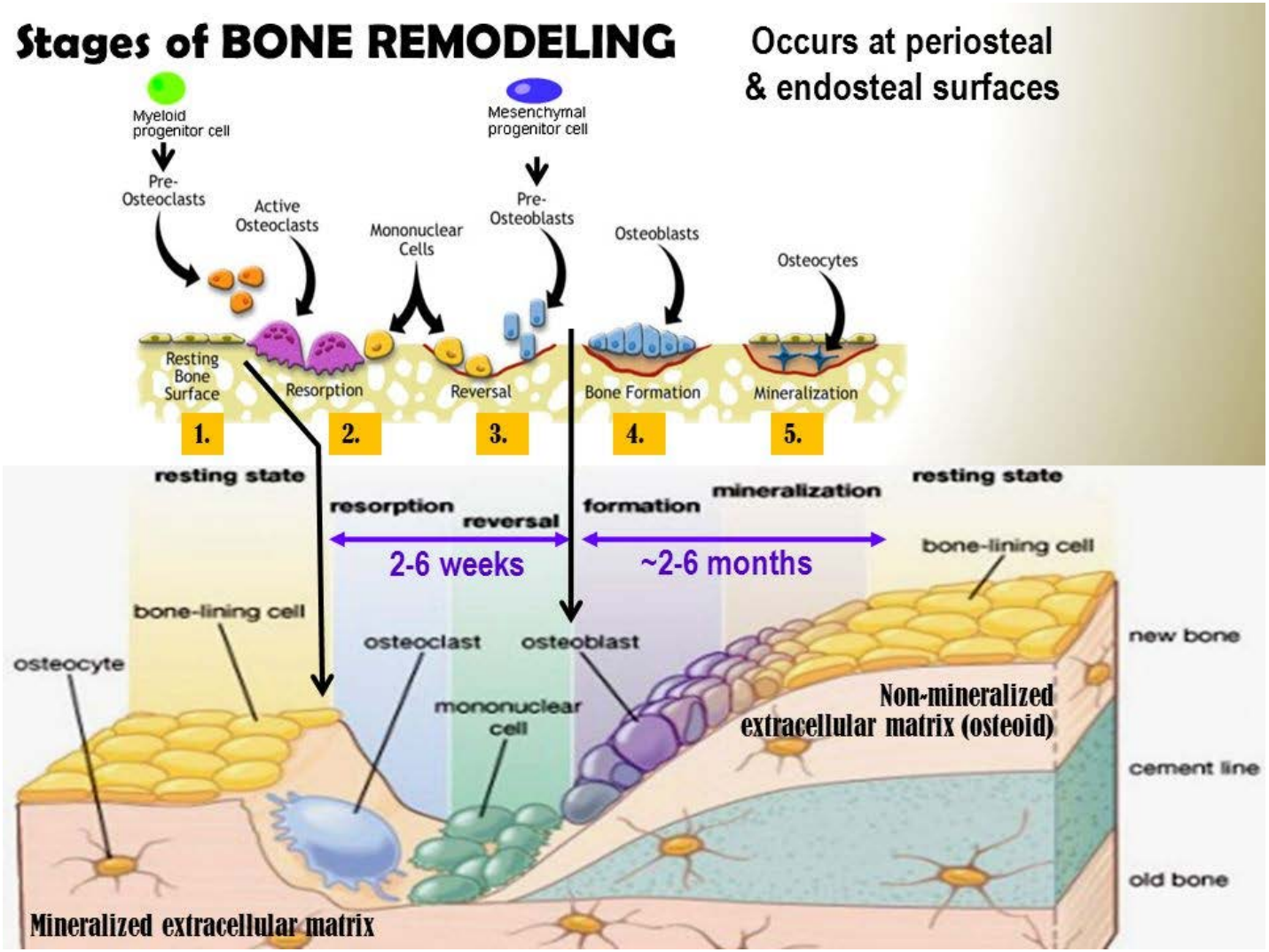
Wnt/β-catenin signaling activates bone morphogenetic protein 2 expression in osteoblasts

Rongrong Zhang, Babatunde O. Oyajobi, Stephen E. Harris, Di Chen, Christopher Tsao, Hong-Wen Deng, Ming Zhao

Bone *(September 2012)*

Introduction



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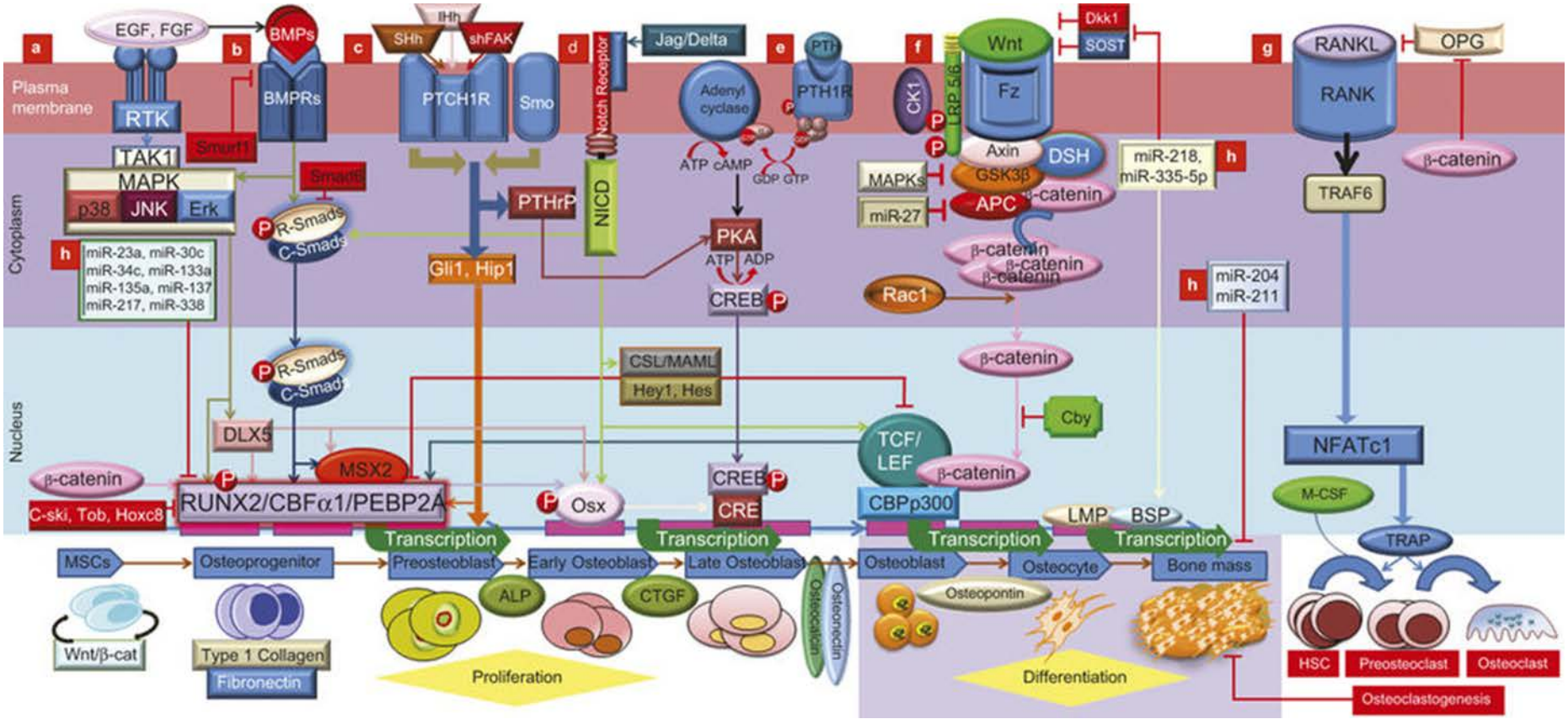
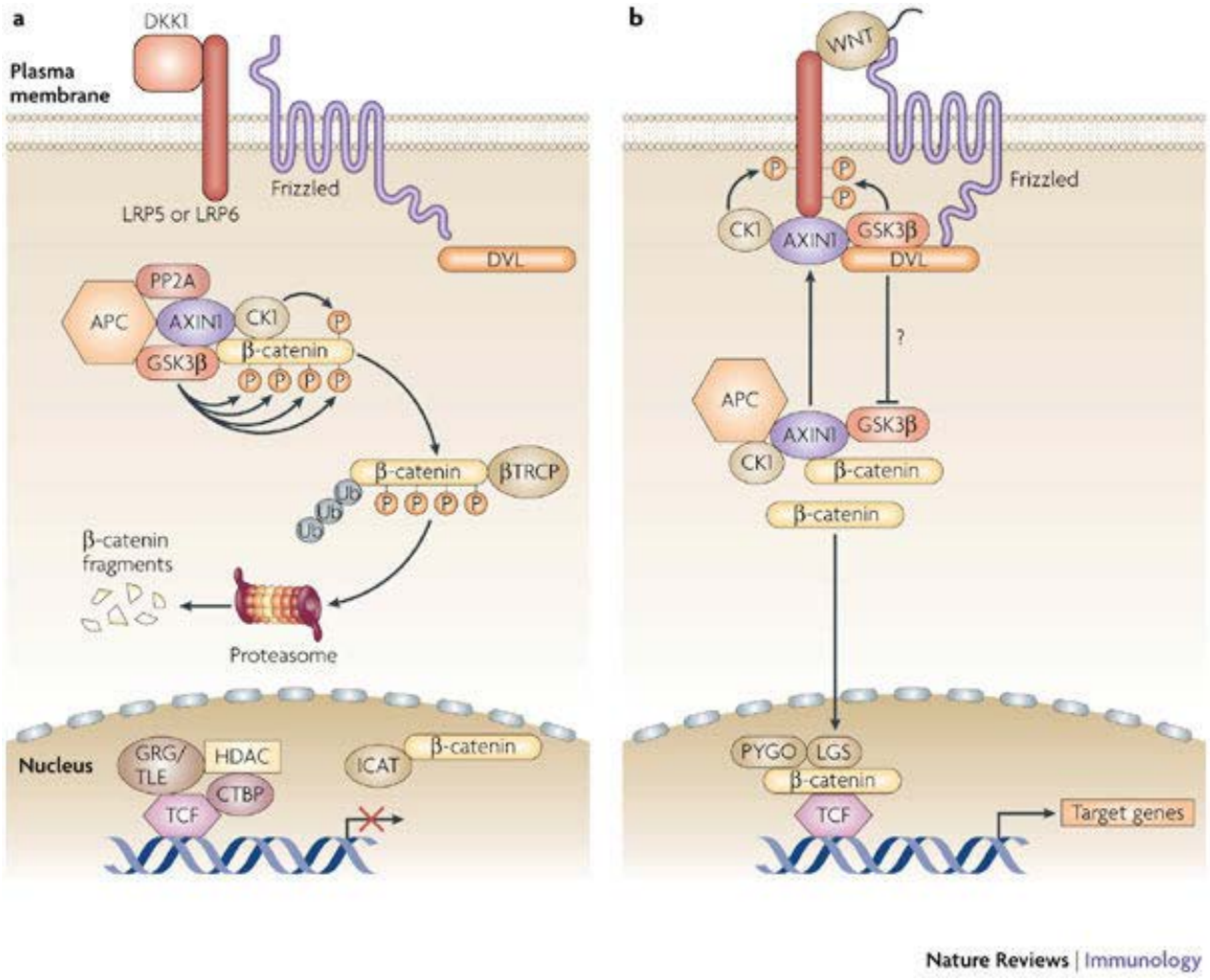
Regulation of osteoblastogenesis and bone formation



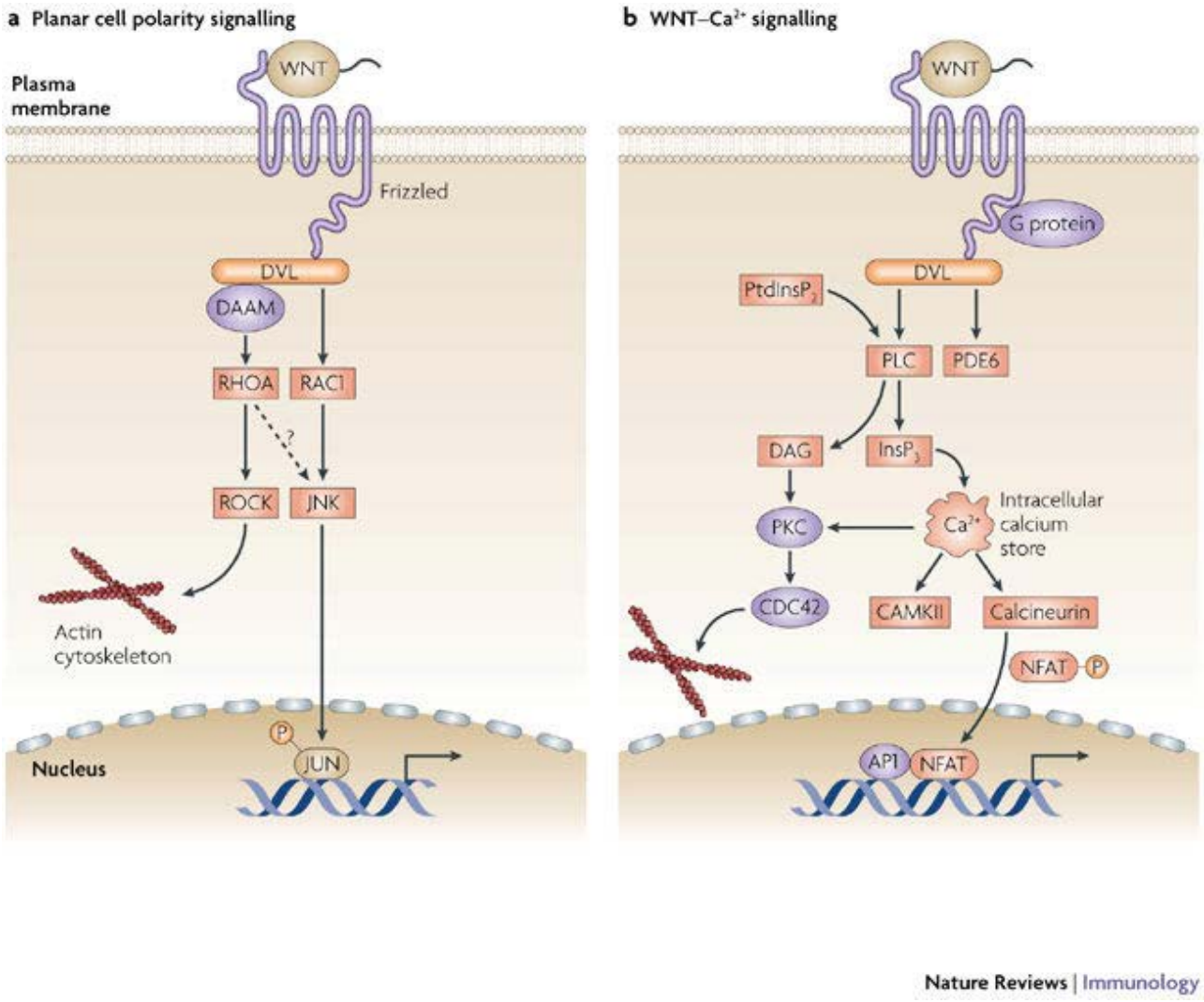
Rahman et al., 2015

Canonical or WNT–β-catenin–TCF/LEF signalling



Staal et al., 2008

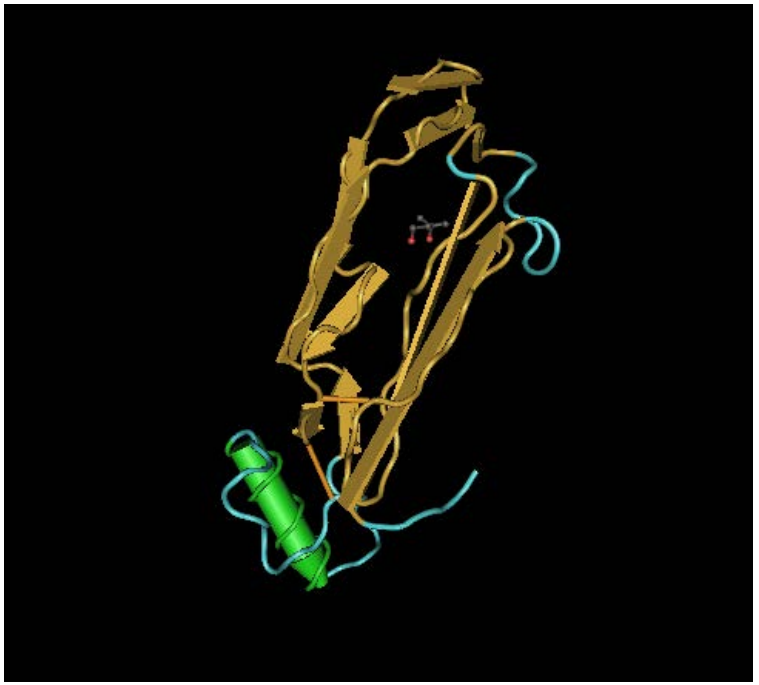
Non-canonical WNT signalling



Staal et al., 2008

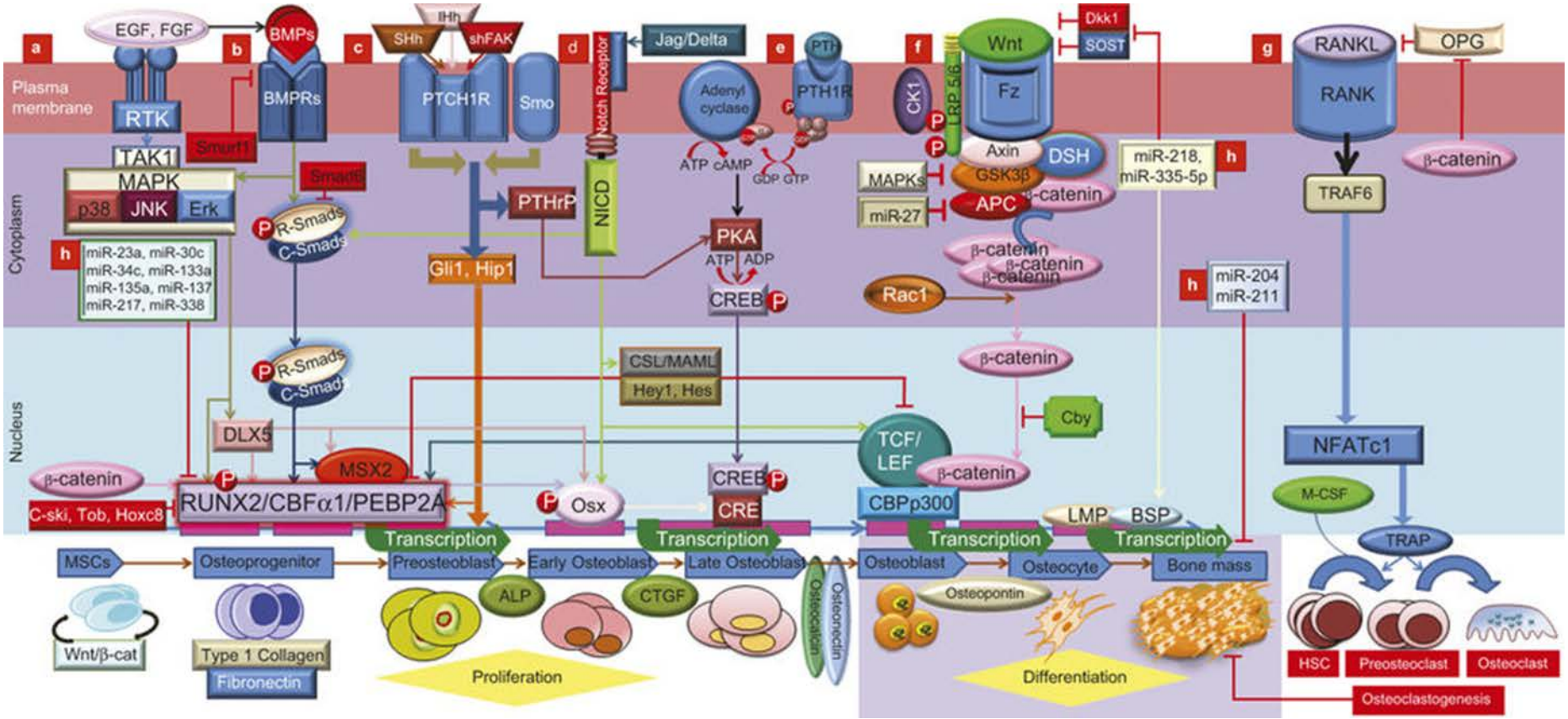
Bone morphogenetic protein

- A group of growth factors (20 proteins)
- Mobilization of SMAD family of proteins
- Development of the heart, central nervous system, and cartilage, post-natal development, early skeletal development
- BMP2: mesenchymal precursor cells → mature osteoblasts



Scheufler et al., 1999

Regulation of osteoblastogenesis and bone formation



Rahman et al., 2015

Material and methods

DNA constructs and recombinant proteins

Expression plasmids:

- pCI-neo-β-catenin for human β-catenin;
- pCI-neo-β-catenin(Δ45) for stabilized β-catenin form in which Ser45 is deleted;
- pCI-neo-β- catenin(S33Y) for stabilized β-catenin form in which Ser33 is replaced by Tyr33;
- pcDNA-Myc-TCF4 for human TCF4;
- pcDNA-Myc-ΔTCF4 for the dominant negative mutant form of TCF4 (ΔTCF4) that lacks amino acids 1–30;
- Wnt signaling reporter TOPFLASH (pGL3-OT)
- Murine inhibitor of β-catenin and TCF (ICAT) was expressed in a vector as pcDNA3.1-Flag-ICAT.
- pcDNA3-Flag-FWD1 for F-box/WD40-repeat protein 1 (FWD1),
- pcDNA3-Flag-FWD1ΔF-dominant negative form of FWD1 in which the F-box is deleted.

- Osteoblast-specific multiple signaling reporter, 9x6-OC-Luc, was constructed by linking a sequence of Tcf/Lef response elements (TRE), Smad binding elements (SBE), Runx2 binding elements (OSE2) upstream of a basal mouse osteocalcin promoter and linked to a luciferase reporter in pGL3 vector.
- The BMP2 promoter reporter −2712/+165-Luc, made by linking mouse BMP2 promoter sequence −2712/+165 to a cDNA for firefly luciferase in pGL3 vector.
- The BMP signaling reporter, 12SBE-Luc was constructed by connecting 12 copies of BMP-specific SBEs upstream of a basal mouse osteocalcin promoter and firefly luciferase coding sequence in pGL3 vector.

Cell culture and transfection

- Osteoblast and osteoblast precursor MC3T3-E1 and 2T3 cells
- Pluripotent mesenchymal C3H10T1/2 cells
- Myoblastic C2C12 cells
- 37 ˚C, 5% CO2, 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% L-glutamine

Bone organ culture assay

- Neonatal mouse calvarial cultures
- Calvariae from 4-day old Swiss pups were dissected and cut in half with the excised hemi-calvariae explanted on metal grids in 1 mL BGJ medium containing 0.1% BSA with glutamine.
- 37 ˚C in a 5% humidified incubator for 24 h, → transferred to wells containing 1 mL of medium with test compounds → further incubation for 72 h.
- The bones were then removed, fixed in 10% buffered formalin for 24 h, decalcified in 14% EDTA overnight, and embedded in paraffin. Sections (7 µm thick) were then cut and stained with hematoxylin and eosin to facilitate assessment of new bone formation and osteoblast proliferation (cellularity of sections).

Western blot

MC3T3-E1, 2T3, C3H10T1/2, and C2C12 cells incubated with Wnt3a at 40 ng/mL or vehicle for 24 hours, or 36 h after transfection of C2C12 cells with the β-catenin expression vector.

Alkaline phosphatase activity

C2C12 cells or primary calvarial cells were incubated with BMP2 at 100 ng/mL or Wnt3a at 40 ng/mL, in the presence or absence of noggin at 500 ng/mL or DKK1 at 100 ng/mL, in medium with 2.5% FCS for 24–48 h.

RT-PCR and quantitative real time PCR

Total RNAs prepared from C2C12, 2T3 or calvarial cells, treated with either Wnt-3a (20–80 ng/mL for 24 h) or transfected with β-catenin/TCF4 expression plasmids for 36 h.

Quantitative real time PCR of mouse BMP2, Col1a1 and Runx2 mRNAs

Mouse GAPDH mRNA served as an endogenous control.

Promoter mutagenesis

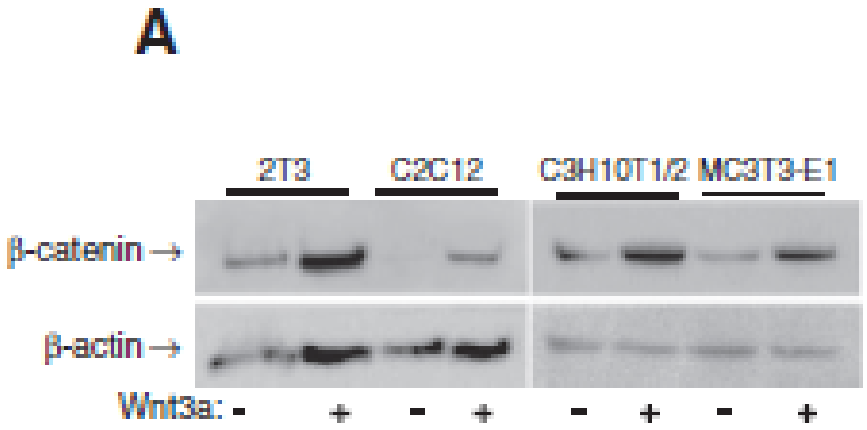
- The putative TREs in the mouse BMP2 promoter were mutated by deleting core nucleotides within the TREs.The mutated BMP2 promoter sequences were recovered and inserted back into a pGL3 reporter vector.
- The luciferase activity of mutant reporters responding to β-catenin/TCF4.

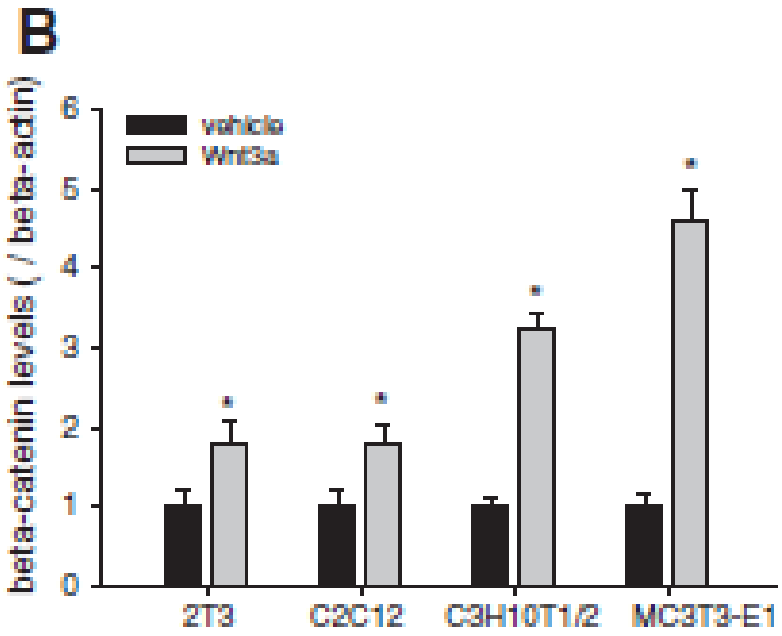
Calvarial cell culture

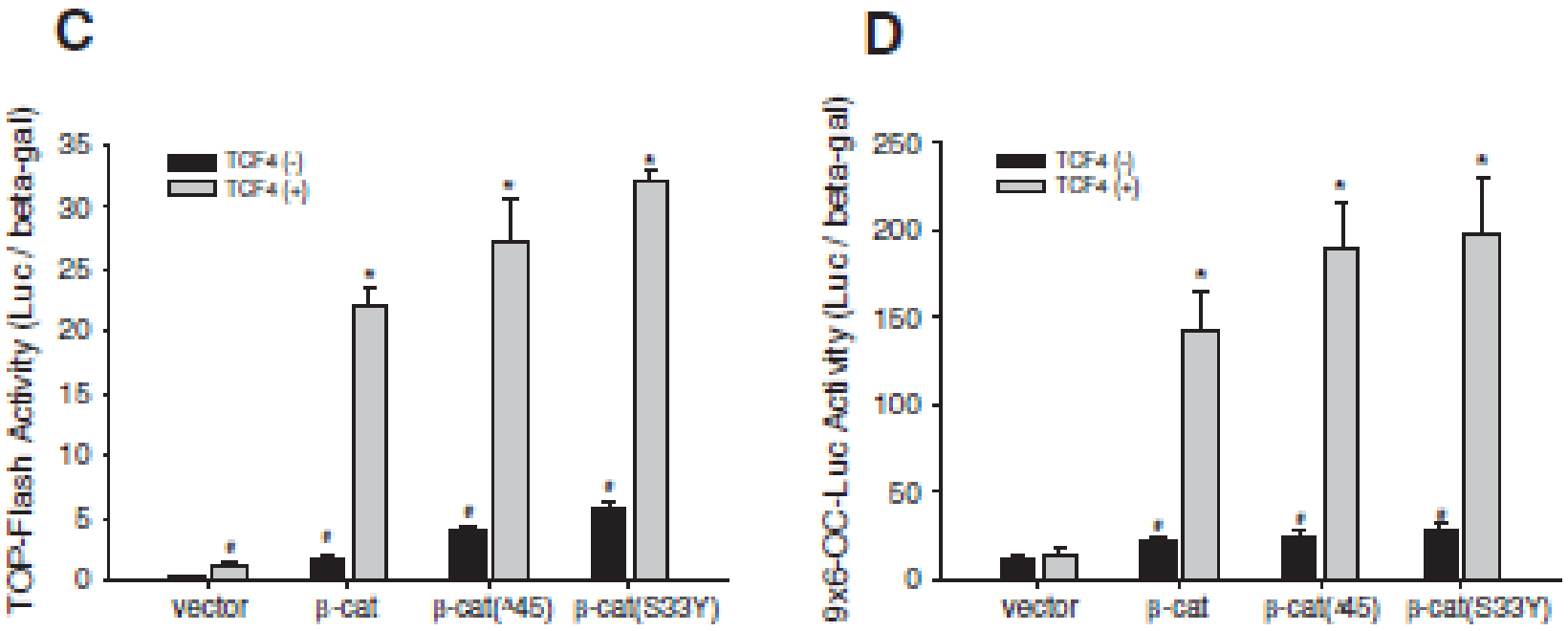
- Calvarial osteoblastic cells from newborn mice (1–4 days).
- Calvarial bone tissues of pups were removed and subjected to multiple 15–25 min digestions in salt solution supplemented with 0.05% trypsin and 1.5 U/ml collagenase at 37 ˚C.
- 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine.
- Treated the growth factors and their inhibitors.
- The osteoblast differentiation and BMP2 mRNA expression in these cells were determined.

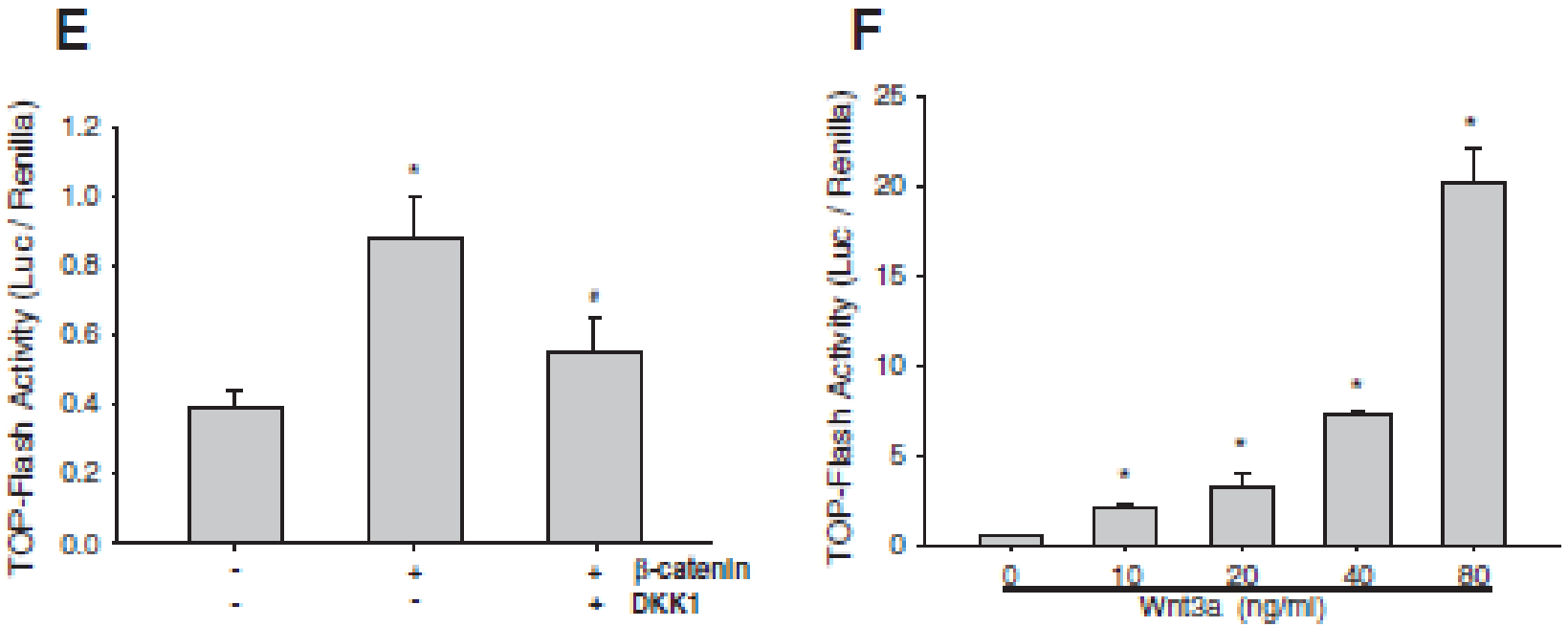
Results

Wnt/β-catenin signaling in osteoblasts

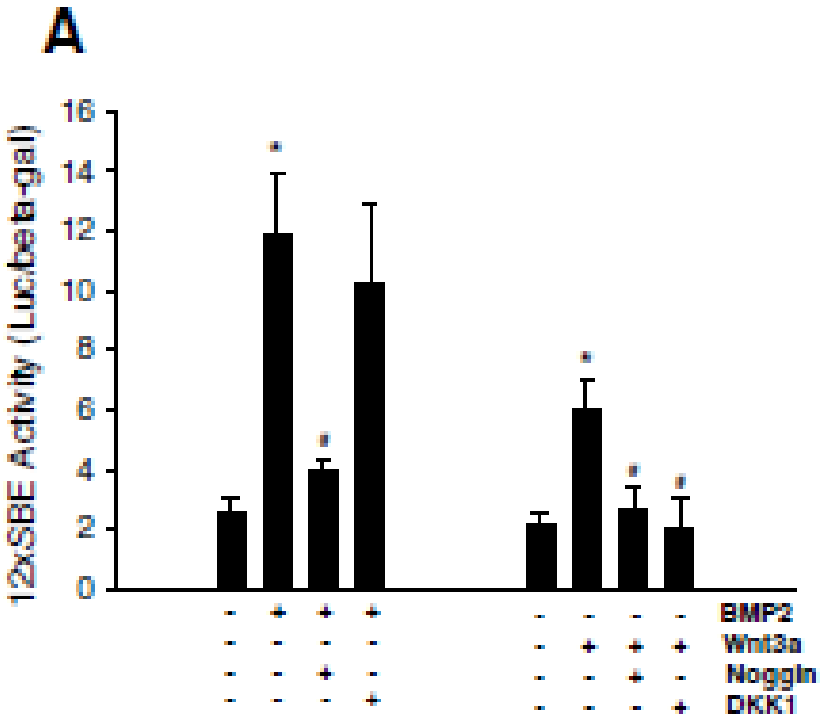


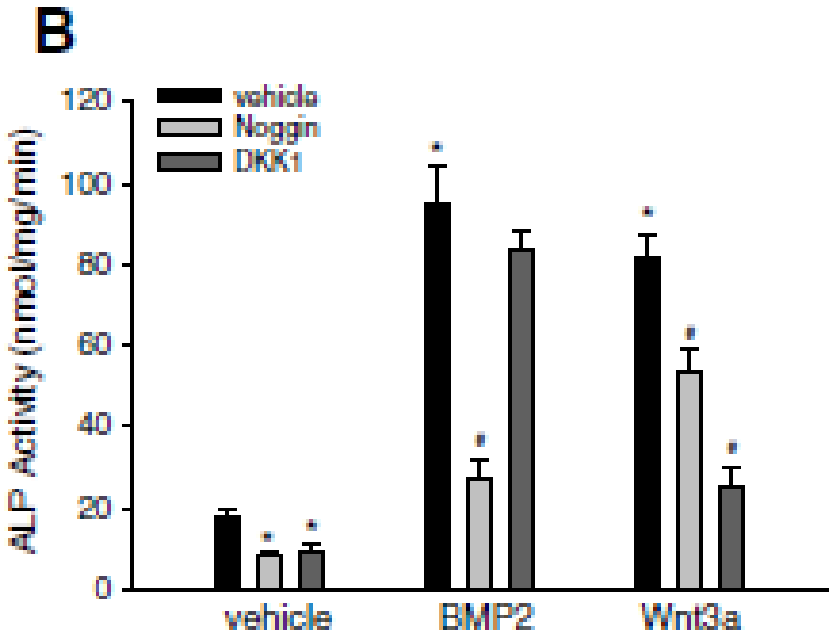


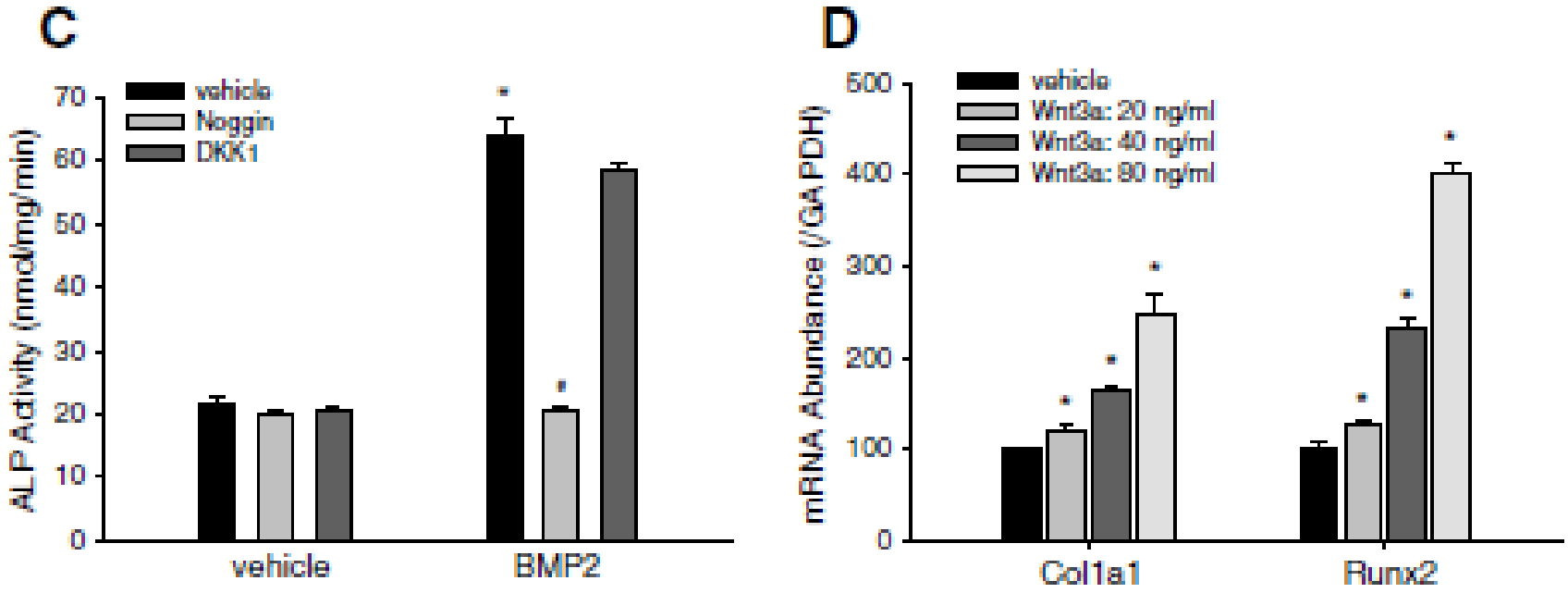

C
TOP-Flash Activity (Luc / beta-gal)
35
30
25
20
15
10
5
0
TCF4 (-)
TCF4 (+)
vector
β-cat
β-cat(Δ45)
β-cat(S33Y)
D
9x6-OC-Luc Activity (Luc / beta-gal)
250
200
150
100
50
0
TCF4 (-)
TCF4 (+)
vector
β-cat
β-cat(Δ45)
β-cat(S33Y)

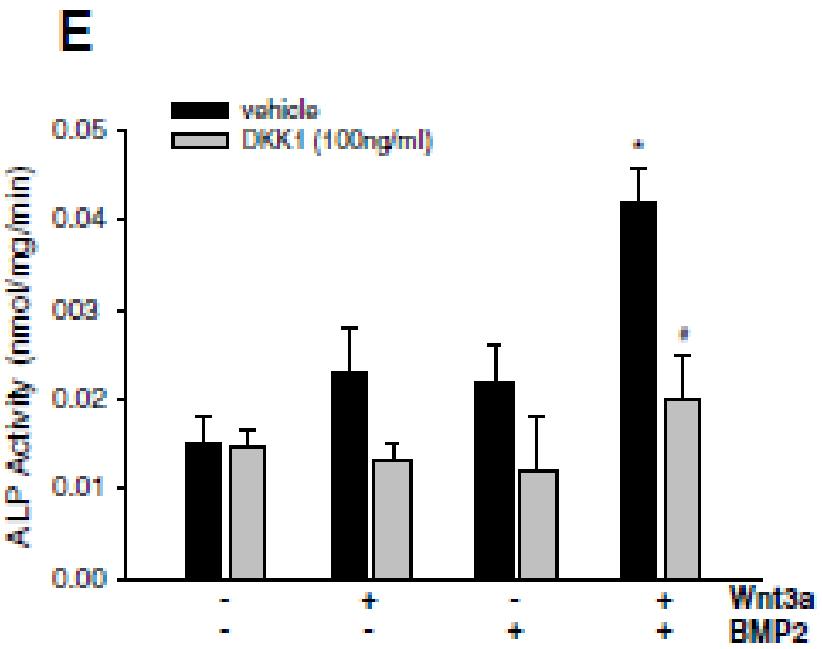

E
TOP-Flash Activity (Luc / Renilla)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-
-
+
-
+
+
β-catenin
DKK1
F
TOP-Flash Activity (Luc / Renilla)
25
20
15
10
5
0
0
10
20
40
80
Wnt3a (ng/ml)

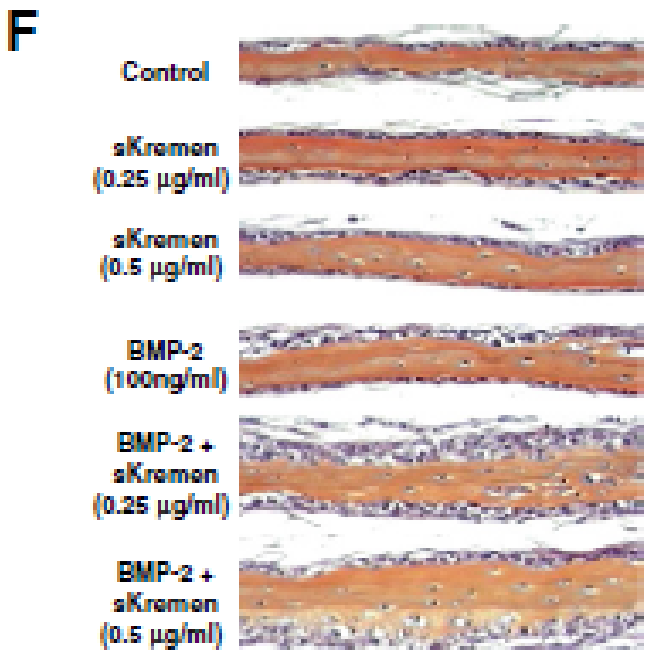
Interaction between the BMP and Wnt signaling pathway in osteoblasts



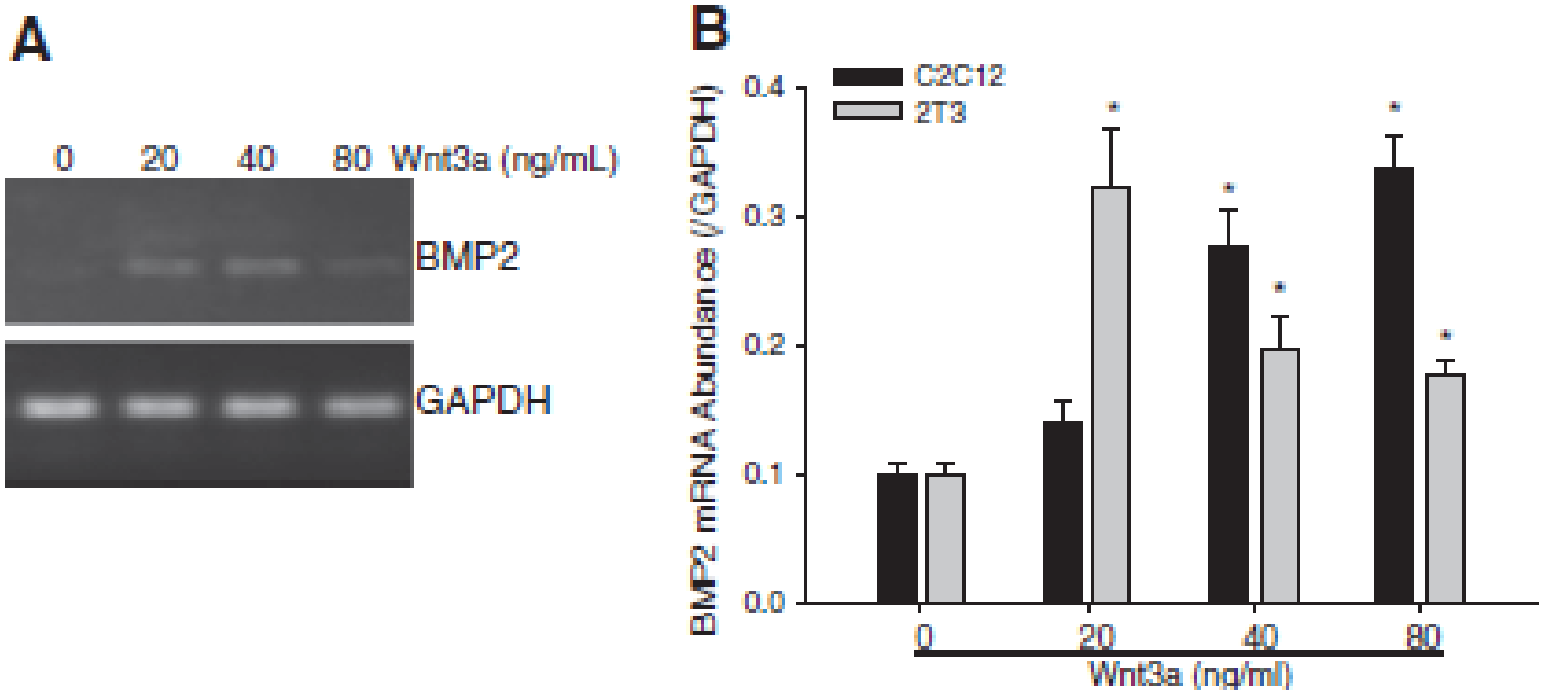


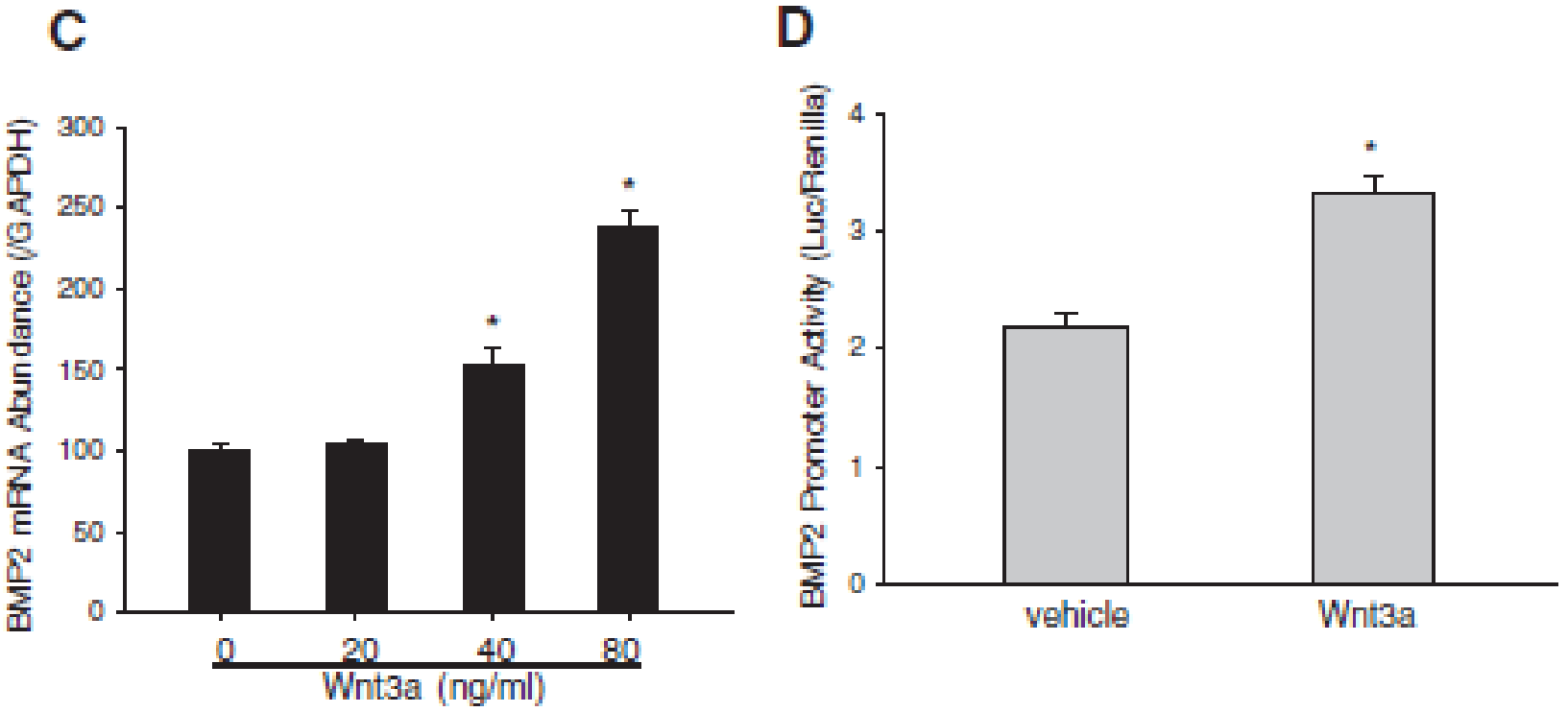

C
vehicle
Noggin
DKK1
ALP Activity (nmol/mg/min)
70
60
50
40
30
20
10
0
vehicle
BMP2
D
vehicle
Wnt3a: 20 ng/ml
Wnt3a: 40 ng/ml
Wnt3a: 80 ng/ml
mRNA Abundance (/GAPDH)
500
400
300
200
100
0
Col1a1
Runx2

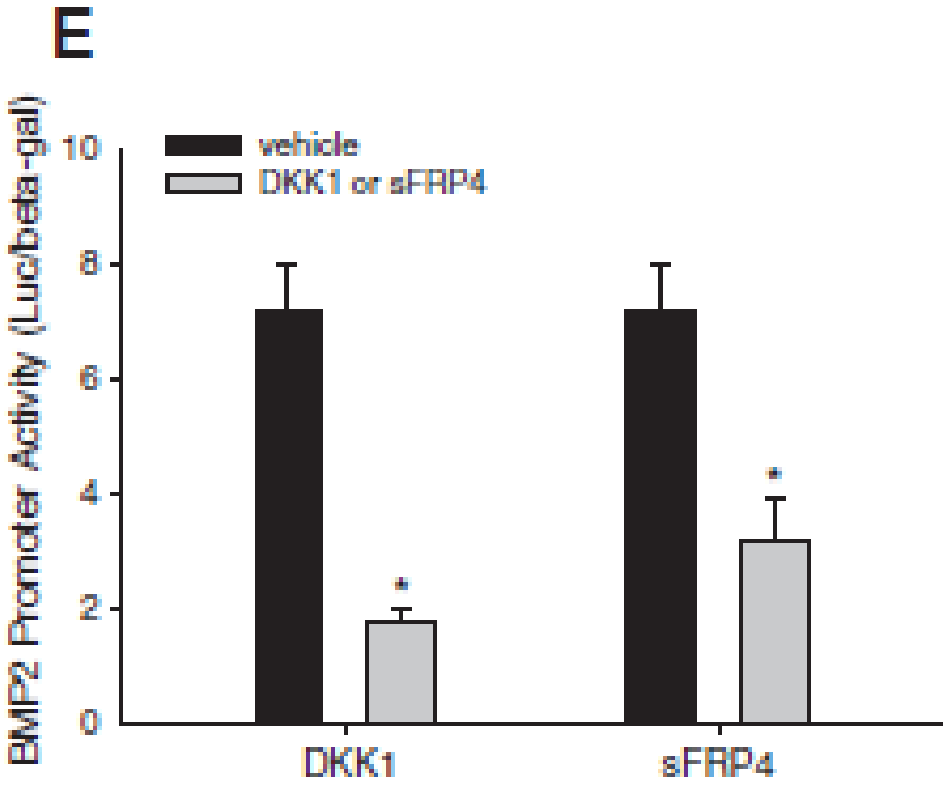

E
vehicle
DKK1 (100ng/ml)
ALP Activity (nmol/mg/min)
0.05
0.04
003
0.02
0.01
0.00
*
#
-
+
-
+
Wnt3a
-
-
+
+
BMP2


F
Control
sKremen
(0.25 µg/ml)
sKremen
(0.5 µg/ml)
BMP-2
(100ng/ml)
BMP-2 +
sKremen
(0.25 µg/ml)
BMP-2 +
sKremen
(0.5 µg/ml)

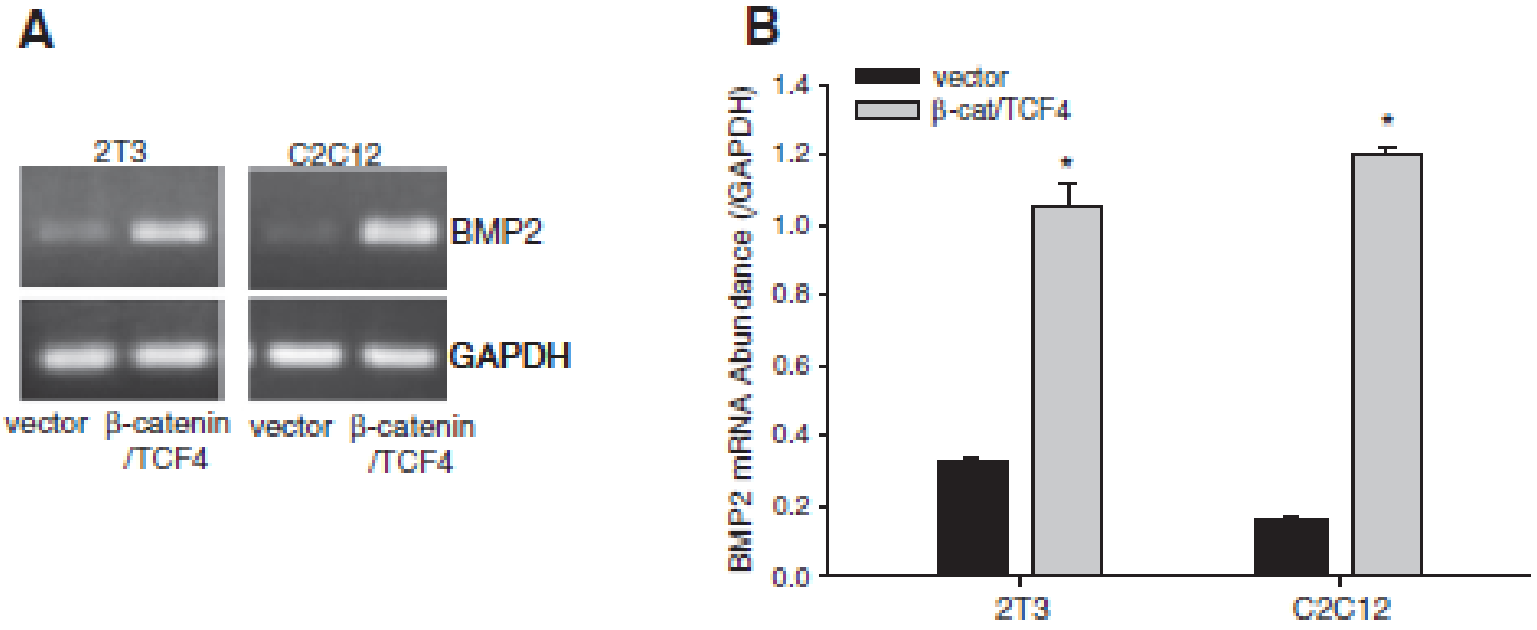
Effects of Wnt ligand and antagonists on BMP2 expression in osteoblasts

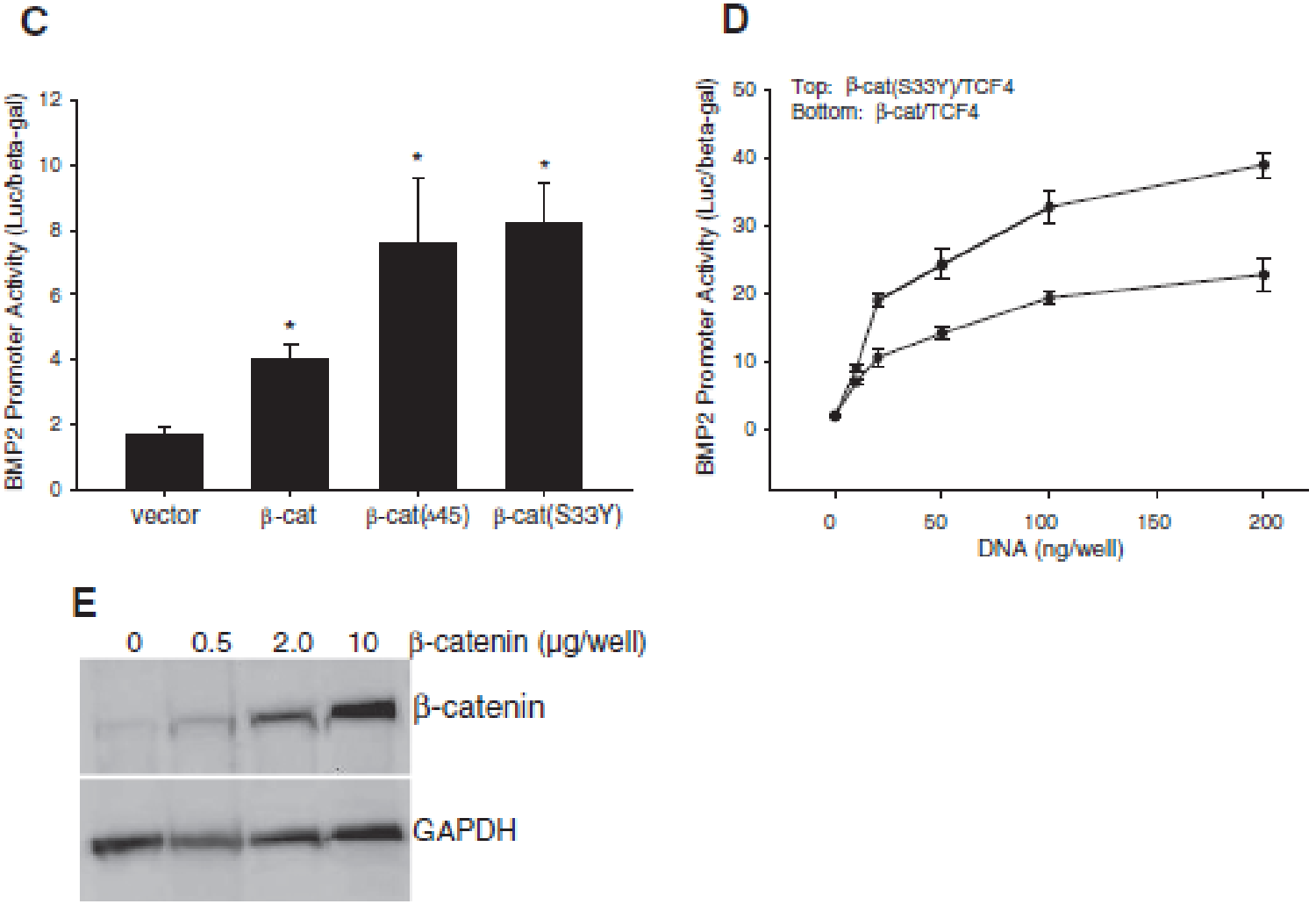



C
BMP2 mRNA Abundance (/GAPDH)
300
250
200
150
100
50
0
*
*
0
20
40
80
Wnt3a (ng/ml)
D
BMP2 Promoter Activity (Luc/Renilla)
4
3
2
1
0
*
vehicle
Wnt3a

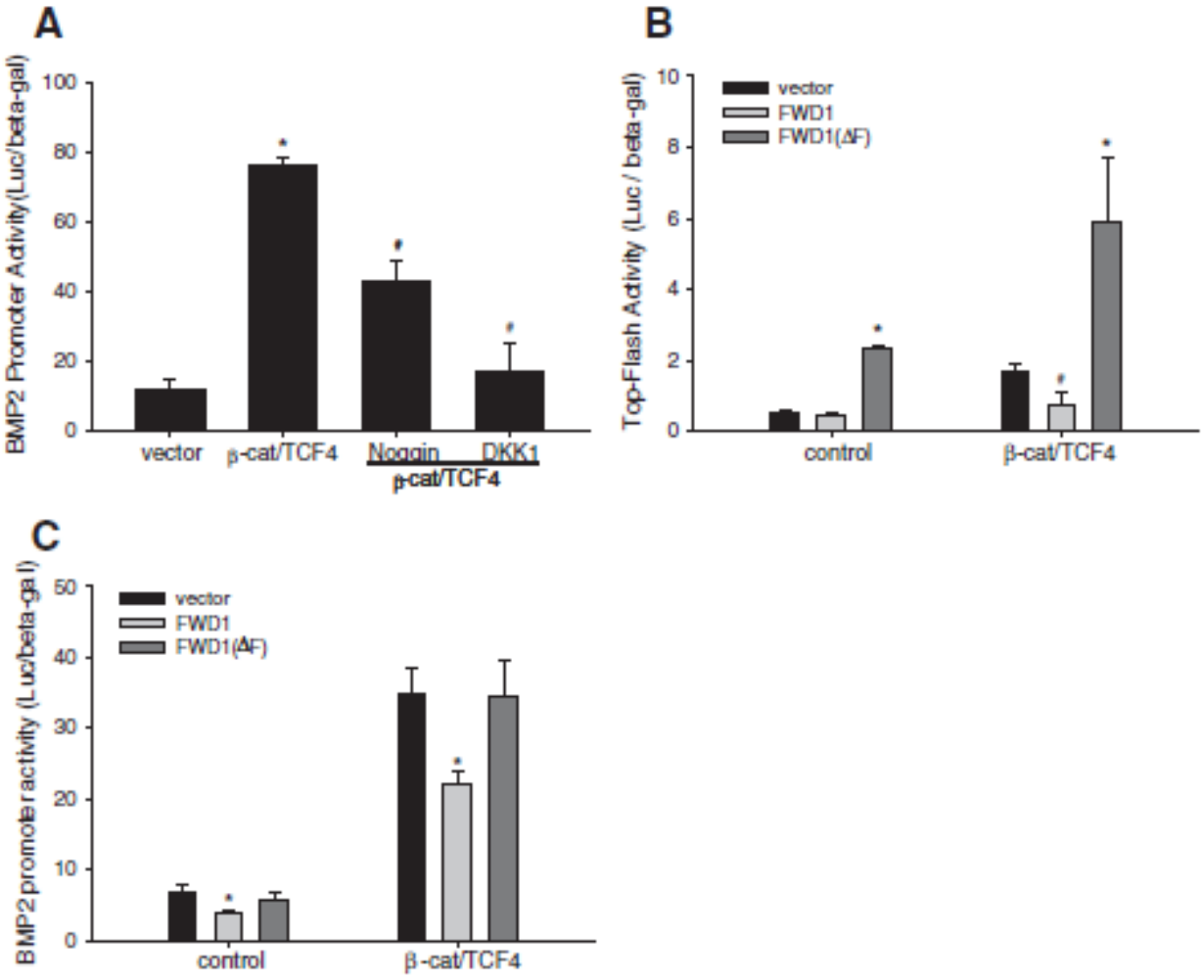

E
BMP2 Promoter Activity (Luc/beta-gal)
10
8
6
4
2
0
vehicle
DKK1 or sFRP4
*
*
DKK1
sFRP4

Effects of overexpression of β-catenin on BMP2 expression in osteoblasts

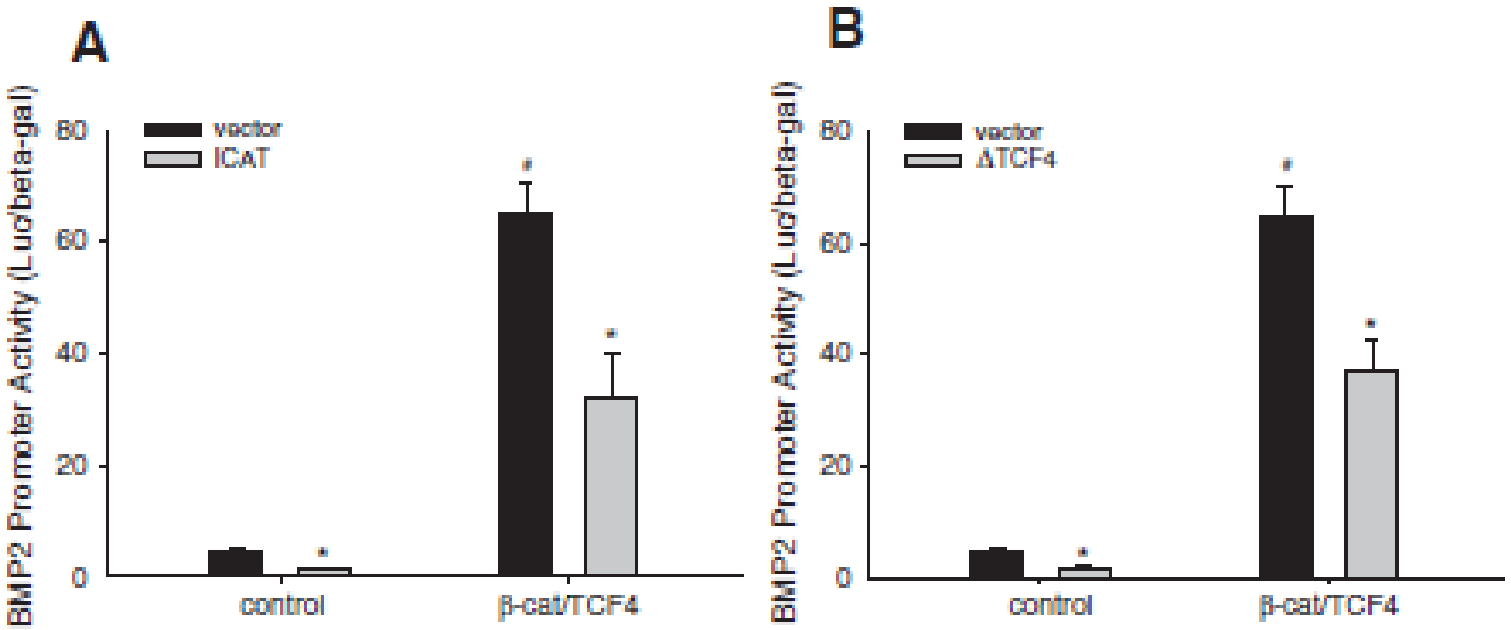



C
BMP2 Promoter Activity (Luc/beta-gal)
12
10
8
6
4
2
0
vector
β-cat
β-cat(Δ45)
β-cat(S33Y)
D
Top: β-cat(S33Y)/TCF4
Bottom: β-cat/TCF4
BMP2 Promoter Activity (Luc/beta-gal)
50
40
30
20
10
0
0
50
100
150
200
DNA (ng/well)
E
0
0.5
2.0
10
β-catenin (μg/well)
β-catenin
GAPDH

Effects of inhibition of β-catenin activity on BMP2 expression in osteoblasts



β-Catenin Transactivation of BMP2 expression through Tcf/Lef binding elements



C

(-2269/-2263)

Wild-type -2712/+165: --TTCGGAGTTTCTT**GCTTTGCT**CCTTCCGCCTCC--

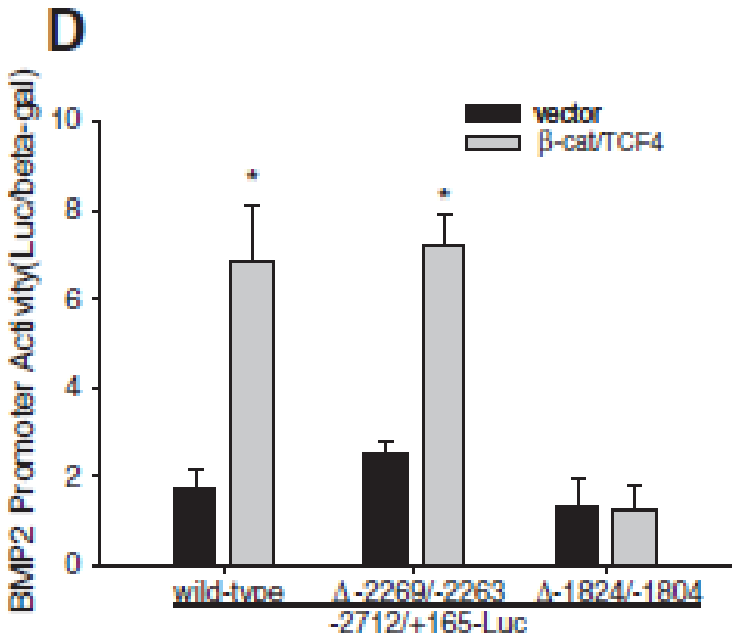
Mutant -2712/+165: --TTCGGAGTTTCTT**GCT--GCT**CCTTCCGCCTCC--

(-1824/-1814)

Wild-type -2712/+165: --CTTCTGGTCTTTCTCGGT**CTTTGCTTTGC**AAACTGGAAAGATCTGGT--

Mutant -2712/+165: --CTTCTGGTCTTTCTCGGT**CT-------GC**AAACTGGAAAGATCTGGT--

D



Discussion

- FWD1ΔF did not increase BMP2 promoter activity.
- Previous studies on Wnt regulation of BMP expression have shown a repressing function of Wnt on expression of the dpp gene, a Drosophila homologue of BMP2.
- Hedgehog/Gli pathway → another possible mechanism

Conclusion

- Wnt signaling is an upstream regulator of BMP2 expression in osteoblasts.
- Wnt/β-catenin transactivation of BMP2 transcription is directly mediated through the Tcf/Lef response elements in the BMP2 promoter.
- Potential therapeutic target for skeletal diseases with bone loss

Thank you for listening!