MICHIGAN MEDICINE
UNIVERSITY OF MICHIGAN

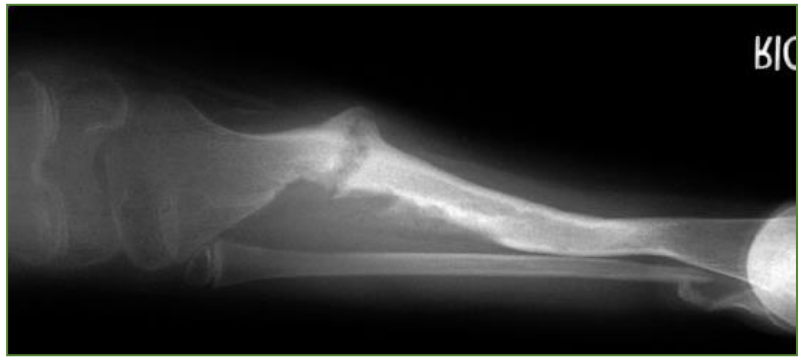
ORTHOPAEDIC SURGERY

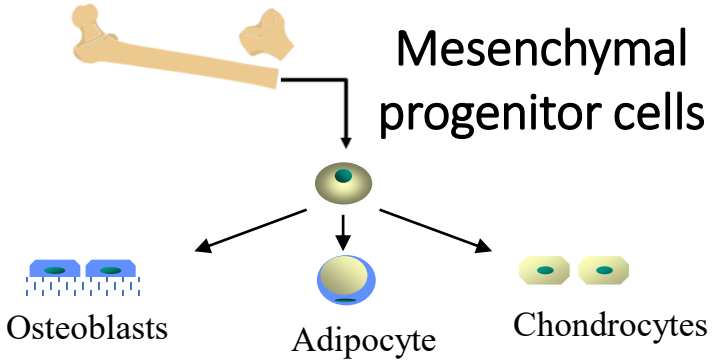
Yadav Wagley, Ph.D

Research Investigator

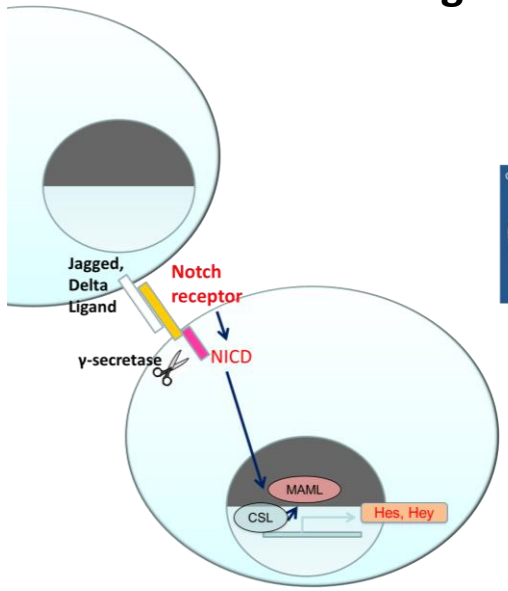
Hankenson Laboratory– Ortho Res Labs (ORL) – Dept. Ortho. Surgery –

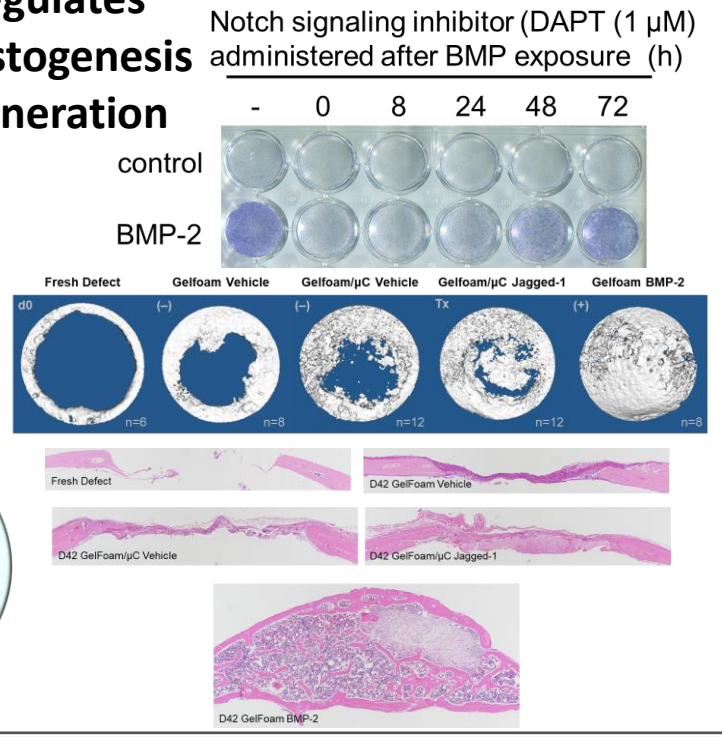
Mesenchymal progenitor biology: Exploring extracellular regulators of bone formation and regeneration

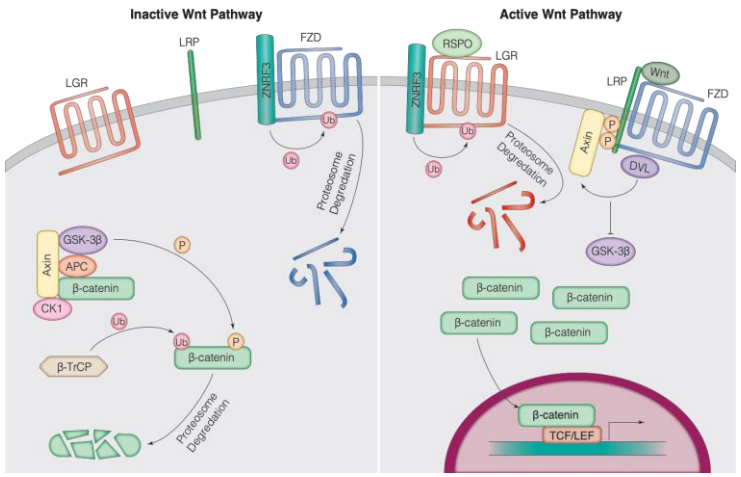




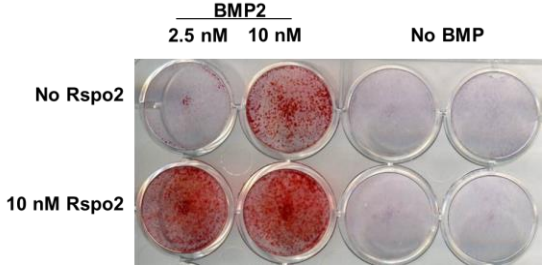
Notch ligand Jagged-1 regulates BMP2 induced osteoblastogenesis and enhances bone regeneration

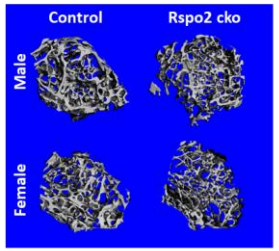


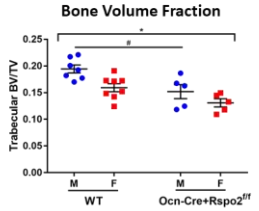




R-spondin-2 regulates Wnt signaling, osteoblastogenesis, and bone formation







Functional Characterization of EPDR1 as Novel Osteoblast Effector Gene at the BMD-GWAS Implicated STARD3NL locus

- **Variant-to-gene mapping for identifying EPDR1 as a novel osteoblast effector gene using functional genomics**
- **CRISPR-Cas9 to confirm regulatory role of proxy SNP in regulating EPDR1 expression during osteoblastogenesis**
- **EPDR1 plays an anti-inflammatory role during human osteoblastogenesis**

Bone Disorders

FRACTURE

Osteoporosis

Osteogenesis imperfecta

Osteopenia

Arthritis

Hyperparathyroidism

Osteopetrosis

Paget’s disease

Craniosynostosis

Bone Metastases

Rickets

Osteomyelitis

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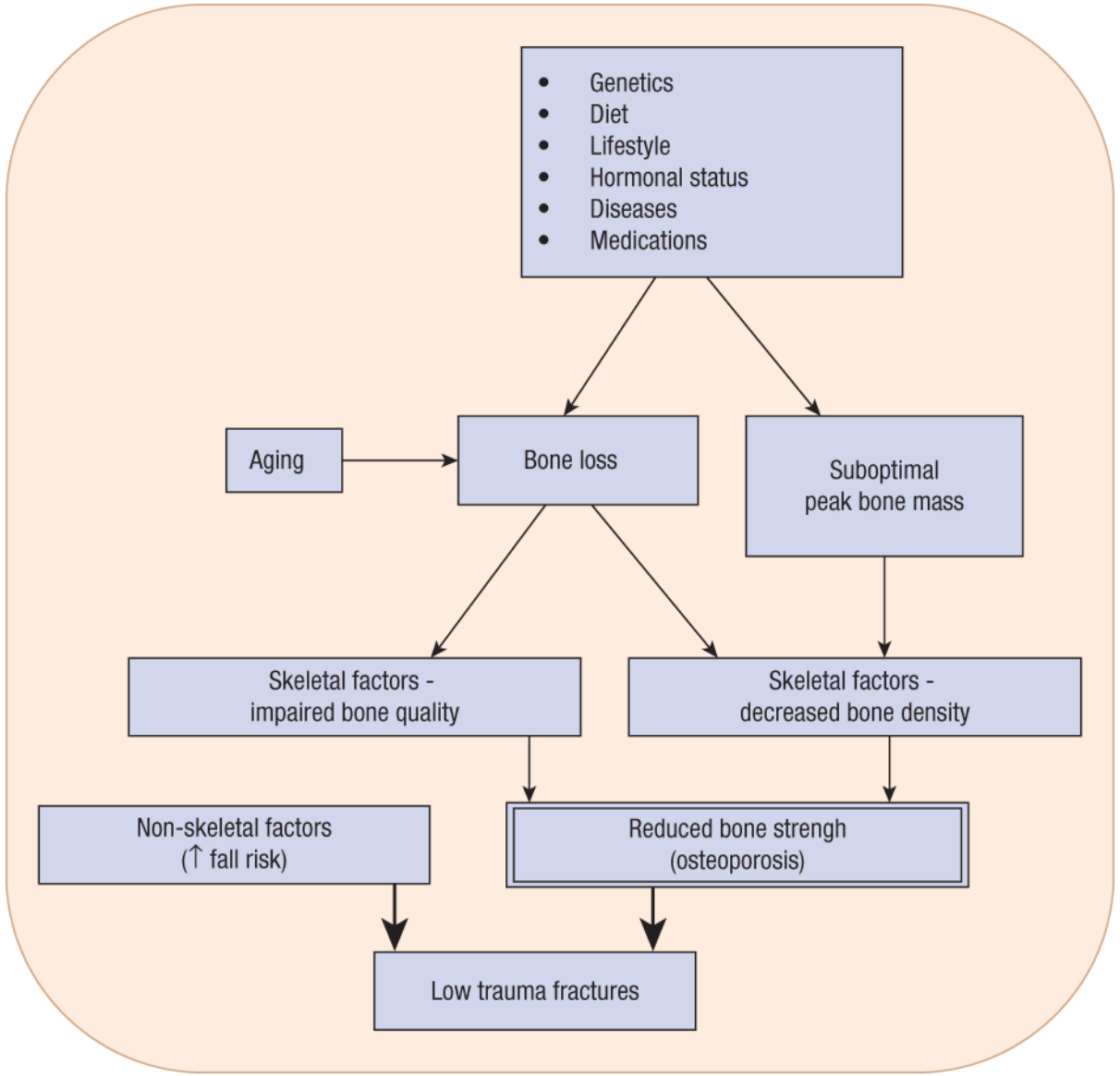
Osteoporosis: A Global Issue

- Characterized by reduced bone mineral density, bone microstructural changes and increased fracture risk
- Over 200 million people worldwide, -- 30% of postmenopausal women in USA and in Europe
- $17 billion to treat fractures in USA $9.45 billion in China
- Significant health problem that is anticipated to cost twice as much to treat by 2035
- Heritable; heritability ranging from 50-80%

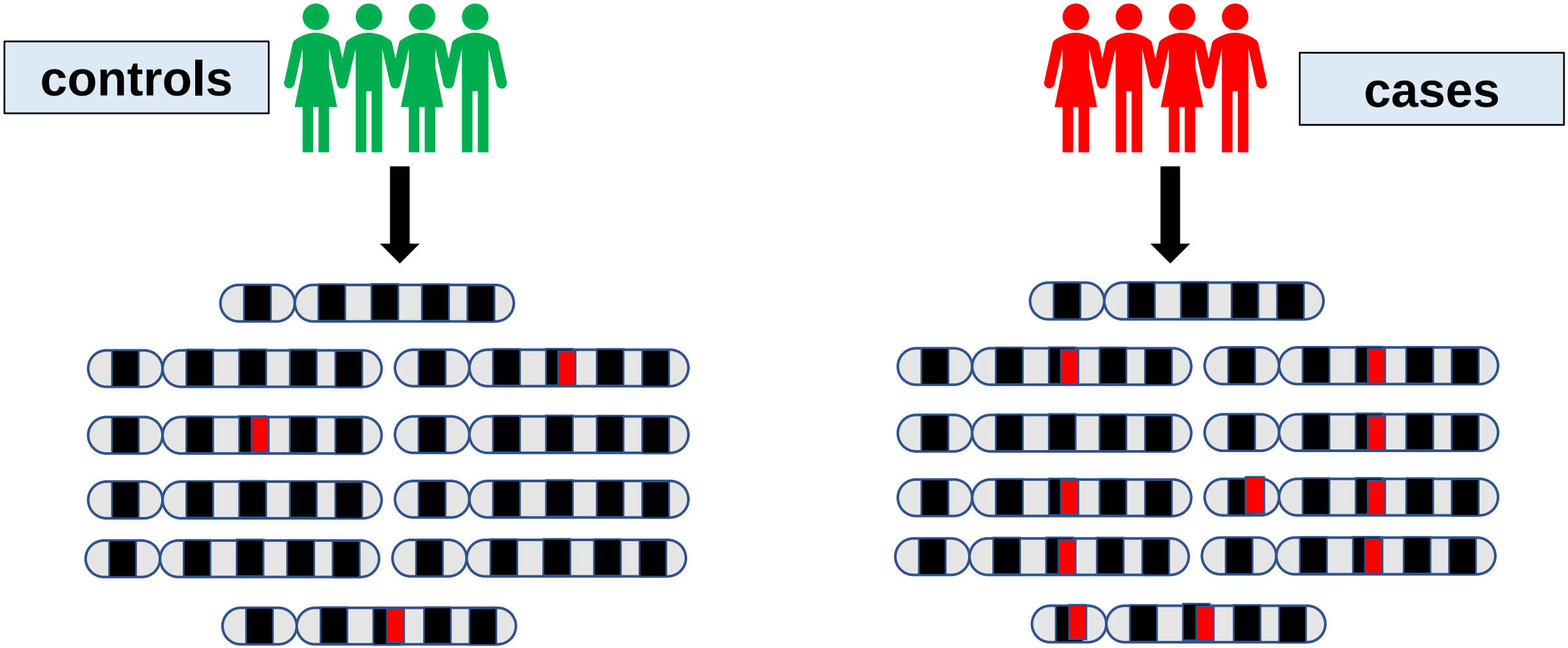


Source: DiPiro JT, Talbert RL, Yee GC, Matzke GR, Wells BG, Posey LM: *Pharmacotherapy: A Pathophysiologic Approach, Ninth Edition:* www.accesspharmacy.com

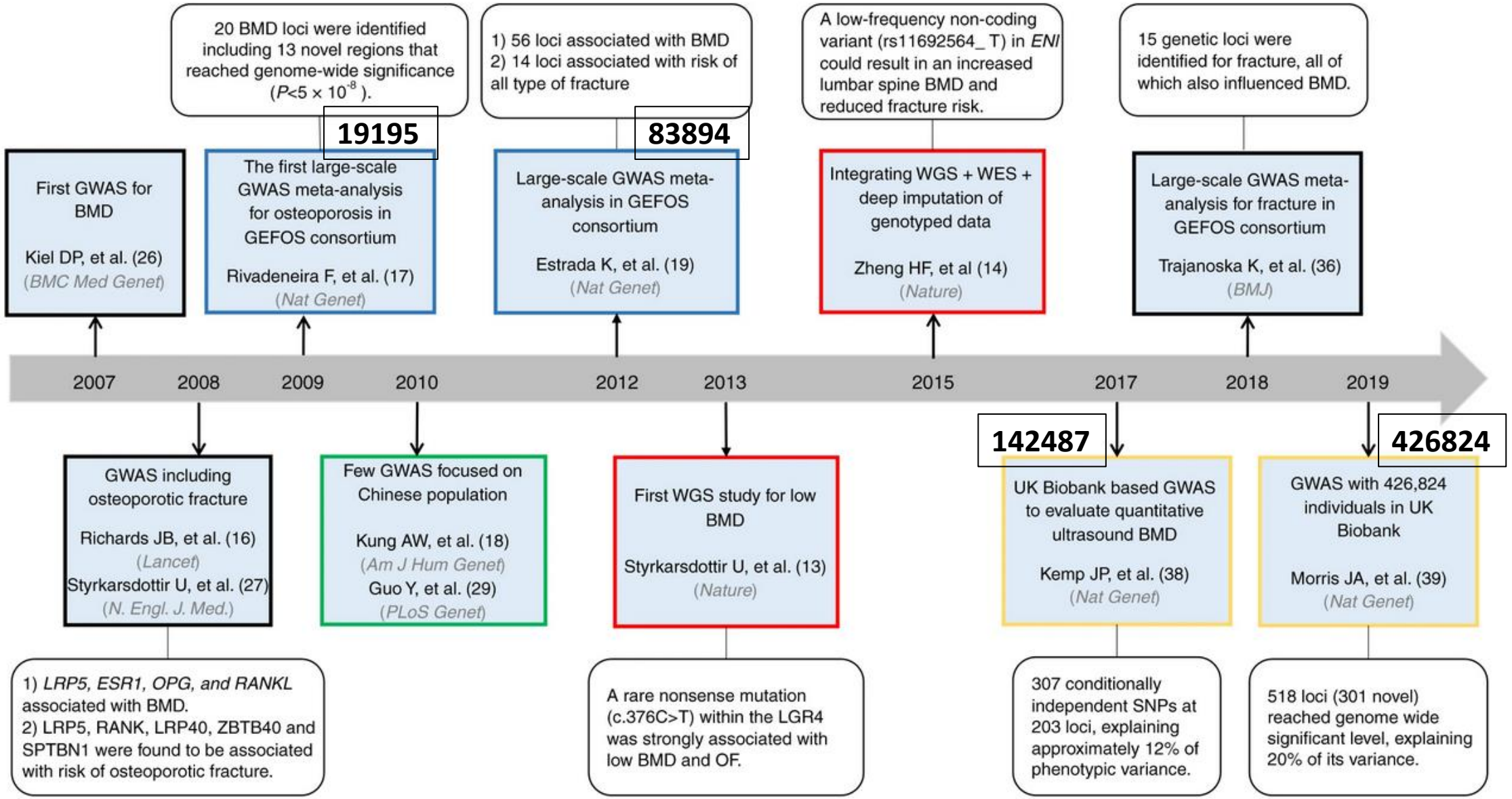
History of Bone Mineral Density (BMD) Genetics

1990s	Up to 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



Timeline highlighting important milestones during GWAS discoveries



Zhu X, Bai W. & Zheng H., *Bone Research 9 (23)*: (2021) https://doi.org/10.1038/s41413-021-00143-3

Five anti-osteoporosis therapeutics currently approved or in advance clinical trials are supported by GWAS data

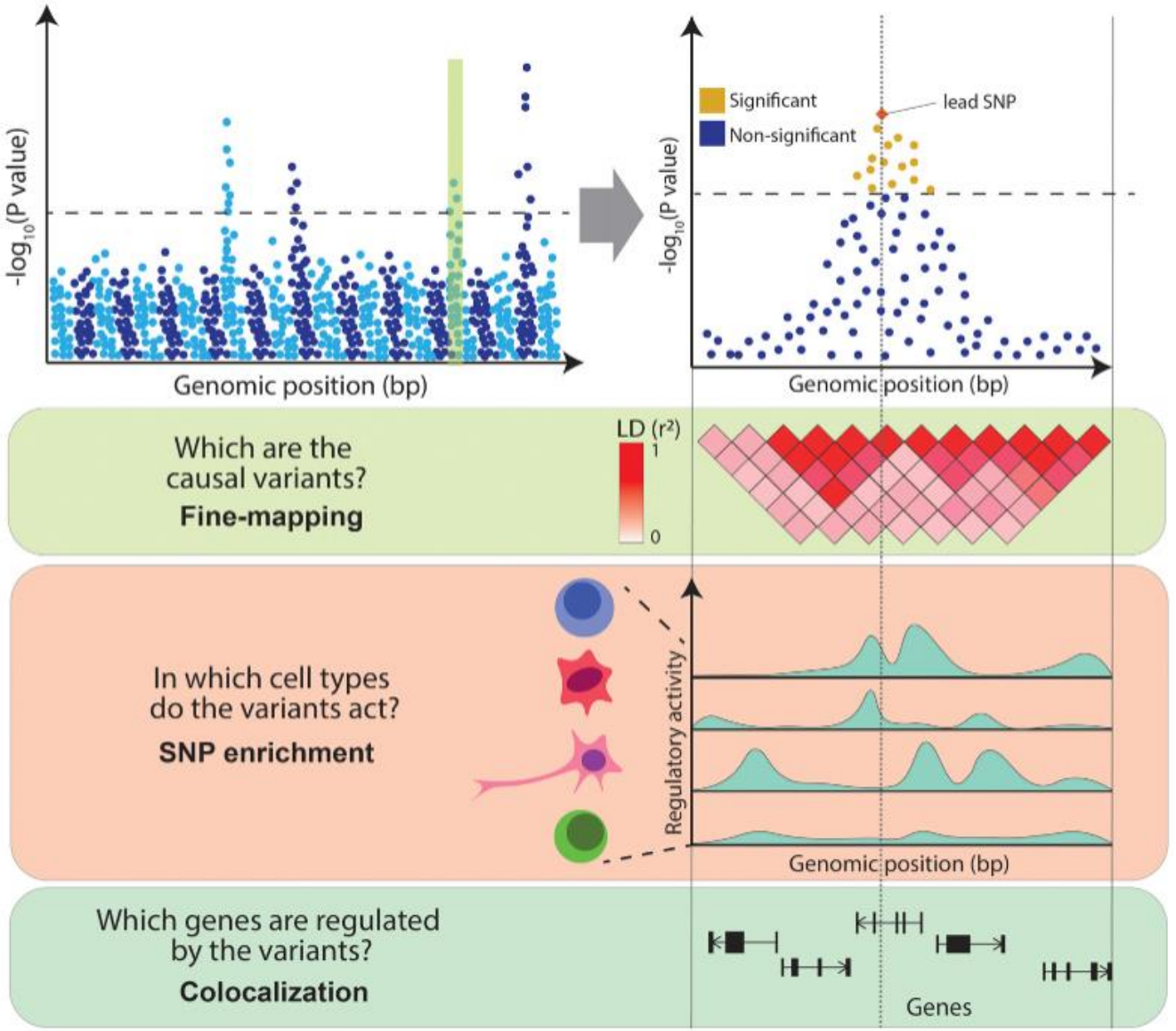
Drug class	Drug target	Principle	Stage	Target locus identified through GWASs
Denosumab	RANKL	Reduces bone resorption by selectively targeting RANKL	Approved for clinical use	RANKL
Sclerostin inhibitors (Romosozumab)	Sclerostin (SOST)	Improve the recruitment and activation of osteoblasts by targeting Wnt/β-catenin signaling pathways	Approved for clinical use in Japan, US and Europe	SOST
Selective estrogen receptor modulators	Estrogen receptor	Reduces bone resorption by targeting the OPG/RANK/RANKL pathway	Approved for clinical use	ESR1
Parathyroid hormone analogs	Parathyroid hormone receptor	Majorly participate in the process of bone formation by targeting the PKA pathway	Approved for clinical use	Not identified, but the pathway has been highlighted through PTHLH (encodes PTHRP)
Bisphosphonates	Farnesyl pyrophosphate	Inhibition of bone resorption	Approved for clinical use	Not identified
Estrogen ESR1	Estrogen receptor	Reduces bone resorption by targeting the OPG/RANK/RANKL pathway	Approved for clinical use	ESR1
Cathepsin K inhibitors	Cathepsin K	Inhibition of bone resorption by targeting the OPG/RANK/RANKL pathway	Terminated	Not identified
DKK1 inhibitors	DKK1	Improve bone formation by targeting the Wnt/β-catenin signaling pathway	In the preclinical phase	DKK1

Zhu X, Bai W. & Zheng H., *Bone Research 9 (23)*: (2021) https://doi.org/10.1038/s41413-021-00143-3

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

GWAS follow up consists of multiple considerations



Cano-Gamez E. and Trynka G: *Front. Genet.* 11:424 (2020) doi: 10.3389/fgene.2020.00424

ATAC-seq and Capture-C can implicate putative effector genes at BMD GWAS loci

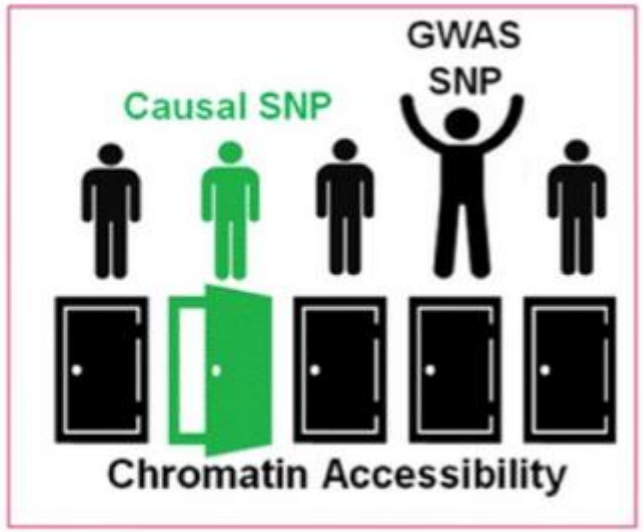
Identify all SNPs in high LD with sentinel associated variant (r^2=0.8)

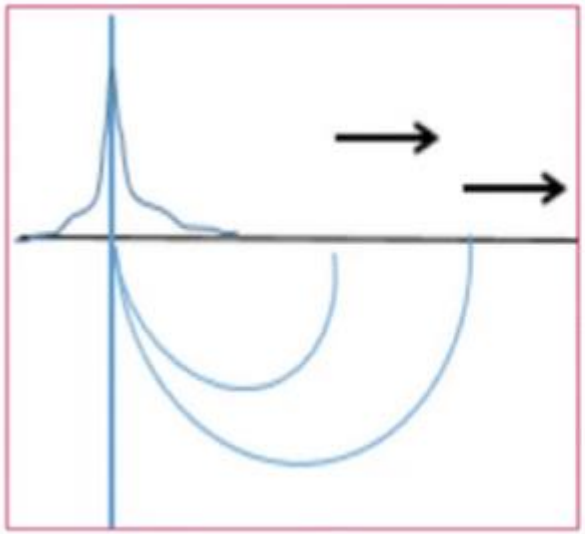
Filter by residing in open chromatin as assessed by ATAC-seq in hMSC-derived osteoblasts

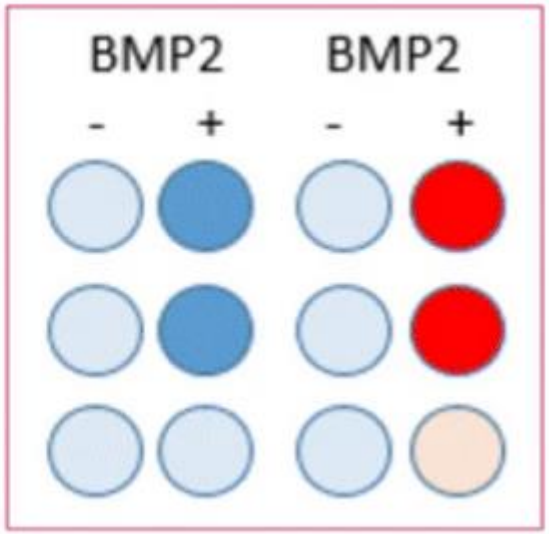
Open chromatin variants are subsequently filtered by being in direct physical contact with gene promoter baits (Promoter-focused Capture-C)

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

(Functional validation)

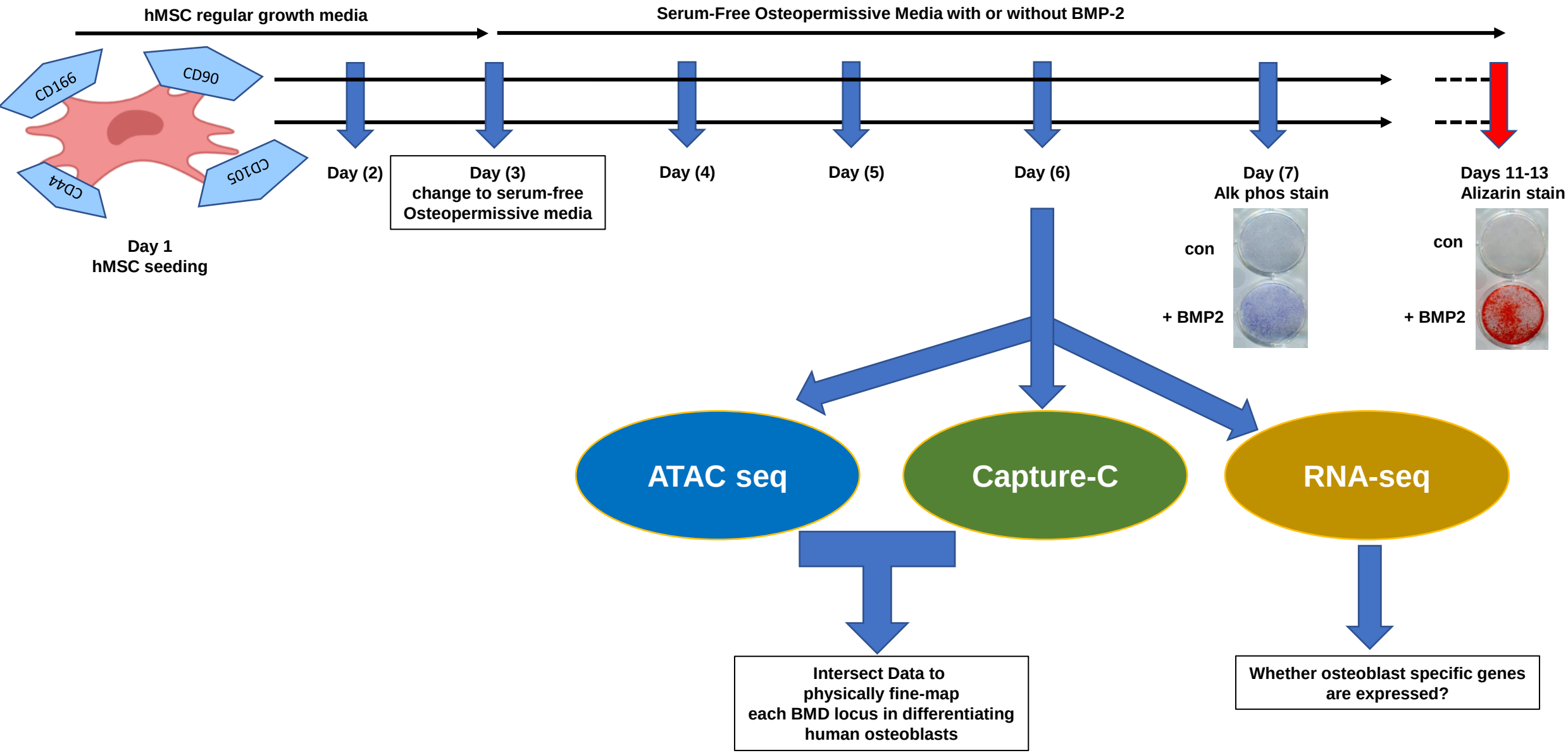






- **Cousminer et al., Genome Biol 2021** 22(1):1 doi: 10.1186/s13059-020-02207-9

Summary of Methods

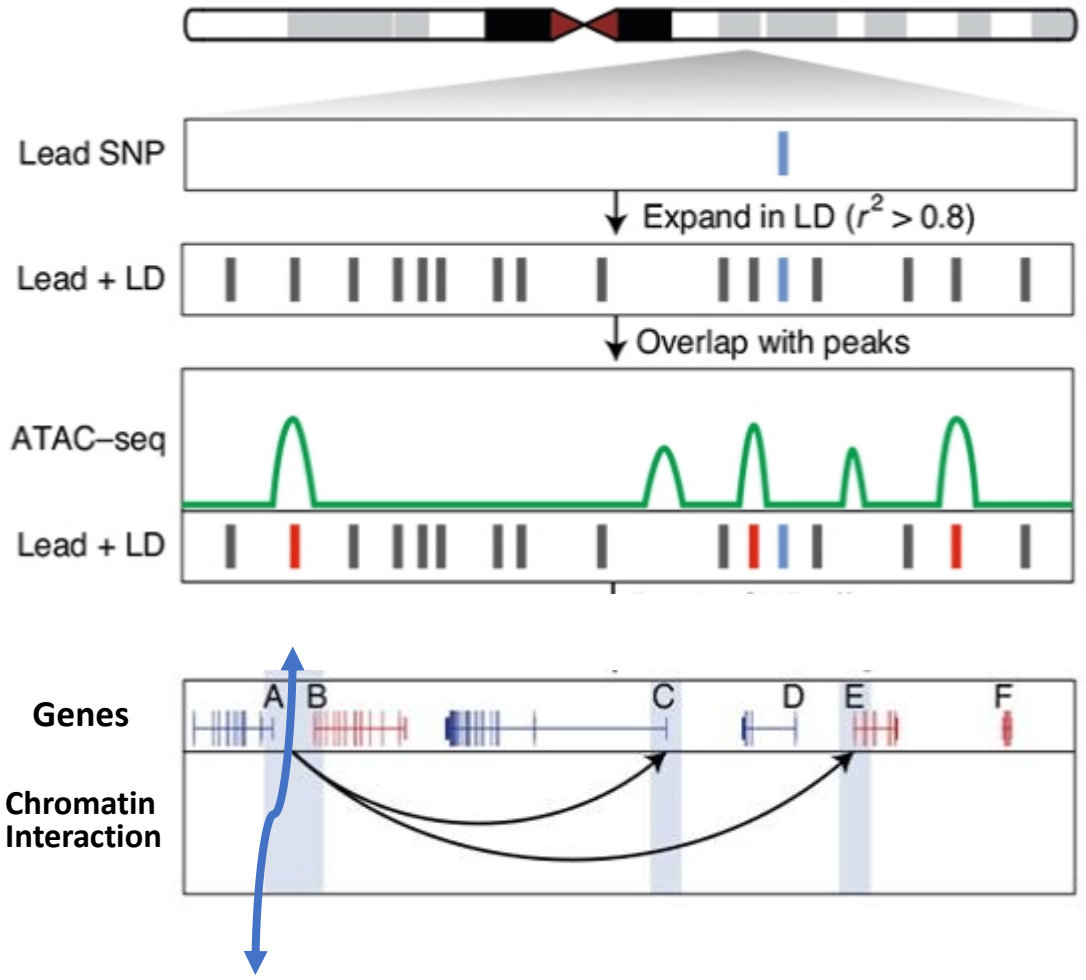


Selection of BMD loci

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

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



Would this proxy SNP affect gene C or gene E?

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)

Article | Open Access | Published: 19 March 2019

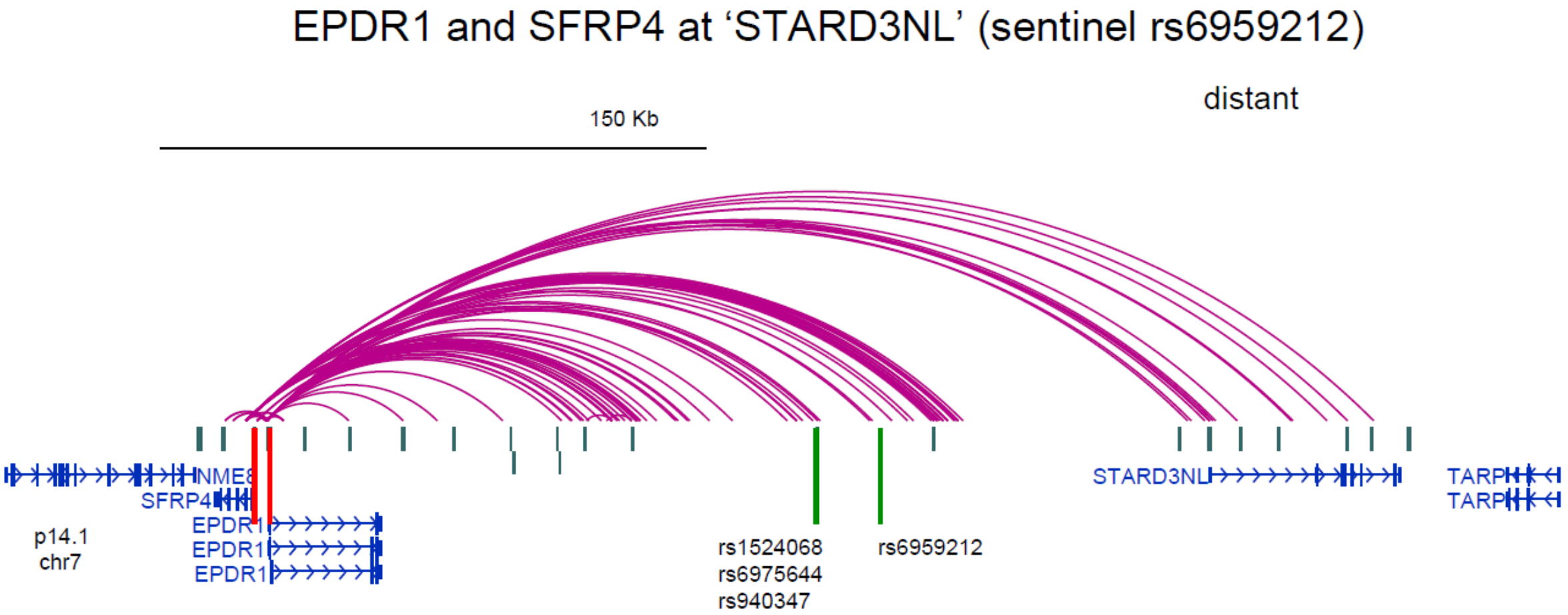
Genome-scale Capture C promoter interactions implicate effector genes at GWAS loci for bone mineral density

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

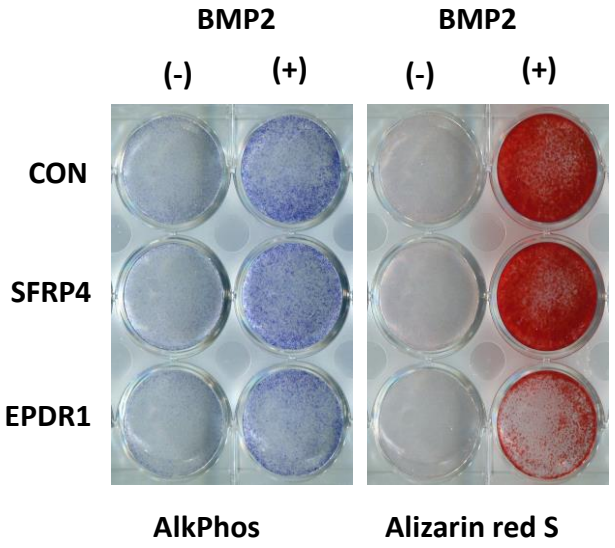
Nature Communications **10**, Article number: 1260 (2019) | Cite this article

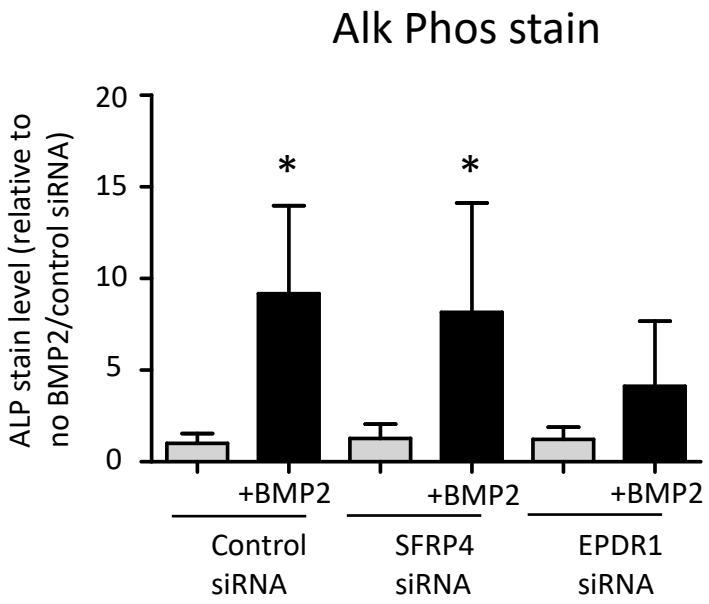
46 GWAS loci revealed at least one or more BMD proxy SNPs in open chromatin (and not residing in a baited promoter region) interacting with an open gene promoter

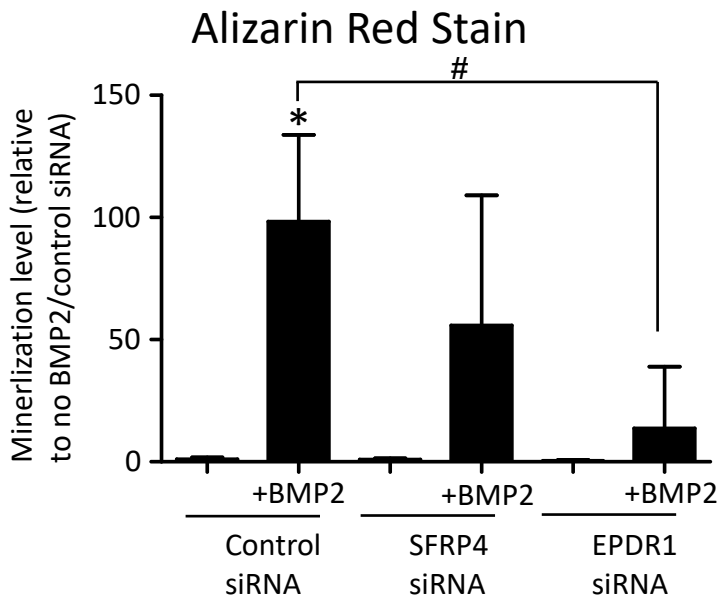
STARD3NL Locus: Variant to gene mapping

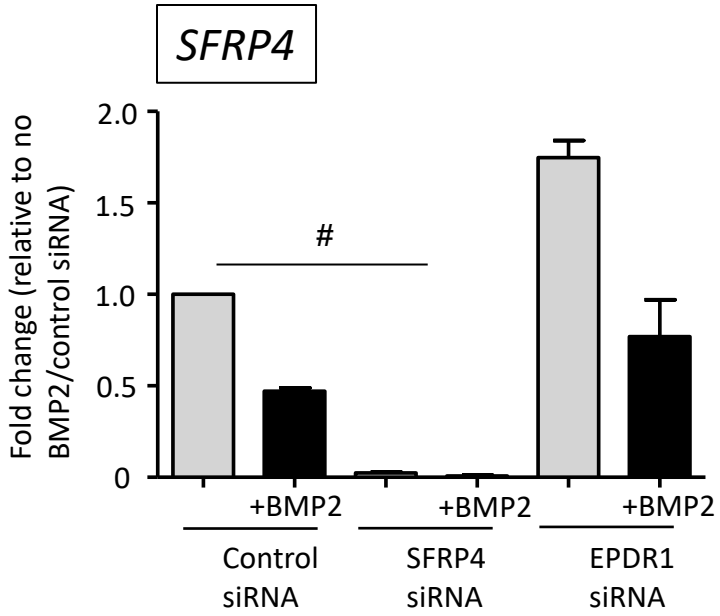


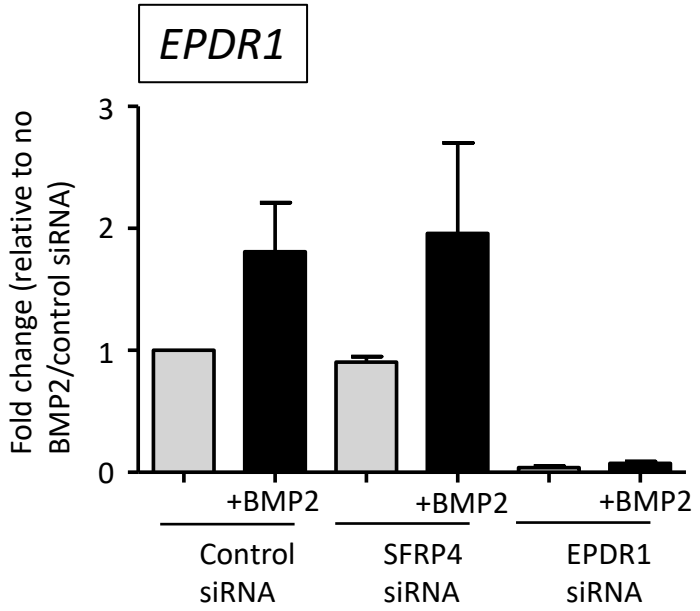
EPDR1 silencing reduces human osteoblast differentiation



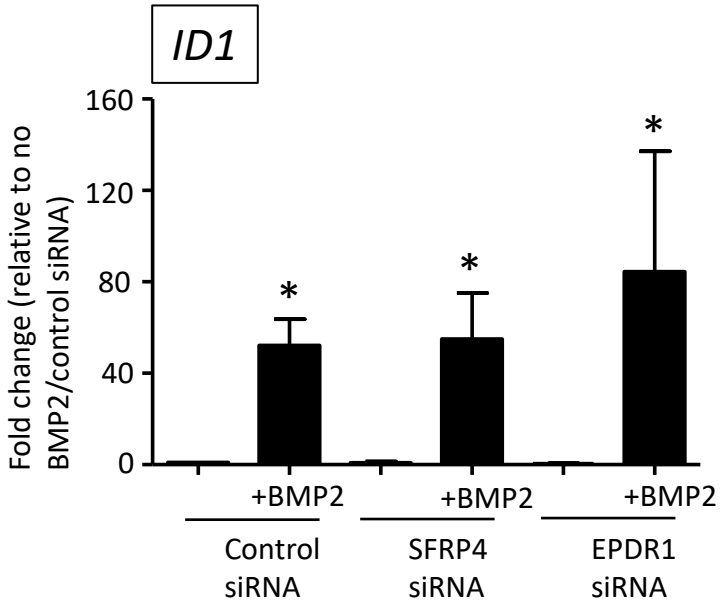


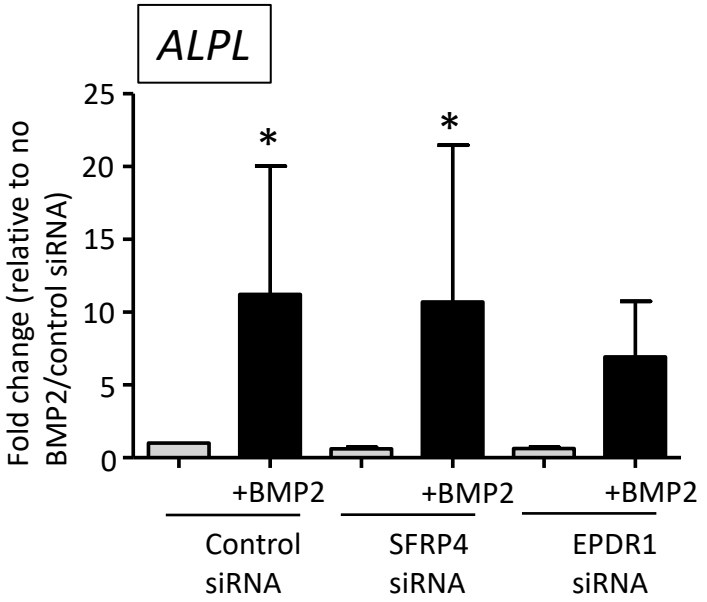


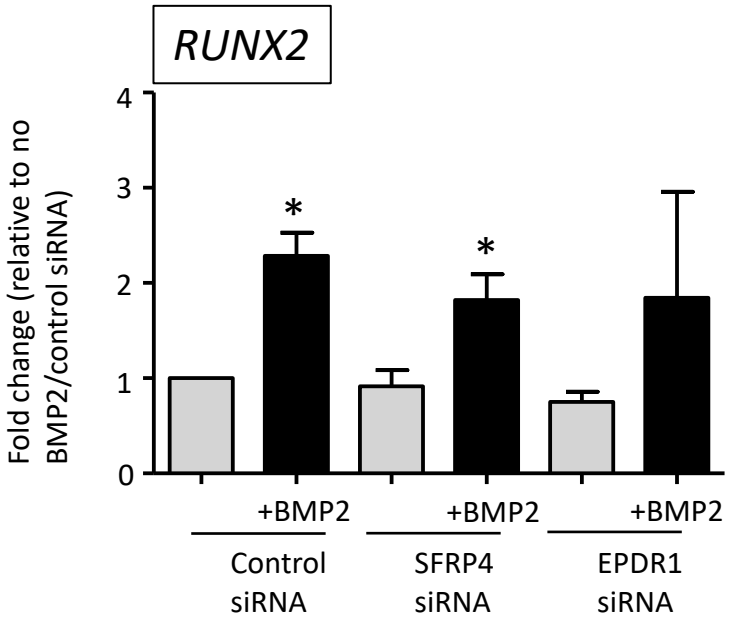


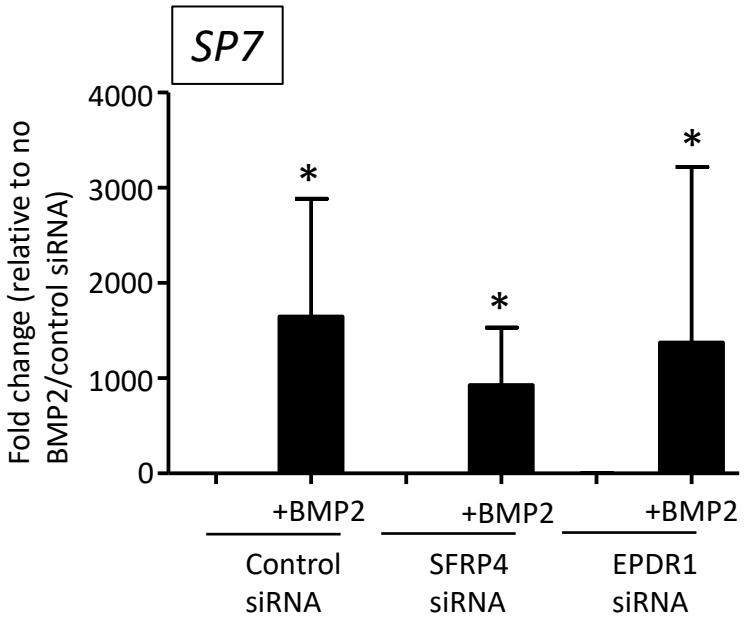


EPDR1 knock-down does not affect canonical BMP signaling





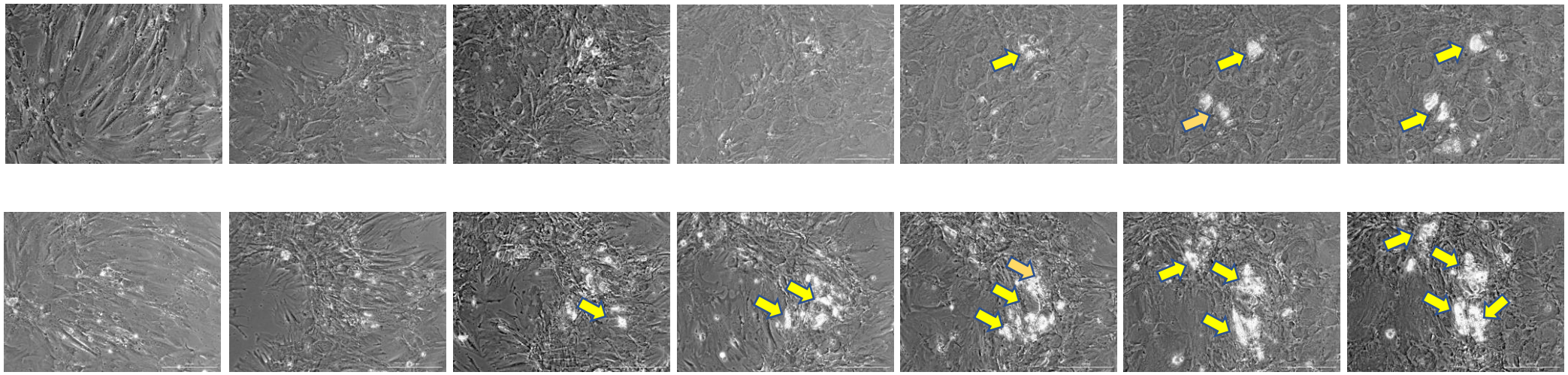




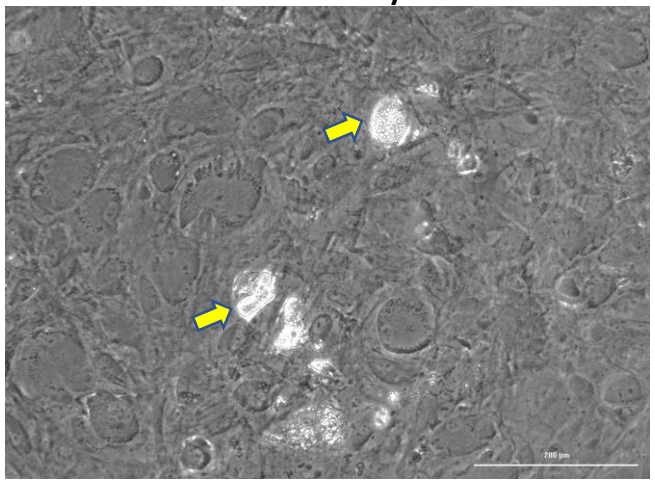
Is *EPDR1* fundamental gene required for general cell metabolism or whether it affects MSC differentiation into adipocytes?

EPDR1 silencing increases adipogenesis

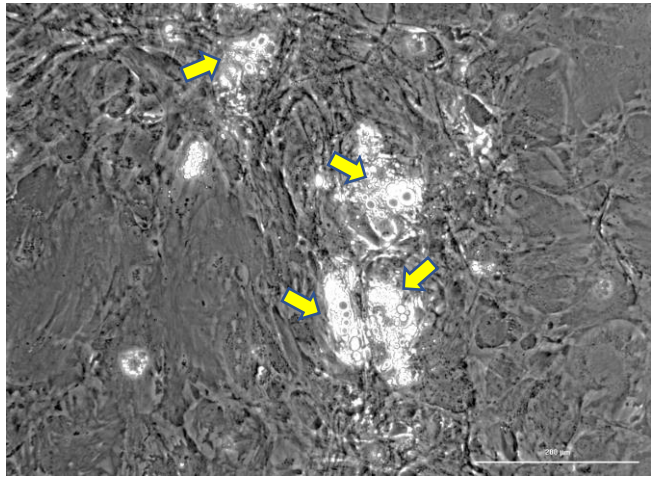
Adipogenic differentiation



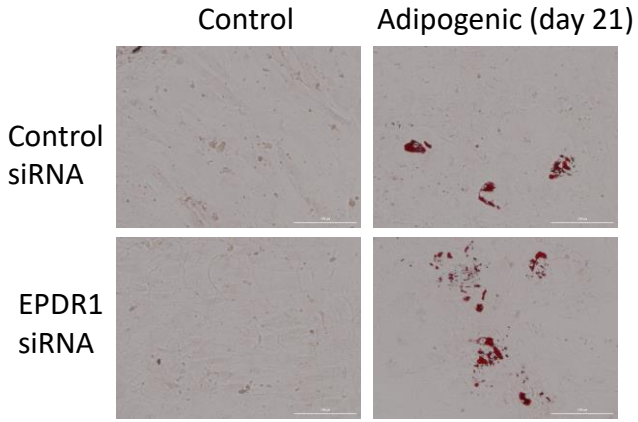
Control siRNA- Day 21

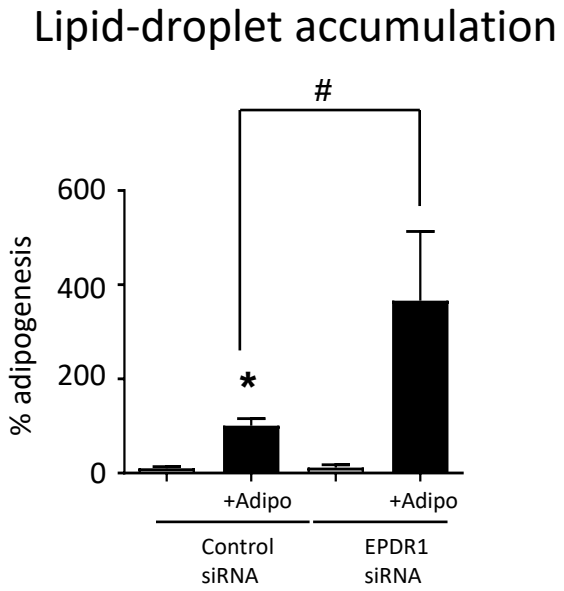


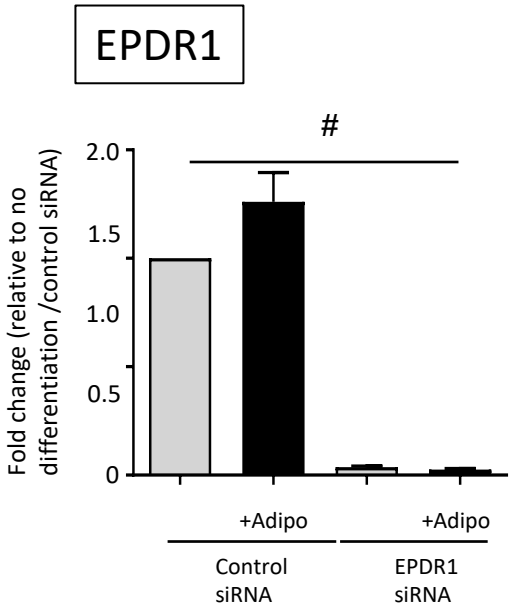
EPDR1 siRNA- Day 21

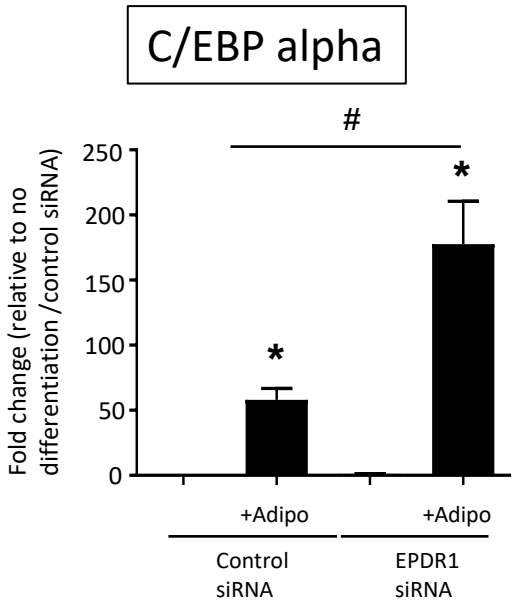


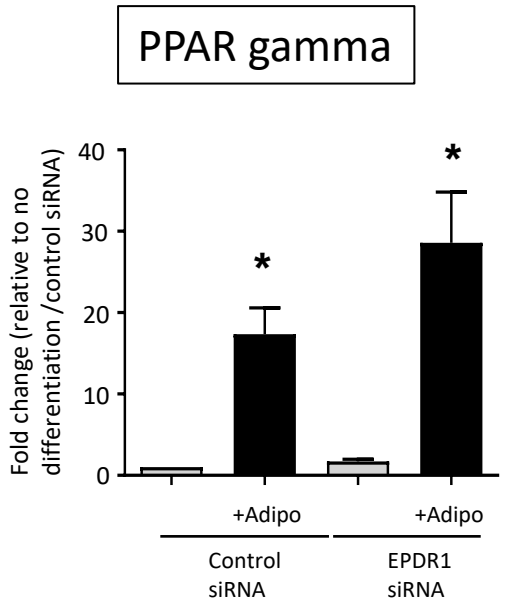
EPDR1 silencing increases adipogenesis









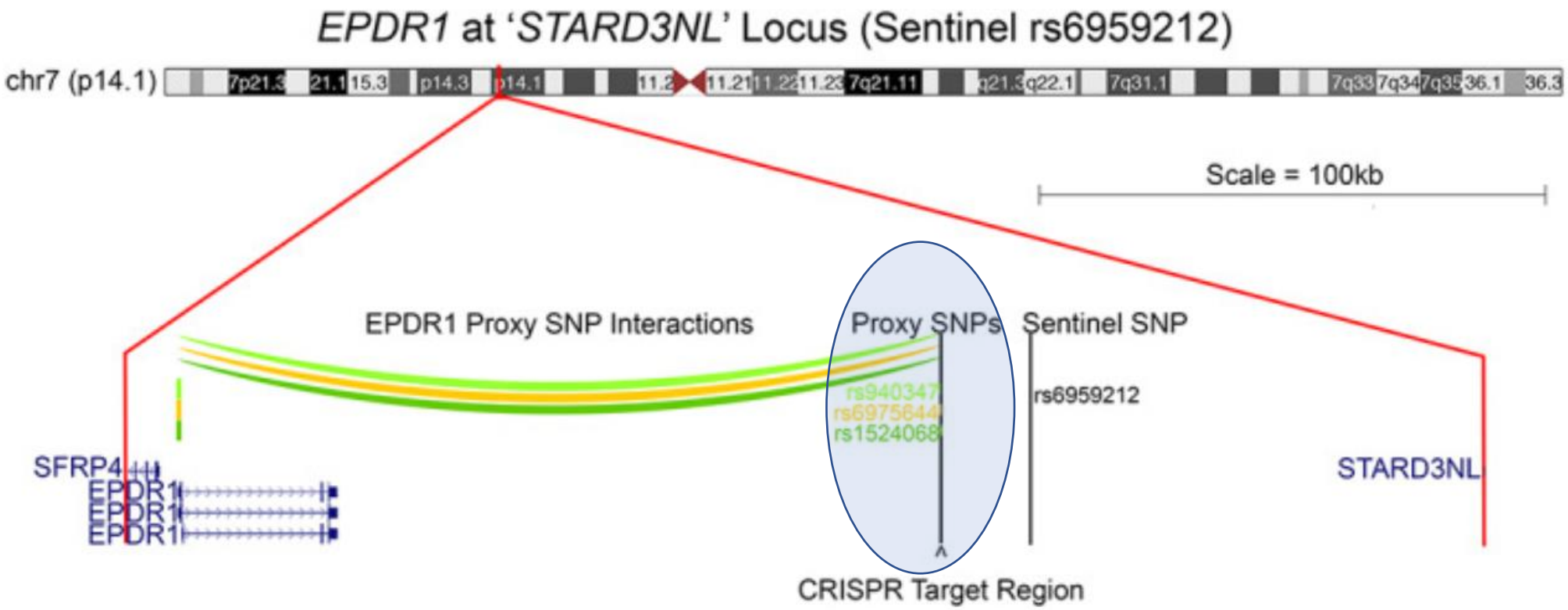


Summary

- **ATAC-seq coupled with chromatin confirmation capture implicates EPDR1 as an osteoblast effector gene at the STARD3NL locus**

- **EPDR1 silencing biases hMSC to adipogensis**

CRISPR-Cas9-Mediated Genome Editing Confirms *EPDR1* as an Effector Gene at the BMD GWAS-Implicated *'STARD3NL'* Locus

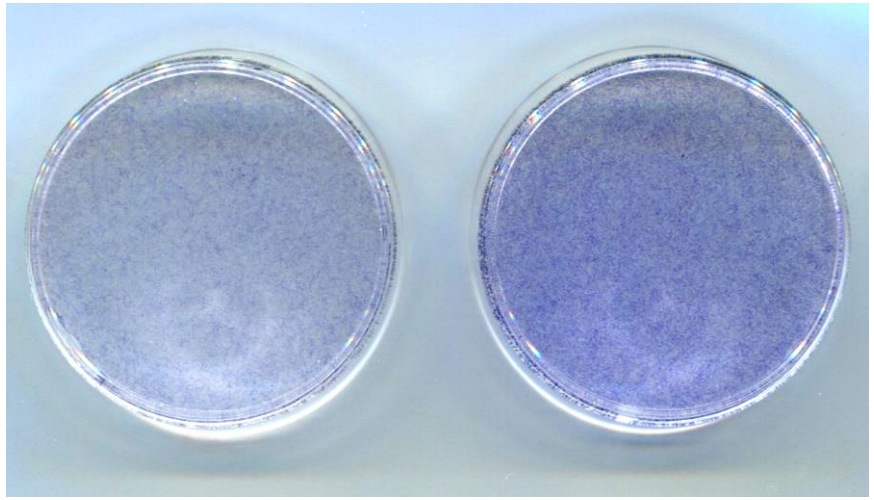


hFOB1.19 cells as an alternate model to study proxy SNP regulation of EPDR1

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

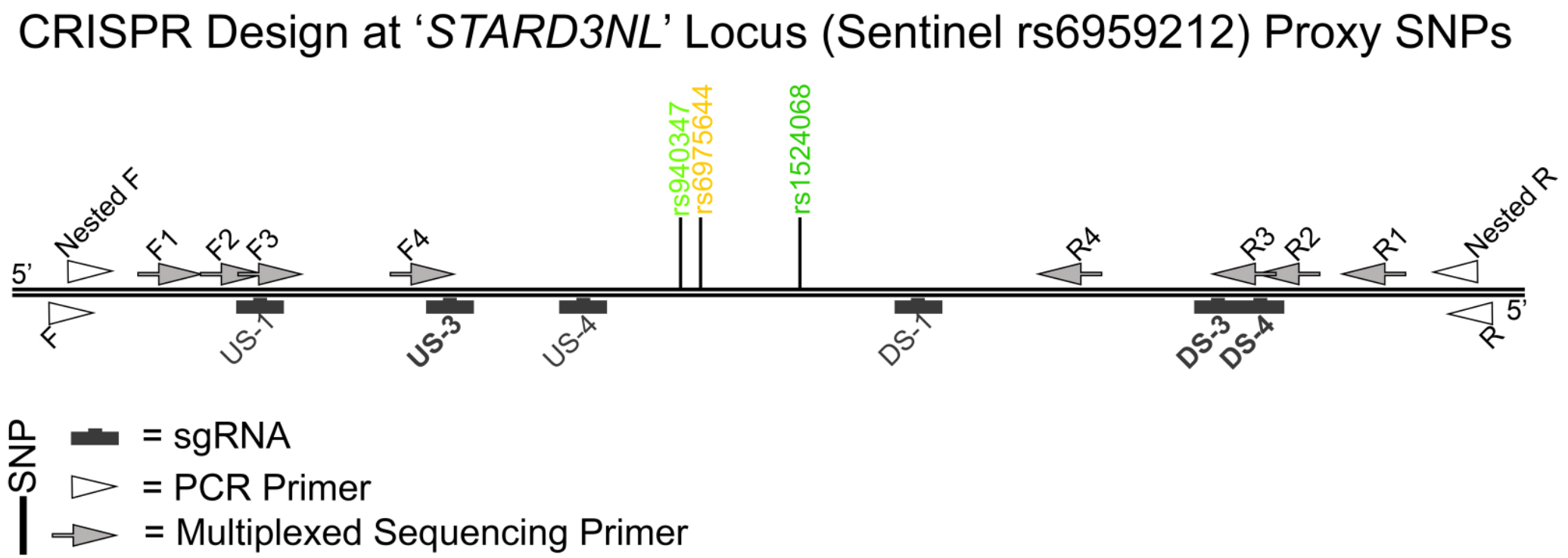
- Contains temperature sensitive mutant, tsA58, of the SV40 large T antigen that allows for the genome-edited cells to proliferate at 33.5˚C and to differentiate at 39.5˚C

33.5˚C 39.5˚C

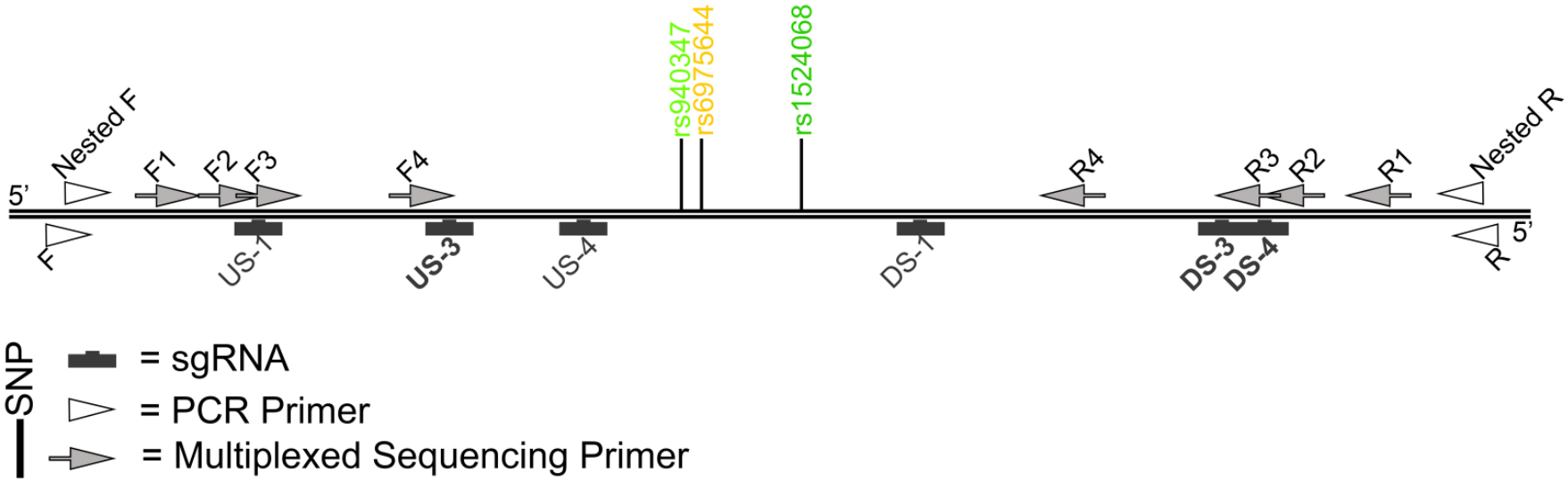


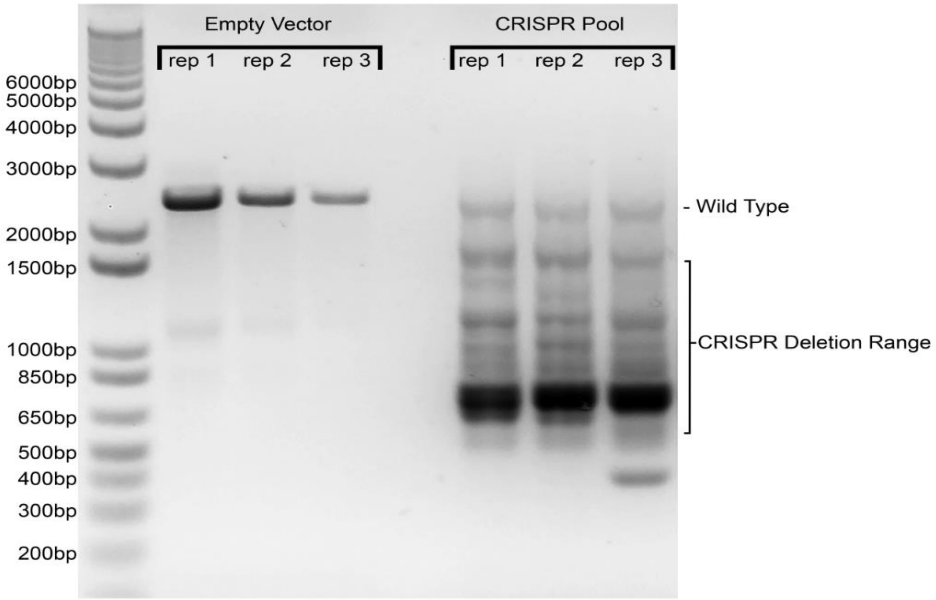
AlkPhos

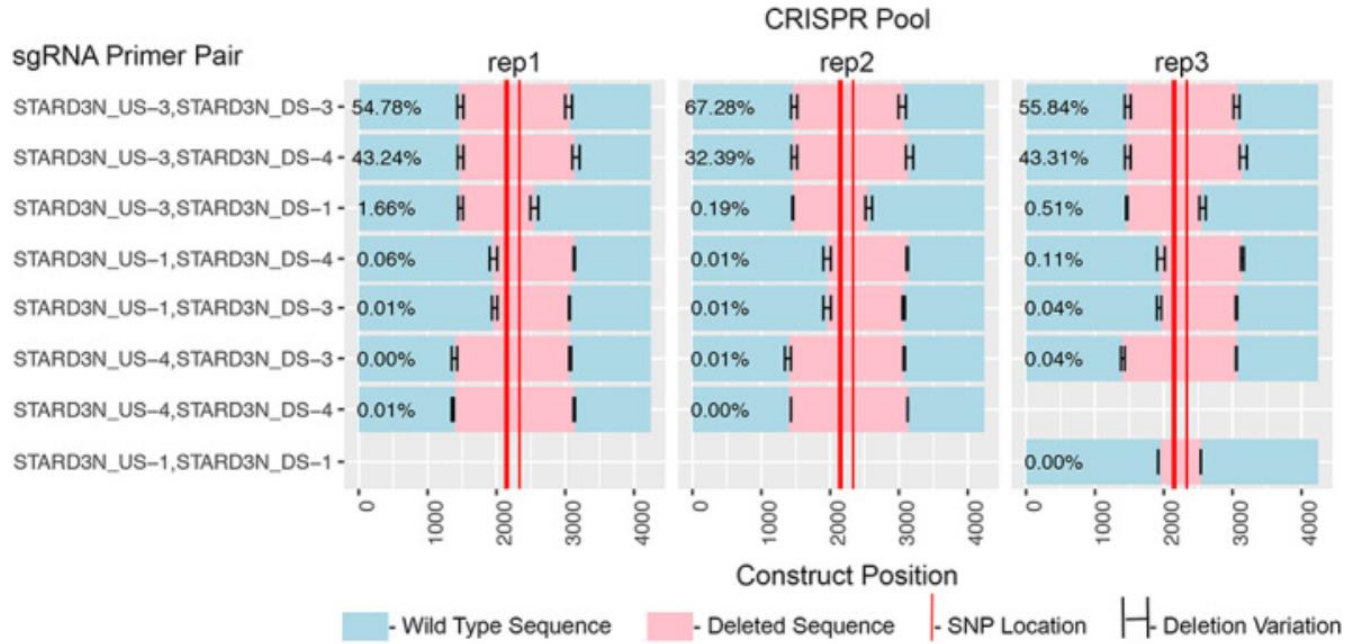
Pooled lentivirus was prepared using three sets of guides on each upstream and downstream site of the proxy SNPs



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

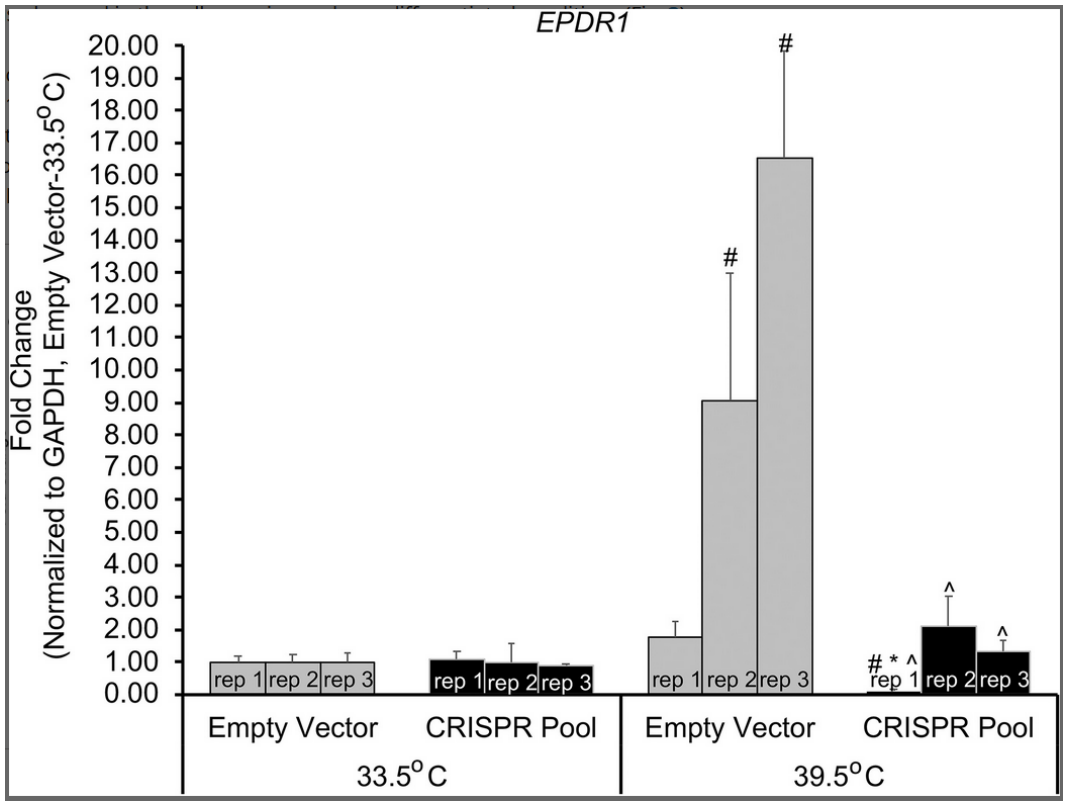




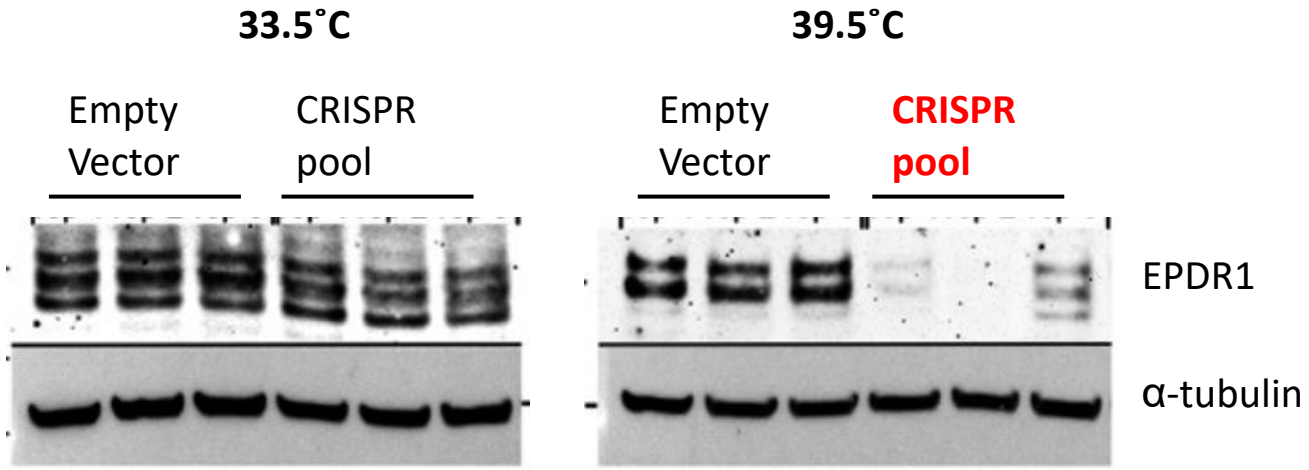


Deletion of proxy SNPs reduces *EPDR1* expression in cells undergoing differentiation

RT-qPCR



Western Blot



EPDR1 proxy SNP deleted cells fails to properly differentiate into osteoblasts

33.5˚C

Empty Vector

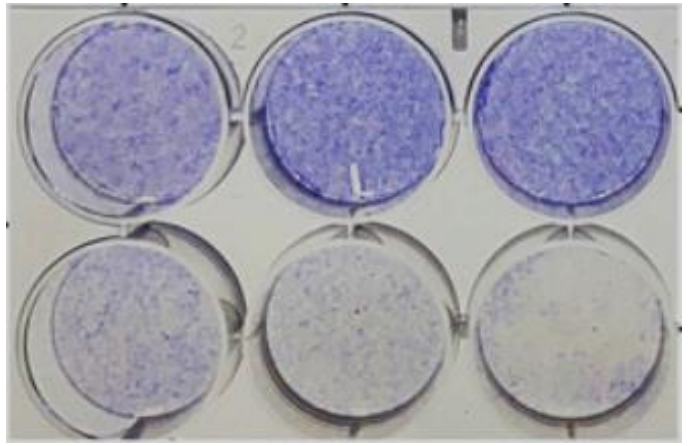
CRISPR pool

AlkPhos

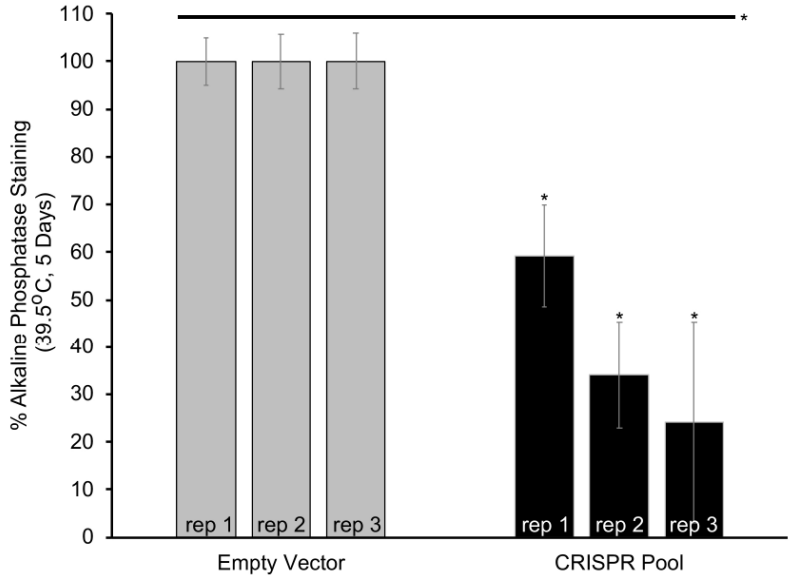
39.5˚C

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 plays an anti-inflammatory role during human osteoblastogenesis

Experimental Outline

3 independent donors

hMSC seeding

Serum-free Osteopermissive Media

Control siRNA

EPDR1 siRNA

Day (0)
siRNA transfection

Day (1)

Day (2)
stimulate with
BMP2

Day (3)

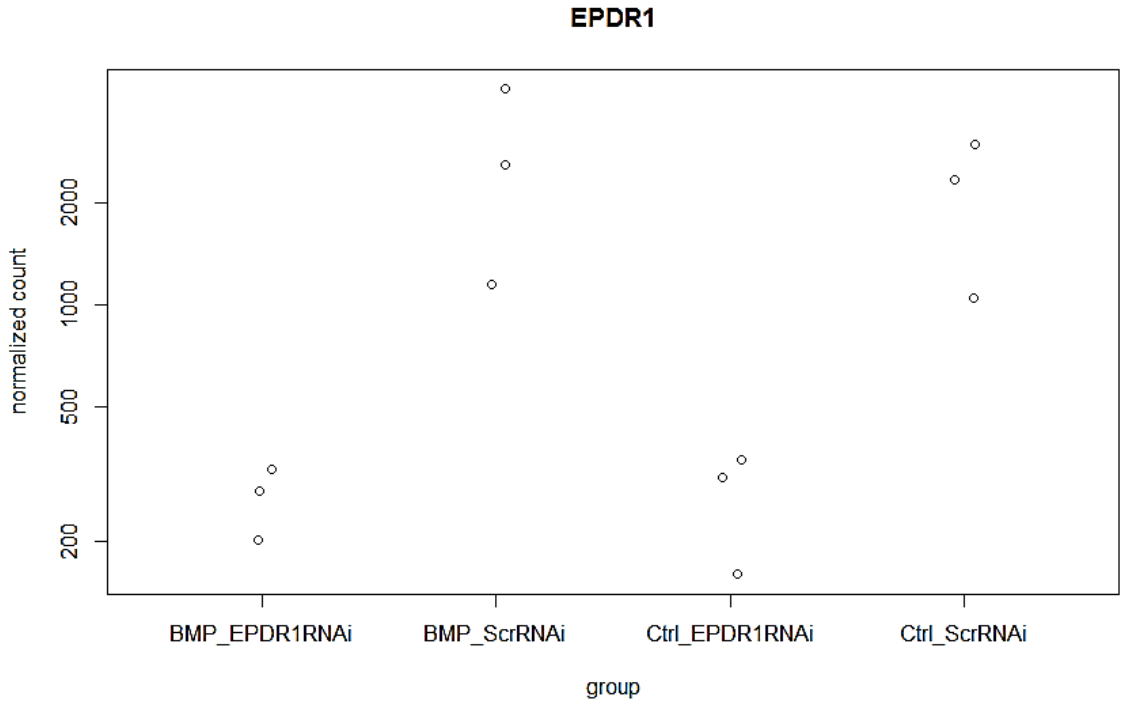
Day (4)

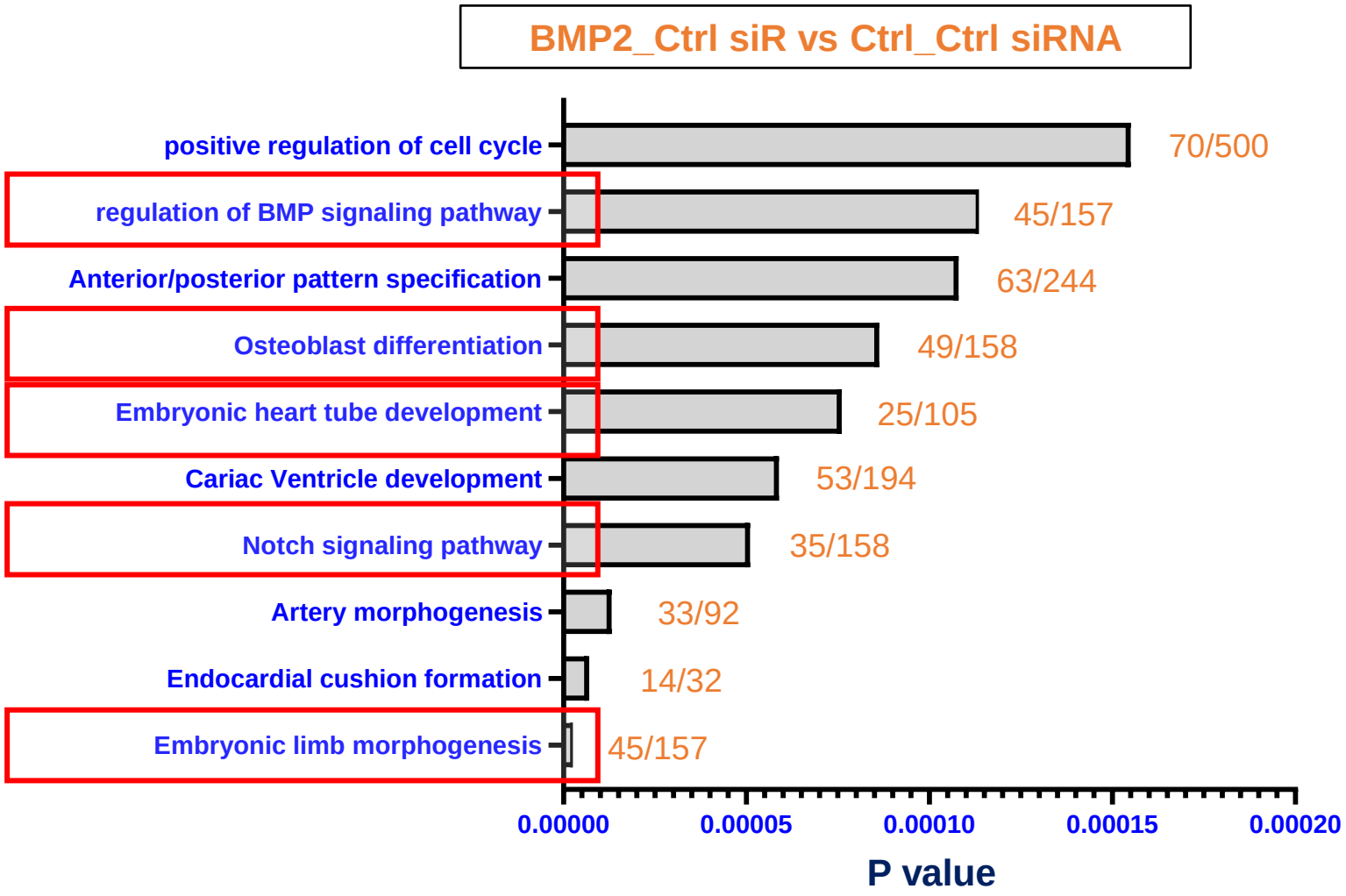
Day (5)

bulk RNA sequencing

1. Ctrl siRNA-Ctrl
2. Ctrl siRNA-BMP2
3. EPDR1 siRNA-Ctrl
4. EPDR1 siRNA-BMP2

BMP2_Ctrl siR vs Ctrl_Ctrl siRNA	BMP2_EPDR1 siR vs Ctrl_EPDR1 siRNA	Ctrl_EPDR1 siR vs Ctrl_Ctrl siRNA	BMP2_EPDR1 siR vs BMP2_Ctrl siRNA
1888	1528	1166	1167
3861	2744	2326	2347
1973	1216	1160	1180





BMP2_EPDR1 siR vs Ctrl_EPDR1 siRNA

FDR >0.05

for all the GO biological processes shown on the left

	Ctrl_EPDR1 siR vs Ctrl_Ctrl siRNA					BMP2_EPDR1 siR vs BMP2_Ctrl siRNA				
GO biological process complete	#	+/-	P value	FDR	Homo Sapiens	#	+/-	P value	FDR
inflammatory response (GO:0006954)	72	+	4.48E-09	4.05E-05	685	79	+	2.93E-08	2.73E-05
cell division (GO:0051301)	59	-	4.62E-06	2.32E-03	598	92	-	8.35E-09	1.11E-05
xenobiotic metabolic process (GO:0006805)	15	+	4.84E-06	2.19E-03	222	17	+	8.81E-04	4.07E-02
cytokine-mediated signaling pathway (GO:0019221)	45	+	4.08E-05	7.84E-03	925	45	+	6.91E-07	2.93E-04
regulation of leukocyte chemotaxis (GO:0002688)	20	+	8.65E-05	1.22E-02	178	27	+	7.76E-04	3.75E-02
regulation of T cell mediated immunity (GO:0002709)	12	+	1.76E-04	2.09E-02	115	11	+	2.74E-04	1.77E-02
regulation of T cell proliferation (GO:0042129)	17	+	2.80E-04	2.88E-02	264	28	+	3.25E-04	1.99E-02
regulation of interleukin-1 production (GO:0032652)	16	+	3.22E-04	3.09E-02	194	21	+	6.65E-04	3.43E-02
negative regulation of T cell activation (GO:0050868)	16	+	3.40E-04	3.20E-02	186	18	+	6.94E-04	3.52E-02
adaptive immune response (GO:0002250)	29	+	4.42E-04	3.99E-02	745	31	+	1.91E-04	1.45E-02

BMP2_EPDR1 siR vs BMP2_Ctrl siRNA

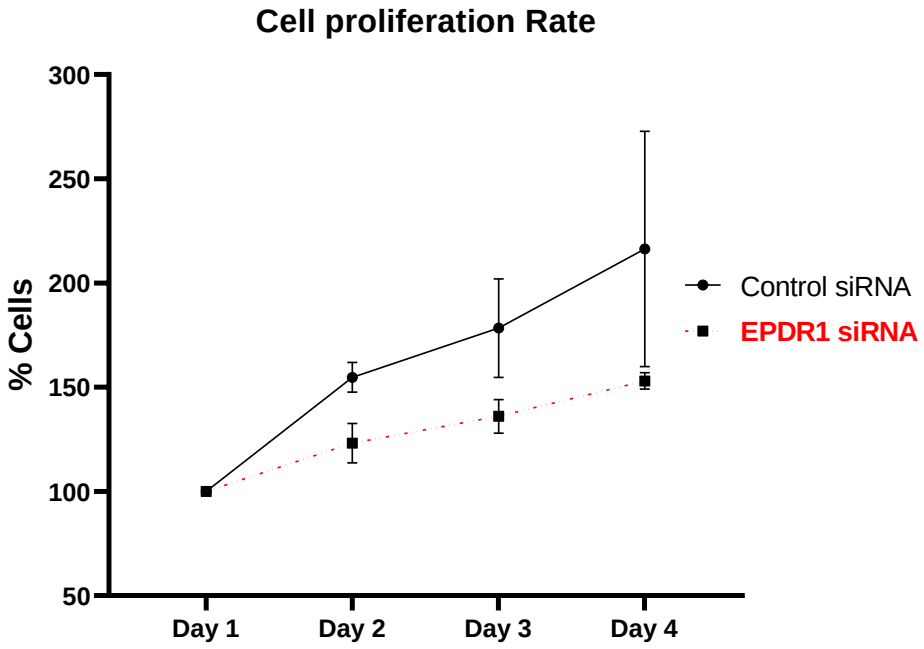
GO biological process complete	#	+/-	P value	FDR	Homo sapiens
regulation of fatty acid metabolic process (GO:0019217)	13	+	3.23E-04	2.00E-02	130
regulation of tumor necrosis factor production (GO:0032680)	27	+	3.48E-04	2.09E-02	240
phospholipid efflux (GO:0033700)	5	+	6.36E-04	3.33E-02	22
negative regulation of collagen biosynthetic process (GO:0032966)	6	+	6.95E-04	3.50E-02	14
regulation of bone resorption (GO:0045124)	10	+	7.44E-04	3.65E-02	44
positive regulation of interferon-gamma production (GO:0032729)	11	+	7.50E-04	3.66E-02	110
regulation of interleukin-8 production (GO:0032677)	13	+	8.46E-04	3.96E-02	135
arachidonic acid metabolic process (GO:0019369)	9	+	9.07E-04	4.15E-02	120
positive regulation of cell adhesion (GO:0045785)	84	+	1.14E-03	4.95E-02	757

EPDR1 knock-down shows ~20-30% reduction in cell proliferation rates of hMSC and mBMSC

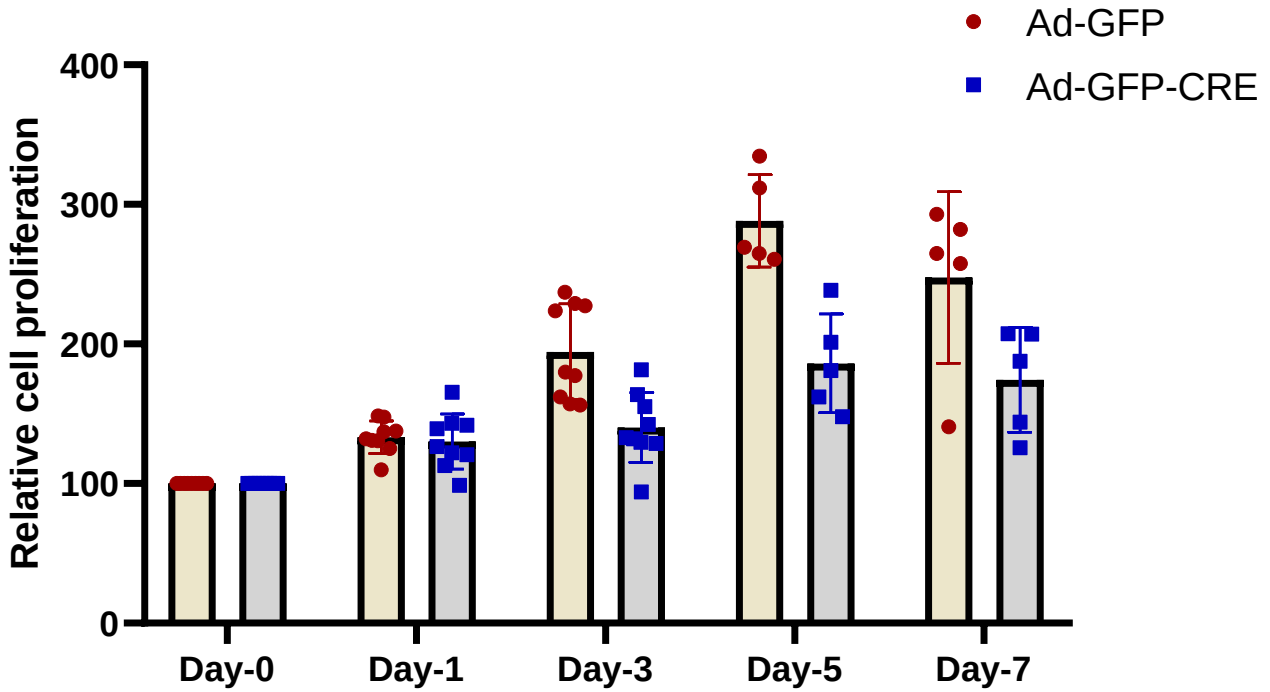
Ctrl_EPDR1 siR vs Ctrl_Ctrl siRNA

GO biological process complete	#	+/-	P value	FDR
cell division (GO:0051301)	59	-	4.62E-06	2.32E-03

hMSC



mBMSC-EPDR1$^{fl/fl}$



BMP2_EPDR1 siR vs BMP2_Ctrl siRNA

GO biological process complete	#	+/-	P value	FDR	Homo sapiens
regulation of tumor necrosis factor production (GO:0032680)	27	+	3.48E-04	2.09E-02	240
positive regulation of interferon-gamma production (GO:0032729)	11	+	7.50E-04	3.66E-02	110
regulation of interleukin-8 production (GO:0032677)	13	+	8.46E-04	3.96E-02	135

hMSC donor 1

rh TNFα (ng/ml)

\- 10 25 50

+BMP2

Alizarin Red

hMSC donor 2

rh TNFα (ng/ml)

\- 10 25 50

+BMP2

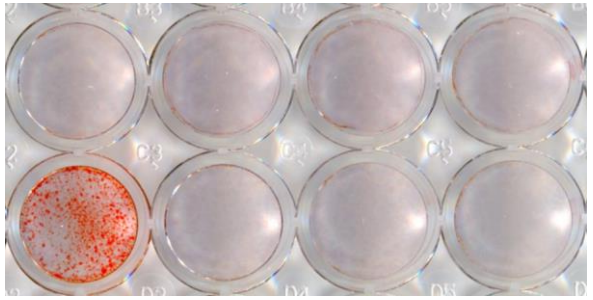
Alizarin Red

hMSC donor 3

rh TNFα (ng/ml)

\- 10 25 50

+BMP2



Alizarin Red

Summary

- **RNA sequencing of EPDR1 silenced cells shows major inflammation response and reduction of cell division**

- **BMP2 mediated human osteoblast differentiation is abrogated in presence of inflammatory cytokines such as TNFα**

MICHIGAN MEDICINE
UNIVERSITY OF MICHIGAN

Orthopaedic Research Laboratories

Children's Hospital of Philadelphia℠

Alessandra Chesi
James A. Pippin
Matthew Pahl
Andrew D. Wells
Struan F. A. Grant
members of Spatial and Functional Genomics Collaborative (CHOP)

Tristan Maerz

Kurt D. Hankenson
Karen Kessel
members of Hankenson Lab

Perelman School of Medicine
University of Pennsylvania

CDMRP

RO1HG010067

RO1AR055607

MEDICINE
TEXAS A&M UNIVERSITY

RO1AG072705

THANK YOU!!