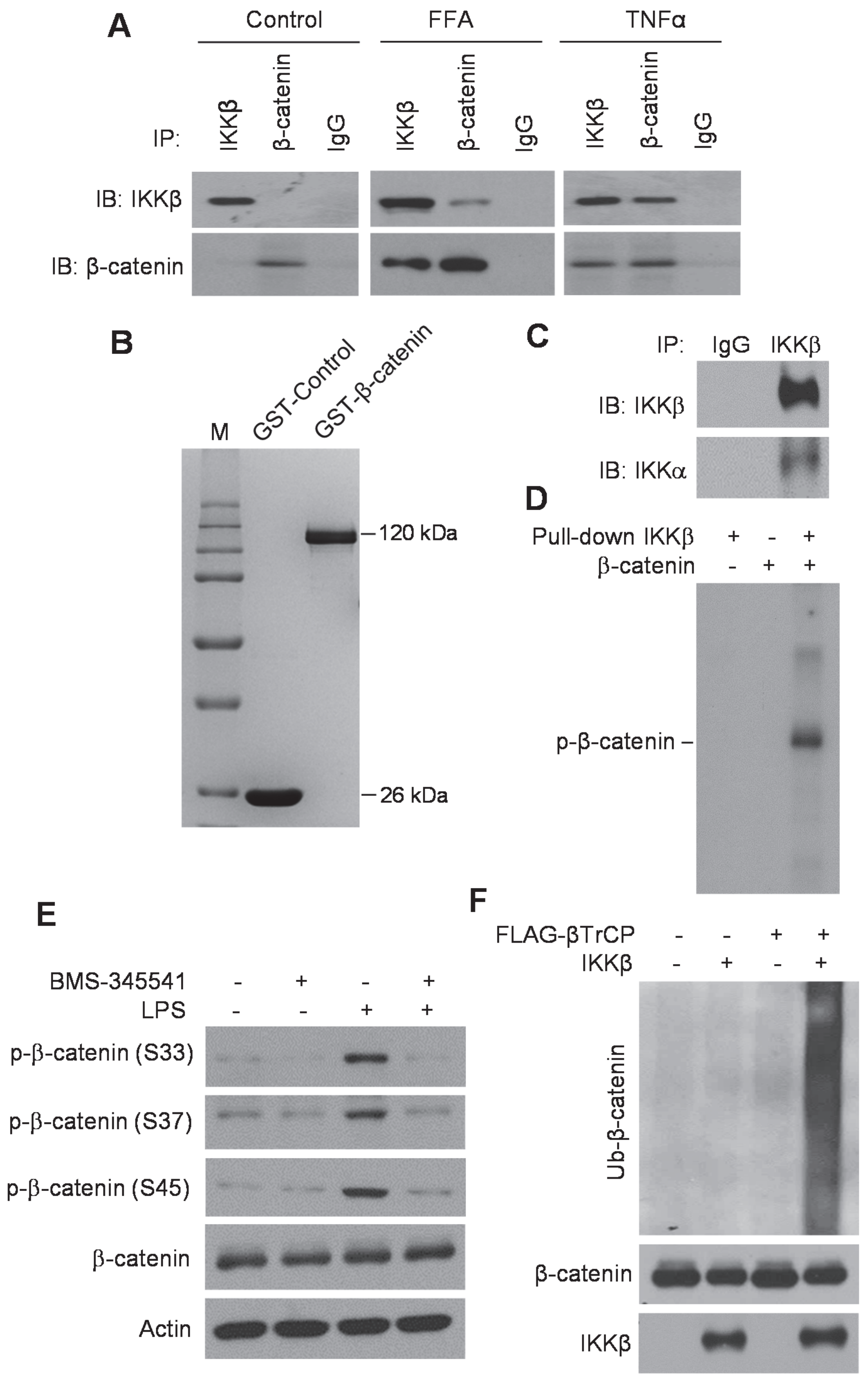
Supplemental Figure 1. Knockdown of IKKβ affects adipogenic and osteogenic potential of murine MSCs.

(**A-E**) C3H/10T1/2 cells were introduced with control siRNA or siRNA against IKKβ. Immunoblotting for IKKβ proteins in control or siIKKβ C3H/10T1/2 cells (**A**). Oil-red-O staining (**B**) and QPCR analysis (**C**) of control or siIKKβ C3H/10T1/2 cells induced by an adipogenic cocktail (n=3). ALP staining (**D**) and QPCR analysis (**E**) of control or siIKKβ C3H/10T1/2 cells induced by an osteogenic cocktail (n=3). Scale bar, 100 μm. (**F-J**) BMMSCs isolated from IKKβ$^{F/F}$ mice were infected with control lentivirus or lentivirus expressing Cre. Immunoblotting for IKKβ proteins in BMMSCs (**F**). Oil-red-O staining (**G**) and QPCR analysis (**H**) of BMMSCs induced by an adipogenic cocktail (n=3). ALP staining (**I**) and QPCR analysis (**J**) of BMMSCs induced by an osteogenic cocktail (n=3). Scale bar, 100 μm. Error bars represent ± SEM. Significance was determined by Student's t test (**H** and **J**) or one-way ANOVA (**C** and **E**). *p <0.05; **p<0.01.

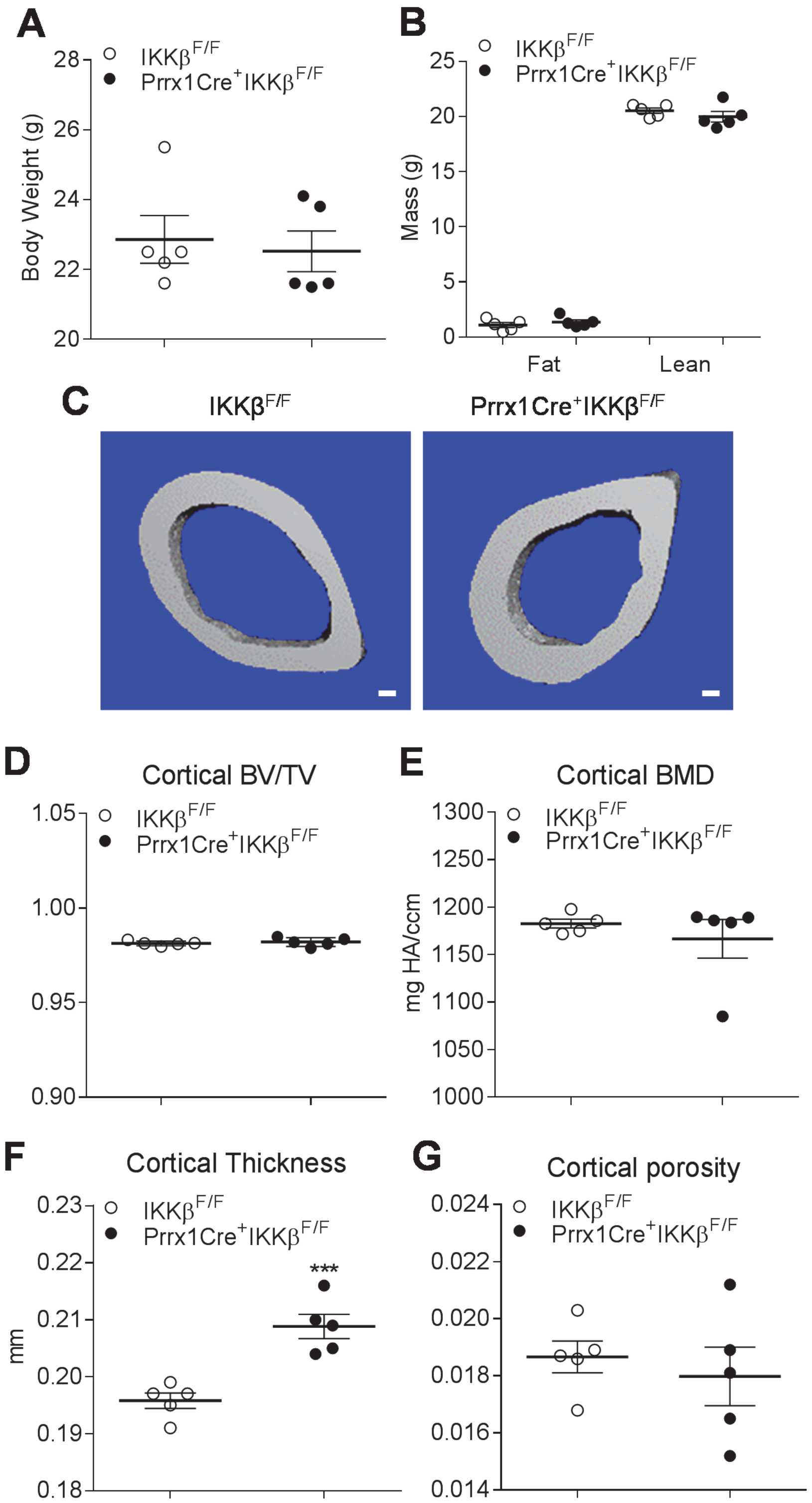


Supplemental Figure 2. Wnt/β-catenin signaling regulates adipogenesis and osteogenesis in MSCs.

(**A-D**) C3H/10T1/2 cells were treated with vehicle control, Wnt3a or LiCl, and were induced by differentiation media. Oil-red-O staining (**A**) and QPCR analysis (**B**) of vehicle, Wnt3a or LiCl-treated C3H/10T1/2 cells induced by an adipogenic cocktail (n=3). ALP staining (**C**) and QPCR analysis (**D**) of vehicle, Wnt3a or LiCl-treated C3H/10T1/2 cells induced by an osteogenic cocktail (n=3). Scale bar, 100 μm. (**E**) Vehicle, Wnt3a, or LiCl-treated C3H/10T1/2 cells were treated with vehicle or 100 nM PS-341. β-Catenin proteins were immunoprecipitated with anti-β-catenin antibodies and then probed with anti-ubiquitin antibodies. The whole cell lysates were probed with anti-β-catenin antibodies as an internal control. (**F**) Immunoblotting for nuclear β-catenin proteins in C3H/10T1/2 cells treated with vehicle, Wnt3a or LiCl. (**G**) Immunoblotting for β-catenin proteins in control or CRISPR/Cas9-mediated β-catenin-deficient C3H/10T1/2 cells. (**H and I**) Oil-red-O staining (**H**) and QPCR analysis (**I**) of control or β-catenin-deficient C3H/10T1/2 cells induced by an adipogenic cocktail (n=3). Scale bar, 100 μm. (**J** and **K**) ALP staining (**J**) and QPCR analysis (**K**) of control or β-catenin-deficient C3H/10T1/2 cells induced by an osteogenic cocktail (n=3). Scale bar, 100 μm. Error bars represent ± SEM. Significance was determined by Student's t test (**I** and **K**) or one-way ANOVA (**B** and **D**). *p <0.05; **p<0.01, ***p<0.001.

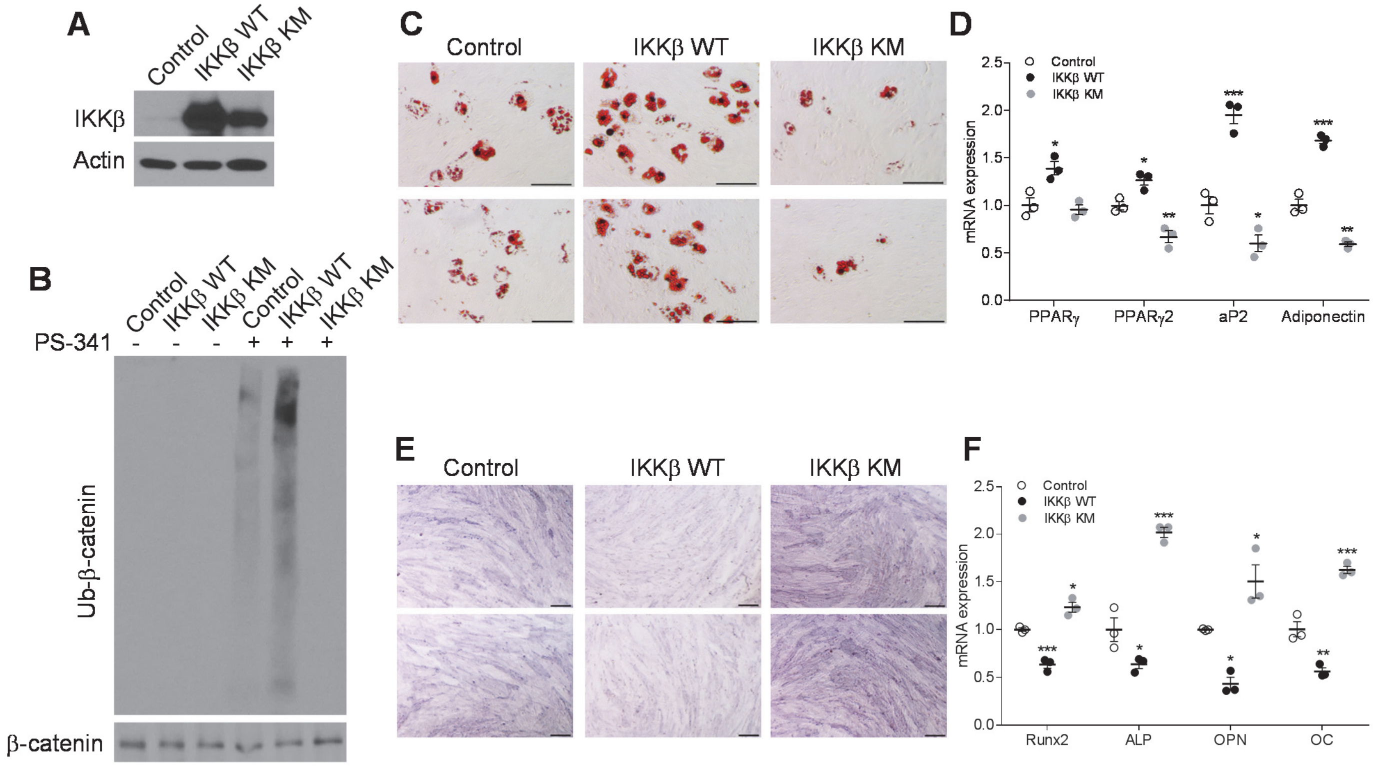


Supplemental Figure 3. IKKβ phosphorylates β-catenin and increases its ubiquitination. (**A**) Immunoblotting for endogenous IKKβ and β-catenin proteins after immunoprecipitation using control IgG or antibodies against IKKβ or β-catenin proteins in C3H10T1/2 cells treated with vehicle control, FFAs or TNFα. (**B**) Monochromatic image of Coomassie blue-stained SDS-PAGE gel for GST and GST-β-catenin proteins. (**C**) Immunoblotting for IKKβ and IKKα proteins after immunoprecipitation using control IgG or antibodies against IKKβ proteins in HEK293T cells infected with virus expressing IKKβ. (**D**) In vitro phosphorylation of GST-β-catenin proteins by the protein complex immunoprecipitated by antibodies against IKKβ proteins in the presence of γ-[^{32}P]ATP. (**E**) Immunoblotting for phosphorylated β-catenin proteins in C3H/10T1/2 cells treated with vehicle control or LPS in the absence or presence of IKKβ inhibitor BMS-345541. (**F**) GST-β-catenin proteins were phosphorylated by IKKβ in vitro in the presence of nonradioactive ATP. The reaction substrates were subjected for cell-free ubiquitination assay in the absence or presence of β-TrCP proteins.

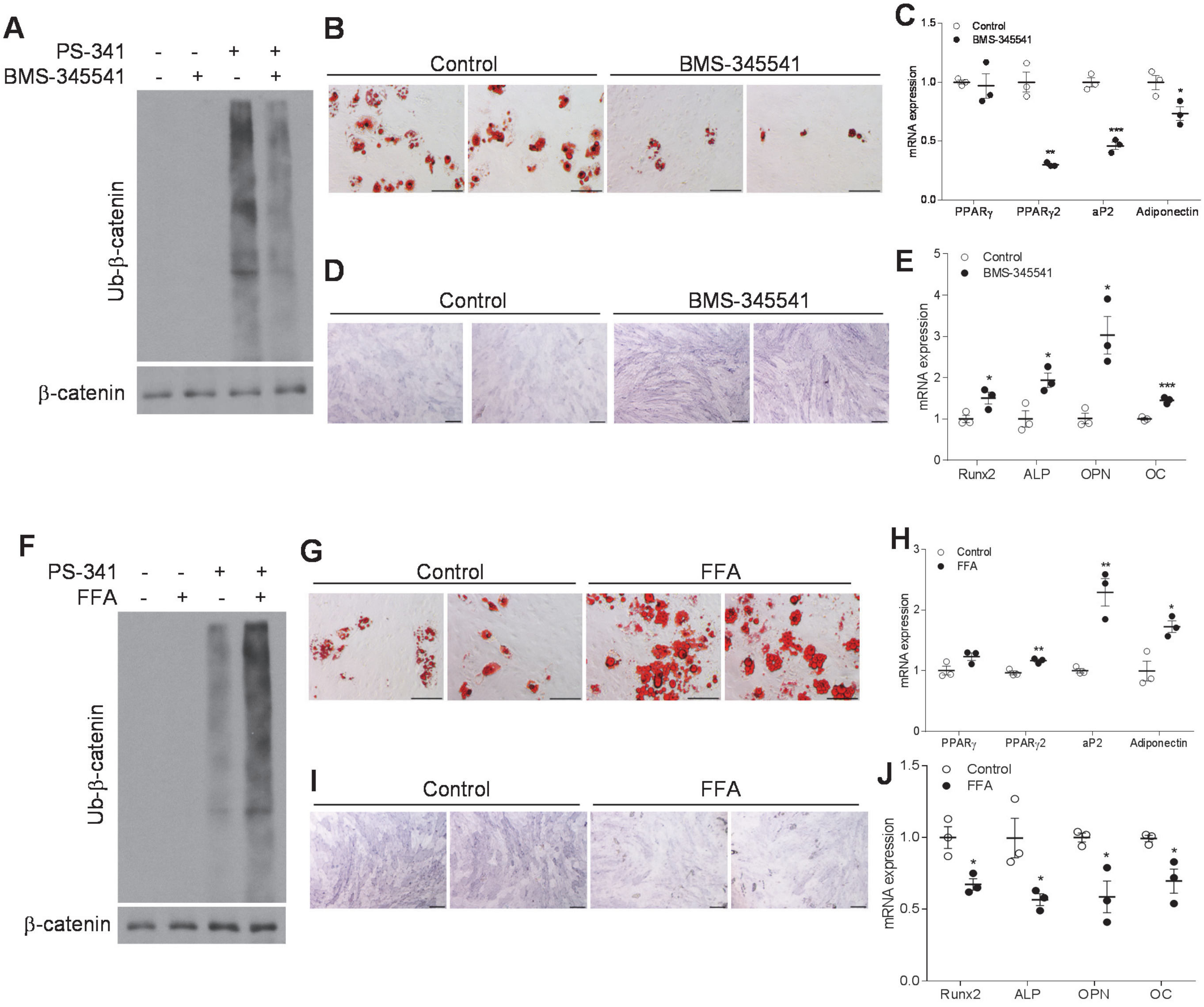


Supplemental Figure 4. Deficiency of IKKβ in BMMSCs has little or no effects on body weight and cortical bone.

(**A** and **B**) Body weight (**A**) and lean and fat mass (**B**) of 20-week-old IKKβ$^{F/F}$ and Prrx1Cre^{+}IKKβ$^{F/F}$ littermate mice. (**C**) MicroCT images of femur cortical bone of 20-week-old IKKβ$^{F/F}$ and Prrx1Cre^{+}IKKβ$^{F/F}$ mice. Scale bar, 100 μm. (**D-G**) MicroCT analyses of cortical bone volume/total volume (**D**), bone marrow density (**E**), thickness (**F**), and porosity (**G**) in the femur mid-diaphysis of IKKβ$^{F/F}$ and Prrx1Cre^{+}IKKβ$^{F/F}$ mice (n=5). Error bars represent ± SEM. Significance was determined by Student's t test (**F**). ***p<0.001.



Supplemental Figure 5. Expression of wild-type or mutant form IKKβ proteins has opposite effects on adipogenesis and osteogenesis of human BMMSCs.
(**A** and **B**) Immunoblotting for IKKβ (**A**) and ubiquitinated β-catenin proteins (**B**) in human BMMSCs infected with control, WT IKKβ and IKKβ KM virus. (**C** and **D**) Oil-red-O staining (**C**) and QPCR analysis (**D**) of human BMMSCs induced by an adipogenic cocktail (n=3). Scale bar, 100 μm. (**E** and **F**) ALP staining (**E**) and QPCR analysis (**F**) of human BMMSCs induced by an osteogenic cocktail (n=3). Scale bar, 100 μm. Error bars represent ± SEM. Significance was determined by one-way ANOVA (**D** and **F**). *p <0.05; **p<0.01, ***p<0.001.



Supplemental Figure 6. Modulation of IKKβ activity affects adipogenesis and osteogenesis of human BMMSCs.
(**A-E**) Human BMMSCs were treated with vehicle control or 5μM IKKβ inhibitor BMS-345541. Immunoblotting for ubiquitinated β-catenin proteins in control or BMS-345541-treated human BMMSCs (**A**). Oil-red-O staining (**B**) and QPCR analysis (**C**) of control or BMS-345541-treated human BMMSCs induced by an adipogenic cocktail (n=3). ALP staining (**D**) and QPCR analysis (**E**) of control or BMS-345541-treated human BMMSCs induced by an osteogenic cocktail (n=3). Scale bar, 100 μm. (**F-J**) Human BMMSCs were treated with vehicle control or 0.5mM FFAs. Immunoblotting for ubiquitinated β-catenin proteins in control or FFA-treated human BMMSCs (**F**). Oil-red-O staining (**G**) and QPCR analysis (**H**) of control or FFA-treated human BMMSCs induced by an adipogenic cocktail (n=3). ALP staining (**I**) and QPCR analysis (**J**) of control or FFA-treated human BMMSCs induced by an osteogenic cocktail (n=3). Scale bar, 100 μm. Error bars represent ± SEM. Significance was determined by Student's t test (**C, E, H** and **J**). *p <0.05; **p<0.01, ***p<0.001.

Supplemental Table 1: Baseline Characteristics of Individual Human Subjects.

Subject	Age	Gender	BMI	SI	FBG	Glucose (2 hr)	IKKβ expression
1	65	F	27.6	9.52	92	172	1.39
2	51	F	26.66	5.02	80	110	1.94
3	29	M	27.44	3.25	91	118	1.80
4	26	F	24	3.45	77	98	0.66
5	29	F	29	N/A	90	92	1.35
6	36	F	26	N/A	77	83	0.10
7	44	F	28	6.95	85	116	0.96
8	23	F	24	3.29	87	108	0.77
9	24	F	27	3.46	76	139	0.41
10	34	M	29	3.87	84	120	2.35
11	26	M	24	4.15	74	69	0.74
12	51	F	29	2.61	80	102	1.41
13	45	M	27	2.30	82	67	0.32
14	39	M	28	4.89	92	124	0.56
15	42	F	30	3.01	N/A	N/A	0.24
16	48	F	40.67	2.18	95	103	3.41
17	56	F	44.22	1.73	113	201	1.55
18	54	M	34.79	N/A	93	105	1.61
19	60	F	35.34	2.16	99	136	1.78
20	42	F	40.32	2.69	106	180	1.45
21	58	F	35.26	N/A	77	122	1.43
22	55	F	32.96	2.71	96	164	0.70
23	43	F	34.78	2.18	82	154	1.89
24	22	F	44.66	1.89	80	91	2.15
25	39	M	32.44	6.55	92	108	0.58
26	55	F	30.2	1.38	83	93	3.78
27	45	F	32.34	1.55	83	94	1.46

M, male; F, female; BMI, body mass index (kg/m^2); SI, insulin sensitivity index; FBG, fasting blood glucose; FBG, fasting blood glucose (mg/dL); Glucose (2 hr), glucose levels 2 hr after standard oral glucose tolerance test (mg/dL); IKKβ expression, IKKβ mRNA levels analyzed by QPCR, arbitrary units.

Supplemental Table 2. Primer Sequences for QPCR.

Gene	Primer sequence	Genes	Primer sequence
	Mouse primers		
IKKβ	5’-GAGCTCAGCCCAAAGAACAG-3’ 5’-AGGTTCTGCATCCCCTCTGG-3’	LacZ	5’-ACGCGCGAATTGAATTATGG -3’ 5’-GTTGACTGTAGCGGCTGATGTT-3’
Runx2	5’-GACGTGCCCAGGCGTATTTC-3’ 5’-AAGGTGGCTGGGTAGTGCATTC-3’	Zfp423	5’-TGGCCTGGGATTCCTCTGT-3’ 5’-CTCTTGACTTGTCACGCTGTT-3’
ALP	5’-AACCCAGACACAAGCATTCC-3’ 5’-GAGACATTTTCCCGTTCACC-3’	PPARγ	5’-GTGCCAGTTTCGATCCGTAGA-3’ 5’-GGCCAGCATCGTGTAGATGA-3’
OPN	5’-CTTTCACTCCAATCGTCCCTA-3’ 5’-GCTCTCTTTGGAATGCTCAAGT-3’	aP2	5’-AAGGTGAAGAGCATCATAACCCT-3’ 5’-TCACGCCTTTCATAACACATTCC-3’
OC	5’-GCAGCTTGGTGCACACCTAG-3’ 5’-GGAGCTGCTGTGACATCCAT-3’	Adiponectin	5’-GCACTGGCAAGTTCTACTGCAA-3’ 5’-GTAGGTGAAGAGAACGGCCTTGT-3’
Osterix	5’-TCTCCATCTGCCTGACTCCT-3’ 5’-AGCGTATGGCTTCTTTGTGC-3’	Gapdh	5’-AACTTTGGCATTGTGGAAGG-3’ 5’-GGATGCAGGGATGATGTTCT-3’
36B4	5’-CCAGGAAGGCCTTGACCTTT-3’ 5’-CTGATCATCCAGCAGGTGTT-3’		
	Human primers		
IKKβ	5’-ATCCCCGATAAGCCTGCCA-3’ 5’-CTTGGGCTCTTGAAGGATACAG-3’	Zfp423	5’-GGCATCAACCACGAGTGTAAGC-3’ 5’-CTTCTGCGGAGAGGTGTCCTGT-3’
Runx2	5’-CCGCCTCAGTGATTTAGGGC-3’ 5’-GGGTCTGTAATCTGACTCTGTCC-3’	PPARγ	5’-TCTCTCCGTAATGGAAGACC-3’ 5’-GCATTATGAGACATCCCCAC-3’
ALP	5’-GGACATGCAGTACGAGCTGA-3’ 5’-GCAGTGAAGGGCTTCTTGTC-3’	aP2	5’-ACCAGGAAAGTGGCTGGCAT-3’ 5’-CAGGTCAACGTCCCTTGGCT-3’
OPN	5’-GAAGTTTCGCAGACCTGACAT-3’ 5’-GTATGCACCATTCAACTCCTCG-3’	Adiponectin	5’-AGCCTCCTTCTCCTGGGTCC-3’ 5’-GTTGCCTCTAGCCTGGTGGG-3’
OC	5’-GGCAGCGAGGTAGTGAAGAG-3’ 5’-CTGGAGAGGAGCAGAACTGG-3’	Gapdh	5’-GGCCTCCAAGGAGTAAGACC-3’ 5’-AGGGGAGATTCAGTGTGGTG-3’
β-actin	5’-CATGTTTGAGACCTTCAACAC-3’ 5’-CCAGGAAGGAAGGCTGGAA-3’		