Variant-to-Gene Mapping and Functional Characterization of Novel Osteoblast Proteins

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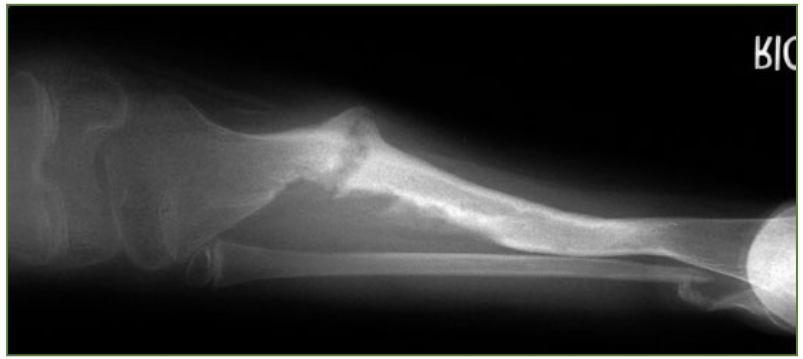
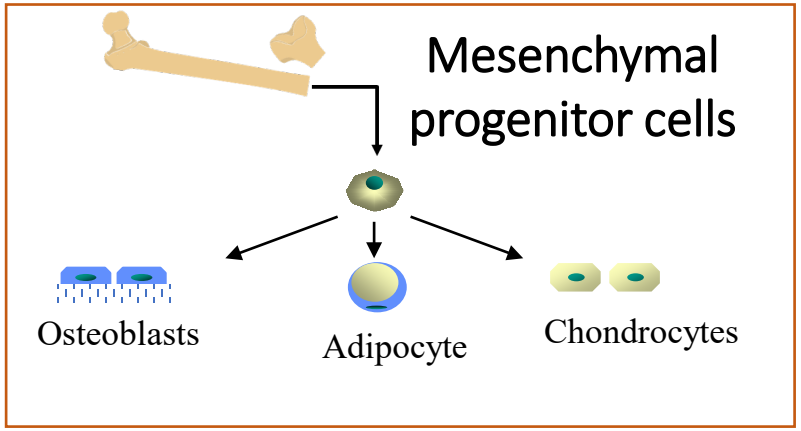
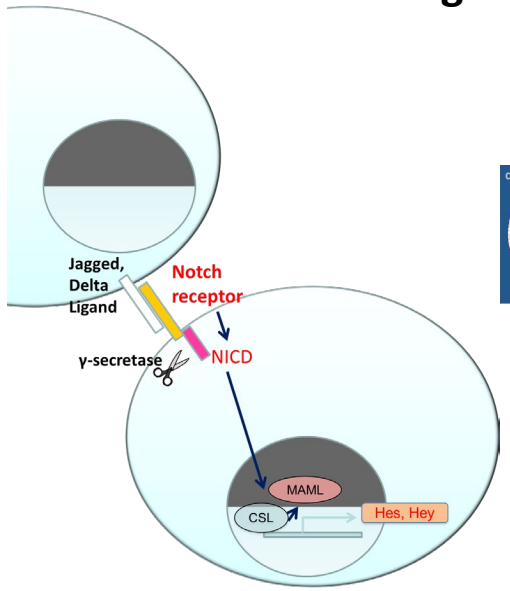
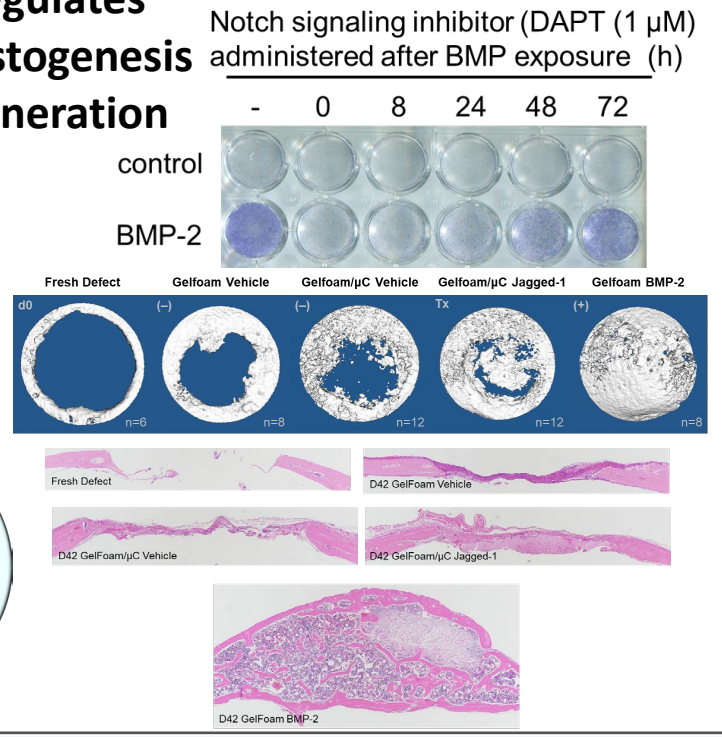
Mesenchymal progenitor biology: Exploring extracellular regulators of bone formation and regeneration

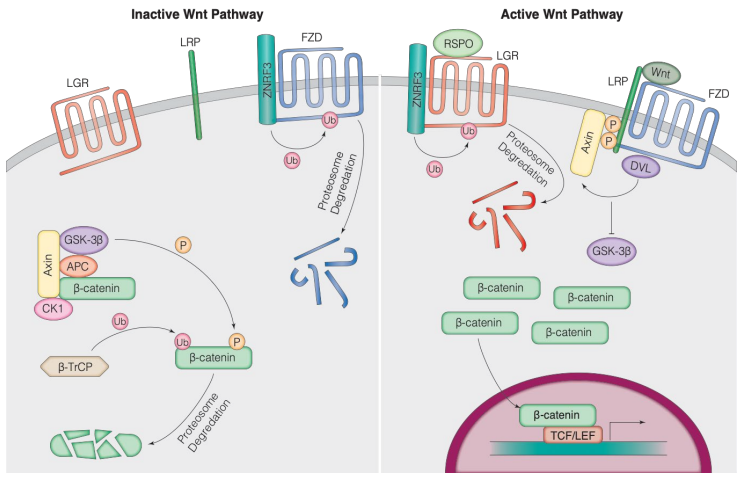




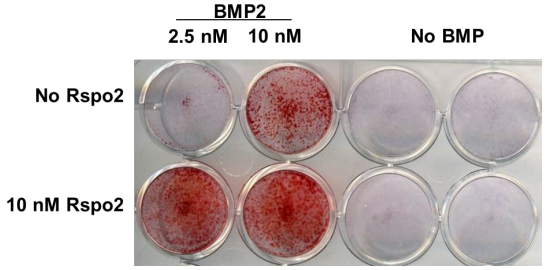
Notch ligand Jagged-1 regulates BMP2 induced osteoblastogenesis and enhances bone regeneration

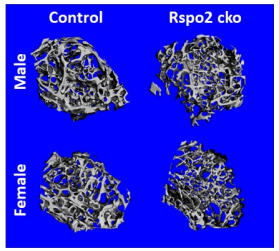


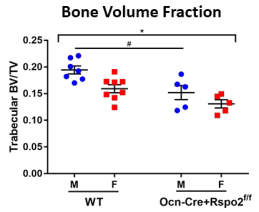




R-spondin-2 regulates Wnt signaling, osteoblastogenesis, and bone formation







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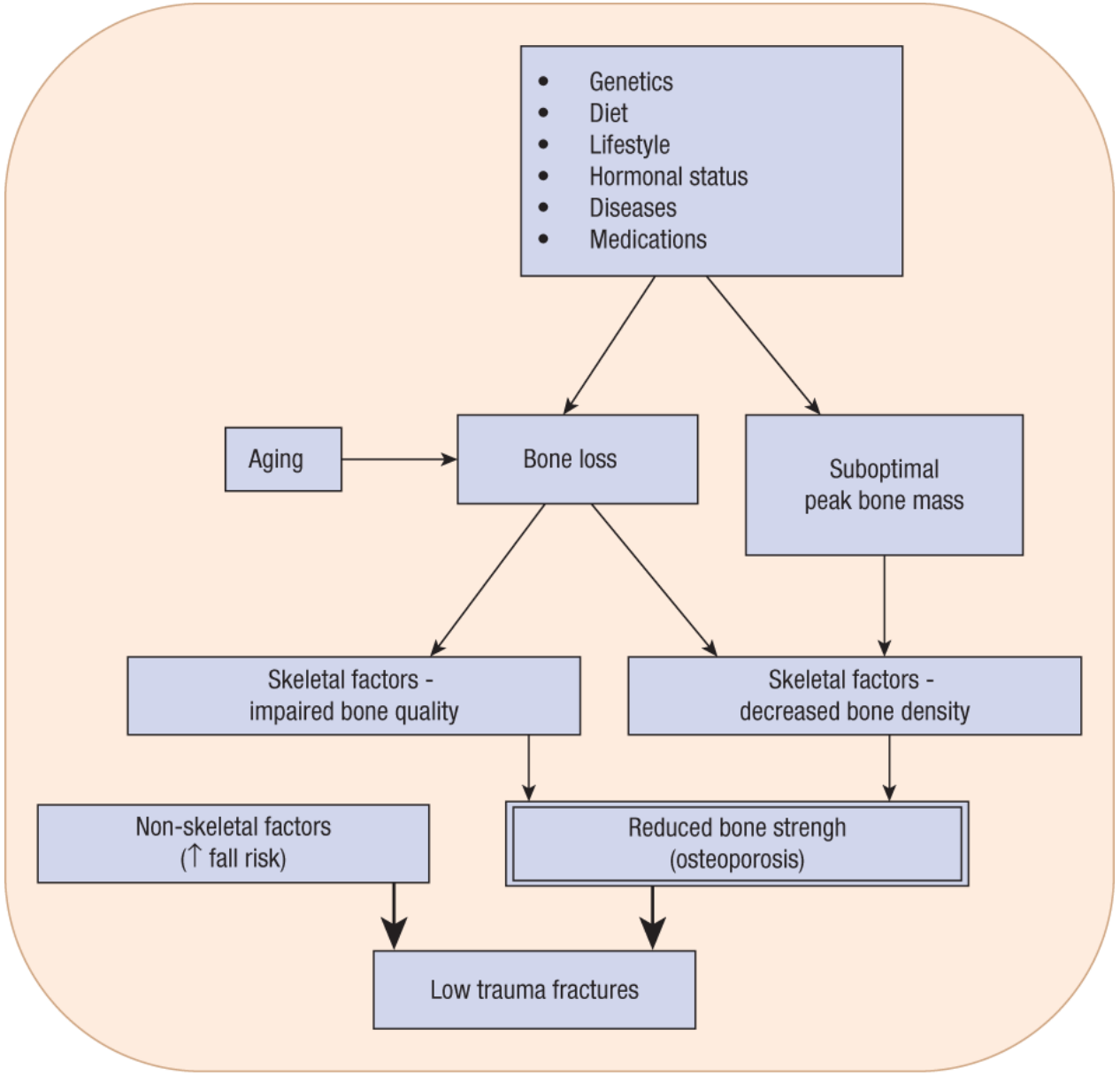
Osteoporosis: a global issue



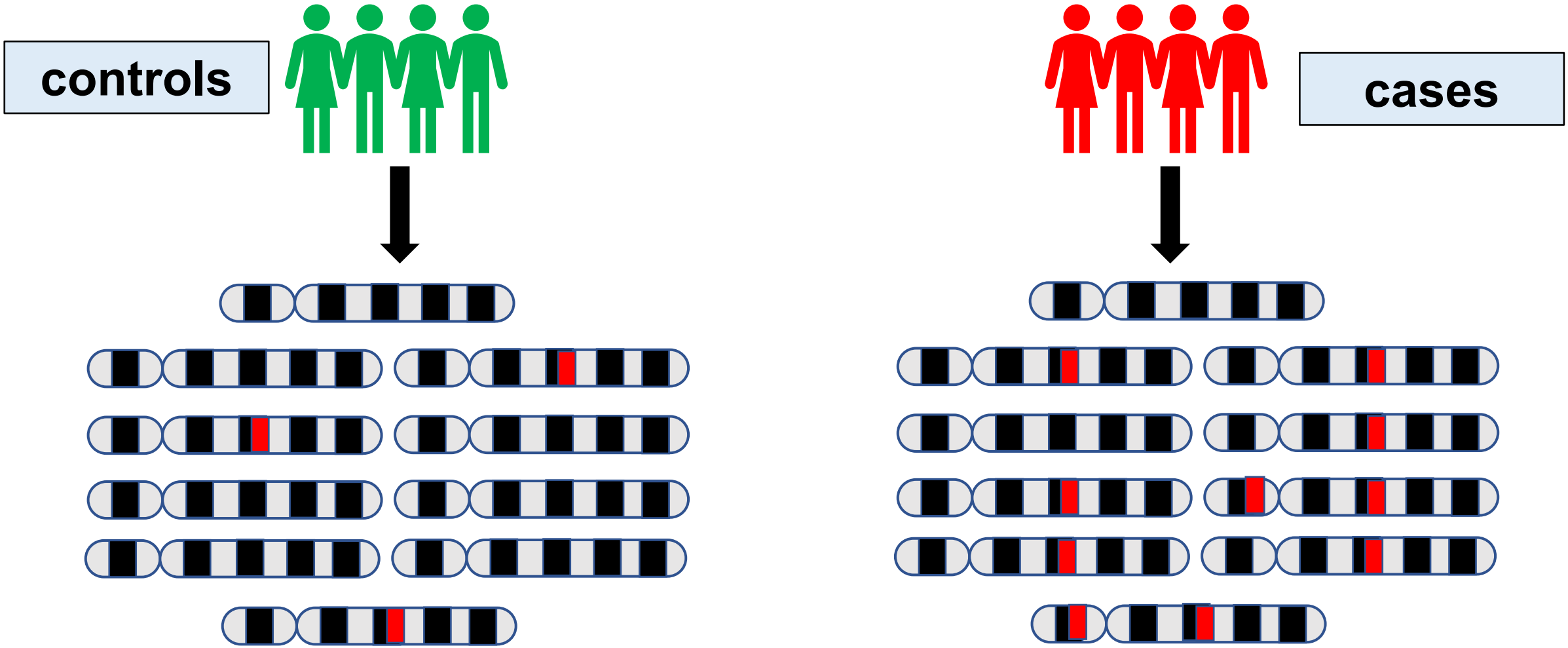
Source: DiPiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM: *Pharmacotherapy: A Pathophysiologic Approach, Ninth Edition:* www.accesspharmacy.com

- Over 200 million people worldwide -- 30% of postmenopausal women in USA and in Europe
- One in three women and one in five men over the age of 50 will suffer osteoporotic fracture
- Significant health problem that will rise by 240% in women and 310% in men over the next 30 years

History of Bone Mineral Density (BMD) Genetics

1990s	Upto mid 2000s	2007 and onwards
• Candidate gene studies that described associations between polymorphism in bone-relevant genes (e.g., vitamin D receptor and type I collagen) and BMD	• Candidate gene investigations and linkage scan in families	• Genome Wide Association Studies (GWAS)

BMD GWAS tests the genotype association of millions of single nucleotide polymorphisms (SNPs) across the genome in hundreds of thousands of individuals



Large GWAS analyses for Bone Mineral Density (BMD) illustrates the increase in identified loci as a function of sample size

Study	Phenotype	Sample size	Association count
Morris et al. (2018)	Estimated heel BMD	426824	1103 independent associations (518 loci[a], 301 novel)
Kemp et al. (2017)	Estimated heel BMD	142487	307 independent associations (203 loci, 153 novel)
Estrada et al. (2012)	Lumbar spine and femoral neck BMD	83894 (32961 discovery, 50933 replication)	64 independent associations (56 loci, 32 novel)
Rivadeneira et al. (2009)	Lumbar spine and femoral neck BMD	19195	20 independent associations (20 loci, 13 novel)

[a]Note that the most recent discovery of 518 loci encompassed nearly all of the previously discovered loci identified in prior studies

Several of the variants identified in individuals with lower BMD are also linked to increased risk of bone fractures

Basel M. Al-Barghouthi and Charles R. Farber *Trends in Genetics* 35(1):55-67 (2019)

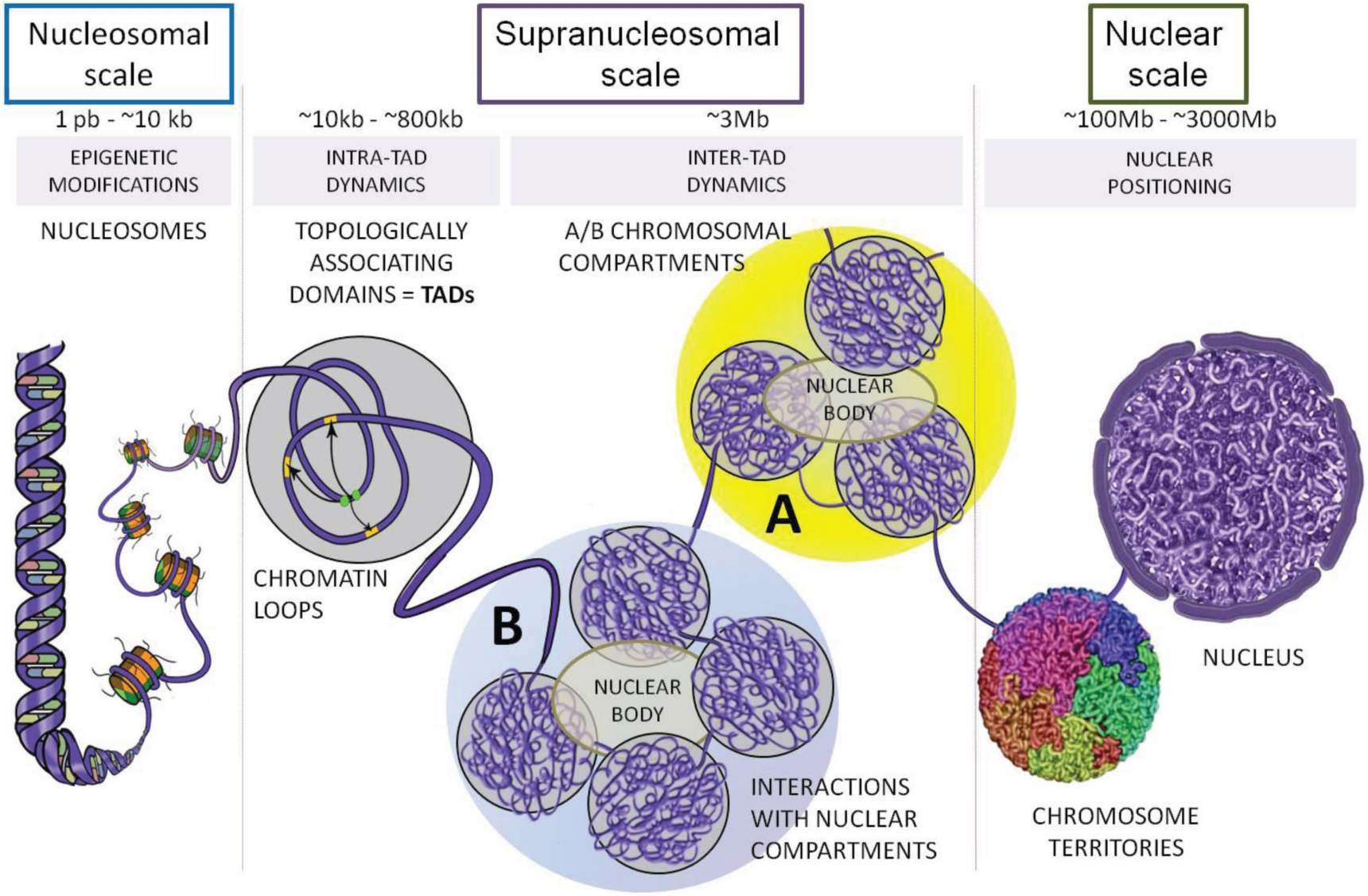
More than GWAS are required to identify causal genes (or gene-networks)

- GWAS are hypothesis free methods to identify associations between genetic regions (loci) and phenotype
- Identifies SNPs across the genome and suggest particular variations that affect a person's response to certain drugs and influence interactions between a person's gene and the environment
- CAN NOT tell if the variant is affecting culprit gene or a gene at more distant loci

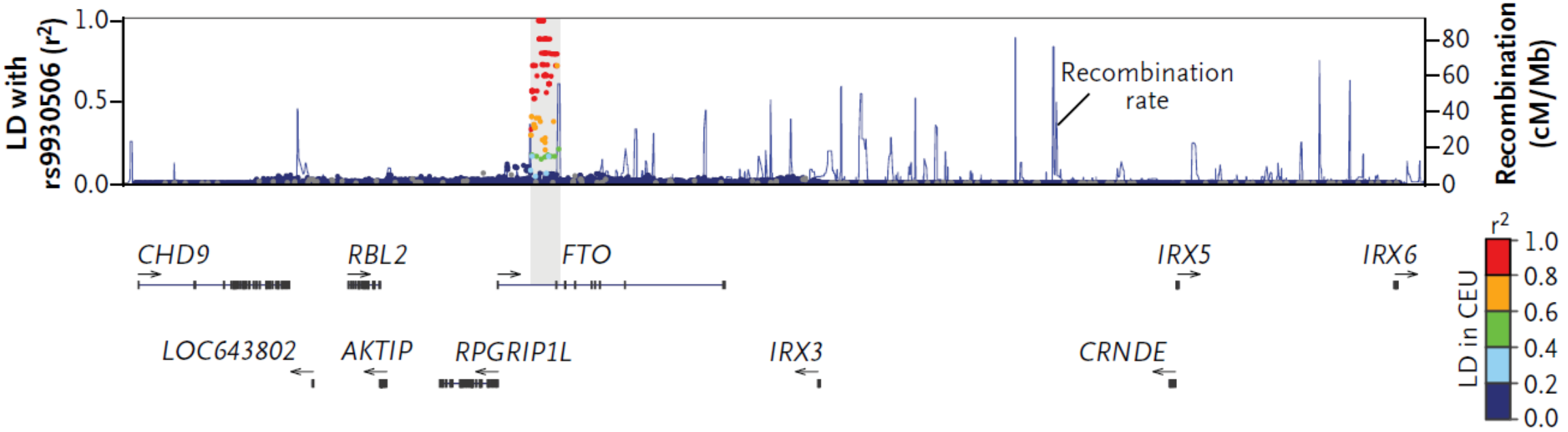
Variant to gene mapping?

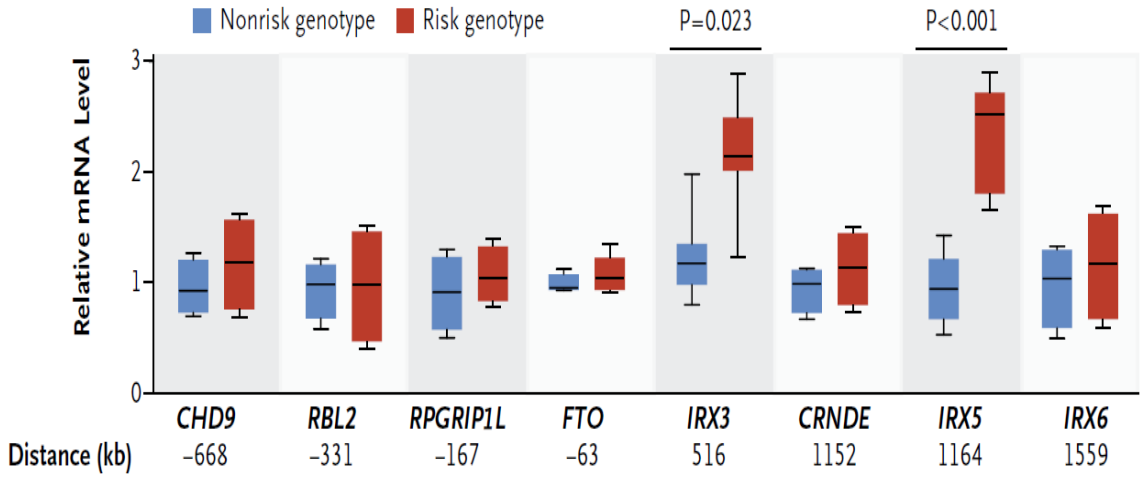
Although GWAS provides valuable insight on affected loci, the causal variants are often embedded in non-coding regions of the genome which contains important regulatory elements such as enhancers and silencers.

Many such regulatory elements do not control the nearest gene and can reside tens or hundreds of kilobases away

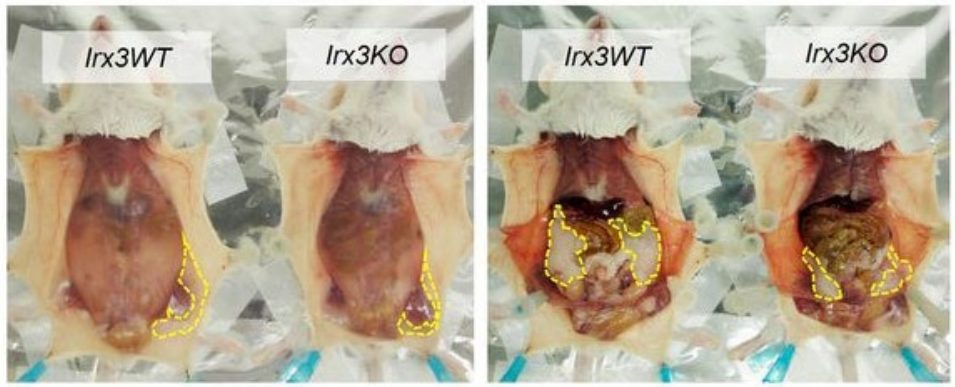


SNP variant does not always affect implicated gene (Obesity model)





Claussnitzer, M. et al. *N Engl J Med* 373, 895-907 (2015)



Smemo, S. *et al. Nature* 507, 271-275 (2014)

How to identify chromatin interactions?

- By resolving the topologically associated chromatin domains (closely interacting chromatin loops) by sequencing approach in human MSC derived osteoblasts
- Are those interacting region accessible for epigenomic (or other) regulations?

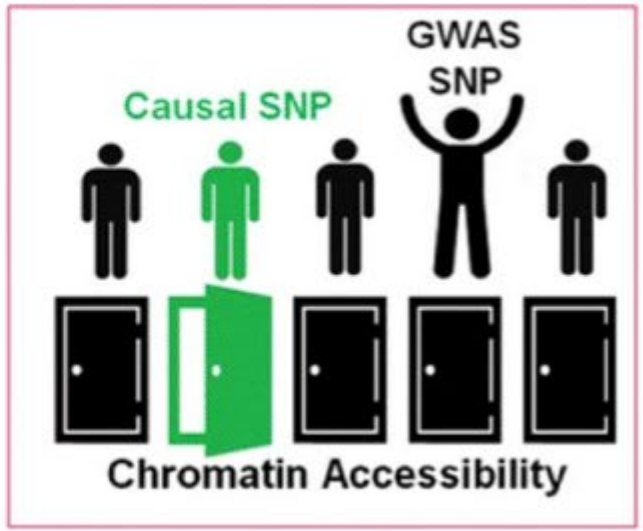
Identify all SNPs in high LD with sentinel associated variant (r^2=0.8)

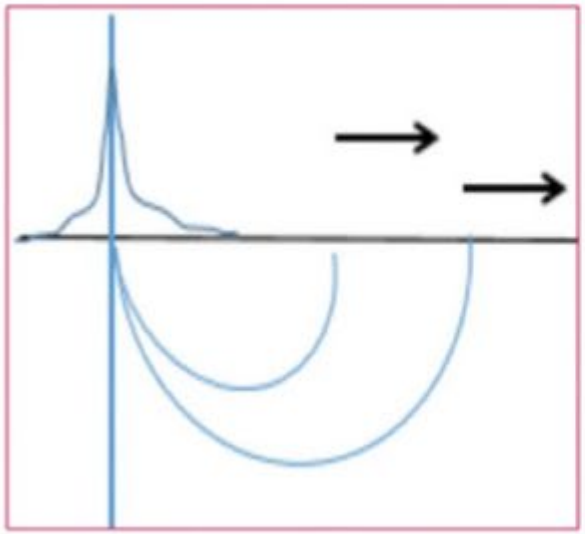
Filter by residing in open chromatin as assessed by ATAC-seq in hMSC-derived osteoblasts

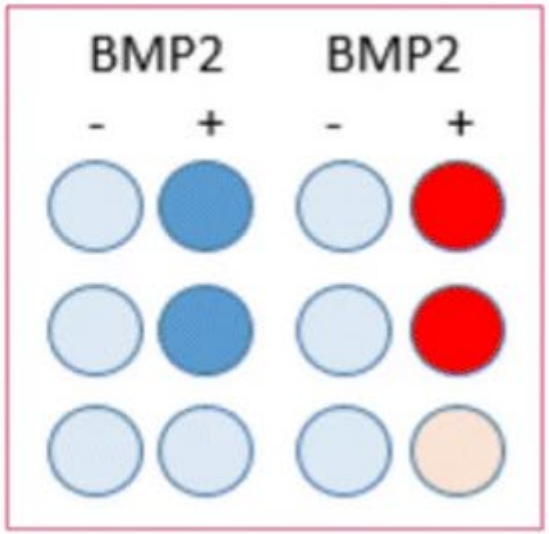
Open chromatin variants are subsequently filtered by being in direct physical contact with gene promoter baits (Promoter-focused Capture-C)

siRNA knockdown experiments are performed for a subset of these contacted genes

(Functional validation)

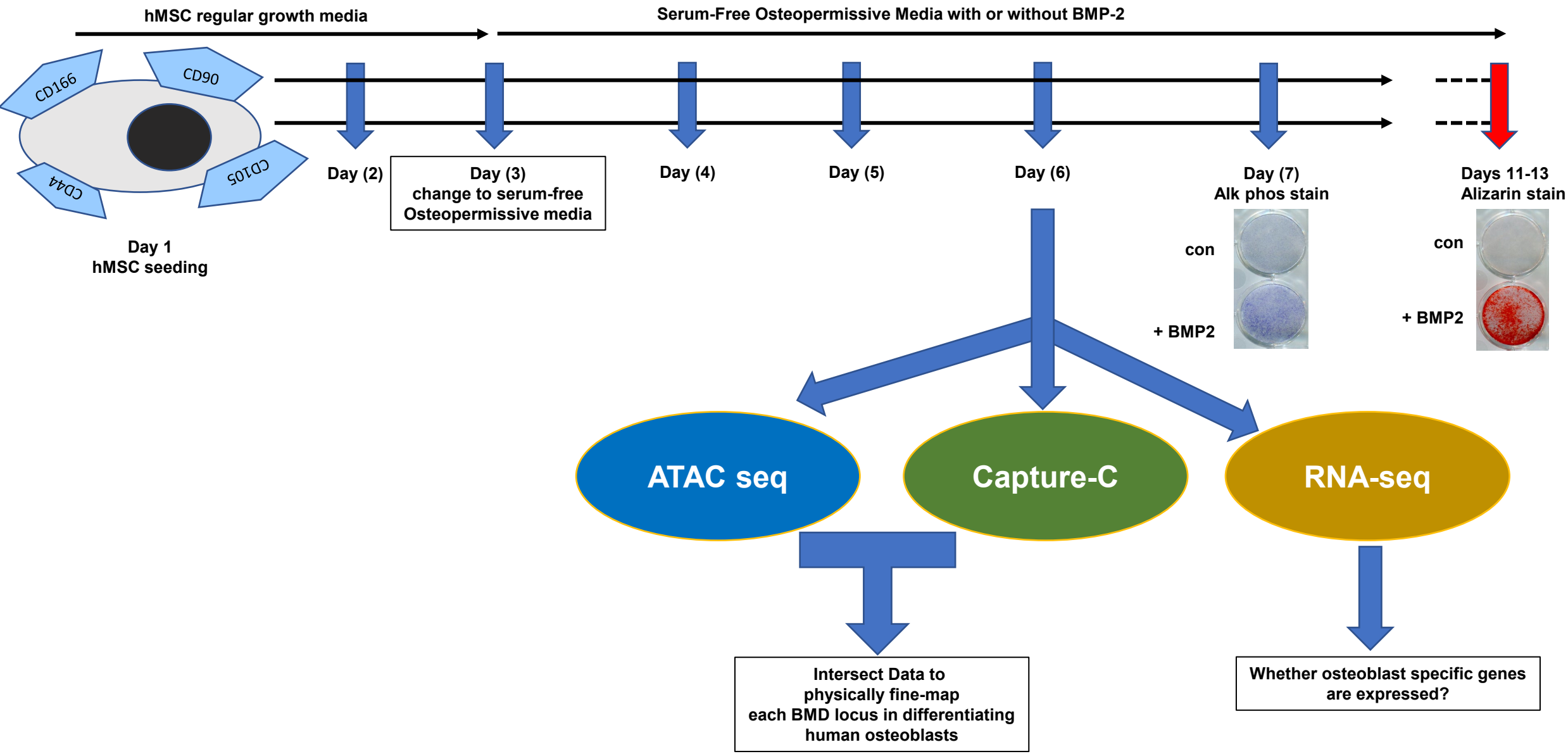




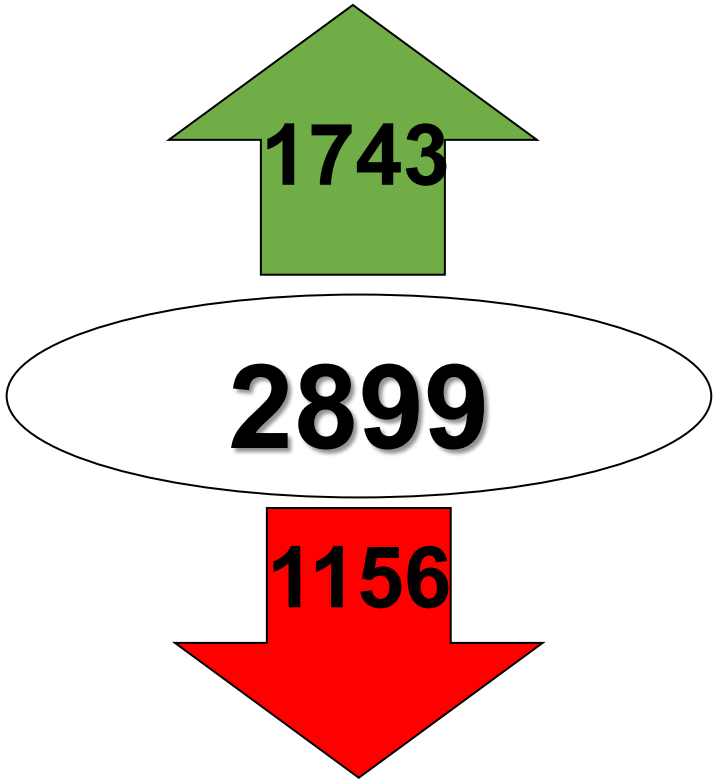


- Cousminer et al., Genome Biol 2021 22(1):1 doi: 10.1186/s13059-020-02207-9

Summary of Methods



RNA sequencing on MSC-derived Osteoblasts



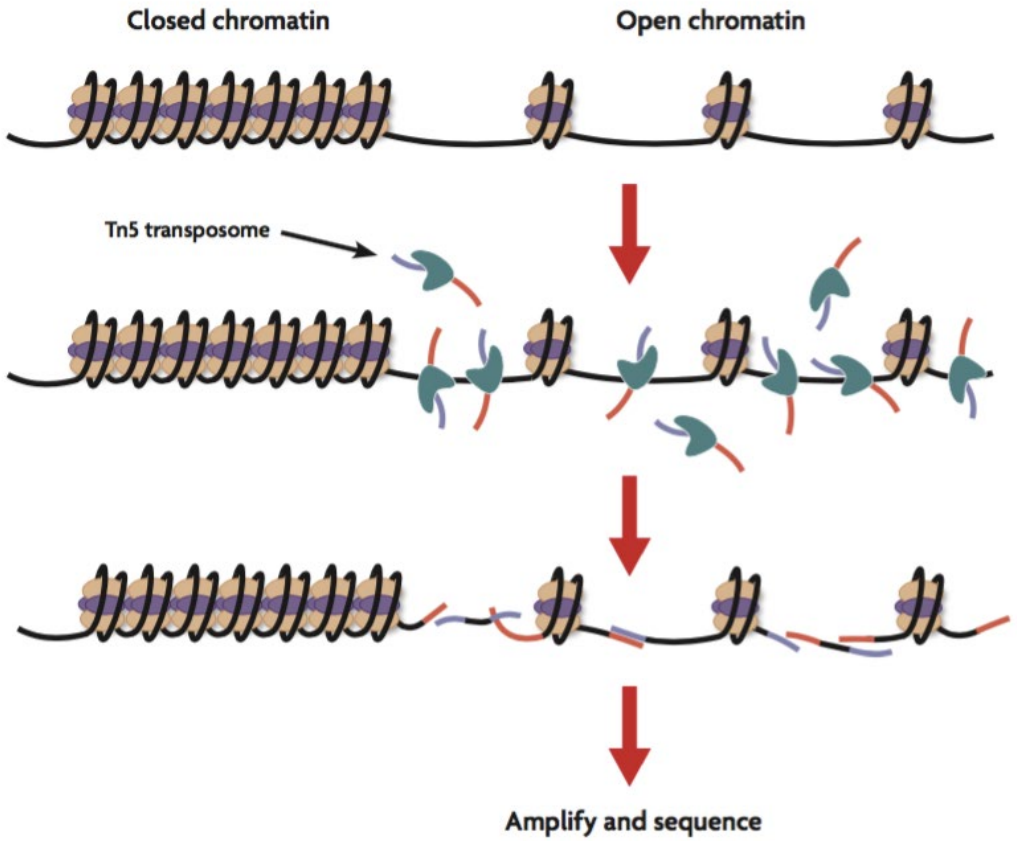
Gene Name	BMP-2 (log2 FC)	Adjusted P. Value
SPP1	3.0496	0.0272
SOST	8.7072	0.0052
DKK1	3.9206	0.0025
SP7	8.3013	0.0004
DMP1	5.5268	0.0229

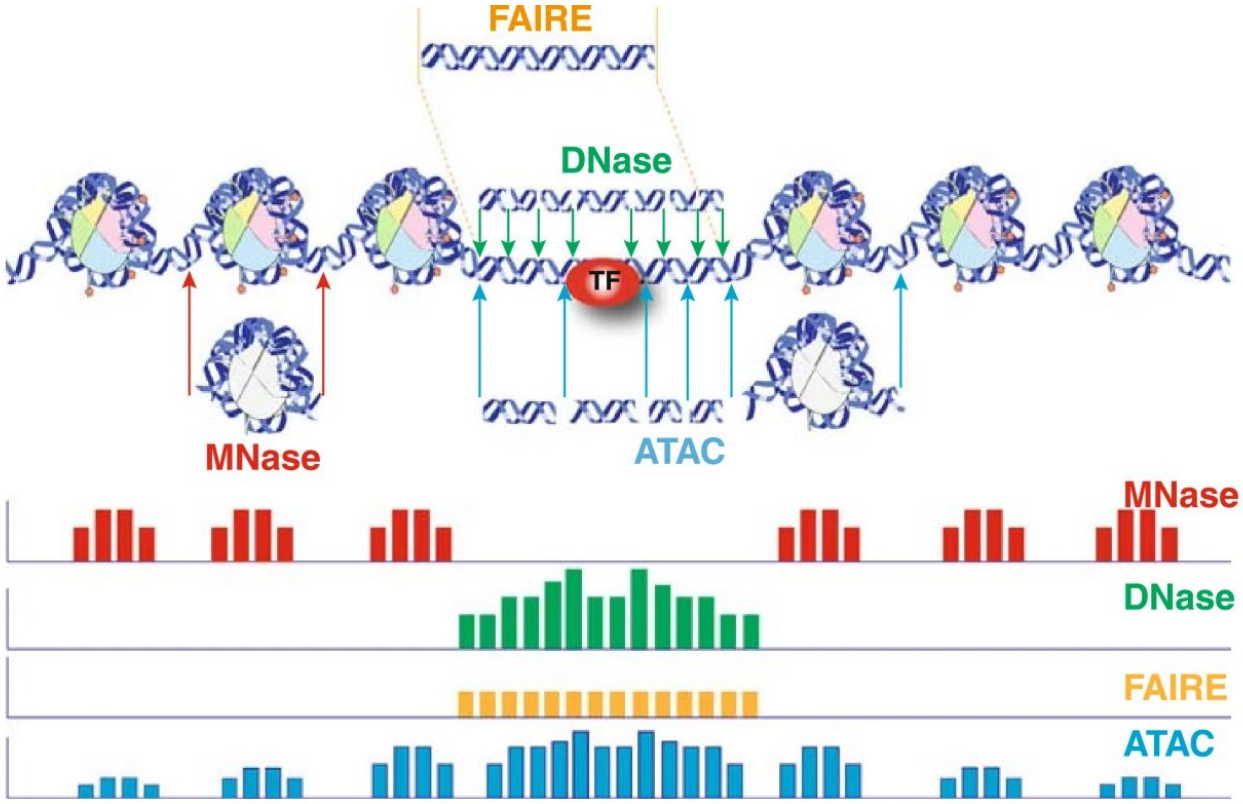
Selection of BMD loci

Estrada et al., Nat. Genet. 44, 491-501 (2012)	**Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture**	56 loci (32 new) $P<5X10^{-8}$
Chesi et al., J Bone Mner Res. 32(6): 1274-1281 (2017)	**A Genomewide Association Study Identifies Two Sex-Specific Loci, at SPTB and IZUMO3, Influencing Pediatric Bone Mineral Density at Multiple Skeletal Sites.**	5 loci (4 new) 2 were sex specific $P<5X10^{-5}$
Chesi et al., Hum Mol Genet. 24(17):5053-5059 (2015)	**A trans-ethnic genome-wide association study identifies gender-specific loci influencing pediatric aBMD and BMC at the distal radius.**	2 loci $P<5X10^{-6}$
Kemp et al., PloS Genet. 10(6):e1004423 (2014)	**Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment.**	13 loci $P<5X10^{-7}$
Zheng et al., Nature 526(7571): 112-117 (2015)	**Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture.**	1 loci $P<5X10^{-6}$
Medina-Gomez et al., Nat Commun. 8(1):121 (2017)	**Bivariate genome-wide association meta-analysis of pediatric musculoskeletal traits reveals pleiotropic effects at the SREBF1/TOM1L2 locus.**	8 loci $P<5X10^{-8}$
Kemp et al., Nat Genet. 49(10):1468-1475 (2017)	**Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis.**	203 loci $P<6.6X10^{-9}$

107 DEXA BMD loci and 203 loci derived from heel ultrasound BMD

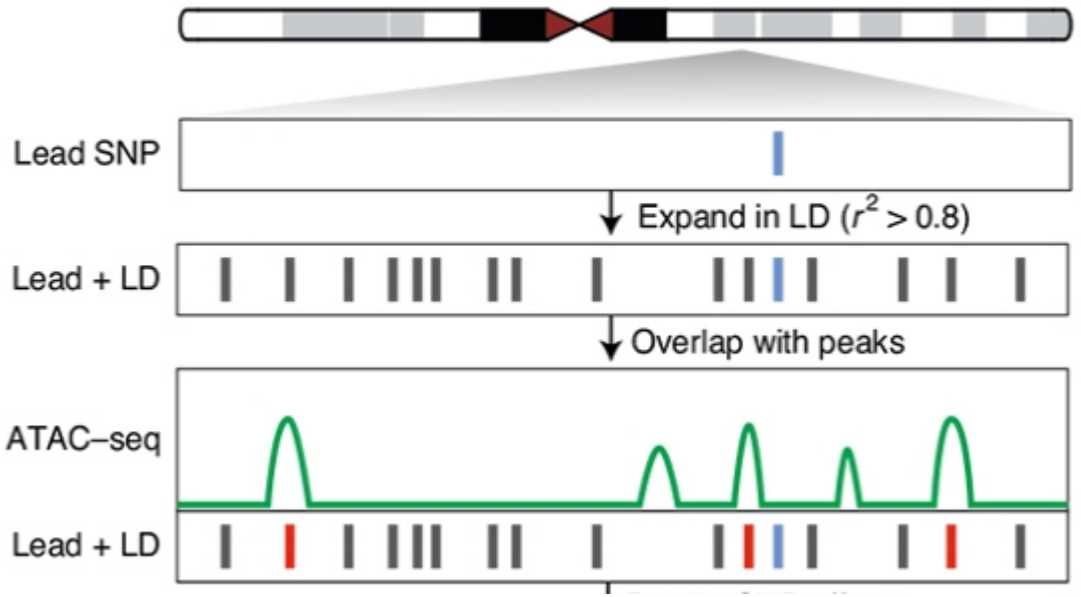
Assay for Transposon Accessible Chromatin (ATAC)





Tsompana & Buck, Epigenetics & Chromatin, 7:33 (2014)

Identifying proxy SNPs in open chromatin and in high LD with GWAS identified sentinel SNP



9 ATAC seq libraries from **4** unique donors

↓

Analyzed using ENCODE ATAC-seq pipeline

↓

156,406 open chromatin peaks

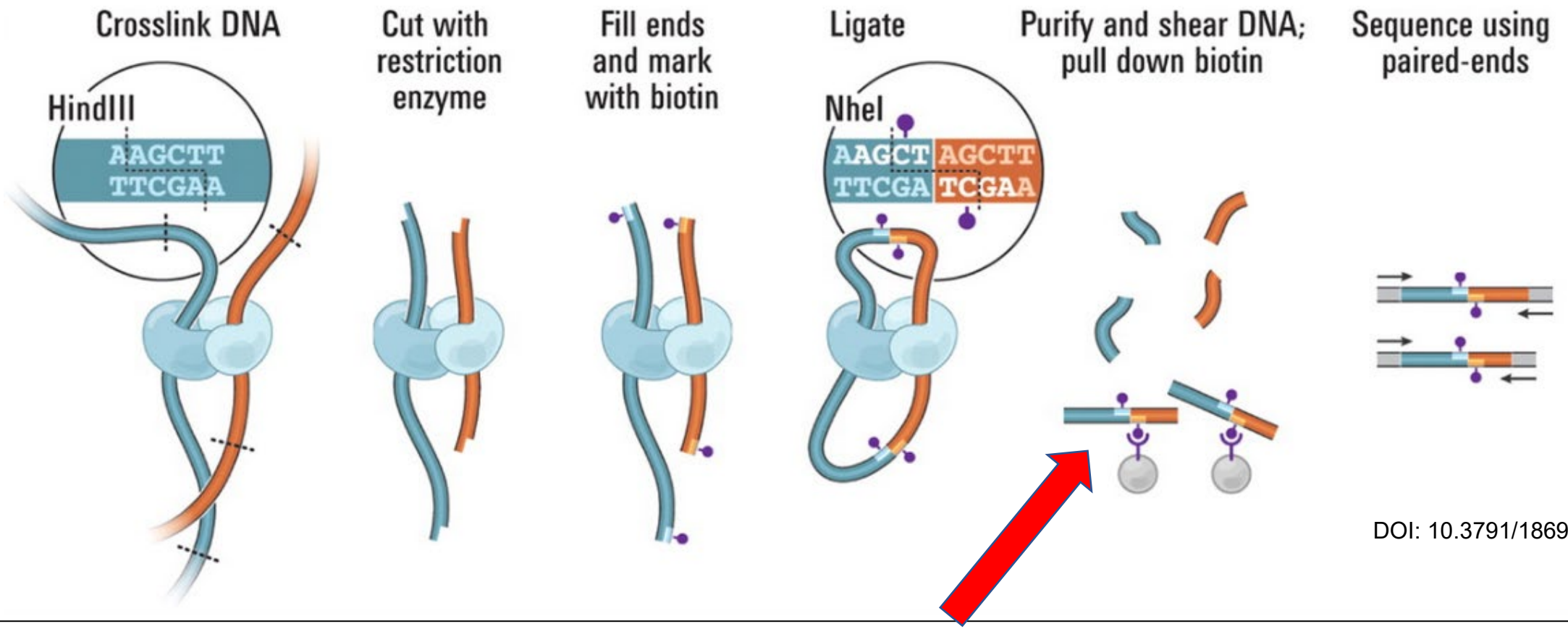
Determine informative proxy SNP for each of the 110 independent signals at **107 DEXA BMD GWAS loci** as well as 307 independent signals at **203 heel ultrasound BMD GWAS loci** by overlapping the positions of the open chromatin regions (peaks) with those of the sentinel and proxy SNPs (r2>0.8 to sentinel SNP in Europeans)

↓

215 informative proxy SNPs corresponding to **58 DEXA loci** and **282 proxy SNPs** corresponding to **112 heel loci** in high LD with the sentinel SNP of the BMD loci investigated

Corces et al., Nature Genetics, 52: 1158-1168(2020)

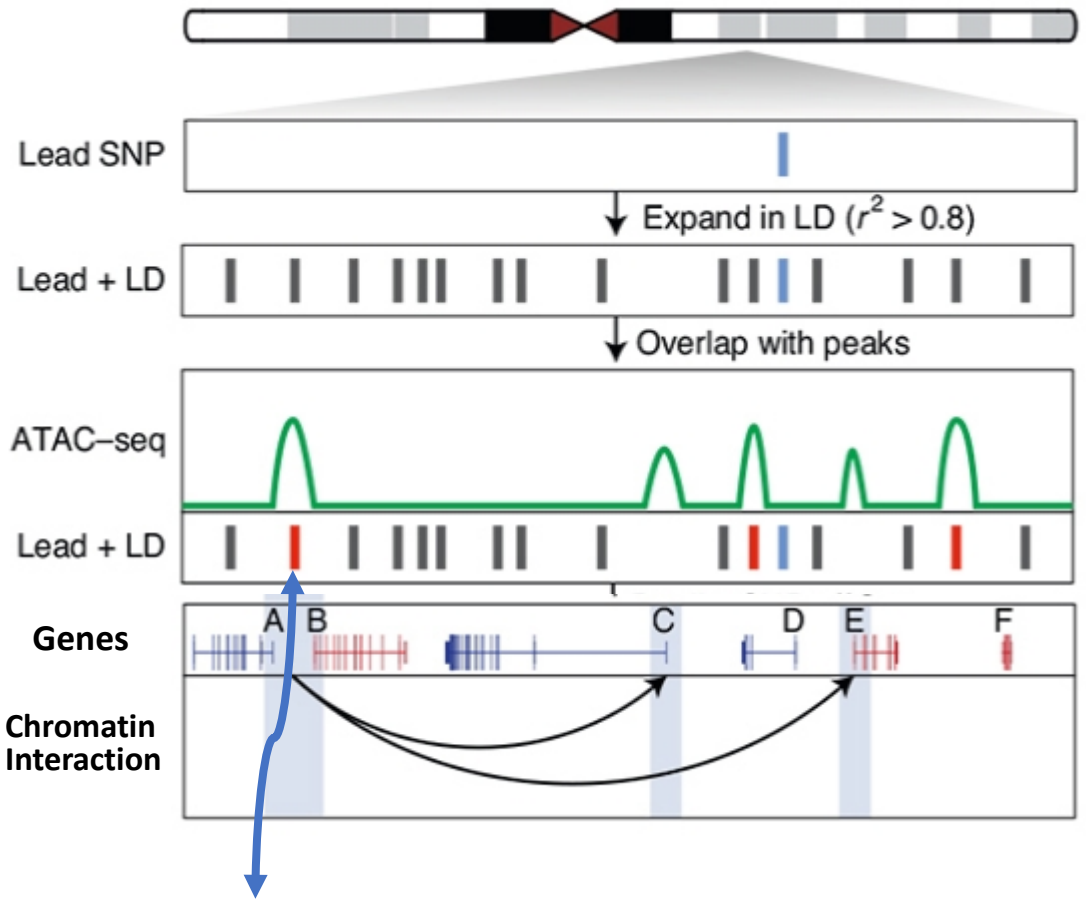
Chromatin Conformation Capture



DOI: 10.3791/1869

ligated library was hybridized with custom-designed Capture-C library consisting of both ends of DpnII restriction fragments encompassing promoters of all human coding genes and non-coding RNAs to enrich promoter interacting region

One of the most comprehensive 3D promoter interactome analyzed !

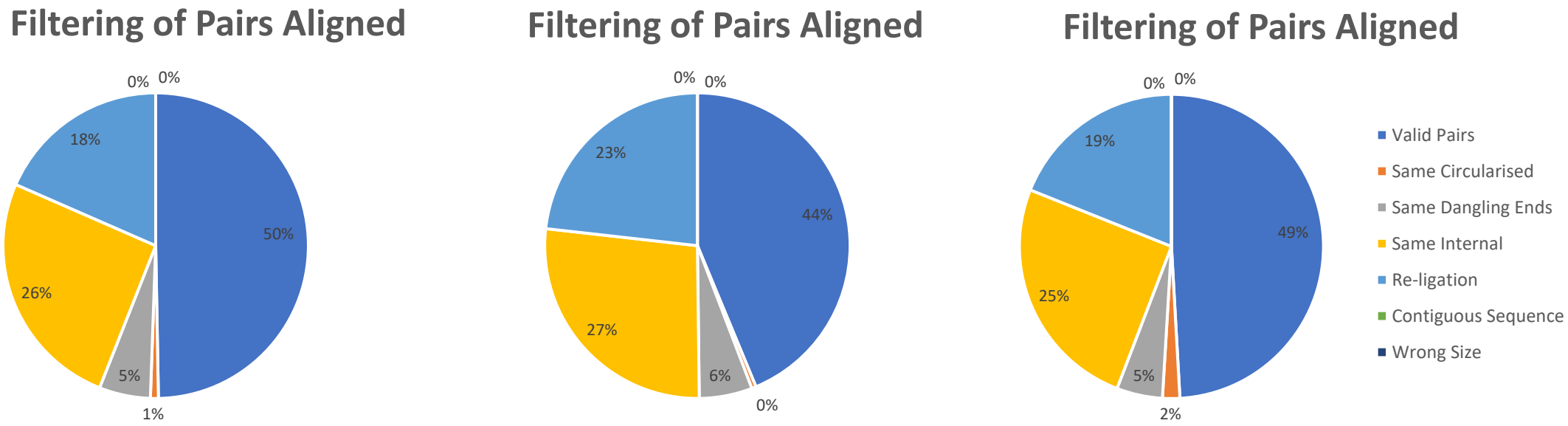


Would this proxy SNP affect gene C or gene E?

Open chromatin variants are subsequently filtered by being in direct physical contact with gene promoter baits (Promoter-focused Capture-C)

Hi-C results for each library

~1.6 billion reads per sample; >40% valid read pairs and >75% capture efficiency

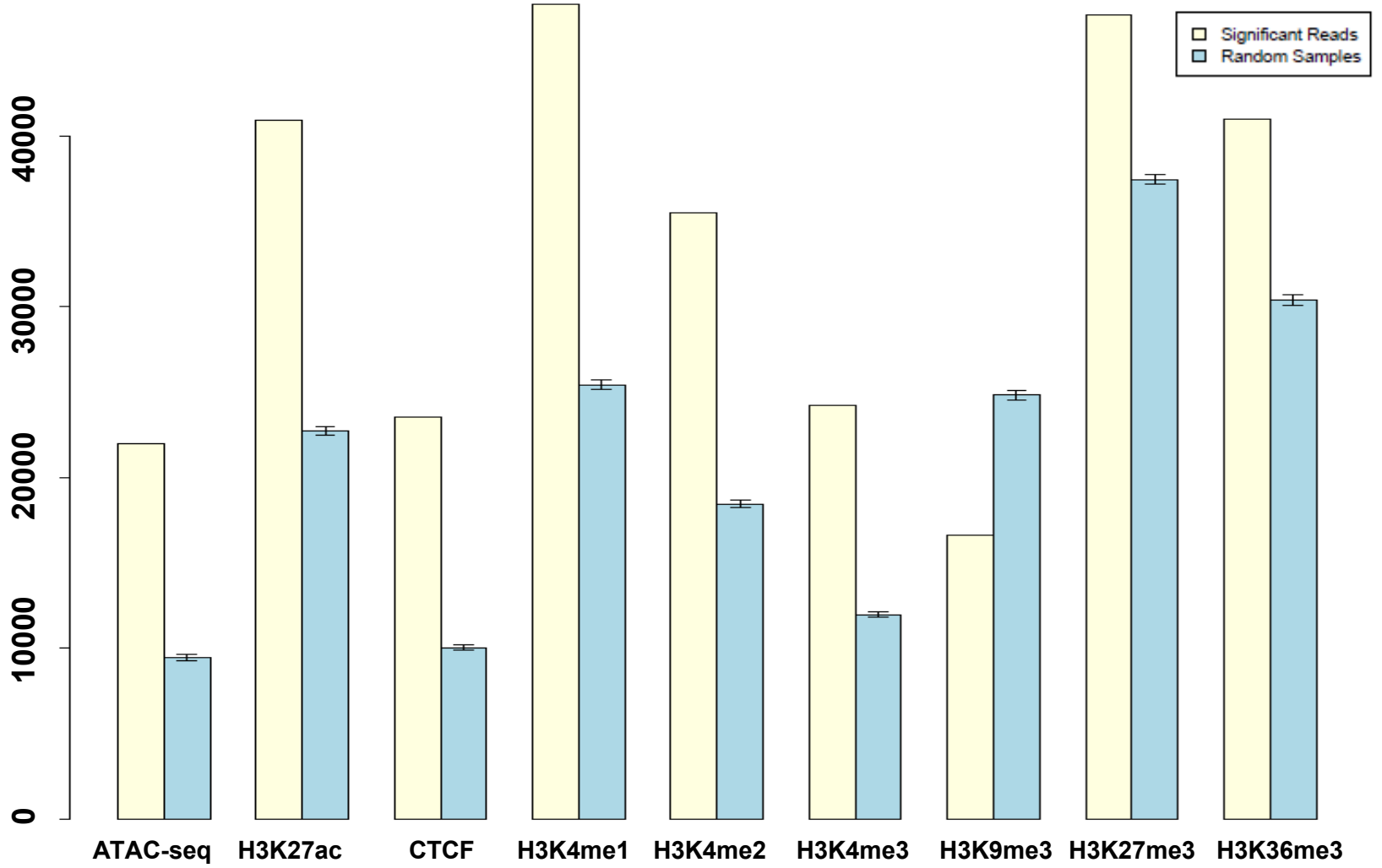


For further analysis: we combined 1 fragment resolution reads and 4 fragments resolution reads to achieve reads for short interactions as well as increased sensitivity

295,422 interactions (~14% were bait to bait; median distance for cis interactions 50.5kb, trans-interactions 1%)

Most of the non-bait promoter-interacting regions (PIRs) had contacts with a single baited region (84%), while only 1% contacted more than 4

Identified Promoter interacting regions (PIRs) were significantly enriched for histone marks associated with active chromatin regions in primary human osteoblasts from the ENCODE project



Combining the open chromatin landscape (ATAC-seq data) with chromatin interaction data (Capture-C) is an efficient approach to link biologically significant genes with their active regulatory elements

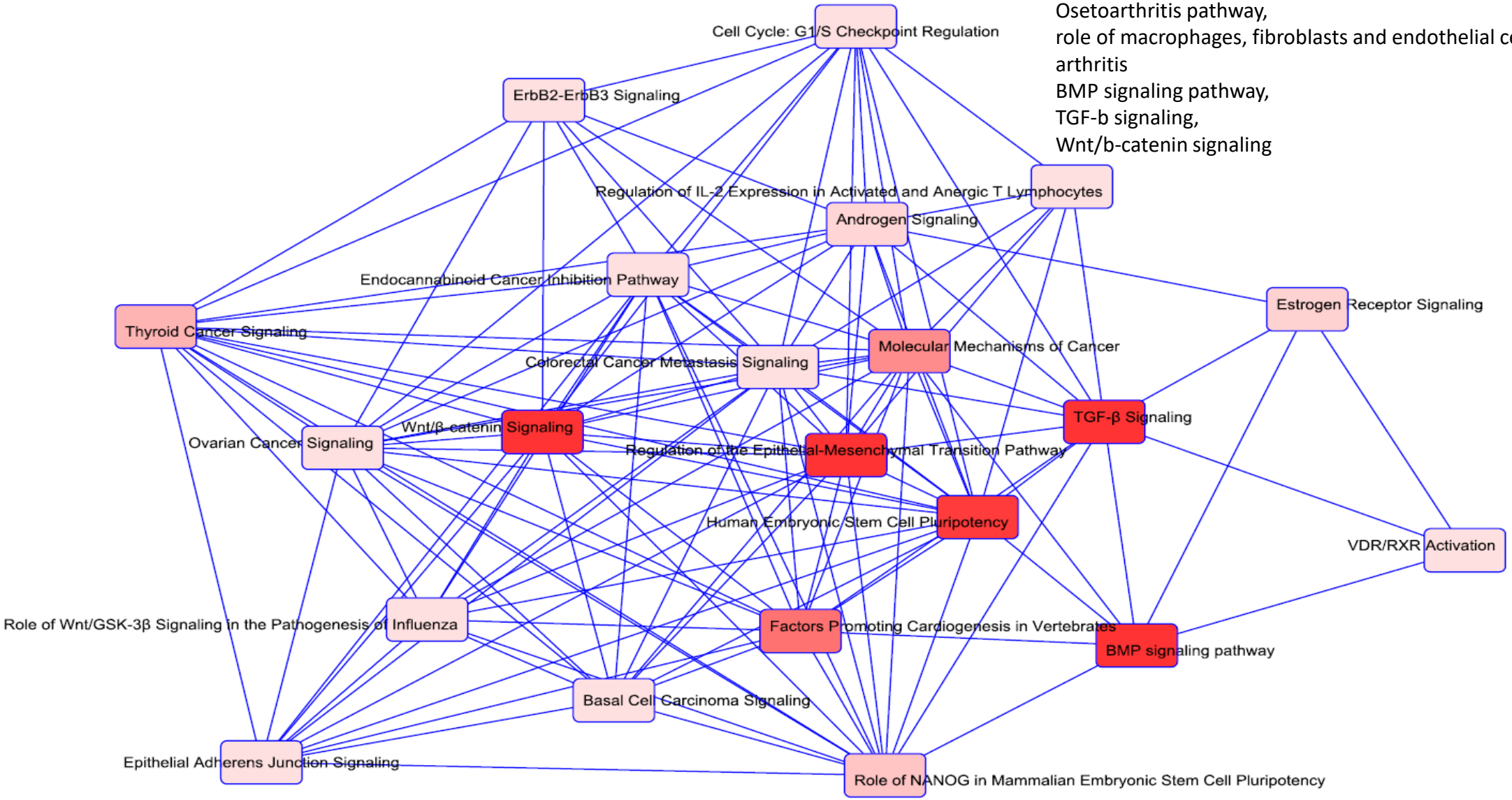
Of the BMD GWAS loci in MSC-derived osteoblasts, 46 GWAS loci revealed at least one or more BMD proxy SNPs in open chromatin (and not residing in a baited promoter region) interacting with an open gene promoter

A total of 77 open baited regions corresponding to 81 gene promoters were linked to 84 open chromatin regions harboring one or more BMD proxy SNPs through 104 distinct chromatin looping interactions

Among the implicated genes in those loci, several have a known role in osteogenesis such as *SMAD3, SMAD9, SPP1, WLS, FRZB, NOG* and *MIR31HG;* while several are completely novel

Ingenuity Pathway Analysis of the implicated genes reveal pathways relevant to osteoblastic differentiation

role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis
Osetoarthritis pathway,
role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis
BMP signaling pathway,
TGF-b signaling,
Wnt/b-catenin signaling



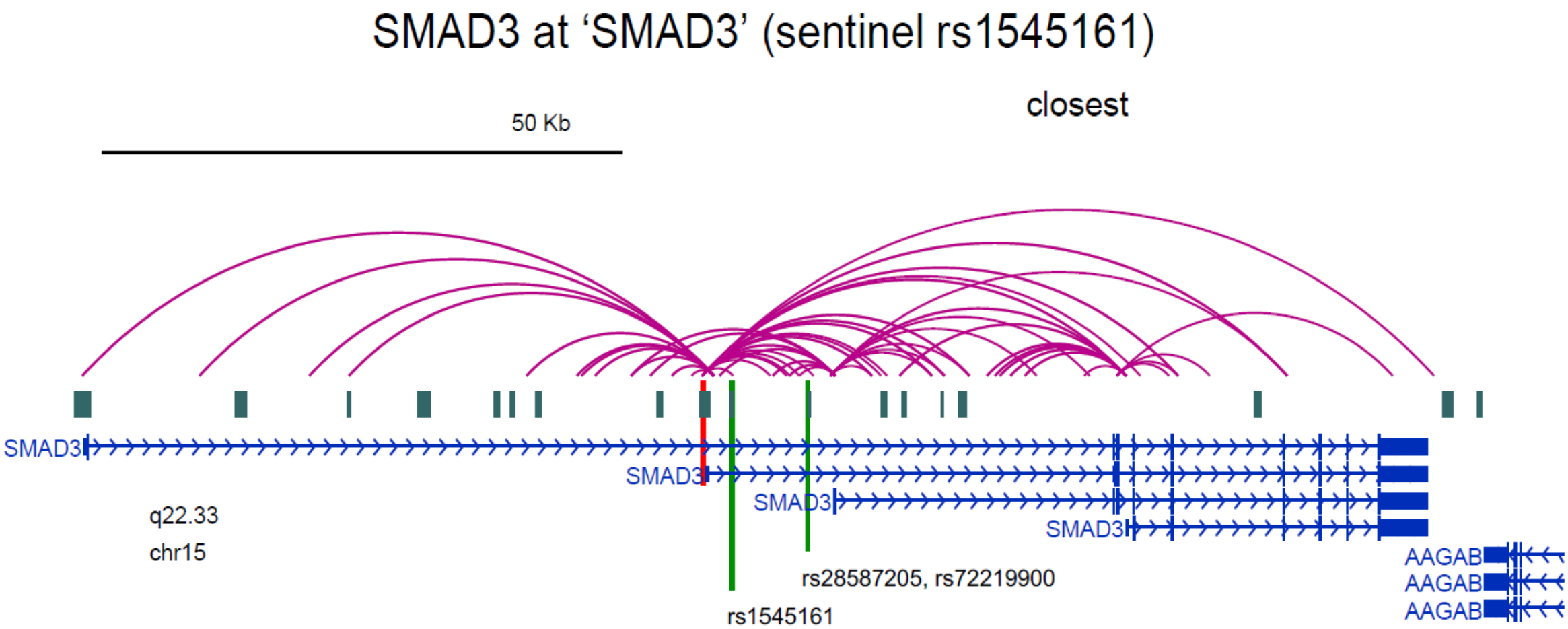
SNP-promoter interactions shows both close and distant genes

SNP-promoter interactions (for SNPs not residing in baits) fell in to three types of observations: 1. to nearest gene only (30%)

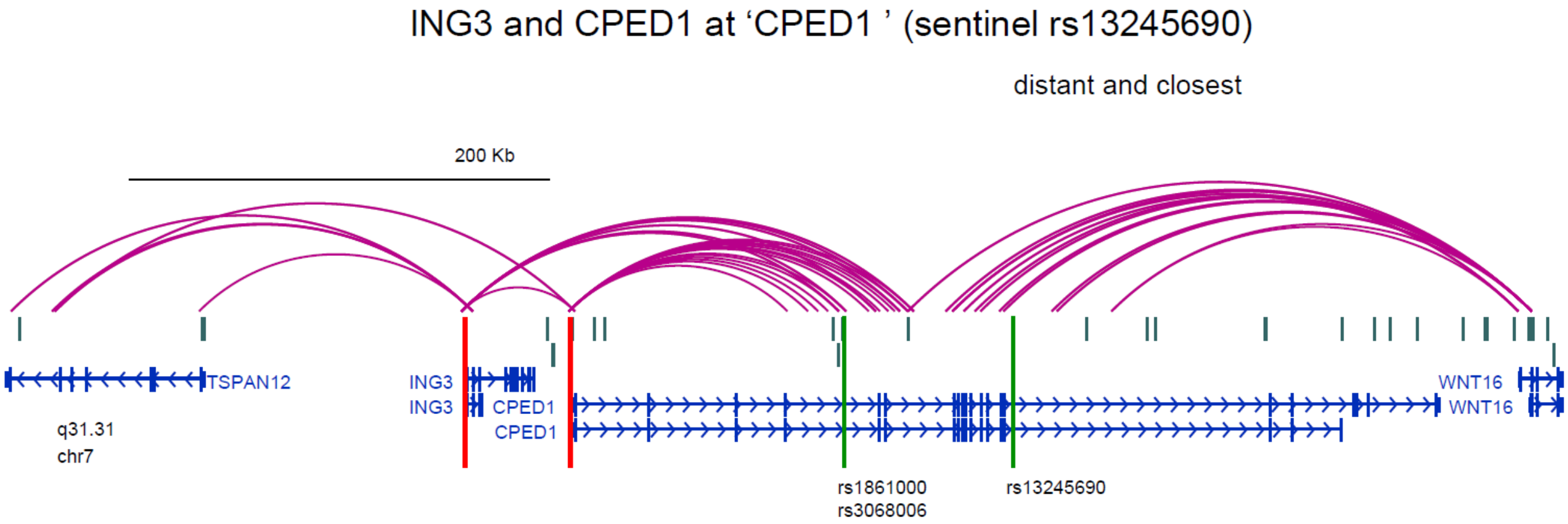
2. to both nearest and more distant genes (13%) and

3. only to distant genes (57%)

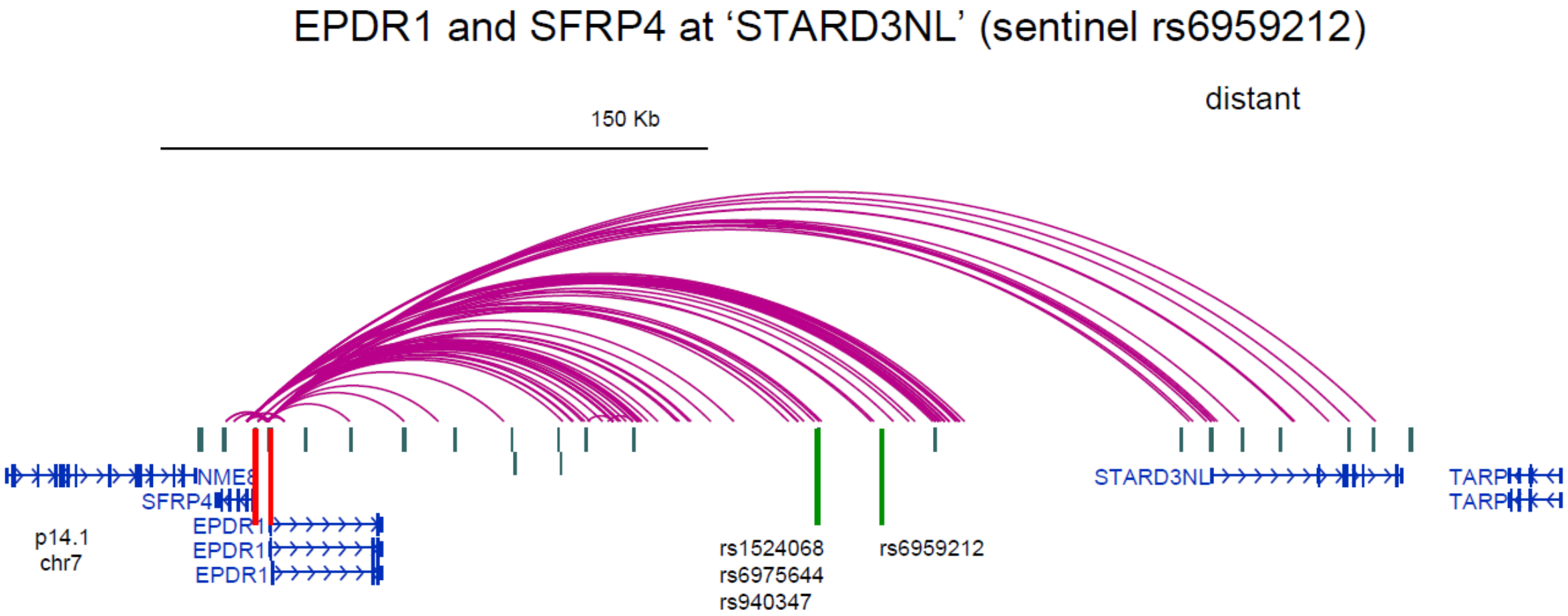
Example of SNP promoter interaction: Nearest



Example of SNP promoter interaction: Nearest and Distant

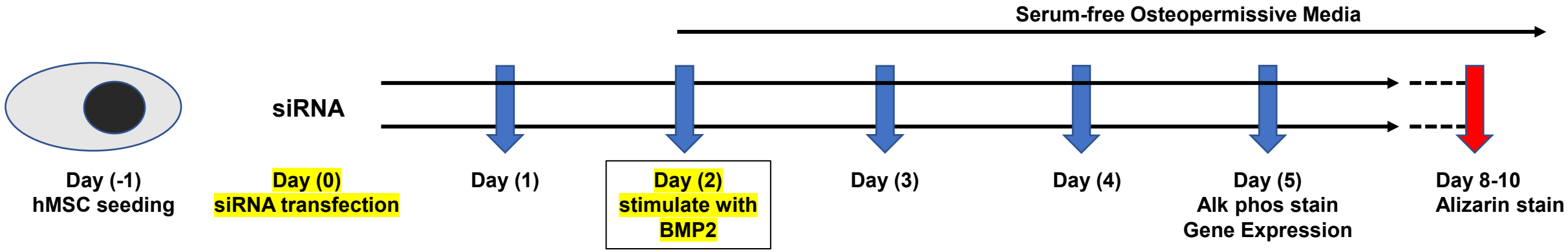


Example of SNP promoter interaction: Distant

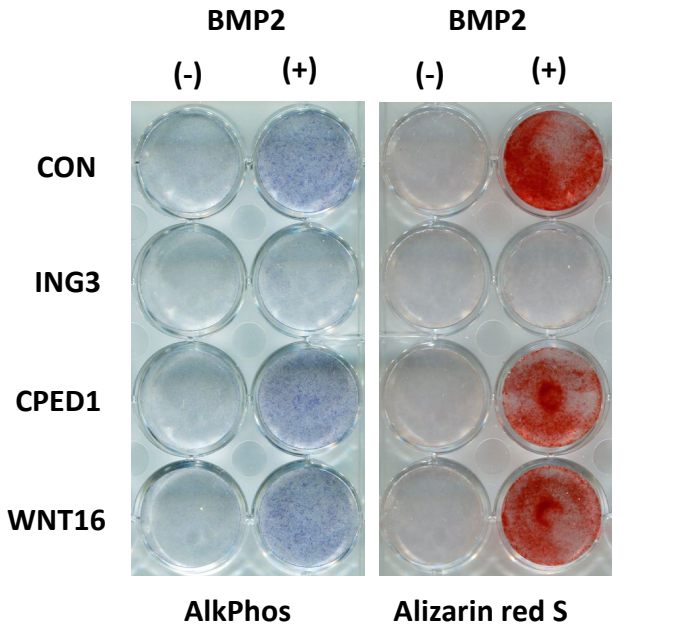


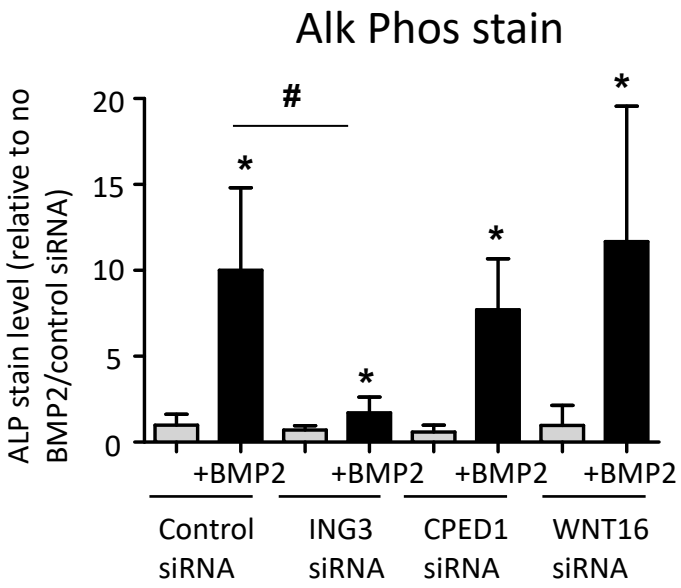
Functional Experiments

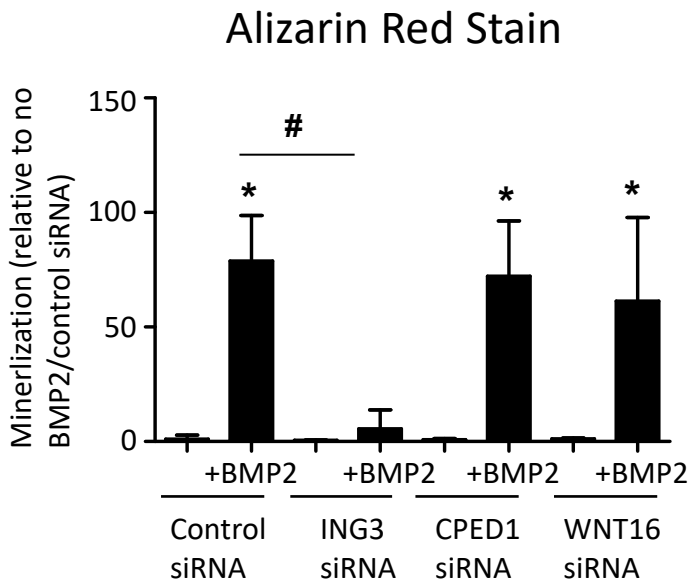
- *CPED1* locus → *ING3, CPED1* and *WNT16*
- *EPDR1* locus → *SFRP4* and *EPDR1*

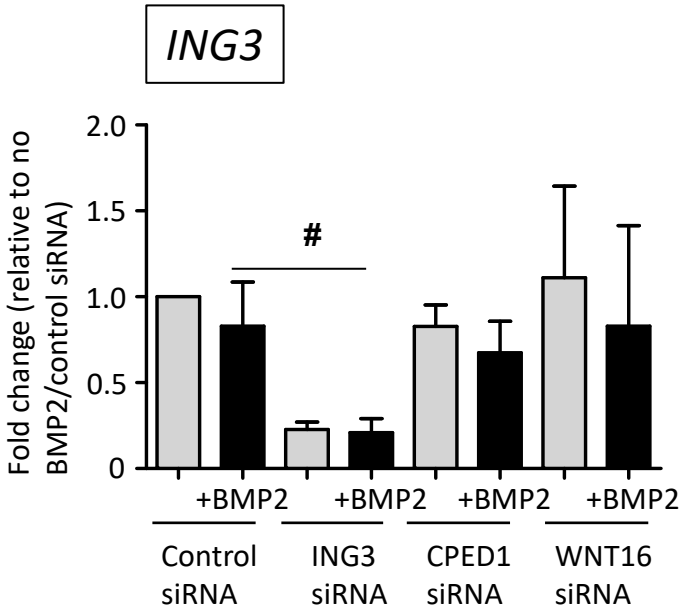


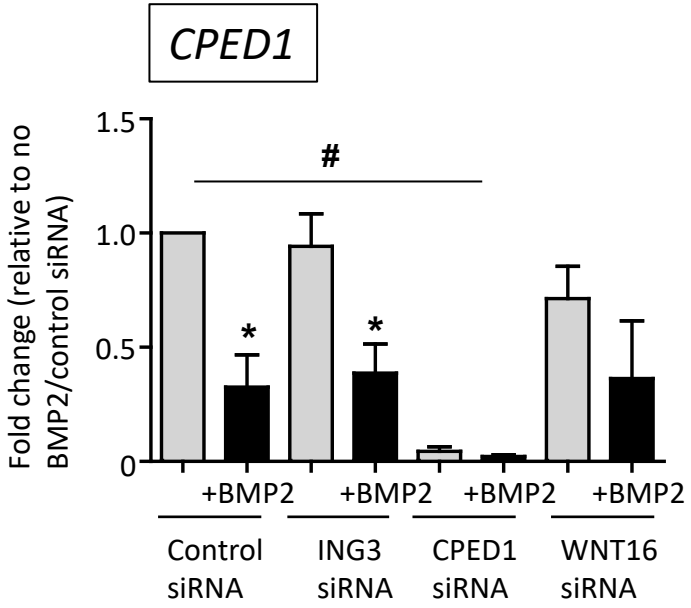
ING3 silencing reduces human osteoblast differentiation

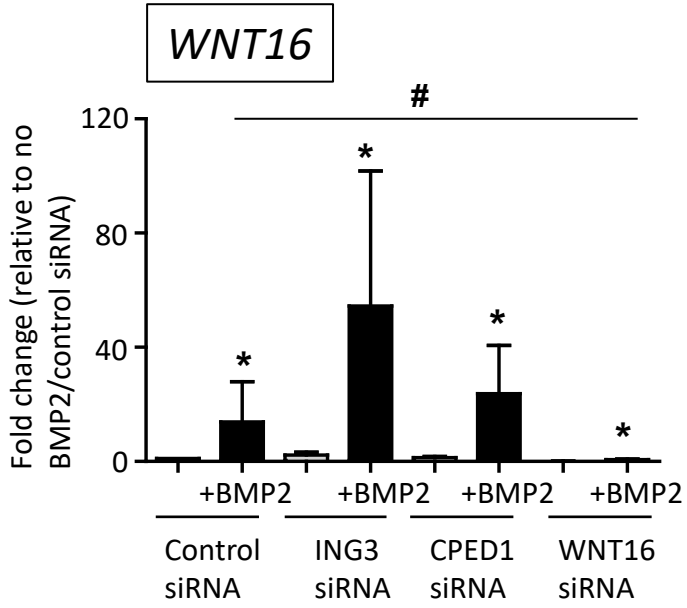




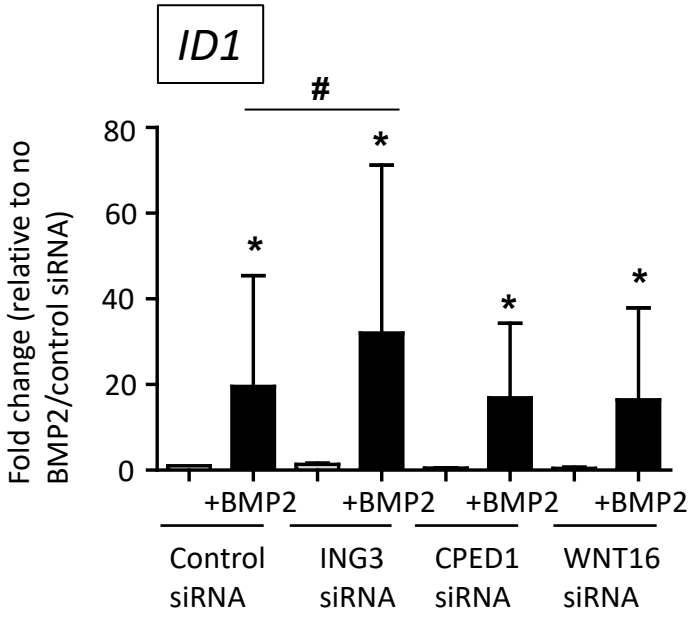


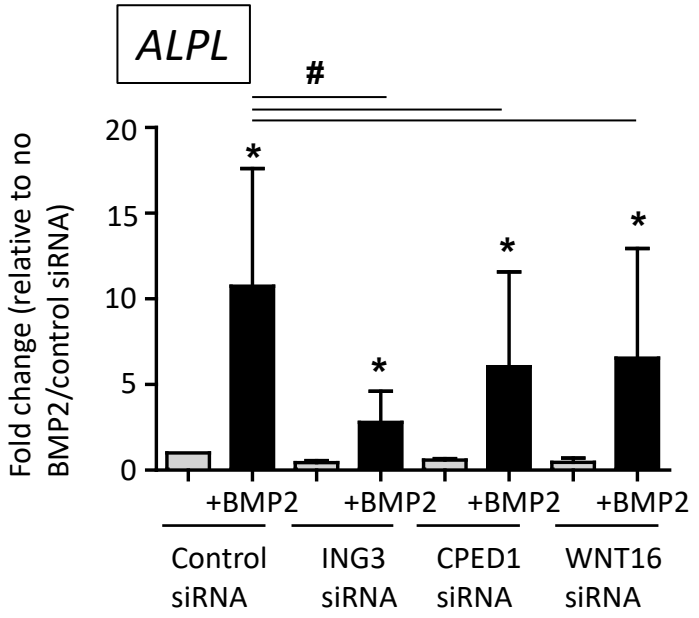


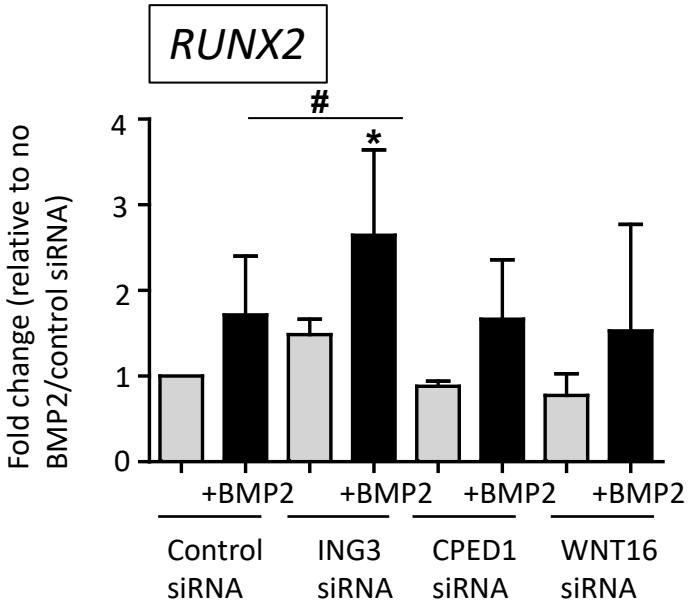


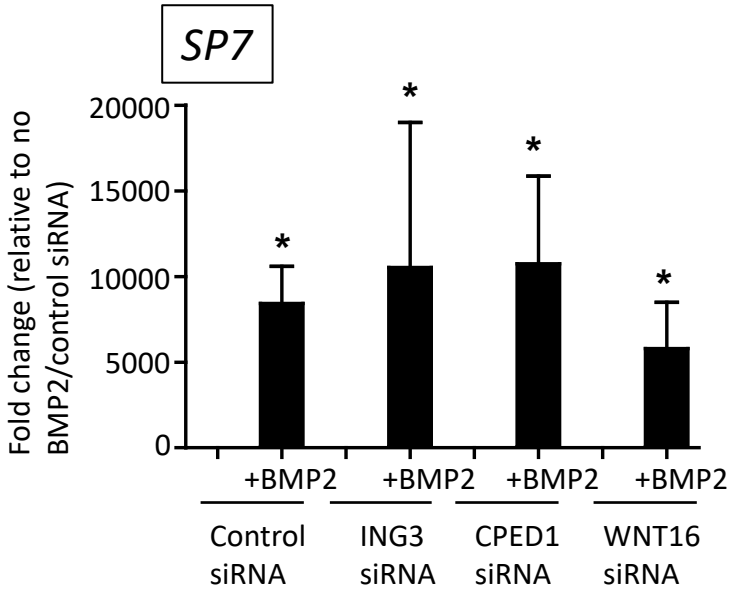


ING3 silencing does not decrease RUNX2 and SP7

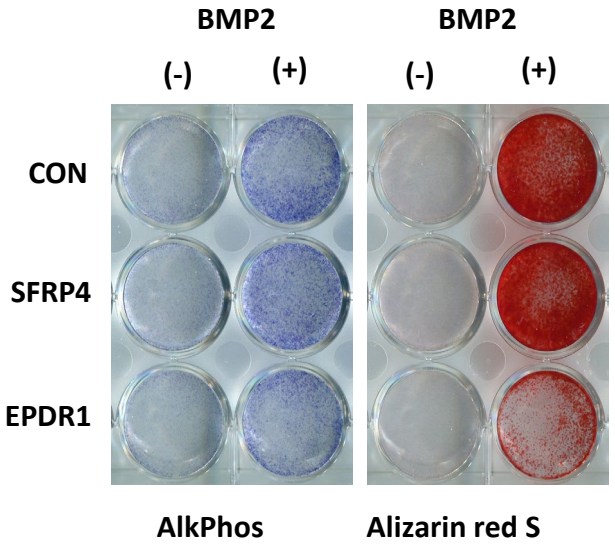


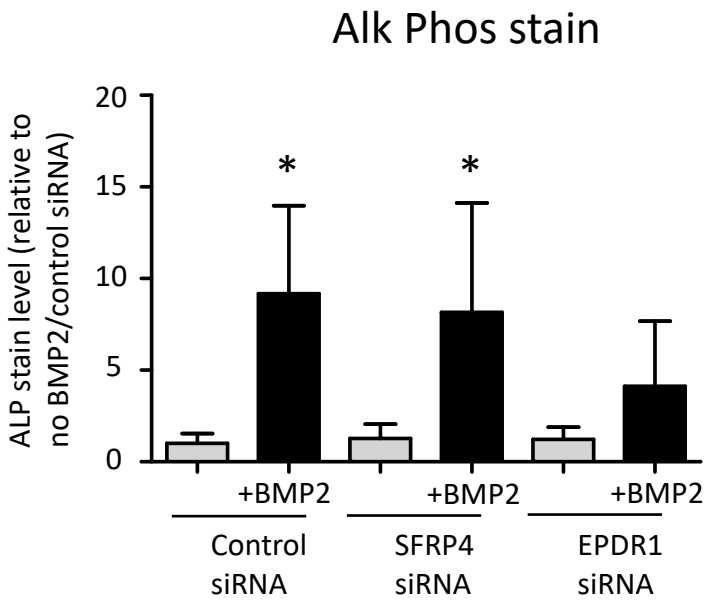


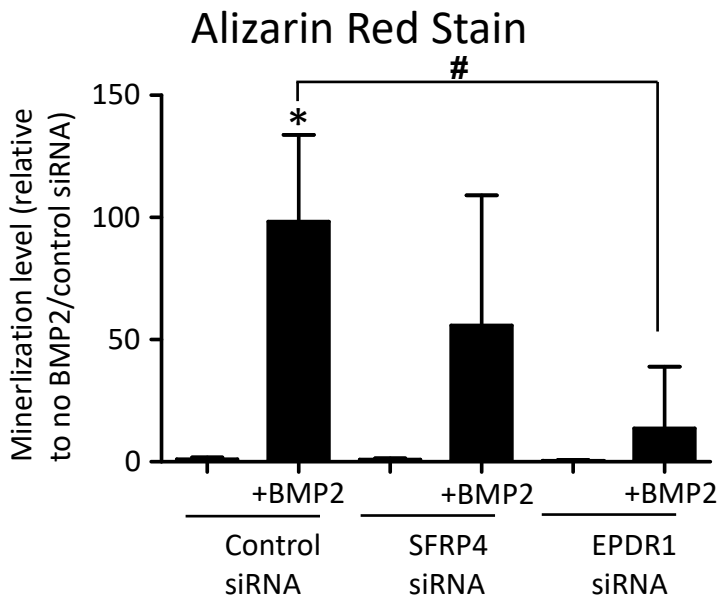


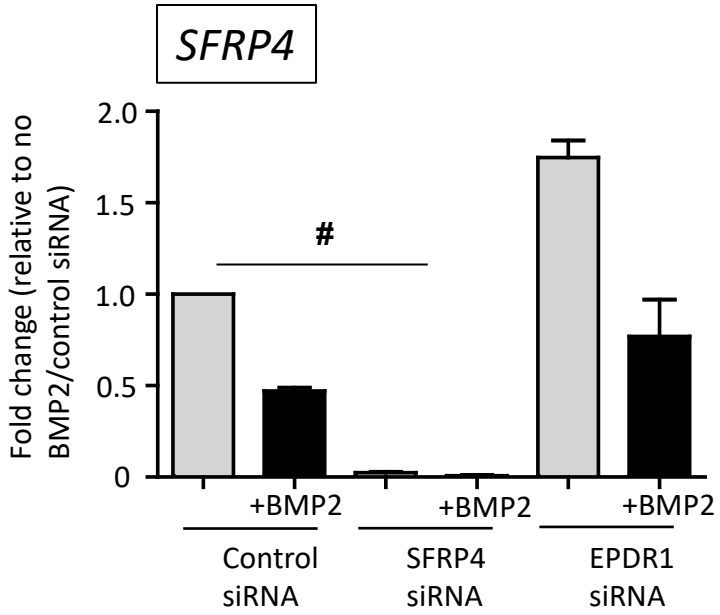


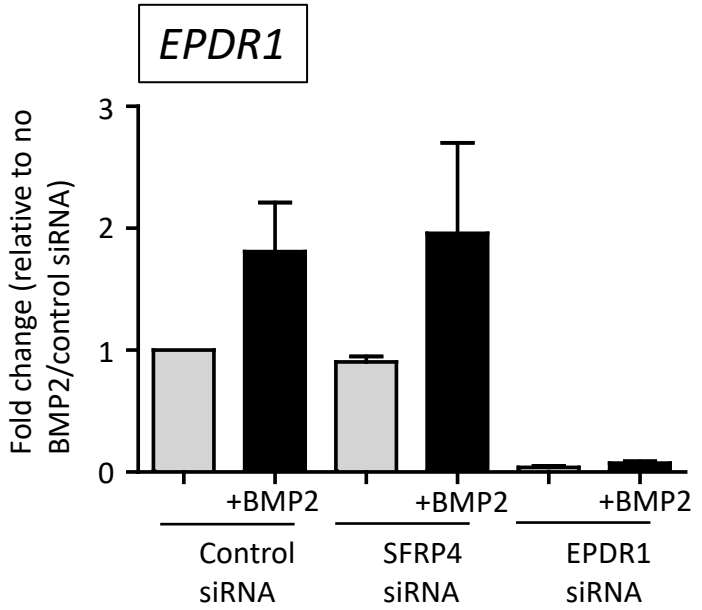
EPDR1 silencing reduces human osteoblast differentiation



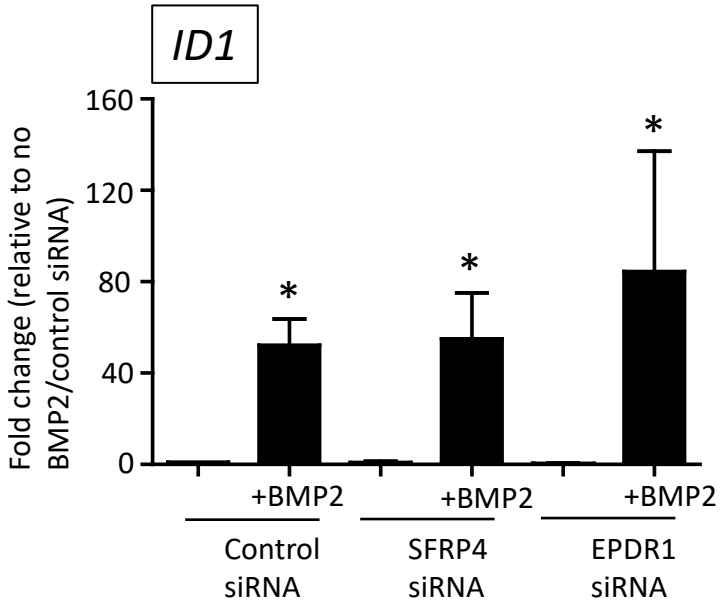


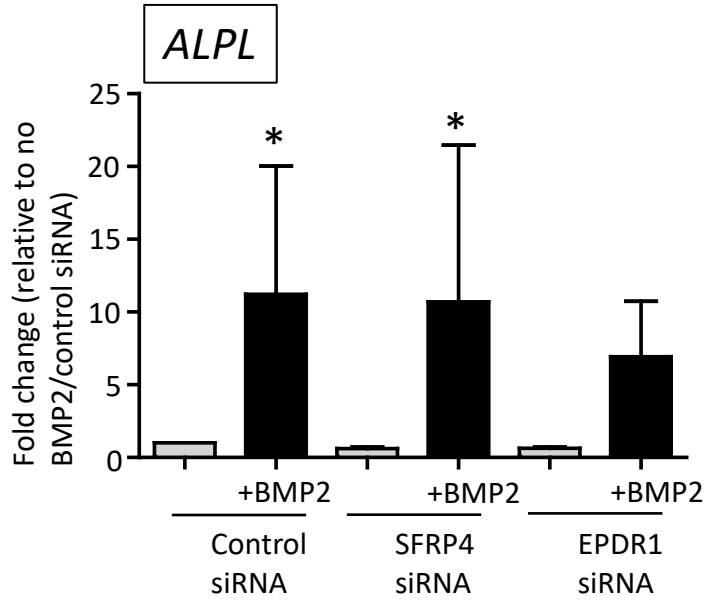


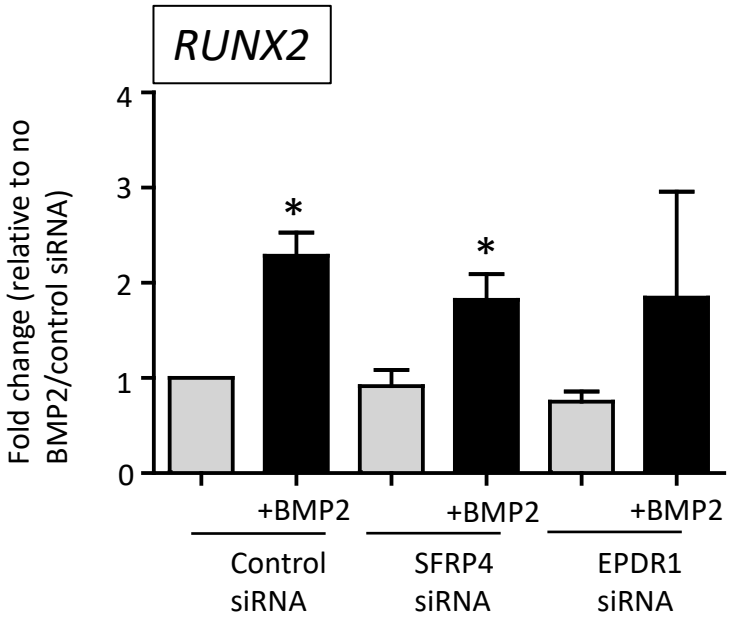


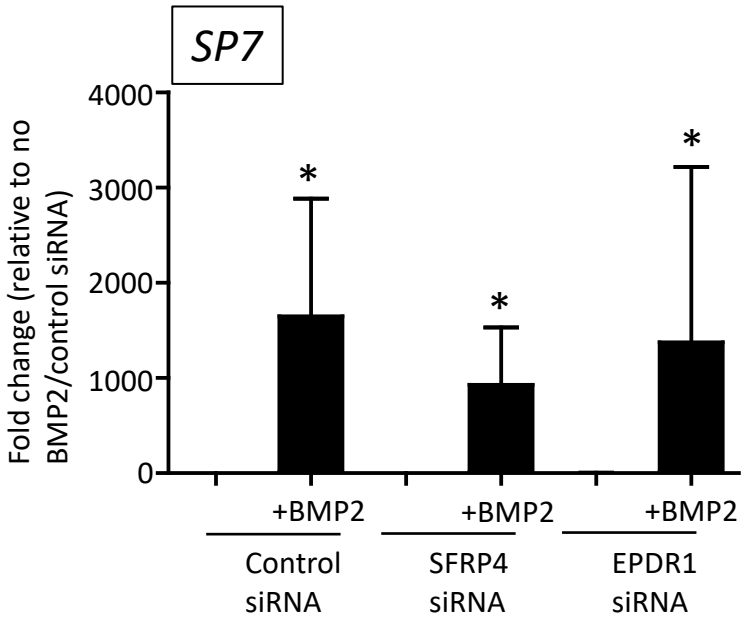


EPDR1 silencing reduces human osteoblast differentiation

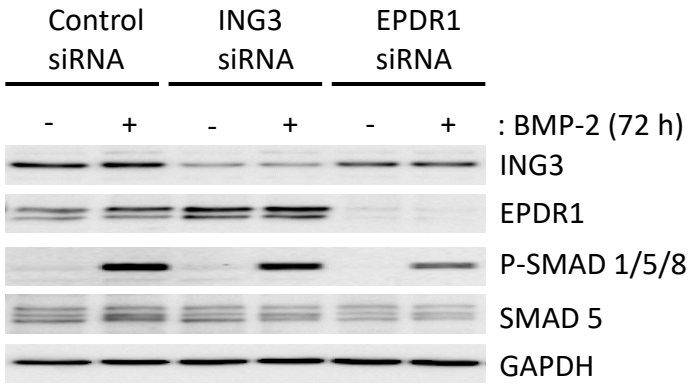


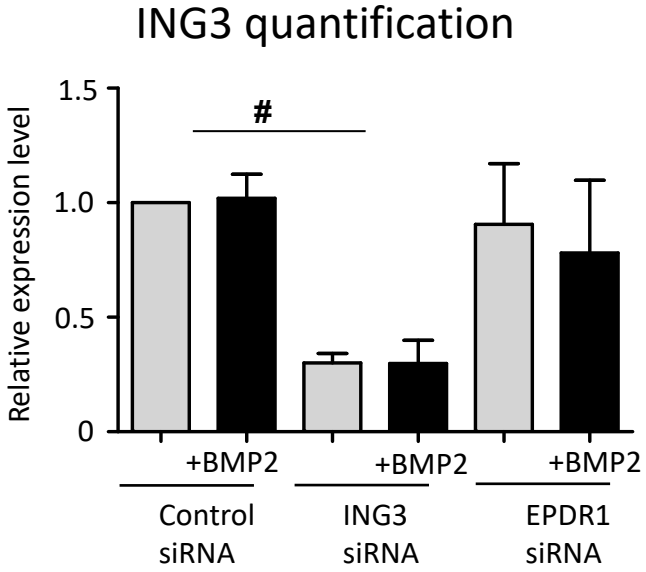


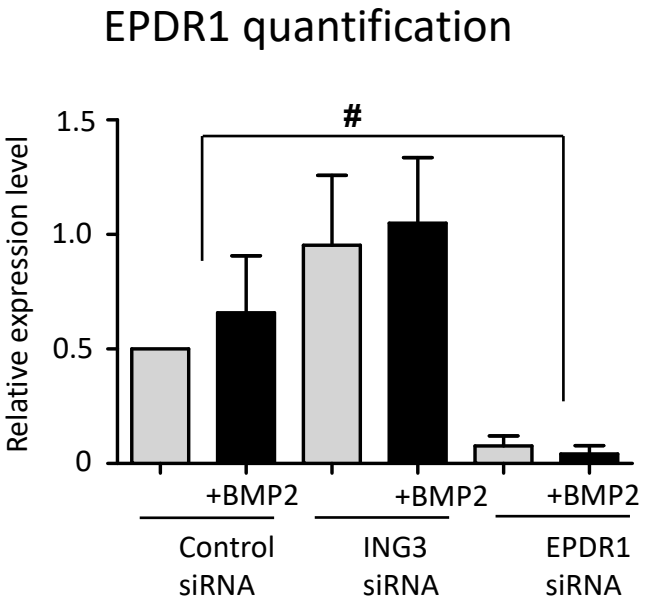


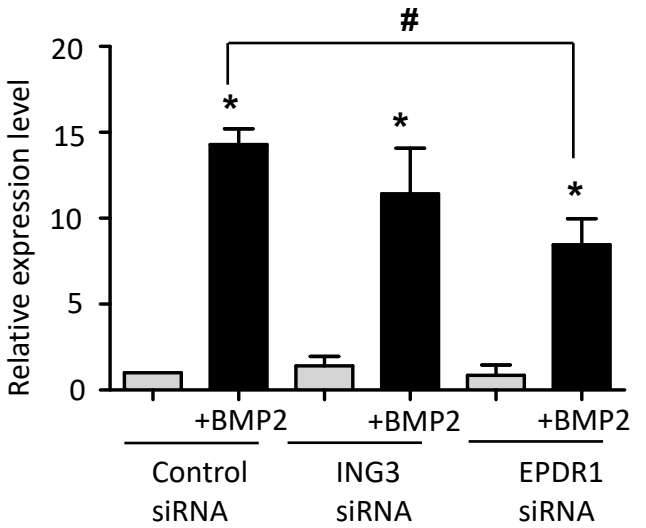


ING3 and *EPDR1* silencing demonstrated at protein levels



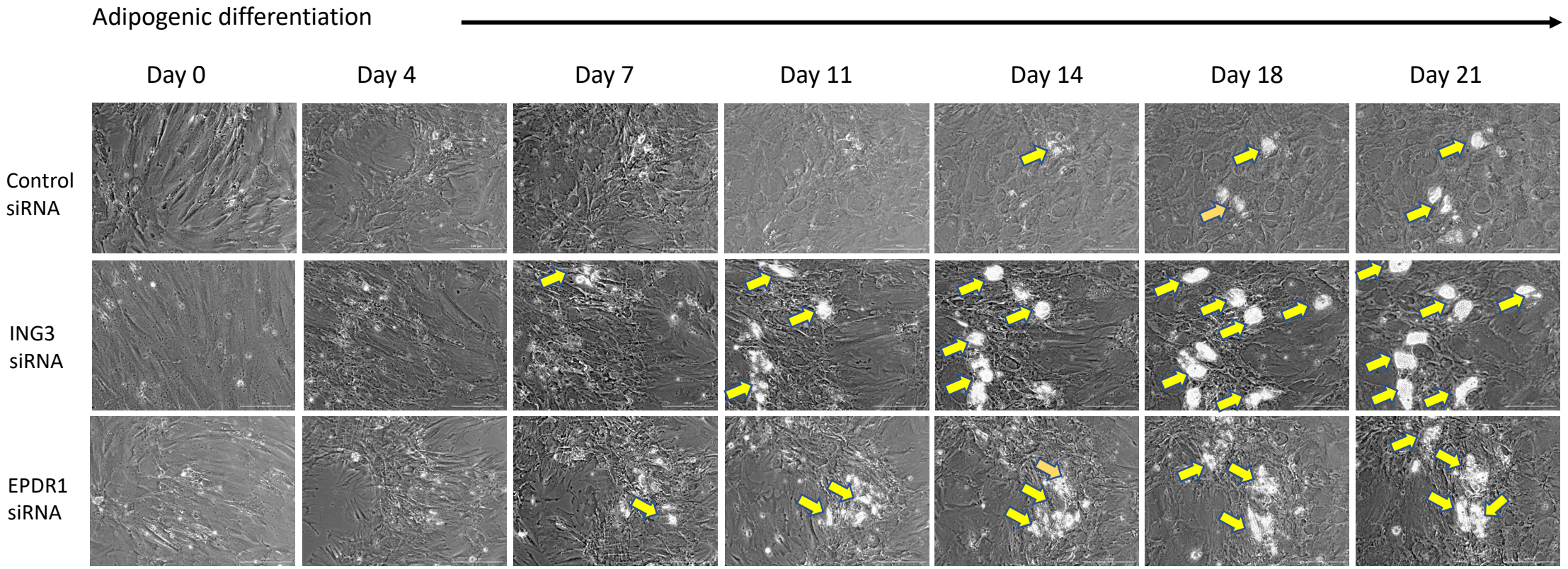




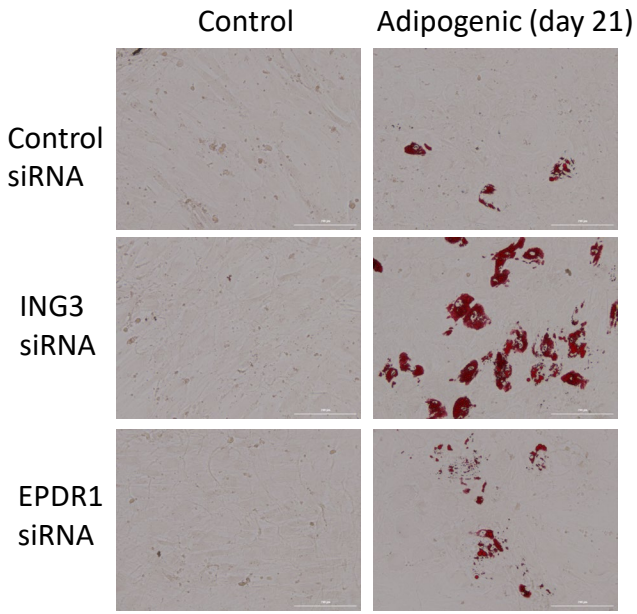


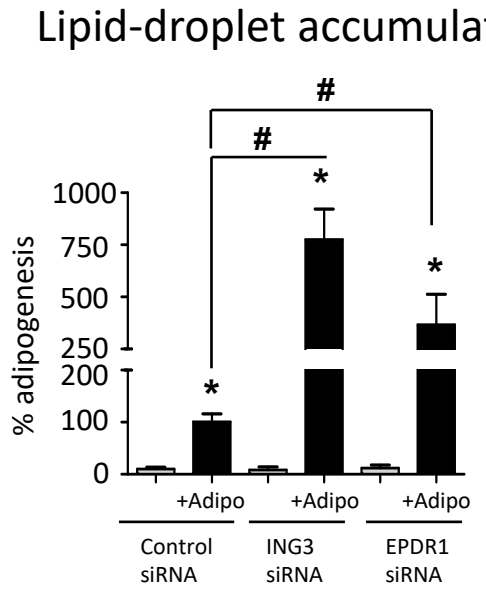
Are *ING3* and *EPDR1* fundamental genes required for general cell metabolism and whether these genes affect MSC differentiation into adipocytes?

ING3 and *EPDR1* silencing increases adipogenesis

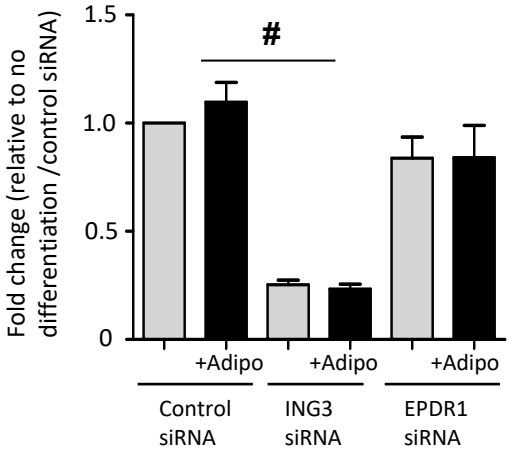


ING3 and *EPDR1* silencing increases adipogenesis

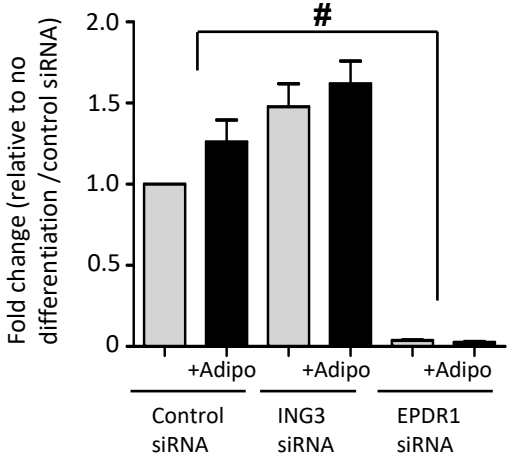




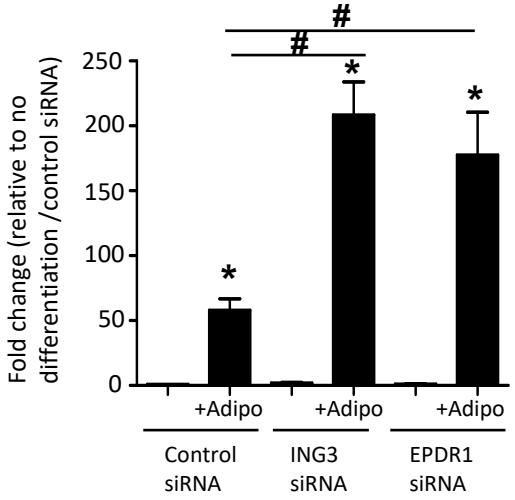
ING3



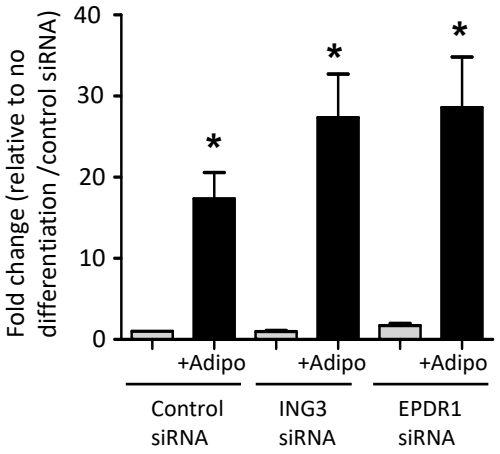
EPDR1



C/EBP alpha



PPAR gamma



Article | Open Access | Published: 19 March 2019

Genome-scale Capture C promoter interactions implicate effector genes at GWAS loci for bone mineral density

Alessandra Chesi, Yadav Wagley, Matthew E. Johnson, Elisabetta Manduchi, Chun Su, Sumei Lu, Michelle E. Leonard, Kenyaita M. Hodge, James A. Pippin, Kurt D. Hankenson, Andrew D. Wells & Struan F. A. Grant

Nature Communications **10**, Article number: 1260 (2019) | Cite this article

46 GWAS loci revealed at least one or more BMD proxy SNPs in open chromatin (and not residing in a baited promoter region) interacting with an open gene promoter

Research | Open Access | Published: 04 January 2021

Genome-wide association study implicates novel loci and reveals candidate effector genes for longitudinal pediatric bone accrual

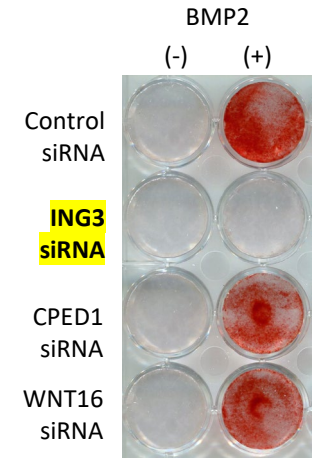
Diana L. Cousminer, Yadav Wagley, [...] Struan F. A. Grant

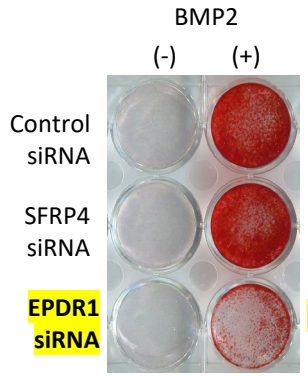
Genome Biology **22**, Article number: 1 (2021) | Cite this article

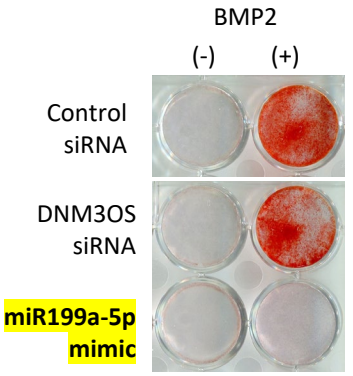
40 prioritized loci using multiple inclusion criteria (traditional GWAS threshold, suggestive significance level) of which 35 signals are novel

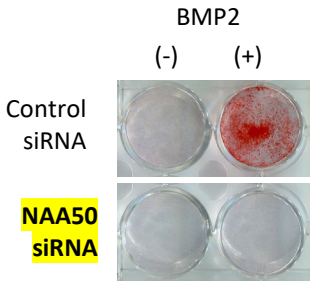
For 7 loci, causal effector genes have been established

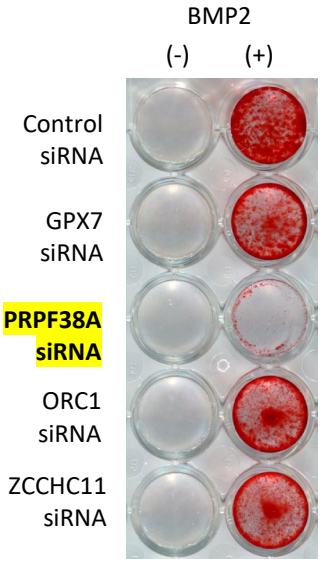
Locus	Implicated genes
CPED1	*ING3, CPED1, WNT16*
STARD3NL	*EPDR1, SFRP4*
DNM3	*DNM3OS, MIR199A2*
KIAA2018	*NAA50*
CC2D1B	*GPX7, PRPF38A, ORC1, ZCCHC11*
TERF2IP	*ADAT1, TERF2IP, KARS, CNTNAP4*
TSPAN9	*FOXM1, C12ORF22, TULP3, TEAD4*

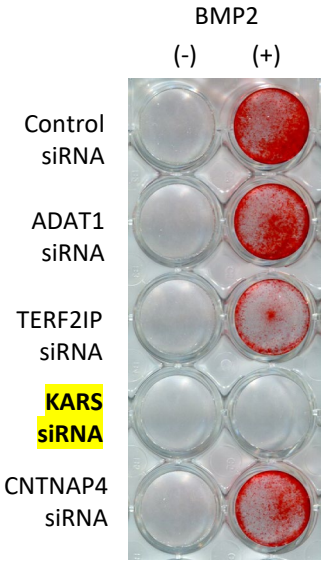


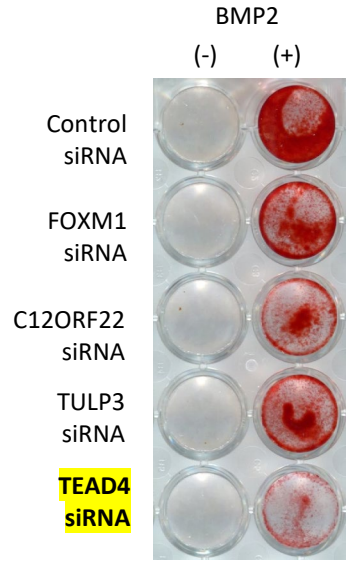












Functional follow up studies with EPDR1 and miR199a-5p are ongoing

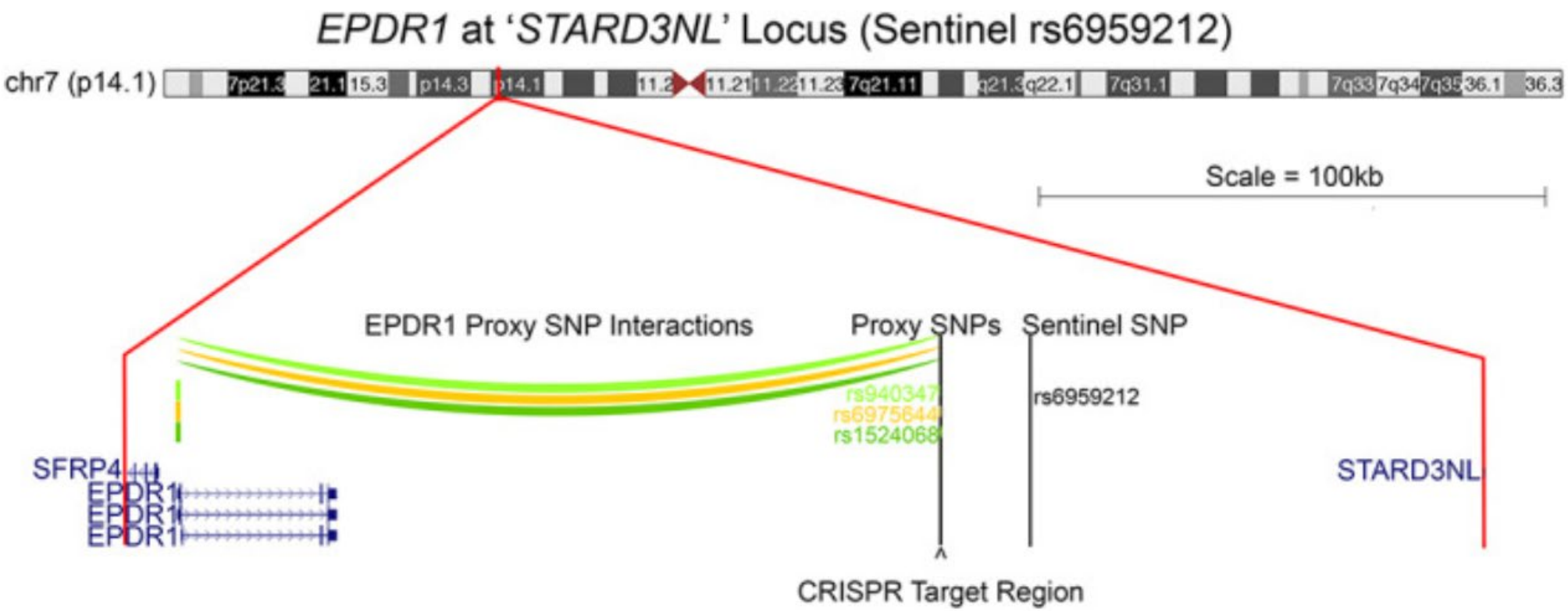
High-throughput CRISPRi assay to identify effector genes at remaining loci is being developed

Summary

- **ATAC-seq coupled with chromatin confirmation capture can be used for implicating gene variants associated with a particular phenotype**

- ***ING3* at the 'WNT16-CPED' locus and *EPDR1* at the 'STARD3NL' locus play a role in human osteoblast differentiation.**

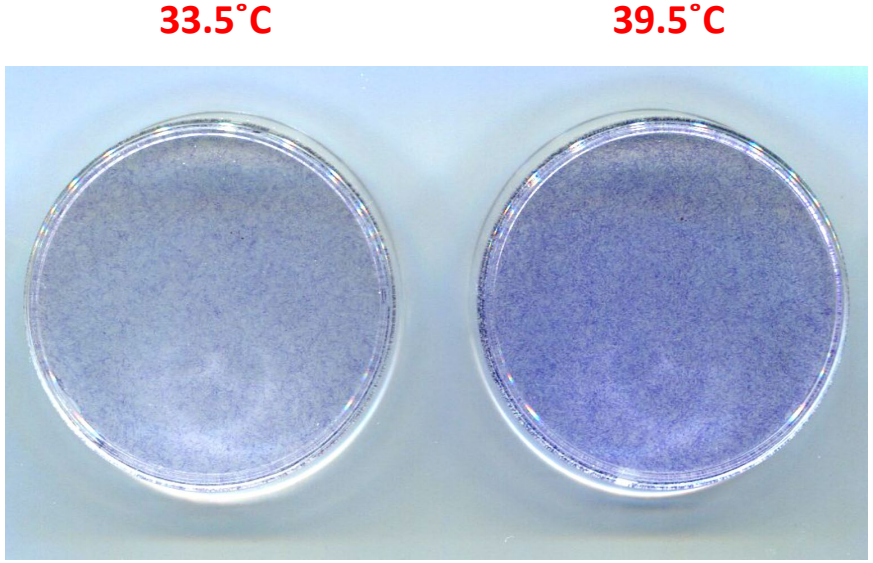
The open chromatin region spanning three proxy SNPs at the ‘STARD3NL’ locus constitutes EPDR1 enhancer and is functional during osteoblast differentiation


EPDR1 at 'STARD3NL' Locus (Sentinel rs6959212)
chr7 (p14.1)
Scale = 100kb
EPDR1 Proxy SNP Interactions
Proxy SNPs
Sentinel SNP
rs940347
rs6975644
rs1524068
rs6959212
SFRP4
EPDR1
EPDR1
EPDR1
STARD3NL
CRISPR Target Region

hFOB1.19 cells as an alternate model to study proxy SNP regulation of EPDR1

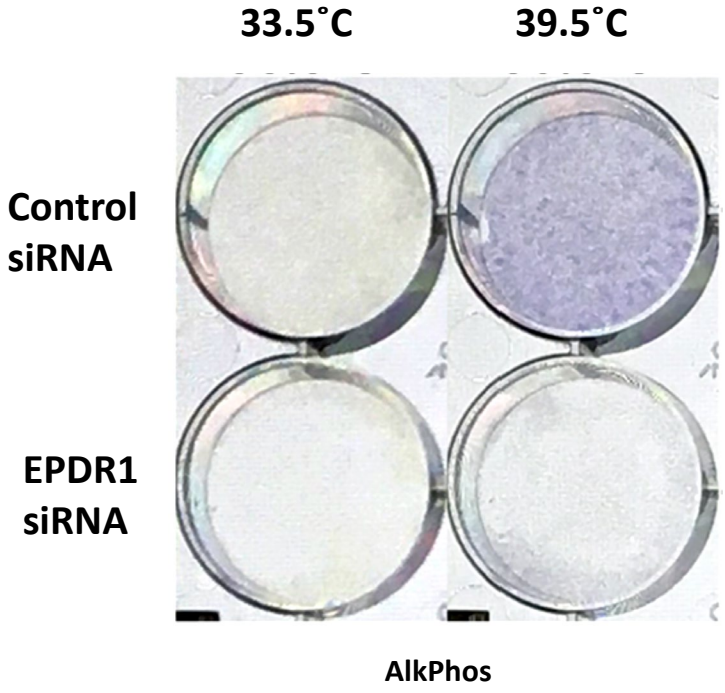
- Immortalized human fetal osteoblastic cell line—easy expanding and passaging

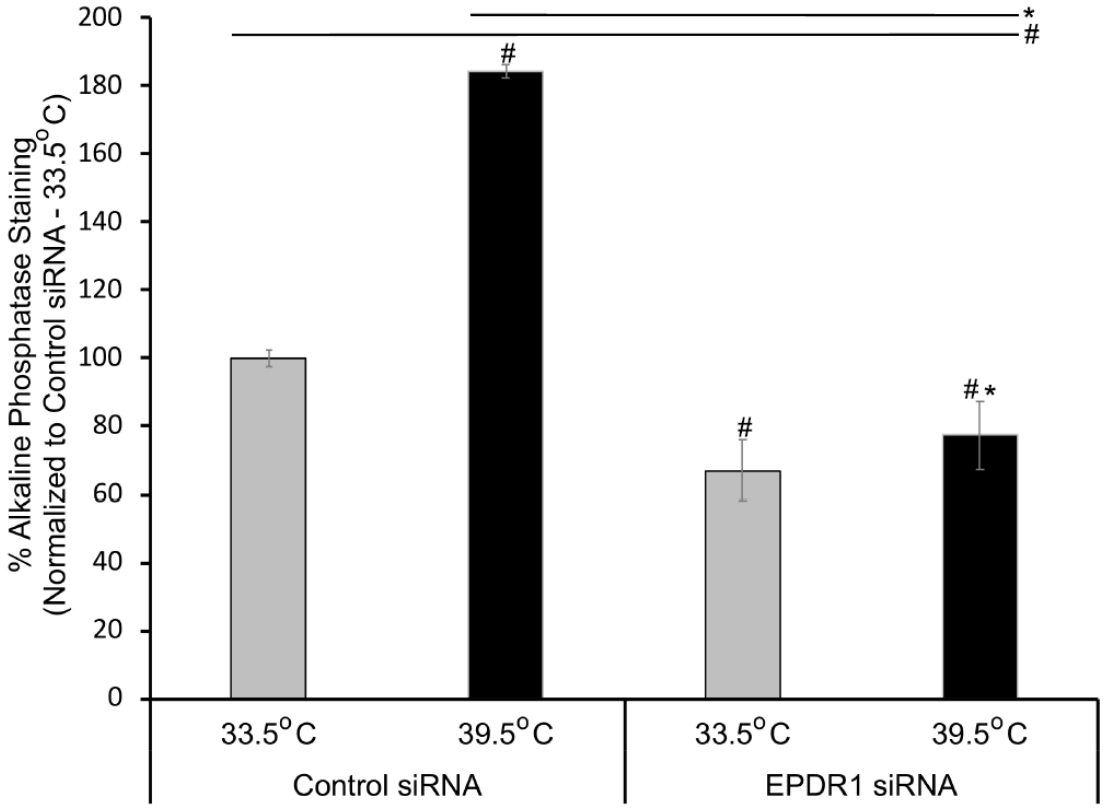
- Contains temperature sensitive mutant, tsA58, of the SV40 large T antigen that allows for the genome-edited cells to proliferate at 33.5˚C and to differentiate at 39.5˚C



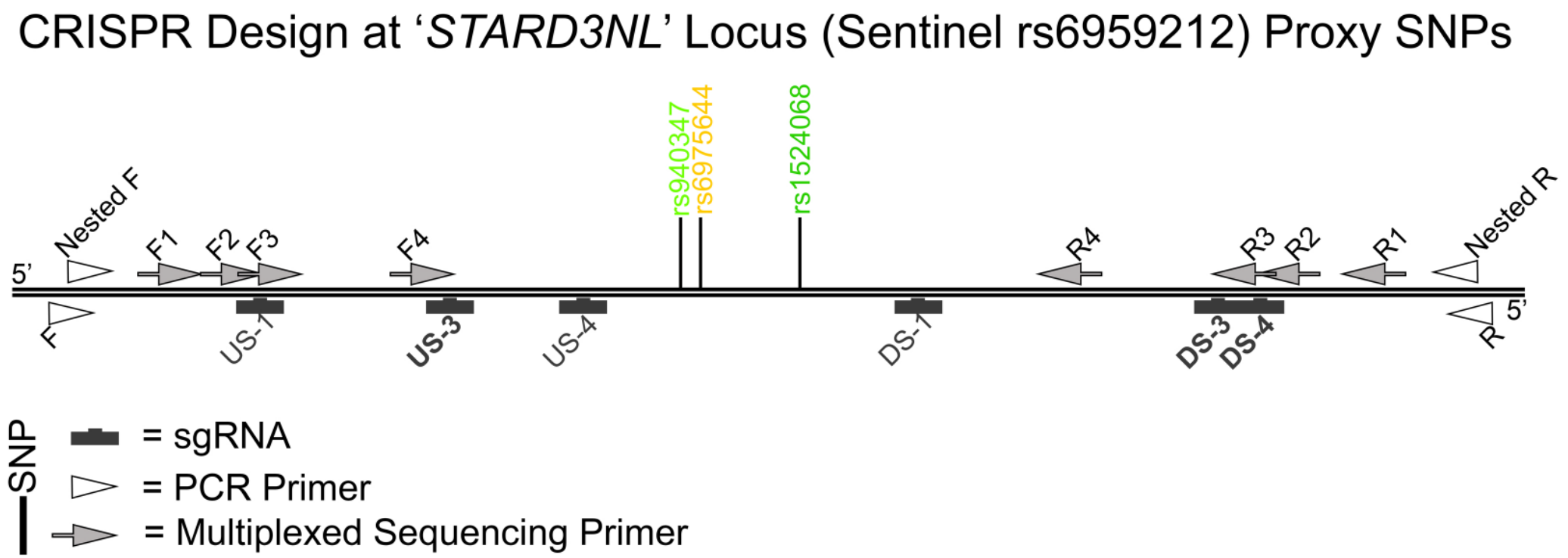
AlkPhos

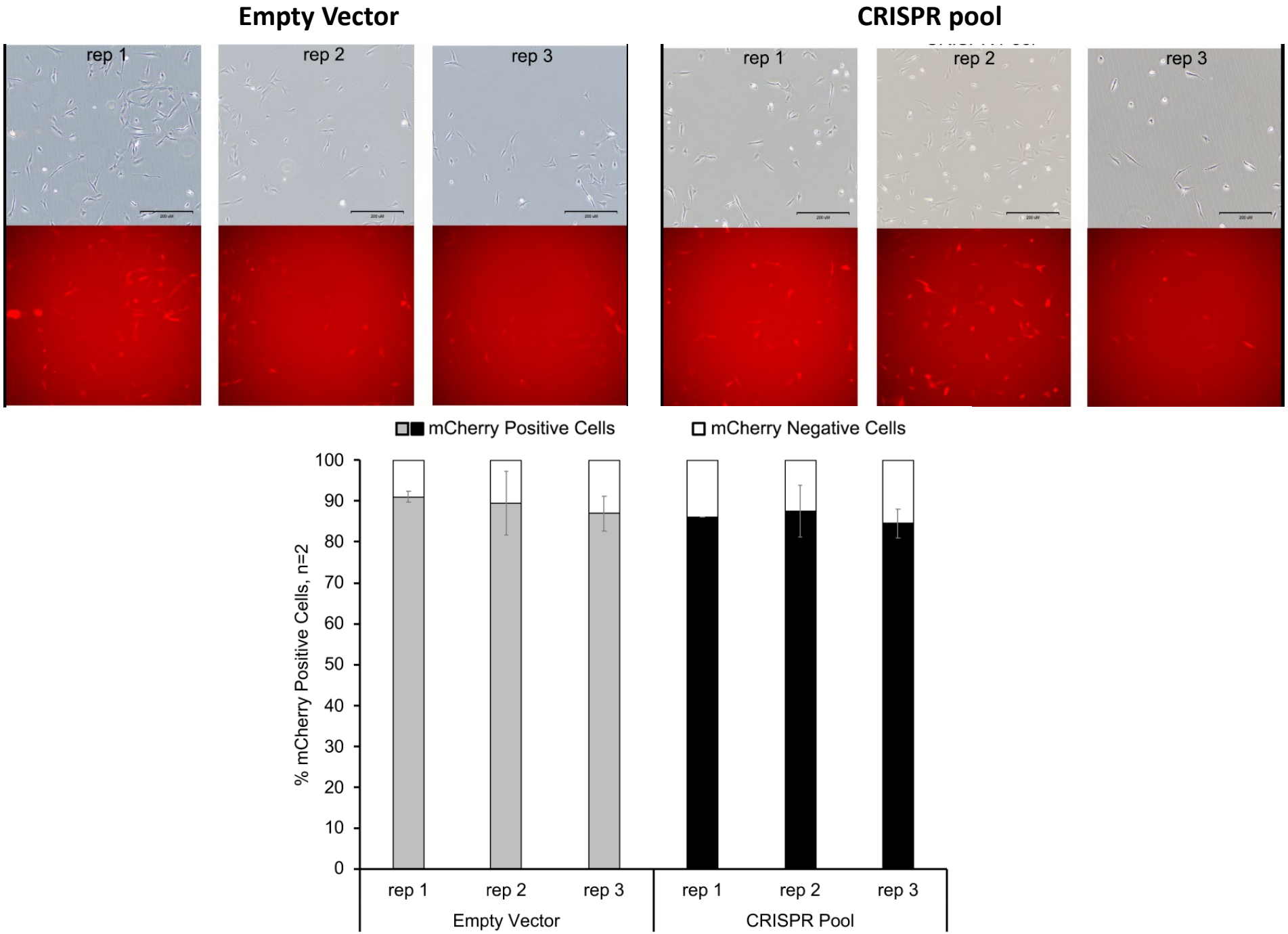
- **EPDR1 knockout in hFOB1.19 cells shows reduced Alk Phos expression and therefore is a suitable cell model to study CRISPR-Cas9 genome editing of proxy SNPs**



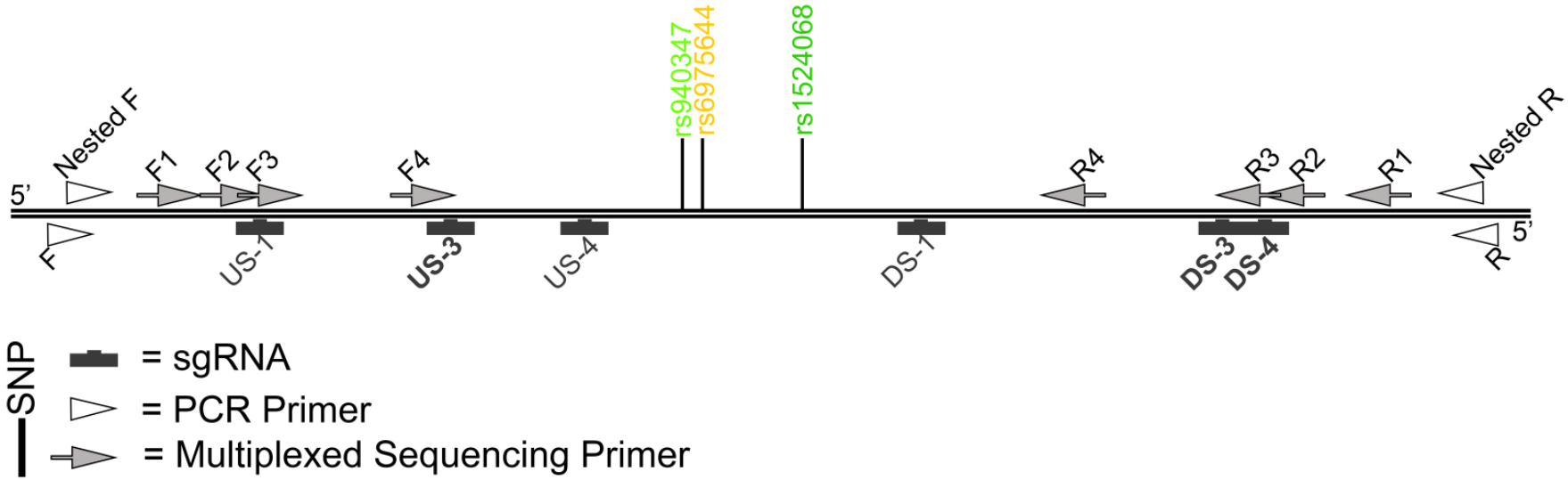


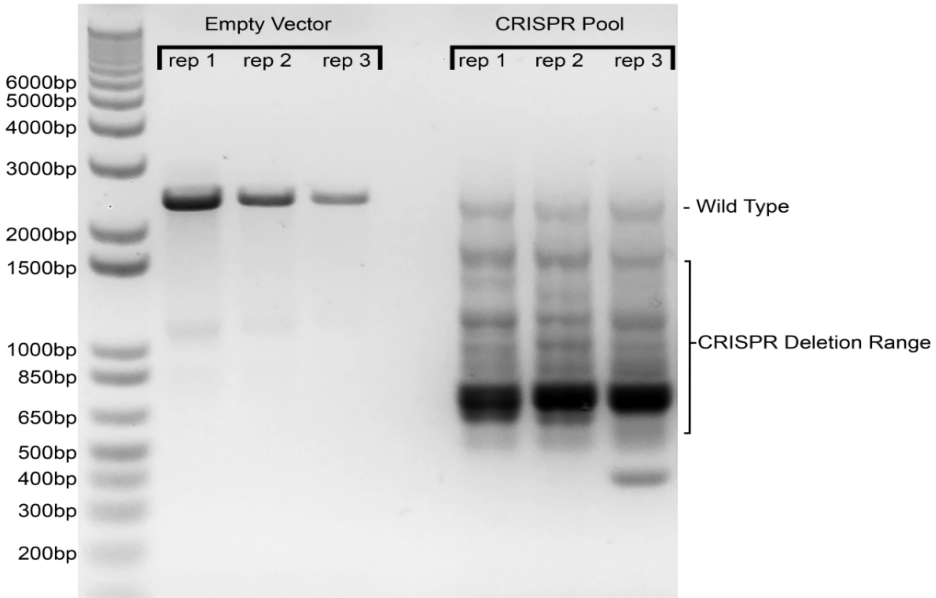
Pooled lentivirus was prepared using three sets of guides on each upstream and downstream site of the proxy SNPs

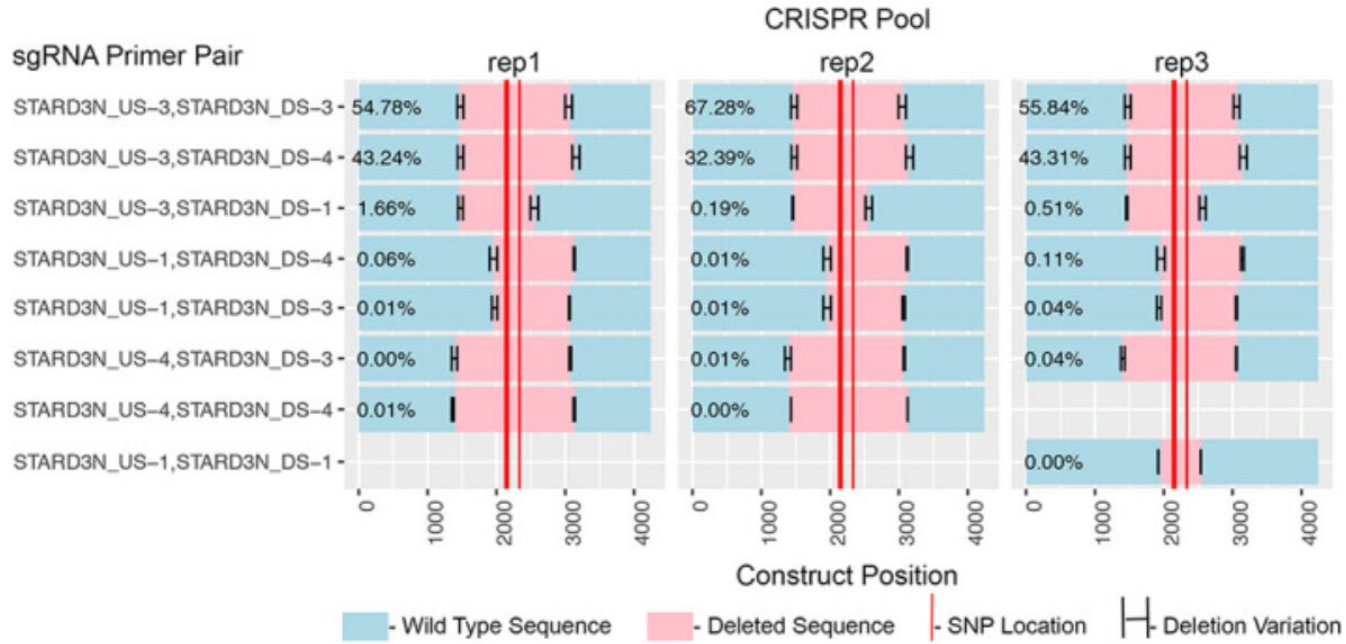



Empty Vector
CRISPR pool
rep 1
rep 2
rep 3
rep 1
rep 2
rep 3
mCherry Positive Cells
mCherry Negative Cells
% mCherry Positive Cells, n=2
100
90
80
70
60
50
40
30
20
10
0
rep 1
rep 2
rep 3
rep 1
rep 2
rep 3
Empty Vector
CRISPR Pool

CRISPR Design at '*STARD3NL*' Locus (Sentinel rs6959212) Proxy SNPs

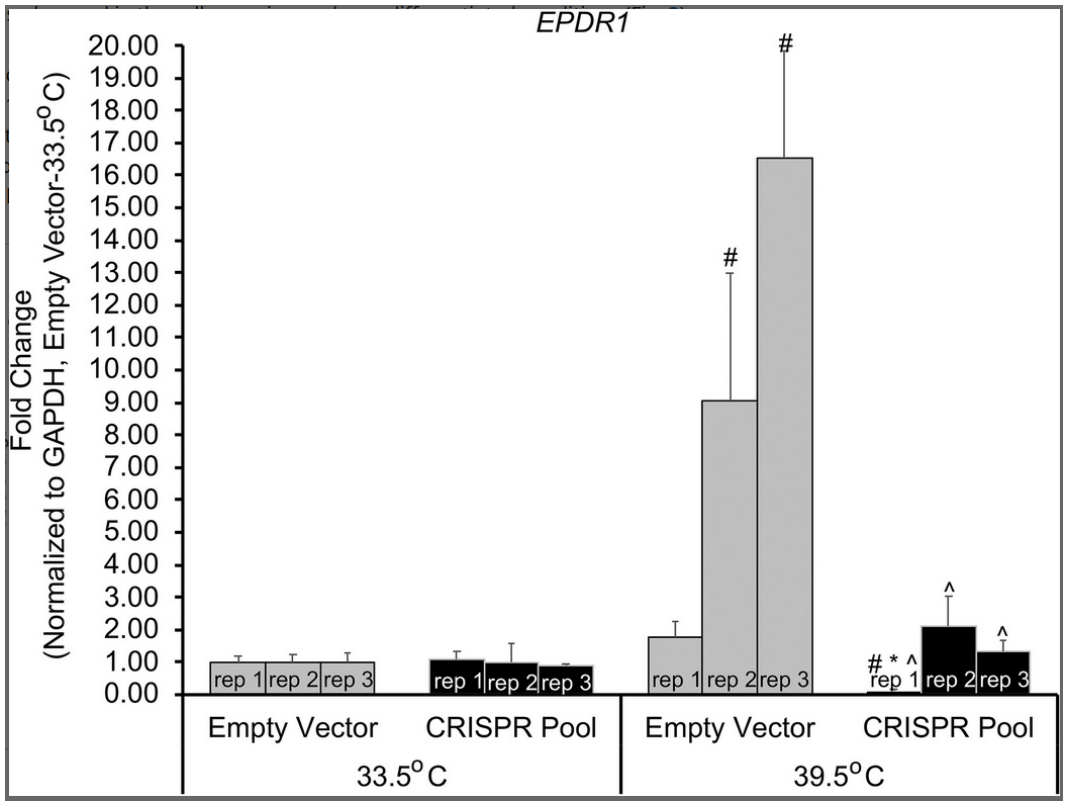




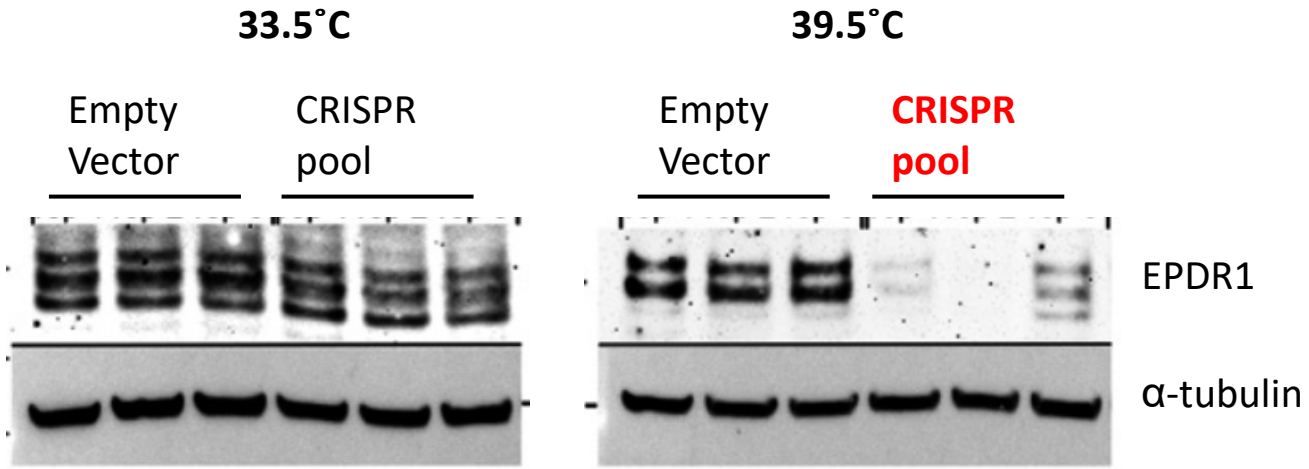


Deletion of proxy SNPs reduces *EPDR1* expression in cells undergoing differentiation

RT-qPCR



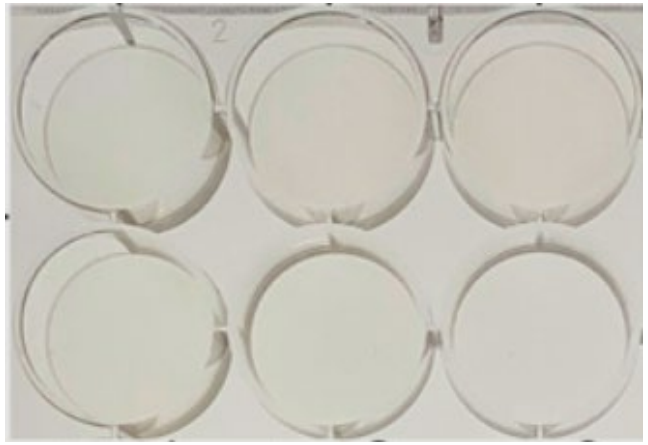
Western Blot



33.5˚C

Empty Vector

CRISPR pool

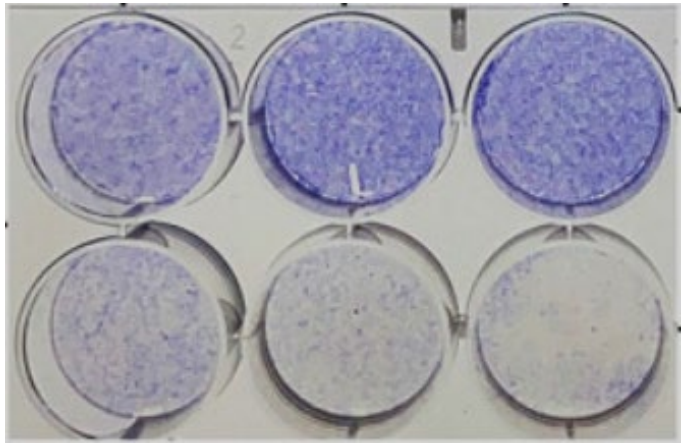


AlkPhos

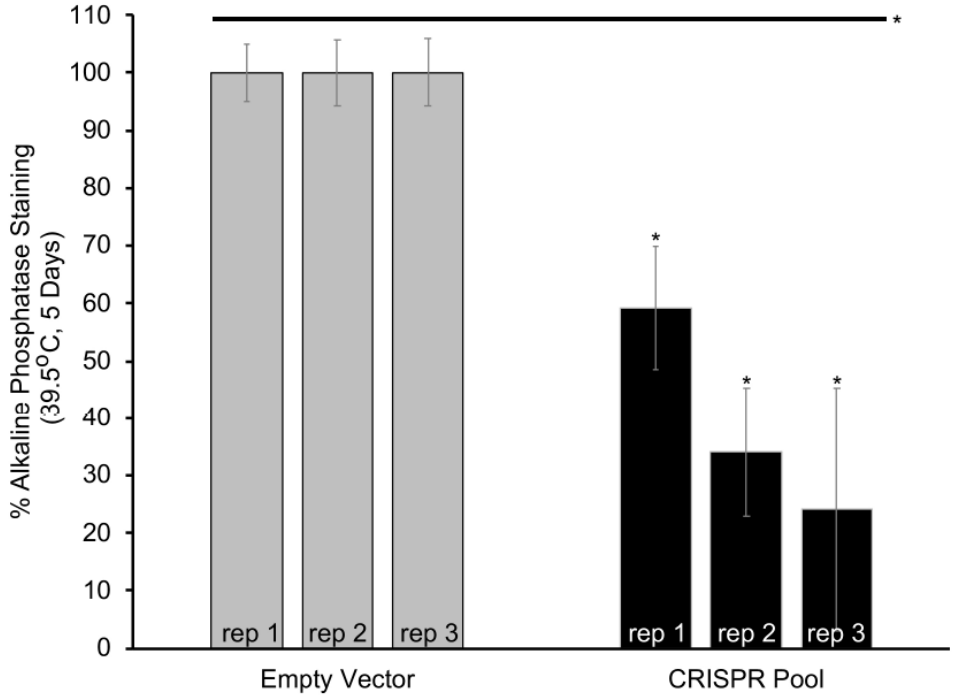
39.5˚C

Empty Vector

CRISPR pool



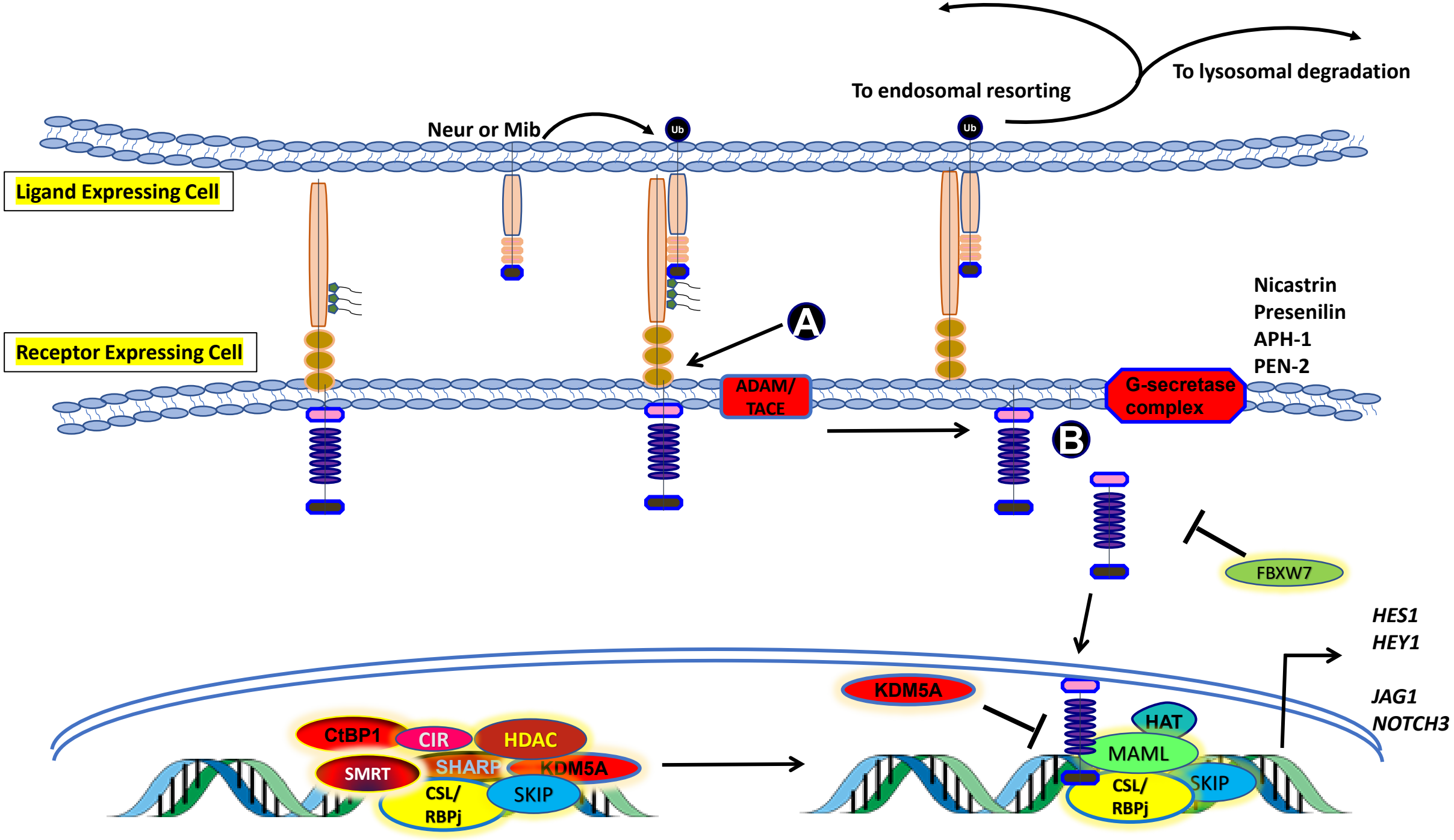
AlkPhos

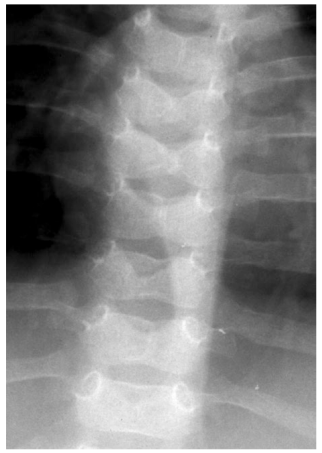


Summary

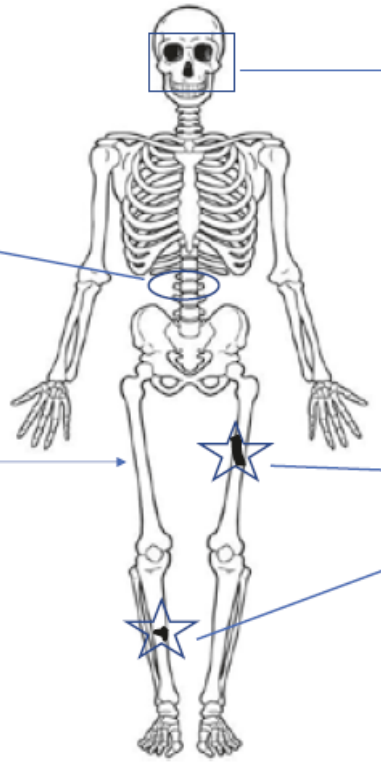
- EPDR1 plays a regulatory role during osteoblast differentiation of hFOB1.19 cells
- CRISPR-Cas9 deletion of three proxy SNPs at the ‘STARD3NL’ locus shows reduced EPDR1 expression during hFOB1.19 osteoblast differentiation

EPDR1 is required for Jagged1 mediated human osteoblast differentiation

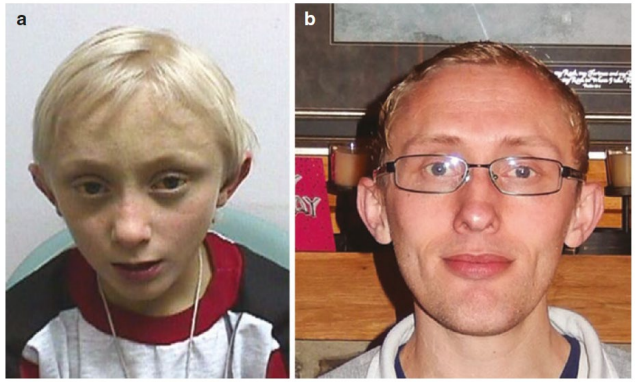

To endosomal resorting
To lysosomal degradation
Neur or Mib
Ub
Ligand Expressing Cell
Receptor Expressing Cell
A
ADAM/
TACE
Nicastrin
Presenilin
APH-1
PEN-2
G-secretase
complex
B
FBXW7
HES1
HEY1
JAG1
NOTCH3
KDM5A
CtBP1
CIR
HDAC
SMRT
SHARP
KDM5A
CSL/
RBPj
SKIP
HAT
MAML
CSL/
RBPj
SKIP



Butterfly vertebrae result from abnormal somite development

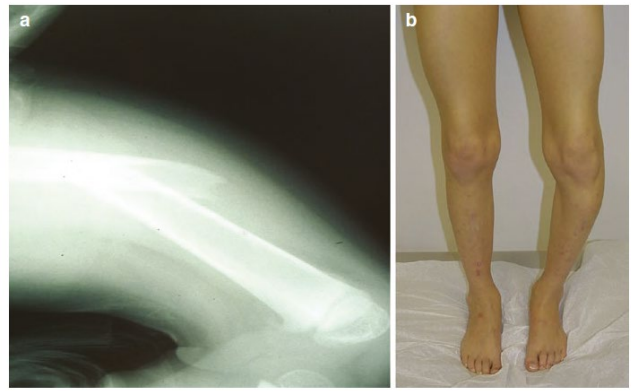


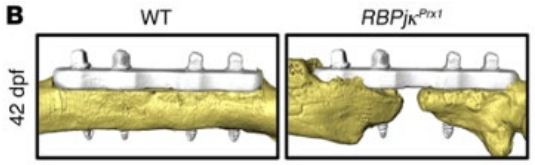
Characteristic facial features
- Prominent forehead w/pointed chin
- Deeply set eyes
- Straight nose with bulbous tip



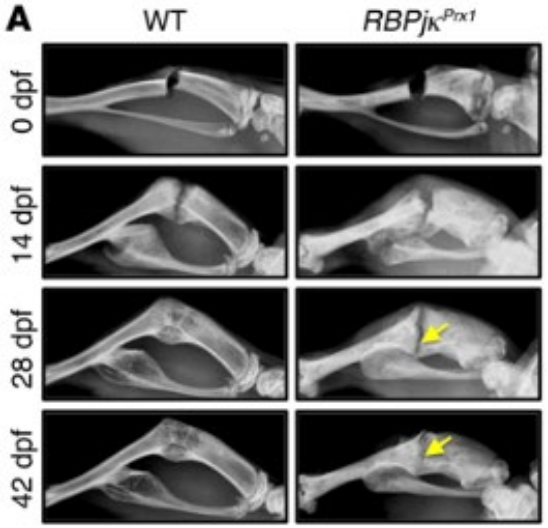
- ALGS patients have reduced bone density.
- Mice with genetic alterations in Notch signaling show highly variable phenotypes.
- Mice with *Jag1* disruption in bone progenitors show reduced bone regeneration.

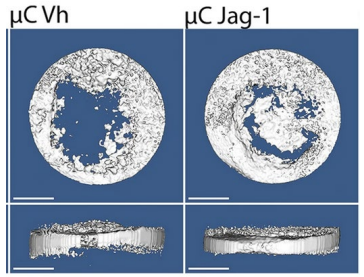
- ALGS patients have increased fracture risk and poor fracture healing.
- Mice with canonical Notch signaling disrupted do not heal bone defects.
- Jagged-1 delivery to bone injuries promotes fracture healing



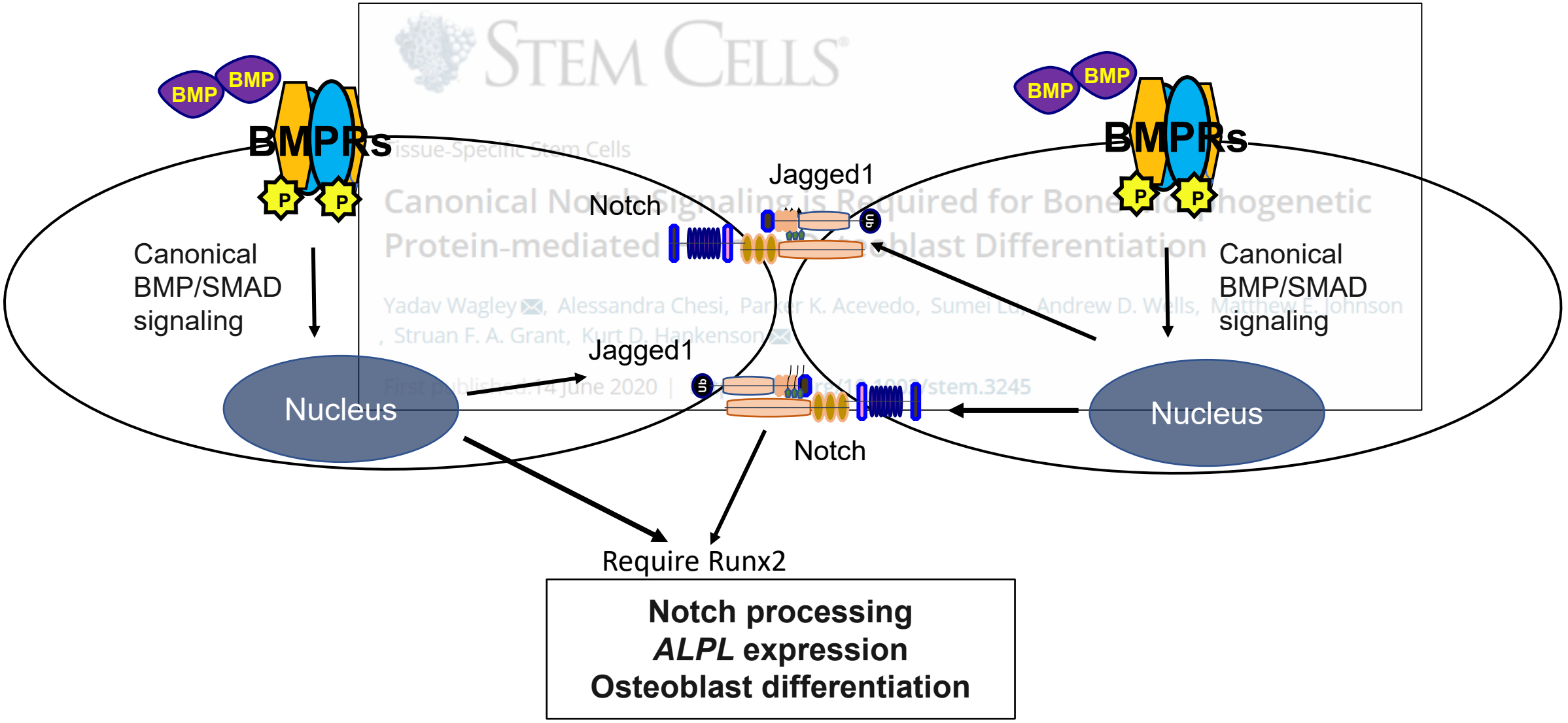


Wang et al., (2016) JCI





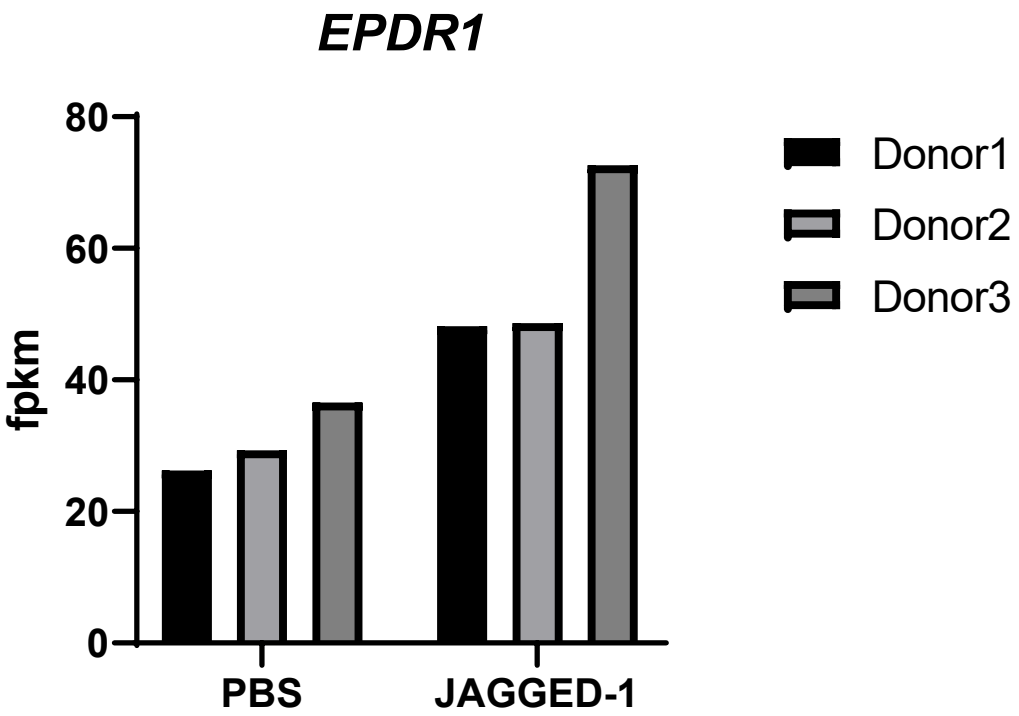
Youngstrom et al. (2017), NPJ Regen Med



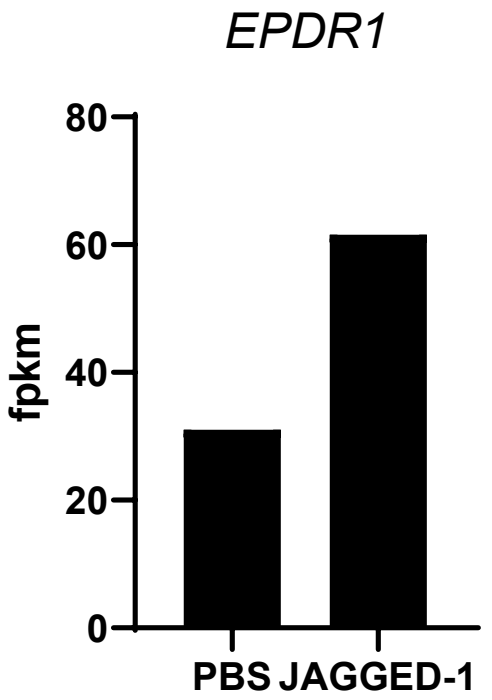
BMP stimulation of bone-marrow derived human mesenchymal progenitor cells (hMSCs) increases Notch proteins via increased Notch ligand Jagged1 expression. Canonical Notch signaling is required for BMP-induced *ALPL* expression and osteoblastic commitment of hMSCs. Both BMP-induced osteoblastogenesis and Notch-induced osteoblastogenesis require Runx2 and RBPj

hMSC upregulates *EPDR1* upon Jagged1 stimulation

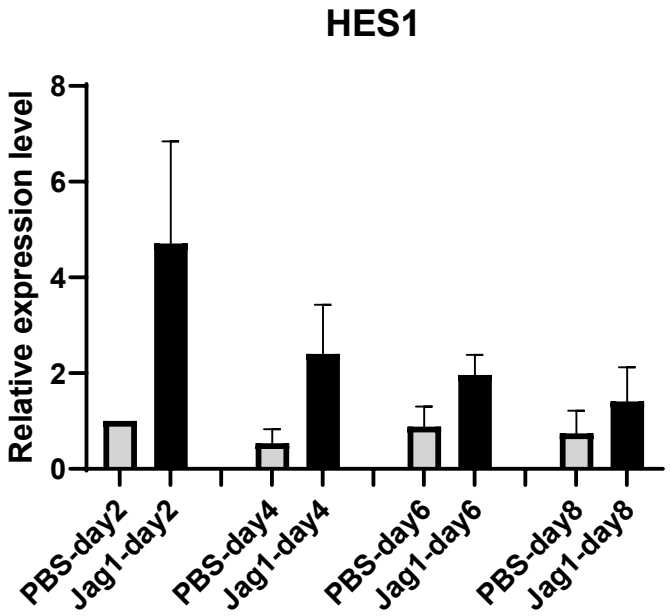
ENSEMBLE_GENE_ID	GENE	FPKM.JAG11	FPKM.JAG12	FPKM.JAG13	FPKM.Contr1	FPKM.Contr2	FPKM.Contr3	FC	FDR
ENSG00000086289.7	EPDR1	48.1494242955653	48.61702414	72.63829631	26.24704744	29.27442245	36.59837206	1.85085841	0.129099636

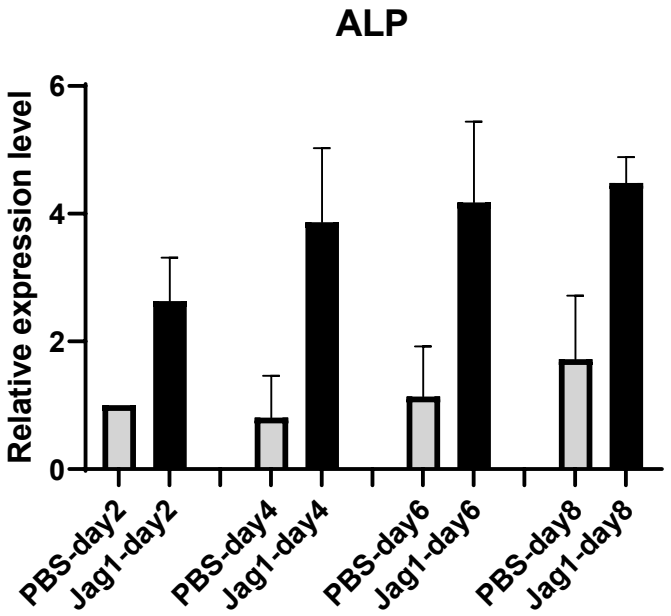


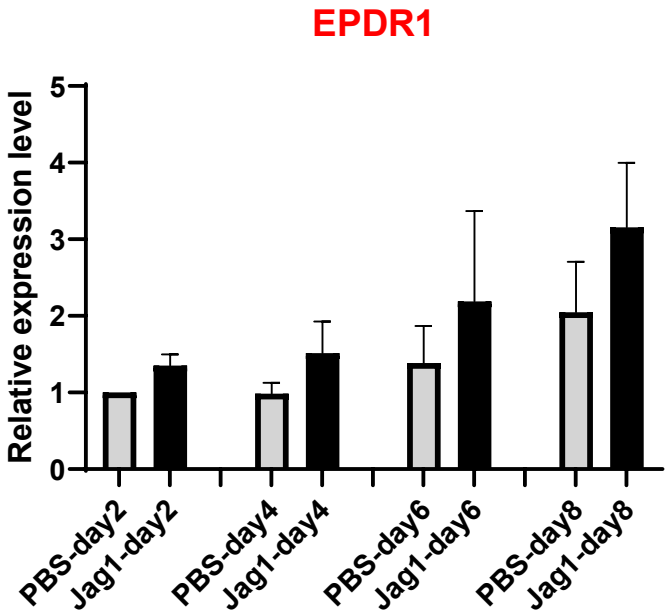
Separate sequencing library for Donor3

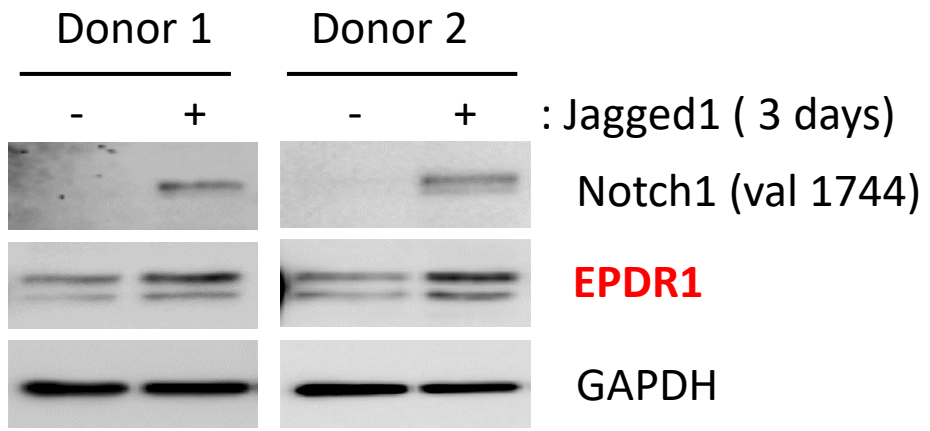


hMSC upregulates *EPDR1* upon Jagged1 stimulation





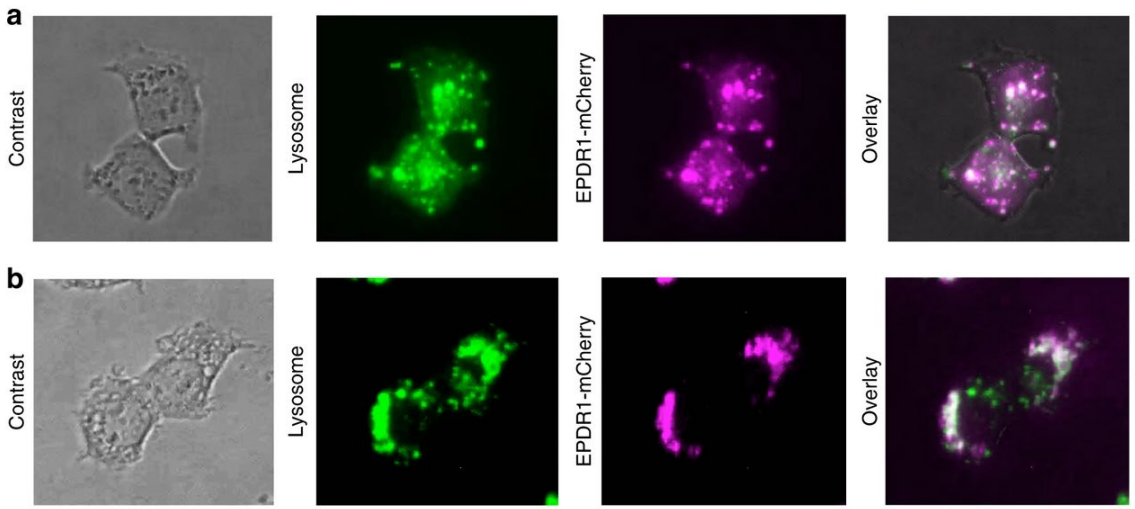




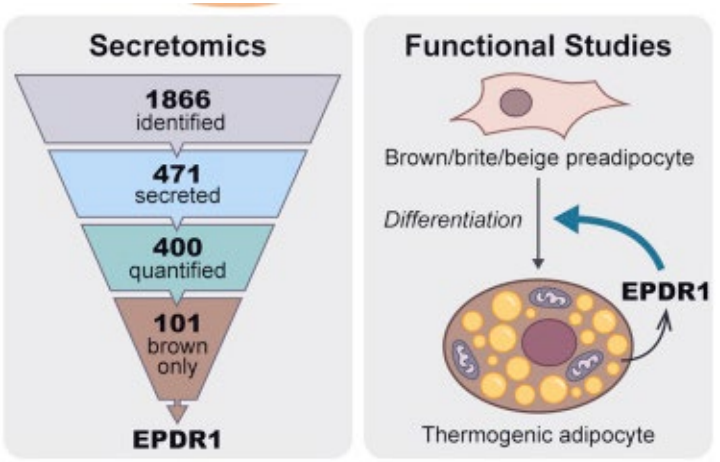
Summaries for EPDR1 Gene

Entrez Gene Summary for EPDR1 Gene

The protein encoded by this gene is a type II transmembrane protein that is similar to two families of cell adhesion molecules, the protocadherins and ependymins. This protein may play a role in calcium-dependent cell adhesion. This protein is glycosylated, and the orthologous mouse protein is localized to the lysosome. Alternative splicing results in multiple transcript variants. A related pseudogene has been identified on chromosome 8. [provided by RefSeq, Aug 2011]



Wei et al, Communications Biology, 2(52): 2019

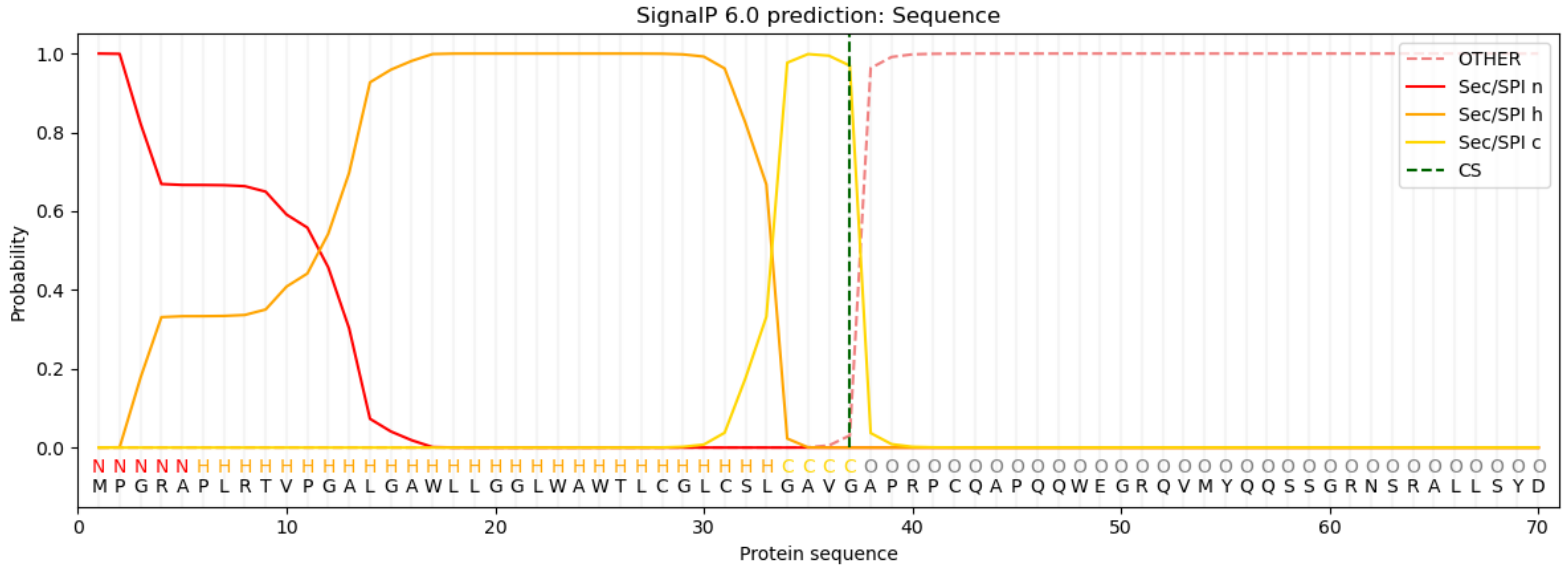


Deshmukh et al., Cell metabolism, 30(5): 963-975.e7, 2019)

Sec/SPI n → Sec/SPI h → Sec/SPI c

MPGRAPLRTVPGALGAWLLGGLWAWTLCGLCSLGAVGAPRPCQAPQQWEGRQVMYQQSSGRNSRALLSYD
GLNQRVRVLDERKALIPCKRLFEYILLYKDGVMFQIDQATKQCSKMTLTQPWDPLDIPQNSTFEDQYSIGGPQE
QITVQEWSDRKSARSYETWIGIYTVKDCYPVQETFTINYSVILSTRFFDIQLGIKDPSVFTPPSTCQMAQLEKM
SEDCSW

Sec/SPI n	1-5
Sec/SPI h	6-33
Sec/SPI c	34-37



Sequence Prediction: Signal Peptide (Sec/SPI)

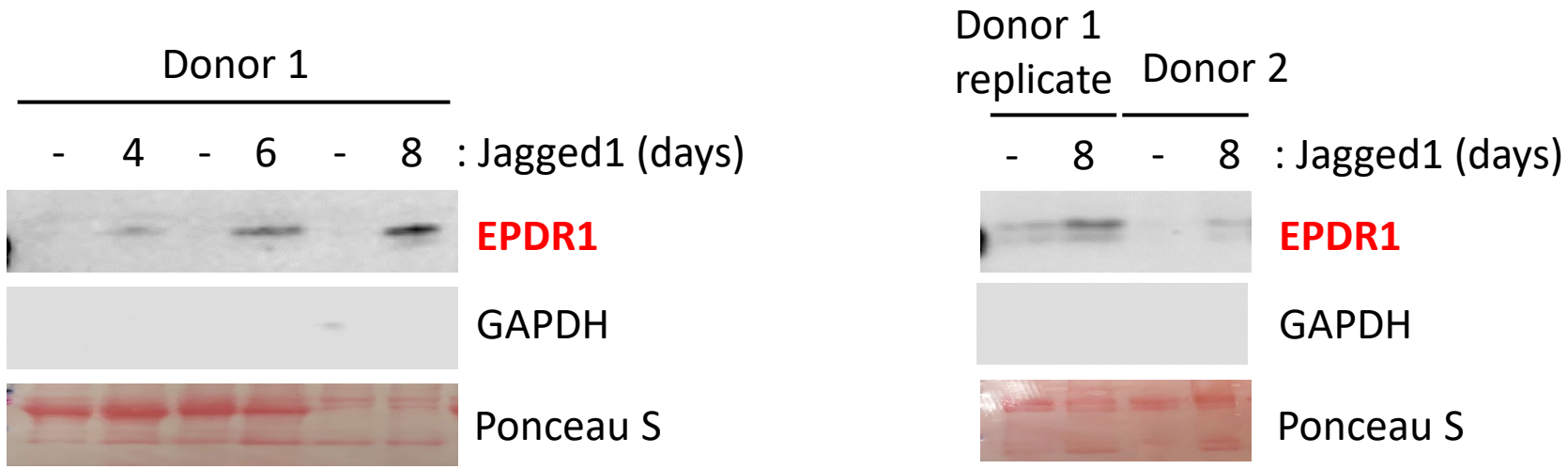
(*"standard" secretory signal peptides transported by Sec translocon and cleaved by Signal Peptidase I (Lep)*

Cleavage site between pos. 37 and 38. Probability 0.969315

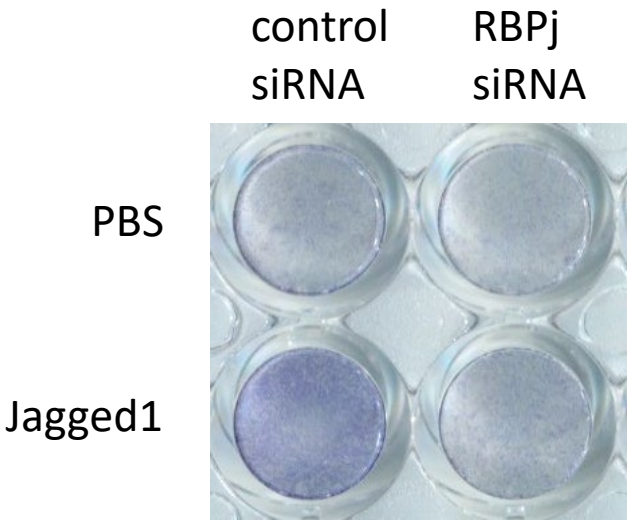
Protein Type	Other	Signal Peptide (Sec/SPI)	Lipoprotein signal peptide (Sec/SPII)	TAT signal peptide (Tat/SPI)	TAT Lipoprotein signal peptide (Tat/SPII)	Pilin-like signal peptide (Sec/SPIII)
Likelihood	0.0002	0.9991	0.0002	0.0002	0.0001	0.0001

https://services.healthtech.dtu.dk/service.php?SignalP

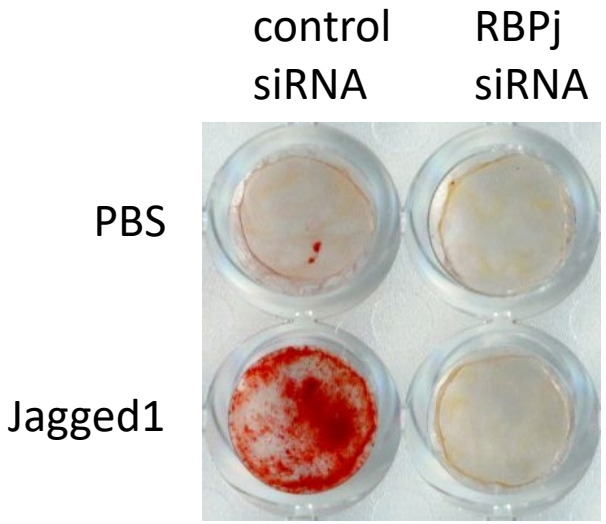
EPDR1 protein secretion is increased in Jagged1 stimulated cells



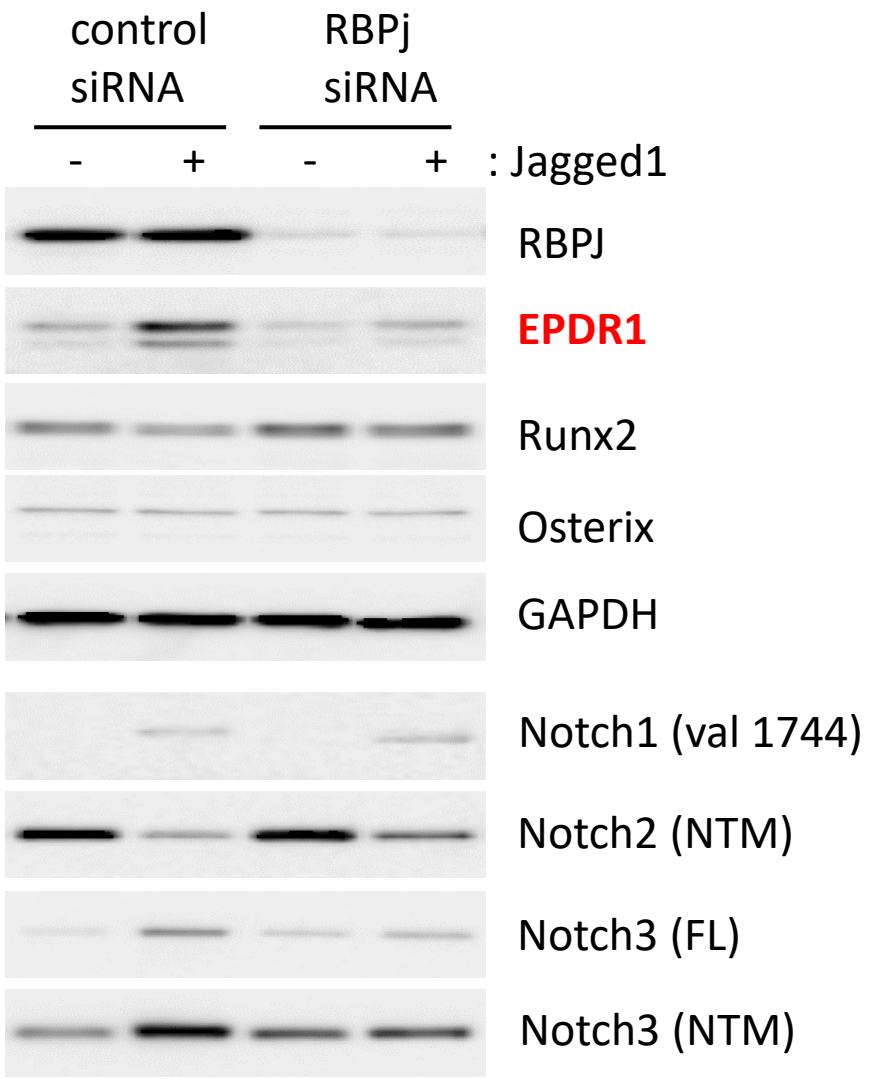
RBPJ silencing using siRNA demonstrates reduction of EPDR1 protein expression



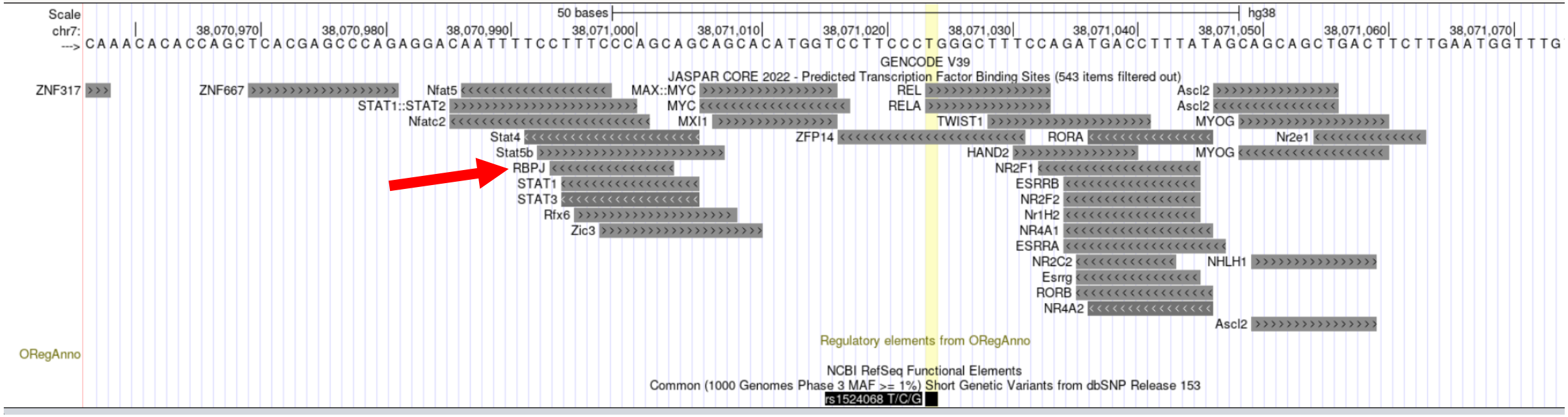
AlkPhos



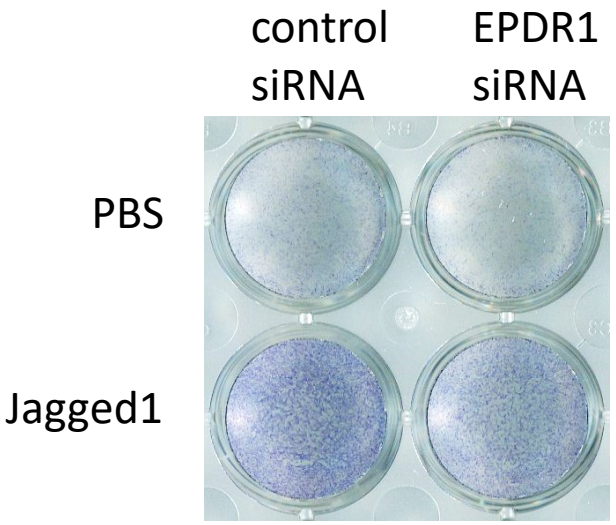
Alizarin Red S



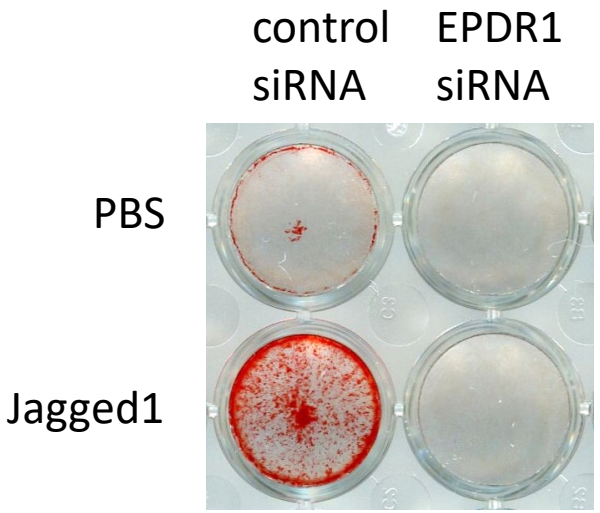
RBPJ binding site is predicted upstream of rs1524069



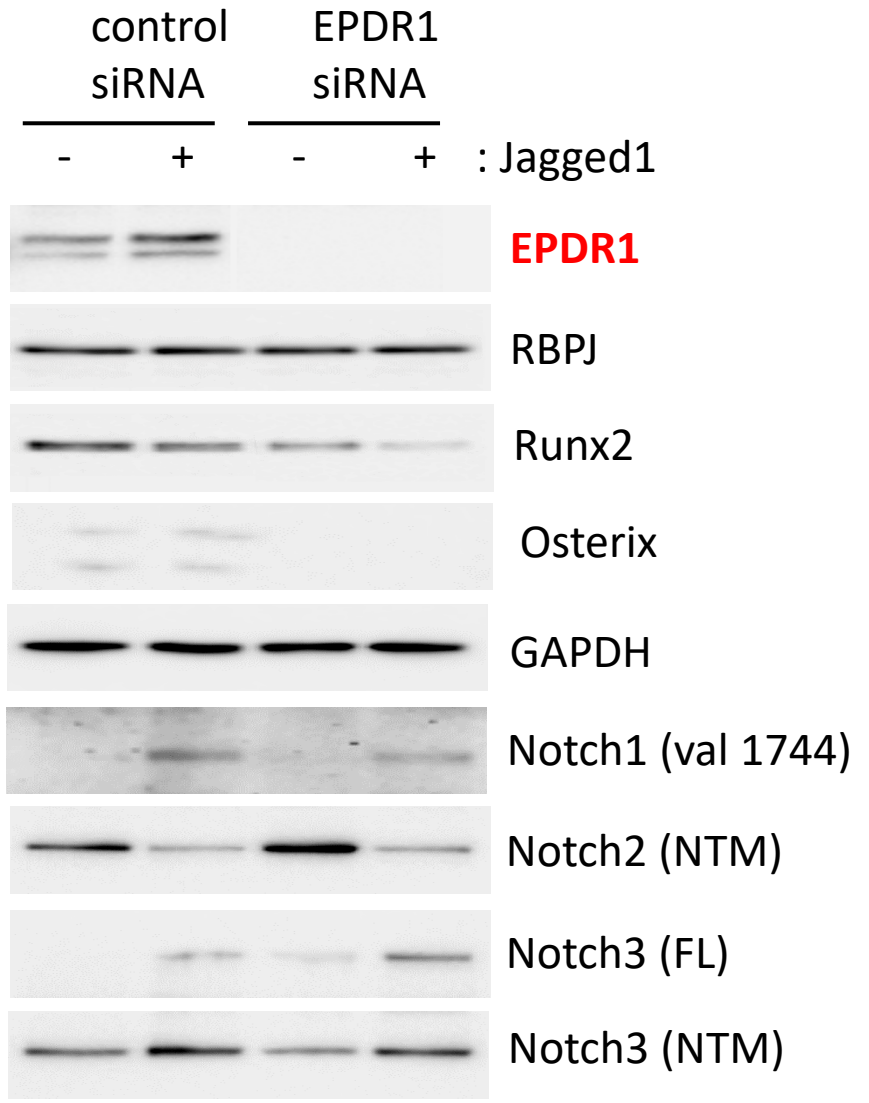
EPDR1 silencing using siRNA impairs Jagged1 mediated osteoblastogenesis



AlkPhos



Alizarin Red S



Summary and future directions

- EPDR1 is a secreted protein that plays a key role during human osteoblast differentiation
- Notch signaling enhances EPDR1 transcriptional activation

- Confirm the binding of RBPJ upstream of proxy SNP rs1524069
- Assess overexpression of EPDR1 during MSC differentiation.
- Evaluate the signaling potential of soluble EPDR1 protein during MSC differentiation.

Thank you

QUESTIONS?