TRANSCRIPTIONAL REGULATION OF CYCLOPHILIN D BY BMP/SMAD SIGNALING AND ITS ROLE IN OSTEOSTEOGENIC DIFFERENTIATION

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SUMMARY:

Cyclophilin D (CypD) promotes opening of the mitochondrial permeability transition pore (MPTP) which plays a key role in both cell physiology and pathology. It is, therefore beneficial for cells to tightly regulate CypD and MPTP but little is known about such regulation. We have reported before that CypD is downregulated and MPTP deactivated during differentiation in various tissues. Herein, we identify BMP/Smad signaling, a major driver of differentiation, as a transcriptional repressor of the CypD gene, Ppif. Using osteogenic induction of mesenchymal lineage cells as a model of BMP/Smad-dependent differentiation, we show that CypD is in fact transcriptionally repressed during this process. The importance of such CypD downregulation is evidenced by the negative effect of CypD ‘rescue’ via gain-of-function on osteogenesis both in vitro and in vivo. In sum, we characterized BMP/Smad signaling as a regulator of CypD expression and elucidated the role of CypD downregulation during cell differentiation.
INTRODUCTION

The mitochondrial permeability transition pore (MPTP) is a non-selective high-conductance channel within the inner mitochondrial membrane (IMM). MPTP opening leads to the increased permeability of the IMM and entry of solutes up to 1.5kDa of molecular mass, i.e. Mitochondrial Permeability Transition (Bernardi, Rasola, Forte, & Lippe, 2015). Although the MPTP molecular identity remains debatable, the ATP synthase converges as a potential locus. The consequences of MPTP opening span from physiological events such as regulation of synthasome assembly, oxidative phosphorylation (OxPhos), membrane potential (Δψm), ROS-induced ROS release, Ca^{2+} homeostasis, and epigenetic regulation (Bernardi & von Stockum, 2012; Beutner, Alanzalon, & Porter, 2017; Boyman et al., 2019; Feissner, Skalska, Gaum, & Sheu, 2009) to pathophysiological processes associated with sustained pore opening, including mitochondrial dysfunction, mtDNA release, inflammation and cell death. Such pathophysiological processes are observed in cancer, aging, injury, and degenerative diseases (Alavian et al., 2014; Bernardi et al., 2015; Du & Yan, 2010; Giang et al., 2013; Rottenberg & Hoek, 2017; Warne et al., 2016). Among some important physiological events under MPTP control, is OxPhos since MPTP opening leads to IMM depolarization, proton motive force dissipation and ATP production decrease. For differentiating cells switching from a glycolytic pathway to higher OxPhos activity, MPTP opening can be particularly detrimental by impairing lineage commitment, differentiation, and functioning of differentiated cells (Eliseev et al., 2007; Hom et al., 2011; Lingan, Alanzalon, & Porter, 2017). Currently, several MPTP opening effectors have been described, but the mitochondrial matrix protein Cyclophilin D (CypD) remains the only genetically proven opener of the MPTP (Briston, Selwood, Szabadkai, & Duchen, 2019).

Encoded by the nuclear gene PPIF, CypD is a chaperone protein - peptidyl-prolyl cis-trans isomerase F (PPlase) - thought to be involved in protein folding. However, very few reports demonstrate such an activity of CypD (Porter & Beutner, 2018). Regarded as the master regulator of MPTP, CypD is imported into mitochondria where it can actively bind to the mitochondrial ATP synthase, decreasing the threshold for pore opening and increasing opening probability. The exact molecular mechanism by which CypD regulates the MPTP and mitochondrial function remains unclear. A range of post-translation modifications, such as acetylation and phosphorylation, is known to exert regulatory properties on CypD activity, and consequently on MPTP opening (Amanakis & Murphy, 2020; Gutierrez-Aguilar & Baines, 2015; Porter & Beutner, 2018). Additionally, it has been
shown that CypD also functions as a scaffold protein, being able to cluster various structural and signaling molecules together to modify mitochondrial physiology and bioenergetic response (Eliseev et al., 2009; Porter & Beutner, 2018). Although several aspects affecting CypD physiologic regulation and its interplay with mitochondrial activity have been elucidated, how the CypD gene is transcriptionally regulated has yet to be described.

Cellular metabolism and mitochondrial function play an important role in cell differentiation, which in turn is a crucial process in organogenesis, tissue homeostasis and damage repair. CypD expression and activity are cell-specific (Laker et al., 2016) and can be regulated during differentiation. As cells become more active and increase their oxygen consumption, higher ROS levels are produced partially driven by an increase in the electron flow within the respiratory chain, and Δψm (Barja, 1999; Drose & Brandt, 2012; Suski et al., 2018; Turrens, 1997). Higher ROS levels due to OxPhos activation can lead to deleterious effects including opening of the MPTP and consequently blunting of mitochondrial function (Carraro & Bernardi, 2016). To inhibit MPTP opening, it is beneficial for differentiating cells moving towards a higher OxPhos usage, to downregulate CypD expression and/or activity to be better protected against oxidative stress and support OxPhos. Such an inhibition due to downregulation of CypD has been reported during neuronal and cardiomyocyte differentiation (Eliseev et al., 2007; Hom et al., 2011; Lingan et al., 2017). Conversely, induced pluripotent stem cell (iPSC) reprogramming is accompanied by a metabolic shift from OxPhos to glycolysis and not surprisingly, by transient MPTP opening and CypD expression upregulation (Ying et al., 2018).

The bone morphogenic protein (BMP) signaling pathway is an important driver of cell differentiation and maturation. BMPs are shown to regulate early processes from embryogenesis and development to adult tissue homeostasis. BMPs are not bone exclusive, the ubiquitous expression of BMP underscores its importance for organogenesis and maintenance in several tissues, such as neurological, ophthalmic, cardiovascular, pulmonary, gastrointestinal, urinary and musculoskeletal systems (Nickel & Mueller, 2019; Wang et al., 2014). Upon BMP receptor activation and trans-phosphorylation, receptor regulated Smads (R-Smads) such as Smad1/5/8 are recruited and phosphorylated. Phospho-Smad1/5/8 binds to Smad4 forming a heterodimeric complex which is subsequently translocated into the nucleus to activate or repress the expression of its target genes. Osteogenesis and osteoblast (OB) differentiation are controlled at least in part via BMP/Smad canonical
signaling. During osteogenic differentiation, activation of the transcription factor Runx2 is mediated by the BMP/Smad signaling pathway initiating a cascade of downstream osteogenic marker genes upregulation (Bianco & Robey, 2015). Our lab and others have demonstrated that bone marrow stromal (a.k.a. mesenchymal stem) cells (BMSCs) shift their bioenergetic profile towards OxPhos during OB differentiation (Colaianni et al., 2018; Feigenson, Eliseev, Jonason, Mills, & O'Keefe, 2017; Shares, Busch, White, Shum, & Eliseev, 2018; Shum, White, Mills, Bentley, & Eliseev, 2016; Smith & Eliseev, 2021; Yu et al., 2019). Since OB function involves bone matrix deposition, an energy-consuming process highly dependent on mitochondrial bioenergetics and OxPhos activity, we hypothesized that MPTP inhibition via CypD transcriptional downregulation is mediated by BMP/Smad signaling and required to sustain OxPhos function and support osteogenic differentiation.
RESULTS

BMP/Smad signaling is a transcriptional repressor of Ppif gene

To determine potential mechanisms involved in CypD regulation on a transcriptional level, we performed an *in silico* analysis of the CypD gene, *Ppif*, promoter for transcription factor (TF) binding sites using PROMO online platform (Messeguer, Escudero et al. 2002). We found multiple BMP-dependent Smad-binding elements (SBE) in the 1.1kb 5’ upstream region of both human *PPIF* and mouse *Ppif* genes (Figure 1A). BMP/Smad signaling pathway is an important driver of differentiation in various lineages including osteogenic lineage. Therefore, to test if BMP/Smad signaling regulates CypD gene transcription, we transfected mouse osteogenic bone marrow- or calvaria-derived cell lines ST2 and MC3T3-e1, respectively, with a pCMV-Smad1 vector. We observed that CypD mRNA was downregulated after Smad1 over-expression, whereas osteogenic marker *Runx2*, a readout of BMP/Smad activity, was upregulated (Figure 1B-C). The efficiency of Smad1 transfection was confirmed by real time RT-qPCR.

To confirm interaction of BMP-dependent Smad1 with the *Ppif* promoter, we performed a chromatin immunoprecipitation (ChIP) DNA-binding assay using nuclear fractions from ST2 cells supplemented with BMP2 or vehicle-control for 24 hours. PCR analysis of the reversed cross-linked protein-DNA immunoprecipitate complexes was done (Figure 2A) using primers to amplify the distal region within the *Ppif* promoter containing multiple SBEs (Figure 2B). Smad1-*Ppif* interaction was found to be present at low levels in the vehicle-treated control cells. This finding is consistent with the fact that intrinsic levels of BMP activity are present even in undifferentiated osteogenic cells. Importantly, BMP2 treatment significantly induced Smad1-*Ppif* interaction. Band density quantification revealed 2.5 fold-increase of Smad1-*Ppif* interaction in the BMP2 treated samples (Figure 2C). These results demonstrate that Smad1 binds the CypD gene (*Ppif*) promoter.

To characterize the functionality of the SBEs found within the *Ppif* promoter, we subcloned the 1.1kb mouse *Ppif* promoter into the pGL4.10 luciferase reporter construct (Figure 3A). We then co-transfected ST2 cells with the above promoter-reporter construct and either pCMV-Smad1 or pCMV-EV and analyzed the luciferase signal. Figure 3C shows that Smad1 significantly decreased the luciferase signal from the 1.1kb *Ppif*-luc promoter-reporter. Furthermore, a promoter bashing approach was used and five other various length promoter constructs were generated (Supplementary Figure S1A): -0.37kb to -0.1kb, or -0.62 to -0.45kb, or -1.1 kb to -0.95kb, or -
0.62kb to -0.1kb, or -1.1kb to -0.45kb deletion mutants that correspond to a cluster of the two most proximal (P), three middle (M), five most distal (D), five middle + proximal (M+P), and eight distal + middle (D+M) SBEs, respectively. We co-transfected ST2 cells with the above promoter-reporter constructs and either pCMV-Smad1 or pCMV-EV and analyzed the luciferase signal for all our constructs. Middle and Distal regions did not show any luciferase signal difference when compared to EV-transfected cells, whereas Proximal, M+P, and D+M showed activation of \textit{Ppif} activity (Supplementary Figure S1B). Thus, only the full-length 1.1kb \textit{Ppif}-luc reporter showed \textit{Ppif} activity repression upon Smad1 transfection (Figure 3C). Knowing that the full-length \textit{Ppif} promoter is the actual region controlling the gene activity and that Smads work by forming oligomeric structures binding various regions of the promoter (Massague, Seoane, & Wotton, 2005; Wang et al., 2014), we considered the data using the 1.1kb \textit{Ppif}-luc reporter as the most relevant. Of note, in all these experiments, activation of Smad1 signaling following transfection was confirmed by the increase in the activity of 12xSBE, a BMP/Smad signaling luciferase reporter (Figure 3B). In sum, our data indicate that BMP/Smad signaling is a transcriptional repressor of CypD.

To further delineate the role of BMP/Smad signaling in \textit{Ppif} repression we used the inhibitory Smad7 which rescued \textit{Ppif} promoter activity in Smad1-transfected cells (Figure 3C). Smad7 competitively inhibits the interaction of R-Smads to the cytoplasmic domain of their respective cell receptors, therefore preventing R-Smad recruitment and phosphorylation, and consequently nuclear translocation. Additionally, cells transfected with the 1.1kb \textit{Ppif}-luc reporter were either treated with BMP2 with or without the BMP inhibitor Noggin, or induced in osteogenic media and assayed for luciferase signal. BMP2 and osteogenic media downregulated the luciferase signal, whereas Noggin rescued \textit{Ppif} activity (Figure 3D). These results were also confirmed in MC3T3e1 cells (Figure 3E-F). Taken together, these data support our hypothesis that BMP/Smad signaling transcriptionally represses \textit{Ppif} promoter activity and consequently downregulates CypD expression during osteogenic differentiation.

**Decreased CypD expression and MPTP activity during osteogenic differentiation**

BMP/Smad signaling is a major driver of differentiation. It is particularly important for osteogenic lineage. During osteogenic differentiation, cell energy metabolism shifts towards OxPhos as was shown by us and others.
Since a higher OxPhos activity, as seen in differentiated OBs, is described to produce more ROS (Barja, 1999; Drose & Brandt, 2012; Suski et al., 2018; Turrens, 1997; Supplementary Figure S2A), potentially leading to oxidative stress and higher probability of the MPTP opening, it is beneficial for actively respiring cells such as OBs to decrease MPTP activity, for example by downregulating CypD. We, therefore measured CypD expression and MPTP activity in several osteogenic cell types. Figure 4 shows that primary BMSCs isolated from mouse long bones, mouse bone marrow-derived osteogenic cell line ST2, and mouse calvaria-derived osteogenic cell line MC3T3-e1 underwent OB differentiation confirmed by Alkaline Phosphate and Alizarin Red staining (Figure 4A-B) and upregulation of osteogenic markers \textit{Alp} and \textit{Bglap}. (Figure 4C-E). In accordance with our hypothesis, osteogenic differentiation was accompanied by downregulation of CypD gene, \textit{Ppif}, mRNA expression in all these cell types (Figure 4C-E). To confirm that this effect is not species-specific, we analyzed human BMSC RNAseq dataset from our previous publication (Shum, White, Mills, et al., 2016) and observed downregulation of CypD gene, \textit{PPIF}, mRNA expression in osteoinduced cells when compared to undifferentiated cells (Supplementary Figure S2B). Protein content of CypD correspondingly decreased during OB differentiation as measured with western blot (Supplementary Figure S3). We then measured MPTP activity using calcein-cobalt assay and flow cytometry (Figure 4F). In this assay, cells are incubated with calcein AM in the presence of CoCl$_2$ which quenches cytosolic but not mitochondrial calcein unless mitochondria are permeabilized due to MPTP opening. The increase in the calcein signal indicates higher resistance to MPTP opening and decreased pore activity (Petronilli et al., 1999). The assay showed that calcein signal increased at day 14 of OB differentiation when compared to day 0 indicating lower MPTP activity (Figure 4F). No changes were detected in the total mitochondrial mass during OB differentiation as labeled by Nonyl Acridine Orange (Figure 4G), therefore confirming that calcein signal increase is in fact caused by a lower pore activity and not by an increased mitochondrial compartment. This is consistent with our previous report showing that mitochondrial mass and mtDNA do not increase during OB differentiation (Shares et al., 2018; Shum, White, Mills, et al., 2016). Altogether, these data indicate that CypD expression is downregulated and MPTP activity is decreased during osteogenic differentiation.
Smad-mediated regulatory effect on CypD is independent from osteogenic signaling downstream of Smad1

Osteogenic differentiation is a complex process that involves the coordination and crosstalk of several signaling pathways. BMP/Smad signaling is a potent driver of osteogenic differentiation in BMSCs. Once the BMP response is activated, other signaling molecules can influence Smad activity and osteogenic differentiation. For instance, after Smad1-Smad4 complex nuclear translocation, its dephosphorylation is accompanied by the dissociation of the complex and export to the cytoplasm. Smad4 then is believed to interact with β-catenin and TCF/LEF1 forming a TF activation complex, now under the control of Wnt signaling. Considering this important crosstalk between BMP/Smad and other signaling pathways, we investigated the role of BMP/Smad signaling in CypD gene, Ppif, repression in a cell line where osteogenic signaling downstream of BMP/Smad is arrested. We previously reported that the human osteosarcoma (OS) cell line 143b cannot undergo osteogenic differentiation due to Runx2 proteasomal degradation (Shapovalov, Benavidez, Zuch, & Eliseev, 2010). Even though Runx2 mRNA expression is upregulated in osteoinduced 143b-OS cells, the osteogenic pathway cascade is blocked by this post-translational regulation. We confirmed the 143b-OS cells inability to fully differentiate upon BMP2 osteogenic induction by comparing their mRNA expression of osteoblastic differentiation markers to that of human osteoblastic cell line, hFOB (Figure 5A). As seen in the ST2 and MC3T3e1 cell lines, pCMV-Smad1 transfection led to downregulation of CypD mRNA expression in 143b-OS cells (Figure 5B) and decreased Ppif promoter activity when co-transfected with the full-length 1.1kb Ppif-luc reporter (Figure 5C). Altogether, our data strongly indicate that BMP/Smad signaling exerts inhibitory effect on the Ppif gene as a direct transcriptional repressor and not as an indirect result of osteogenic differentiation (Figure 5D).

Downregulation of CypD is important for osteogenic differentiation

We previously reported that CypD knock-out (KO) mice, a loss-of-function (LOF) model of the MPTP, present with higher BMSC OxPhos function and osteogenic potential (Shum, White, Nadtochiy, et al., 2016). Moreover, RNAseq transcriptome analysis showed that CypD KO BMSCs present pro-osteogenic gene signatures (Shares et al., 2020). However, it has not yet been established that CypD downregulation is necessary for OB differentiation. To address this question, we studied the effect of CypD re-expression and CypD/MPTP gain-of-
function (GOF) on OB differentiation. It is known that acetylation of CypD at K166 increases CypD activity and MPTP opening and can be mimicked by K166Q mutation (Alkalaeva et al., 2009; Porter & Beutner, 2018). We, therefore used the pCMV6-*Ppif*<sup>K166Q</sup> vector that expresses this constitutively active CypD (caCypD) mutant tagged with c-Myc and DDK-Flag peptides to stably transfect MC3T3e1 cells. Expression of caCypD mutant was confirmed by western blot (Figure 6A). Cells expressing caCypD showed impaired osteogenic differentiation as evidenced by lower expression of OB marker genes, *Runx2*, *Alp*, and *Bglap*, at both day 7 and 14 in osteogenic media (Figure 6B).

As mentioned above, CypD LOF mouse models were created before and in our hands demonstrated the beneficial effects of CypD deletion on bone (Shares et al., 2020; Shum et al., 2020; Shum, White, Nadtochiy, et al., 2016). However, CypD GOF mouse model was not previously created except for one model that is no longer available (Baines et al., 2005). We, therefore designed a tissue specific knock-in mouse model of CypD GOF that expresses the above caCypD mutant in the presence of Cre recombinase. Briefly, *Ppif*<sup>K166Q</sup> cDNA preceded by the floxed ‘Stop’ codon and followed by an IRES sequence and eGFP cDNA was inserted into the Rosa26 locus. The presence of this transgene was confirmed with genotyping (Supplementary Figure S4). To achieve OB-specific caCypD expression, we crossed these mice with tamoxifen-inducible Col1-Cre<sup>ER<sup>2</sup></sup> mice (final cross is Col1-Cre<sup>ER<sup>2</sup></sup>; *Ppif*<sup>K166Q-eGFP</sup>). BMSCs were isolated from these skeletally mature 3 mo old male mice, expanded and induced to differentiate in osteogenic media. Addition of 4'-OH-Tamoxifen to osteoinduced BMSCs prompted recombination at day 11 (Figure 6C), coinciding with *Col1a1* expression time-point during the course of OB differentiation induced in vitro (Safadi et al., 2009). Translation of caCypD mutant was confirmed by western blot (Figure 6D). Cells were harvested at day 14 of osteoinduction and analyzed. Osteoinduced caCypD-expressing cells presented with decreased osteogenic differentiation and OB markers when compared to control cells (Figure 6E, F). Altogether, our results indicate that CypD gain-of-function impairs OB differentiation and thus downregulation of CypD is an important part of osteogenic program.

**CypD re-expression and gain-of-function reverse the beneficial effects of CypD deletion on bone phenotype in aged mice**
Higher MPTP activity and CypD expression are shown to be present in aging (Farr & Almeida, 2018; Rottenberg & Hoek, 2017; Sun, Youle, & Finkel, 2016). Additionally, CypD/MPTP upregulation are involved in several pathophysiological conditions including premature aging and degenerative diseases (Bernardi et al., 2015). Conversely, CypD deletion produces protective effects against several degenerative processes, including bone loss in aged mice (Shum, White, Nadtochiy, et al., 2016). To confirm our in vitro results and obtain further insight into the CypD/MPTP regulation of bone cell function, we investigated the effects of CypD re-expression and gain-of-function in vivo. For this, we used a viral delivery of the above caCypD mutant (Ppif\textsuperscript{K166Q}) in a mouse model where CypD was initially deleted. In our experimental design, OB-specific CypD deletion (Col1-Cre\textsuperscript{ER\textsuperscript{R2}; CypD\textsuperscript{fr}) was induced beginning at 2 months of age. These mice were aged to 22 mo while maintaining CypD deletion with bimonthly tamoxifen boosts (Figure 7A). Cre\textsuperscript{-} mice (CypD\textsuperscript{fr}) were also injected with tamoxifen and used as controls. To rescue CypD expression and achieve CypD GOF, we designed a CRE-DIO (Saunders, Johnson, & Sabatini, 2012) viral system carrying Ppif\textsuperscript{K166Q} and eGFP cDNA. The CRE-DIO system allows expression of the gene of interest only in Cre\textsuperscript{+} cells in the animal, therefore providing specificity. The AAV2-CRE-DIO-Ppif\textsuperscript{K166Q-eGFP} vector (AAV-DIO, Figure 7B) was introduced via intra-bone marrow injection in the tibiae of the above mice. Contralateral tibiae were injected with sterile PBS solution as an intra-mouse control. Two months after the viral infection and recombination, eGFP signal confirming a successful CypD re-expression was present in the AAV-DIO injected tibiae of Cre\textsuperscript{+} but not Cre\textsuperscript{-} mice (Figure 7C and Supplementary Figure S5A). CypD re-expression and GOF also led to decreased OB function in vivo as confirmed by decreased osteocalcin immunofluorescence (Figure 7D), recapitulating the results we have observed in vitro (Figure 6E and 6F). Since OB function is described to partially regulate osteoclast formation, recruitment and maturation, we analyzed osteoclast activity by tartrate-resistant acid phosphatase (TRAP) staining (Figure 7E), and found no differences between the AAV-DIO injected tibiae of Cre\textsuperscript{+} and Cre\textsuperscript{-} mice. An important observation in this experimental design is that OB-specific CypD deletion is in fact protecting against bone loss in aging, corroborating our previous results when we investigated the effects of CypD global deletion in mice (Shum, White, Nadtochiy, et al., 2016). For instance, biomechanical testing showed increased torsional rigidity in control tibiae of Cre\textsuperscript{+} (CypD\textsuperscript{-/-}) mice when compared to Cre\textsuperscript{-} (CypD\textsuperscript{+/+}) mice (Figure 7H), indicative of an increased bone strength. Bone volume fraction measured with μCT, a strong predictor of bone strength (Nazarian, von Stechow, Zurakowski, Muller, &
Snyder, 2008), provided further evidence that OB-specific CypD deletion effectively protects against bone degeneration in aging (Figure 7G). Although bones with AAV-DIO-delivered CypD re-expression showed decreased cortical thickness and torsional rigidity when compared to their respective contralateral PBS-injected tibiae (Figure 7F and 7H), we further analyzed our data using data normalization. In both Cre− and Cre+ mice, AAV-DIO injected tibiae were normalized to the contralateral PBS-injected limb to account for unspecific differences in bone phenotype between animals, differences caused by CypD deletion among experimental and control mice, and unforeseen effects of virus infection. Normalized data showed a significant decrease in cortical thickness in the virus-infected Cre+ mice (Figure 7I) and a decrease in tibial bone volume fraction in these mice which did not reach statistical significance (Figure 7J). These changes were sufficient to decrease tibial biomechanical properties and increase bone fragility in virus-injected bones from Cre+ mice when compared to Cre− mice (Figure 7K-M). Since bone homeostasis is achieved by the interplay of distinct tissues and cell populations such as BMSCs, OBs, osteoclasts, myeloid, lymphoid, endothelial, sensory, and myogenic cells; as expected, some bone morphological parameters are not affected by CypD/MPTP manipulation targeted in OBs (Supplementary Figure S5B-S). Nonetheless, our aggregated data present strong evidence that OB-specific CypD re-expression and GOF detrimentally affect OB function and it is sufficient to impair bone phenotype in aging.
DISCUSSION

Cellular differentiation usually challenges cells with significantly increased energetic demands when compared to the undifferentiated state. Fully differentiated cardiomyocytes and neuronal cells are characterized by a higher demand of energy and therefore an elevated mitochondrial activity when compared to their undifferentiated pairs. Such energetic shift requires OxPhos activation which relies on a higher ΔΨm and proton motive force, fueled by increased electron flow in the electron transport chain. There is a mounting evidence that an important part of the above-mentioned bioenergetic switch is a closure of the MPTP. As cells become more active, OxPhos activation leads to increases in ROS, byproducts of active respiration. ROS can then trigger MPTP opening, leading to loss of integrity of the IMM and of OxPhos function and ultimately prompting mitochondrial dysfunction, inflammation, and catabolic pathways (Carraro & Bernardi, 2016; Fayaz, Raj, & Krishnamurthy, 2015). Although the energetic state of mitochondria can be regulated through several pathways, CypD is an important indirect regulator of OxPhos and the key opener of MPTP (Alavian et al., 2014; Beutner et al., 2017; Porter & Beutner, 2018). MPTP closure is beneficial for OxPhos activity, desensitizing mitochondria to higher ROS levels, and preserving mitochondrial membrane integrity.

OB function involves bone matrix deposition, an energy-consuming process, which according to our and others’ data, is highly dependent on mitochondrial bioenergetics. In fact, our lab and others have demonstrated that BMSCs shift their bioenergetic profile towards OxPhos during OB differentiation (Colaianni et al., 2018; Feigenson et al., 2017; Shares et al., 2018; Shum, White, Mills, et al., 2016; Smith & Eliseev, 2021; Yu et al., 2019). Not surprisingly, we recently reported that higher OxPhos in BMSCs correlates with better spinal fusion outcomes in both human patients and in a mouse model (Shum et al., 2020). Taken together, the evidence collected in other groups’ studies, our previous studies, and the present study points that OxPhos is activated while MPTP is inhibited via CypD downregulation during OB differentiation. Our data corroborate reports of CypD downregulation and MPTP closure during neuronal differentiation in developing rat brains, as well as during cardiomyocyte differentiation in developing mouse hearts (Eliseev et al., 2007; Hom et al., 2011; Lingan et al., 2017).

The effects of CypD on cell functioning have been studied for 35 years. Firstly, CypD was exclusively attributed to regulate cell death, however later studies provided further understanding of CypD function within
mitochondria and its importance for cell physiology and bioenergetic response. The CypD KO mouse model has been vastly applied to study the effect of MPTP downregulation in vivo. CypD deletion is shown to present a protective phenotype against aging in several tissue-specific mouse models. Additionally, CypD ablation in mice also resulted in diminished disease progression for some pathologies, such as reperfusion injury of the heart and brain, axonopathy, and other neurodegenerative and demyelinating diseases (Ahier et al., 2018; Baines et al., 2005; Gauba, Chen, Guo, & Du, 2018; Giorgio et al., 2010; Halestrap & Richardson, 2015; Schinzel et al., 2005). In contrast, CypD over-expression or over-activation is related to the onset and progression of several pathological states, either during development or in aging. Cardiomyocytes unable of downregulating CypD during embryonic development can cause congenital heart defects, secondary to defects in MPTP activity and myocyte differentiation (Hom et al., 2011; Lingan et al., 2017). The progression of Alzheimer’s disease is accelerated by higher CypD levels and MPTP activity, leading to further mitochondrial stress and neuronal exhaustion (Alaviani et al., 2014; Gauba et al., 2018). Collagen VI myopathies are well described and established pathologies where CypD over-expression plays a major role, and as expected, treatment with CypD inhibitors can reverse the disease (Giorgio et al., 2010; Zulian et al., 2014).

In bone tissue, we have shown that BMSCs from CypD KO mice have higher osteogenic potential and OxPhos activity. Moreover, mice with CypD ablation presented with less osteoporosis burden, stronger bones in aging, and improved fracture healing (Shares et al., 2020; Shum, White, Nadtochiy, et al., 2016). However, the importance of CypD/MPTP downregulation for OB differentiation and bone maintenance was not totally clear. In this study, we used a CypD/MPTP GOF model that stably expressed caCypD (PpiK166Q mutant) in either MC3T3-e1 cell line or in primary cells from a novel OB-specific CypD GOF mouse model. Acetylation of CypD at K166 position increases CypD activity and MPTP opening and can be mimicked by K166Q mutation (Alkalaeva et al. 2009; Porter and Beutner 2018). The development of this mouse model allowed us to investigate the importance of CypD downregulation during osteogenic differentiation in primary mouse cells. Both MC3T3e1 cells and BMSCs showed decreased osteogenic markers, impaired OB differentiation and function at the time caCypD was expressed (from D0 in caCypD-transfected MC3T3e1 cells, and from D11 in osteoinduced BMSCs from CypD GOF mouse). In our in vivo model, CypD re-expression and GOF reversed the beneficial effects of CypD deletion on bone phenotype in aged mice. We have shown that CypD KO mice are protected against bone loss
in aging, and present stronger bones when compared to wild type control littermate mice (Shum, White, Nadtochiy, et al., 2016). CypD re-insertion in OBs in mice with OB-specific CypD deletion by tibial intra-bone marrow viral infection, delivering the \( Ppif^{K166Q} \) transgene, rescued bone mechanical properties observed in aging. Our data suggests that OB-specific CypD/MPTP overactivation \textit{in vivo} is related to a weaker bone phenotype as seen in advanced bone loss and osteoporosis. This result goes along with studies showing that CypD/MPTP upregulation is present in several tissues during aging and as discussed, such upregulation plays a role in the onset and progression of several degenerative pathologies. However, a limitation in our \textit{in vivo} model is the fact that we have not analyzed vertebral bones, which are more relevant in the context of osteoporosis.

CypD is regarded as the master regulator of MPTP and a range of post-translation modifications have been described to exert regulatory properties on CypD activity and, consequently, on MPTP opening. However, how CypD expression is transcriptionally regulated has never been described. We found through an \textit{in silico} analysis that \( PPIF \) promoter has multiple SBEs in the 1.1kb 5’ upstream region of both human \( PPIF \) and mouse \( Ppif \) gene. Smads are TFs downstream of the BMP signaling pathway and since OB differentiation is controlled at least in part via BMP/Smad signaling, we hypothesized that in the case of \( Ppif \), Smads are inhibitory TFs. BMPs are a group of cytokines belonging to the Transforming Growth Factor-\( \beta \) (TGF-\( \beta \)) superfamily of proteins, which among other functions, induce stem cell differentiation. The BMP canonical pathway is Smad dependent, which upon phosphorylation and Smad4 binding, forms a heterodimeric complex. The complex translocates into the nucleus and activates or represses expression of a variety of genes (Nickel & Mueller, 2019; Wang et al., 2014). As expected, our DNA-protein binding ChIP analysis revealed stronger Smad1-\( Ppif \) interaction when ST2 cells were supplemented with BMP2 for 24 hours compared to vehicle-control treated cells. Although Smad1-\( Ppif \) interaction was found to be present at lower levels in the non-BMP treated ST2 cells, this finding is consistent with the fact that intrinsic levels of BMP activity are present in undifferentiated osteogenic cells. Since we have shown that pCMV-Smad1 transfection in ST2 and MC3T3e1 cells downregulated CypD mRNA expression, our results demonstrate that increased Smad1 interaction with CypD gene (\( Ppif \)) promoter leads to \( Ppif \) transcriptional repression. The role and functionality of BMP/Smad signaling in \( Ppif \) transcriptional repression was further confirmed and delineated using the \( Ppif \)-luc reporter with or without the addition of BMP/Smad inhibitors. However, the cross-talk between BMP and Wnt signaling could indirectly repress \( Ppif \) activity by other
means than just Smad1-\textit{Ppif} binding. Our \textit{in silico} analysis revealed potential TCF/LEF1 binding sites within the \textit{Ppif} promoter. Moreover, Runx2 is a downstream target of BMP/Smad signaling known to regulate several genes responsible for growth control and differentiation. Other TFs downstream Runx2 activation could also directly/indirectly exert regulatory function on \textit{Ppif} transcription. Therefore, we investigated the role of BMP/Smad signaling in CypD downregulation and \textit{Ppif} repression in 143b-OS cells, where osteogenic signaling downstream of BMP/Smad is arrested. Confirmatory results for 143b-OS cells strongly indicate that BMP/Smad signaling is in fact a direct transcriptional repressor of \textit{PPIF} and is sufficient to repress \textit{PPIF} without any additional downstream differentiation signals.

To our knowledge, this is the first evidence for TF-mediated regulation of \textit{PPIF} transcription. Although our main focus was on osteogenic cells, our findings can be potentially extrapolated and studied in other cell types. 143b-OS cells results indicate that Smad-mediated CypD downregulation/\textit{Ppif} repression is not exclusive nor indirect to osteogenic differentiation. As previously discussed, BMP is ubiquitously expressed and described to induce cellular differentiation and maturation in various tissues. Accordingly, some cells induced by BMP/Smad signaling, reprogram their metabolic profile to a higher energetic state during differentiation and are shown to decrease CypD mRNA expression and/or activity, such as cardiomyocytes and neuronal cells. We previously reported that BMP2 induction stimulate mitochondrial OxPhos during OB differentiation and that such activation is at least, partially driven by a metabolic signaling (Smith and Eliseev 2021). Myocardial differentiation from cardiac progenitors is regulated by BMP/Smad signaling. BMP2/4 conditional KO impairs myocardial differentiation by drastically reducing sarcomeric myosin (Wang et al., 2014). In neuronal cells, BMP signaling is pivotal for cellular fate specification in both neurogenesis and astrogliogenesis by regulating transcriptional activity (Bond, Bhalala, & Kessler, 2012). Several BMP ligands, including BMP2, are described to regulate central nervous system development and patterning. Therefore, it is logical to suggest that CypD downregulation mediated by BMP-dependent, R-Smad (Smad1, 5, 8) transcriptional repression of \textit{Ppif} gene can be present in other tissues during development. Overall, our findings establish BMP/Smad signaling as a transcriptional regulator of \textit{PPIF} expression. We also provided evidence that CypD downregulation is important during osteogenic differentiation and that restoring CypD expression in OBs during aging can be detrimental to bone phenotype and strength. Even though aged skeleton is characterized by a very low turnover rate, notably, CypD
rescue for a total period of only 2 Mo was sufficient to decrease bone mechanical properties. These results highlight the importance of CypD regulation for a proper bone maintenance, and positions CypD as a potential target for bone health. In fact, there are plenty of evidence showing that CypD can be therapeutically targeted to treat some pathological conditions. Studies in the heart and brain have shown that CypD inhibitors can protect against injury after reperfusion in animal models (Halestrap & Richardson, 2015; Warne et al., 2016). However, the lack of target validation and better understanding of CypD regulatory pathway has contributed to failed Phase III clinical trials (Briston et al., 2019; Nighoghossian et al., 2015; Ong et al., 2017). Therefore, our findings can be applied to develop new strategies to downregulate CypD expression. At first, targeting CypD overexpression can enhance bone biomechanical properties in aging, and the similar strategy can be used to other fields of mitochondrial-mediated human pathologies characterized by CypD/MPTP deregulated activity.
METHODS

**Mouse strains:** C57BL6/Tg(Rosa26-Ppif\textsuperscript{K166Q-eGFP})/J, CypD GOF, mice were generated by the combined effort of our lab and Dr. George Porter’s lab in the University of Rochester Gene Targeting and Transgenic Core Facility. We used the CTV vector (CAG-STOP-eGFP-Rosa targeting vector, Addgene) described by Dr Changchun Xiao (a modification from the original vector in the Klaus Rajewsky lab) to knock-in the transgene \textit{Ppif}\textsuperscript{K166Q} construct in embryonic stem (ES) cells. In brief, cDNA containing Myc-DDT(Flag) tag on its N-terminal was cloned into the CTV vector containing NeoSTOP cassette flanked by loxP sites. This vector also contains IRES-eGFP for easy detection of a transgene expression. This vector was designed for CypD gain-of-function while inserted at Rosa26 locus. Transformed ES cells were injected into the blastocysts to produce chimeric mice followed by breeding the chimeras to germline transmitted offspring containing the knock-in \textit{Ppif}\textsuperscript{K166Q} gene.

After the creation of first transgenic hemizygous mouse littermates, the colony was expanded and later bred in house with OB-specific tamoxifen-inducible Col1-Cre\textsubscript{ERT2} mice (2.3kb variant, final cross is Col1-Cre\textsuperscript{ERT2}; \textit{Ppif}\textsuperscript{K166Q-eGFP}). Col1-Cre\textsuperscript{ERT2} mice were a kind gift from Dr. Ackert-Bicknell (formerly of University of Rochester, originated in the Karsenty Lab at Columbia University Department of Genetics). CypD floxed (CypD\textsuperscript{lof}) mice were acquired from Jackson Laboratories (originated in the Korsmeyer lab at Dana Farber Cancer Institute, RRID: IMSR_JAX:005737) and bred in house with Col1α1-Cre\textsubscript{ERT2} mice to allow OB-specific CypD deletion. C57BL/6J mouse strain was obtained from the Jackson Laboratory (RRID: IMSR_JAX:000664) and bred in house. All mice were housed at 23°C on a 12-h light/dark cycle with free access to water and PicoLab Rodent Diet 20 (LabDiet #5053, St. Louis, MO). Mice were in group housing when applicable based on weaning. Testing naïve mice with an average weight of 28 g were used for experiments. The assessments of animal studies were performed in a blinded and coded manner.

**Isolation of BMSCs:** Primary bone marrow cells were harvested from femurs and tibiae bone marrow from Col1-Cre\textsuperscript{ERT2}; \textit{Ppif}\textsuperscript{K166Q-Tg}\textsuperscript{0} and Cre negative control littermates or from C57BL/6J mice. Cells were plated at a density of 20x10\textsuperscript{6}/10 cm dish in physiological ‘low’ glucose (5 mM) DMEM (LG-DMEM) supplemented with 1 mM L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and incubated at 37 °C, 5% CO\textsubscript{2}, and physiologically relevant 5% O\textsubscript{2}. BMSCs were selected by plastic adherence, and detached from plates using 0.25% trypsin/1 mM EDTA treatment, as previously described (Shum, White, Mills, et al., 2016).
Mouse cell lines: Mouse long bone-derived ST2 cells were a gift from Dr. Clifford Rosen. Mouse calvarial bone-derived MC3T3-E1 cells were acquired from ATCC. Cells were expanded in sterile conditions and maintained in a 37°C incubator at 5% CO₂ in low glucose αMEM media (Gibco A10490–01) containing 1 mM L-glutamine, ribonucleosides (0.01 g/L each), deoxyribonucleosides (0.01 g/L each), no ascorbic acid, 10% FBS (Gibco 10437–028) heat inactivated for 30 min at 55°C, and 1% Penn/Strep (Gibco 15140–122). Cells were maintained at passage numbers <20 as recommended by the supplier and from previous handling experience.

Human osteoblastic cell line, hFOB: hFOB cells were purchased from ATCC and expanded in DMEM supplemented with 1 mM L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and incubated at 37 °C, 5% CO₂, and physiologically relevant 5% O₂. We used passage number between 3 and 7 for our experiments (Giang et al., 2013; Shapovalov et al., 2010).

Human osteosarcoma cells: 143b human osteosarcoma cells were acquired from ATCC (Manassas, VA). Cells were expanded in sterile conditions and maintained in a 37°C incubator at 5% CO2 in DMEM supplemented with 1 mM L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin.

Osteoinduction: Cells were osteoinduced at confluency in their appropriate media either supplemented with 50 μg/mL ascorbate (TCI A2521) and 2.5 mM β-glycerolphosphate (USB Corp Cleveland, OH, 21655) for 7 and 14 days, or 50ng/mL BMP2 (R&D systems 355-BM-050/CF) for 5 days. OB differentiation was assessed by Alkaline Phosphatase (Thermo NBT/BCIP 1-step 34042) and Alizarin Red staining. Crystal Violet stain (Sigma C3886) was used at 0.5% to determine total cell count as previously described (Shares et al., 2018).

Real-time RT-qPCR: Total RNA was isolated using the RNeasy kit (Qiagen 74106) and reverse transcribed into cDNA using the qScript cDNA synthesis kit (Quanta 95048–500). cDNA was subjected to real-time RT-PCR. The primer pairs used for genes of interest are outlined in Key Resources Table. Real-time RT-PCR was performed in the RotorGene system (Qiagen) using SYBR Green (Quanta 95072–012). Alp and Bglap gene expression was used to confirm osteogenic differentiation.

MPTP opening Calcein-cobalt assay: To assess the MPTP activity, mitochondrial membrane integrity was measured using a method of calcein quenching by cobalt as described by Petronilli et al. in 1999. The assay is based on the fact that calcein accumulated in the cytosol is quenched after co-loading with cobalt, whereas calcein accumulated in mitochondria is not accessible to cobalt and therefore not quenched unless mitochondrial
membranes are permeabilized. Cells were incubated with 1μM calcein-AM (acetoxymethyl ester) in the presence of 1mM cobalt chloride for 30 min at 37 °C, washed, lifted from plates using a cell lifter, resuspended in phosphate buffered saline solution (PBS), and assayed using the BD Biosciences LSRII flow cytometer. Ionomycin at 1μM was used as negative control in a set of calcein/cobalt-loaded cells to dissipate calcein signal.

**Mitochondrial mass assay:** Cells were stained with nonyl acridine orange (NAO) at 100 nM, a fluorescent probe that labels cardiolipin present primarily in mitochondrial membranes (Beutner, Eliseev, & Porter, 2014), for 15 min at 37 °C. Stained cells were then lifted from plates with cell lifter, washed, and resuspended in PBS. NAO signal was detected in BD Biosciences LSRII flow cytometer.

**Western blot:** Cells were lysed in a lysis buffer containing protease inhibitors and subjected to 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes and blocking in 5% dry milk reconstituted in PBST (PBS supplemented with Tween 20 at 0.05%), as previously described (Shum, White, Mills, et al., 2016). All antibodies were diluted in 2.5% dry milk in PBST. For CypD detection, blots were probed with monoclonal CypD antibody (RRID: AB_478283) diluted 1:1000 and HRP conjugated goat anti-mouse antibody diluted 1:3000. To verify equal loading, blots were re-probed with β-actin antibody (RRID: AB_476697) diluted 1:2000 and HRP conjugated goat anti-mouse antibody diluted 1:5000. CypD and β-actin signals were developed with West Pico Substrate. Band densitometry was measured with Image Lab™ Software. CypD signal was normalized to β-actin.

**Tibial intra-bone marrow AAV2(serotype 2)-Cre-DIO; Ppif<sup>K166Q</sup>-eGFP infection:** Transgene Ppif<sup>K166Q</sup> construct was cloned into the Double-floxed Inverted Orientation (DIO) Cre-On vector pAAV-Ef1a-DIO-eGFP-WPRE-pA (Addgene plasmid # 37084 ; http://n2t.net/addgene:37084 ; RRID:Addgene_37084 - gift from Bernardo Sabatini - (Saunders et al., 2012)). DIO vector was cloned using the Ascl and Nhel restriction site, and transgene introduced through transgene-specific primers and PCR amplification. The Ascl site was N-Terminal and the Nhel site C-Terminal with respect to the transgene. Cloning and sequence confirmation was performed. Cloned vectors were amplified with recombination deficient bacteria (OneShot Stbl3, Invitrogen) and tested functionally for Cre-On expression by calcium phosphate transfection (Invitrogen) into HEK 293 cells constitutively expressing Cre. Col1-Cre<sup>ERT2</sup>; CypD<sup>ff</sup> and Cre-negative CypD<sup>ff</sup> littermate mice were anesthetized using 100 mg/kg Ketamine and 10 mg/kg Xylazine IP at a rate of 0.1ml per 10 grams of body weight. Hair was shaved...
around the joint area and 70% alcohol and iodine were used to clean the area. A 1 ml syringe with a 26 (3/8 length) gauge needle was inserted into the intra-bone marrow tibial space by gentle twisting and pressuring at the top of the tibiae (proximal epiphyses) in the knee joint. The hole created by the 26G needle was expanded by lateral and whirling movements. The 26 gauge needle was removed and a Hamilton syringe with a 22S needle gauge (Z15364-8, 100μl Hamilton, Gastight 1800, needle Gauge 22S) was inserted into the expanded hole created by the 26G needle. Ten microliters of viral solution (1.8x10^{13} viral particles/mL AAV2-Cre-DIO; Ppir^{K166Q}) was slowly injected by free hand into the marrow space of the tibiae (injection technique and viral load adapted from Selenica et al. (Selenica et al., 2016)). Contralateral tibiae was injected with 10μL of sterile PBS solution as an intra-mouse control. Immediately after the viral injection, 100μL of 3mg/mL tamoxifen was intra-peritoneally injected 5 days in a row to induce Cre activation. eGFP signal confirming viral infection and subsequently OB recombination was captured by in vivo image system IVIS® 30 days and 60 days post-infection.

**In Vivo Imaging System (IVIS®):** Under isoflurane anesthesia, lower limbs from virus-infected mice were shaved and imaged for eGFP signal using IVIS® Spectrum (Caliper Life Sciences). A negative control mouse, which received only PBS intra-bone marrow injection on both tibiae, was used to subtract non-specific eGFP signal arising from bone autofluorescence.

**Bone micro-computed tomography (μCT):** following euthanasia, virus and PBS-injected tibiae were isolated and cleaned of excess soft tissue. Tibiae were stored at −80 °C prior to μCT. Bones were imaged using high resolution acquisition (10.5 μm voxel size) with the VivaCT 40 tomograph (Scanco Medical). Scanco analysis software was utilized for volume quantification.

**Biomechanical torsion testing:** immediately following μCT scanning, tibiae were subjected to biomechanical testing. The ends of the tibiae were cemented (Bosworth Company) in aluminum tube holders and tested using an EnduraTec TestBench™ system (Bose Corporation, Eden Prairie, MN). The tibiae were tested in torsion until failure at a rate of 1°/sec. The torque data were plotted against rotational deformation to determine maximum torque and torsional rigidity. Data from virus-injected tibiae were normalized to the contralateral PBS-injected (intra-mouse control) tibiae data.

**Histology:** After biomechanical testing, tibiae bones were NBF-fixed and processed for histology via decalcification in Webb-Jee 14% EDTA solution for one week followed by paraffin embedding. Sections were
cut to 5 μm in three levels of each sample, and then stained with either TRAP or immunofluorescence (IF).

**Immunofluorescence:** NBF-fixed tibiae were processed as above. IF was carried out using a primary anti-osteocalcin antibody (RRID:AB_10540992) diluted 1:400 or anti-GFP antibody (RRID:AB_303395) diluted 1:500, followed by incubation with anti-rabbit IgG secondary antibody conjugated with Alexa Fluor®647 (RRID:AB_2722623) diluted 1:2000 or with anti-rabbit IgG secondary antibody conjugated with Alexa Fluor®488 (RRID:AB_2630356), respectively. The primary antibody solution was composed of PBS, 0.1% Tween-20 and 5% Goat Serum. Prior incubation at 65°C in 10mM sodium citrate (pH 6.0) for 3 hours was performed for antigen retrieval. Fluoroshield Mounting Medium with DAPI (ab104139) was used to counterstain and coverslip IF-slides.

**Histomorphometry:** TRAP or IF-stained slides were scanned in an Olympus VSL20 whole slide imager at 40x magnification and evaluated with VisioPharm automated histomorphometry software. TRAP-stained slides were analyzed to measure the TRAP positive area relative to total bone area in the tibiae shaft. IF-stained slides probed for osteocalcin were analyzed to measure the fluorescence signal intensity relative to total bone area of tibiae shaft. Three different levels were counted per mouse and averaged.

**Chromatin immunoprecipitation assay:** Chromatin immunoprecipitation assay was performed using SimpleChIP® Enzymatic Chromatin IP Kit - Magnetic Beads (Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's instructions. Briefly, cells were fixed and DNA cross-linked with formaldehyde and homogenized. Nuclei were pelleted and chromatin was digested enzymatically, sonicated and immunoprecipitated with either anti-Smad1 antibody (RRID:AB_628261), or negative control immunoglobulin G, or positive control Histone H3 antibody using protein G magnetic beads. Chromatin was eluted from immunoprecipitate complexes and cross-links reversed with NaCl and proteinase K. After purification, DNA was amplified by a PCR reaction using primers to amplify the distal SBE region (-1107 to -947) within the *Ppif* promoter.

**Cloning of *Ppif* promoter into luciferase reporter and reporter activity assay:** Mouse *Ppif* promoter fragments containing the -1.1kb to -0.1kb, or -0.37kb to -0.1kb, or -0.62 to -0.45kb, or -1.1 kb to -0.95kb, or -0.62kb to -0.1kb, or -1.1kb to -0.45kb region were PCR amplified from purified C3H/HeJ mouse DNA. Primers were designed to introduce CTCGAG Xhol 5’ and AAGCTT HindIII 3’ flanking sequences. The fragments were then subcloned into the Xhol 3’/HindIII 5’ site of the promoterless pGL4.10 luciferase reporter vector. The correct
Insert orientation of the resulting promoter reporters was verified by sequencing. To evaluate promoter activities, the constructed Ppif-Luc reporters were transfected into ST2, MC3T3e1, or 143b-OS cells at 0.8µg per well in twelve-well plates. The promotorless renilla luciferase vector pRL (Promega, Madison, WI, USA) was cotransfected at 50ng per well as a reference. Smad1 activity was further activated with either 0.8µg/mL pCMV-Smad1 co-transfection, 50ng/mL BMP2, or osteogenic media induction. The role of BMP/Smad was delineated by co-transfecting inhibitory Smad7 using 0.8 µg/mL pCMV-Smad7 vector, or using BMP inhibitor, Noggin (R&D systems 1967-NG-025/CF) at 0.1 µg/mL. Firefly and renilla luciferase activities were measured using an Optocomp 1 luminometer and a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol. The firefly luciferase signal was normalized to renilla luciferase signal and expressed as relative luminescence units fold change to pCMV empty vector control.

Statistics: A power analysis on normalized biomechanical data was performed since it showed the highest variance. It was determined that some quantitative outcomes would require 6 mice per group. We set the significance level at 5% (α=0.05) and Type II error (β) to ≤20%. For statistical analysis, we compared the difference of two simple groups independently, therefore an unpaired t-test was used when the frequency distribution of the differences between the two groups fitted a normal distribution. When left and right tibiae from the same animal was compared, we used a paired t-test. Although we analyzed independent variables and therefore independent hypothesis in the multi-group graphs (CypDf/f: Cre+ or Cre- x AAV-DIO or PBS), we performed Ordinary one-way ANOVA using Dunnett’s multiple comparisons test with a single pooled variance to further validate our statistical findings significance. Since no differences in significance were found, we maintained our t-test results.
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FOOTNOTES
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The abbreviations used are:

AAV2 - adeno associated virus serotype 2

Alk P - alkaline phosphatase

Ar - area

AR - alizarin red

ATP - adenosine triphosphate

BM - bone marrow

BMP - bone morphogenic protein

BMSC - bone marrow stromal (a.k.a. mesenchymal stem) cell

BV/TV - bone over total volume

caCypD - constitutively active CypD

Calcein AM - calcein acetoxymethyl

ChIP - chromatin immunoprecipitation

Cross-Sec - cross-sectional

Ct - cortical area

CV - crystal violet

CypD - cyclophilin D

D+M - distal + middle

DIO - double-floxed inverted orientation

EV - empty vector

f/f - floxed/floxed
GOF - gain-of-function
IMM - inner mitochondrial membrane
iPSC - induced pluripotent stem cell
K166Q - substitution of lysine at position 166 for glutamine
KO - knock-out
LOF - loss-of-function
Luc - luciferase
MOI - moment of inertia
MPTP – mitochondrial permeability transition pore
M+P - middle + proximal
N - number
OB - osteoblast
OxPhos - oxidation phosphorylation respiration
ROS - reactive oxygen species
SBE - smad binding element
Sp - space
Tb - trabecular
TF - transcription factor
Tg/0 - hemizygous transgene
Th - thickness
TRAP - tartrate-resistant acid phosphatase
VDAC1 - voltage-dependent anion channel 1
Δψm – mitochondrial membrane potential
**FIGURE LEGENDS**

**Figure 1.** CypD gene, *Ppif*, promoter contains multiple Smad binding elements and Smad1 overexpression leads to downregulation of CypD. A) Several Smad-binding elements (SBE) were found on both human (*PPIF*) and mouse (*Ppif*) CypD gene promoter. ST2 or MC3T3e1 cells were transfected with pCMV-Smad1 vector or empty vector (EV) control; B) and C) Real-time RT-PCR data demonstrating the efficiency of Smad1 transfection and that Smad1 overexpression upregulated *Runx2* while downregulated *Ppif* mRNA expression. Plot shows the actual data points (biological replicates), calculated means and *P* value determined by an unpaired *t*-test.

**Figure 2.** BMP signaling promotes Smad1 interaction with *Ppif* promoter. ChIP assay of nuclear fractions from ST2 cells incubated either in the presence of 50 ng/ml BMP2 for 24h, or vehicle. A) PCR analysis of the ChIP assay was performed using primers to amplify the distal SBE-containing region within the *Ppif* promoter (B); Positive control Histone H3 signal showing that proper DNA fragmentation was achieved (A); C) Band density quantification for Smad1 immunoprecipitation was adjusted by background subtraction and normalized to total DNA input. Unspecific signal from IgG band was also subtracted from Smad1-specific signal. Plot shows the actual data points representing the mean of two technical replicates from three independent experiments, calculated means and *P* value determined by an unpaired *t*-test.

**Figure 3.** BMP-dependent Smad1 transcriptionally represses *Ppif* promoter activity. Dual luciferase reporter assay was performed on either ST2 or MC3T3e1 cells 48h after luciferase (Luc) reporter transfection. A) Diagram: 1.1kb full-length *Ppif* promoter region containing several SBEs was cloned into the pGL4.10 vector; B) and E) pCMV-Smad1 co-transfection highly activated the BMP/Smad signaling reporter 12xSBE; C) pCMV-Smad1 co-transfected with the 1.1kb *Ppif* full length luc-reporter downregulated the luciferase signal. Inhibitory Smad7 rescued *Ppif* promoter activity; D) Osteogenic media or 50 ng/ml BMP2 was used to activate BMP/Smad signaling. The BMP inhibitor, Noggin, rescued *Ppif* promoter activity; F) MC3T3e1 cells showed similar effects...
on *Ppif* promoter activity after BMP/Smad signaling activation. Plot shows the actual data points (biological replicates), calculated means and *P* value determined by an unpaired *t*-test.

**Figure 4. CypD expression and MPTP activity are downregulated during osteogenic differentiation.** ST2 stromal cells, MC3T3e1, or mouse BMSCs were cultured in osteogenic media; RNA collected and staining done at day 0, day 7, and day 14. A) Staining representative image at day 14 for Crystal Violet (CV) a proxy of total cell content, Alkaline Phosphatase (Alk P), and Alizarin Red (AR); B) Staining quantification confirming osteogenic commitment; C), D), and E) Real-time RT-PCR data: osteogenic markers (*Alp*, and *Bglap*) are upregulated, whereas *Ppif* mRNA expression is downregulated; F) BMSCs subjected to Calcein-Cobalt assay, showed decreased MPTP activity upon osteogenic differentiation; G) BMSCs stained with Nonyl Acridine Orange (NAO) show no difference in mitochondrial mass. Plot shows the actual data points (biological replicates), calculated means and *P* value determined by an unpaired *t*-test.

**Figure 5. Smad1-mediated CypD downregulation/Ppif repression is not dependent on signaling downstream of BMP/Smad.** Immortalized human osteoblastic cells (hFOB) or osteosarcoma cells (143b) were treated with 50 ng BMP2 for 5 days to induce osteogenic differentiation. BMP2 induced early (*RUNX2, ALP*) but not late (*IBSP, BGLAP*) OB markers in 143b-OS cells, reflecting differentiation-arrested phenotype; B) Real-time RT-PCR data demonstrating the efficiency of Smad1 transfection and that Smad1 overexpression downregulated *Ppif* mRNA expression in 143b cells; C) pCMV-Smad1 co-transfected with the -1.1kb *Ppif* full length luc-reporter downregulated the luciferase signal; D) Schematic representation of our summary of results. To maintain BMSC commitment to the osteogenic lineage, closure of the MPTP is required, which is achieved by CypD downregulation through Smad1 transcriptional repression of *Ppif* gene. Plots show the actual data points (biological replicates), calculated means and *P* value determined by an unpaired *t*-test.
Figure 6. CypD re-expression and gain-of-function impair osteogenic differentiation. MC3T3e1 stably transfected cells with pCMV6-Ppif^K166Q vector to express constitutively active CypD mutant (Ppif^K166Q) and thus achieve CypD GOF, or BMSCs from OB-specific, inducible Col1-CreERt2; caCypD^Tg/0 mice were cultured in osteogenic media for 14 days. A) Representative western blot of stably transfected MC3T3e1 cells (EV: pCMV6-empty vector; KQ: pCMV6-Ppif^K166Q vector; VDAC1: loading control; caCypD: mutant CypD; CypD: endogenous CypD); B) and F) Real-time RT-PCR data of osteogenic markers: CypD GOF cells were incapable to complete OB differentiation; C) Diagram showing the Ppif^K166Q mouse transgene construct. Images show eGFP signal in cell culture confirming recombination induced in vitro; D) Representative western blot BMSCs from OB-specific, inducible Col1-CreERt2; caCypD^Tg/0 mice; E) Staining representative image at day 14 for Crystal Violet, Alkaline Phosphatase, and Alizarin Red: CypD GOF (tamoxifen-induced Cre^+ ) BMSCs showed decreased OB activity and mineralization capacity. Plot shows the actual data points (biological replicates), calculated means and P value determined by an unpaired t-test.

Figure 7. CypD re-expression and gain-of-function in vivo decreases bone mechanical properties. A) Experimental design: CypD deletion was induced in 2 mo-old OB-specific inducible (Col1-CreERt2; CypD^f/f) CypD loss-of-function (LOF) mice. Virus intra-bone marrow injection performed in the right tibia at 22 mo; B) Diagram showing the AAV2-Cre-DIO; Ppif^K166Q-eGFP construct. The gene of interest is inserted in the vector in antisense orientation and is flanked by double floxed sites. In cells expressing Cre recombinase, the gene of interest and eGFP reporter are flipped and “turned-on”; C) eGFP signal captured by IVIS in vivo confirming the successful viral infection and recombination in the tibia of Cre^+ mice 2 mo after intra-bone marrow injection; CypD re-expression decreased osteocalcin immunofluorescence signal (D) but it did not change osteoclast activity measured by TRAP staining (E) - (BM: bone marrow); Bone volumes and biomechanical properties of CypD deletion and CypD re-expressing bones were measured by μCT and a torsion test, respectively. CypD conditional deletion did not affect cortical thickness (F) however, it showed protective effect against trabecular bone volume loss in aging (G) and improved torsional rigidity (H) - (PBS: CypD^+/+ X CypD^−/−). For analysis of bones with AAV-DIO-delivered CypD re-expression, data was normalized to the contralateral PBS-injected limb to account for differences in bone phenotype between animals. Cre^+ mice showed decreased bone volumetric
parameters and mechanical properties when compared to Cre− mice; I) Cortical thickness (Ct Th); J) Bone over total volume (BV/TV); K) Torsional rigidity; L) Yield torque; M) Maximum torque. Plots show the actual data points from six independent mice per group, calculated means and $P$ value determined by an unpaired $t$-test. Paired $t$-test was used when left and right tibia from the same mouse were compared. Specimens' genotype guide:  

- **PBS_CypD**+/+: wild type;  
- **PBS_CypD**−/−: CypD conditional KO;  
- **AAV-DIO_CypD**+/+: wild type caCypD rescue;  
- **AAV-DIO_CypD**−/−: CypD conditional KO caCypD rescue.
A. PPIF promoter (human)

B. Smad1

C. Smad1

ST2 stromal cells

MC3T3e1 cells

Logfold change to EV normalized to Bm

Smad1, Runx2, Ppif
A - BMP activation

B - Smad1

C - -1.1kb Luc Reporter

D - BMP activation

- Runx2, ALP, IBSP, BGLAP

- Smad1

- Luciferase, RLU - Fold Change

- hFOB cells

- 143b OS cells

- p-values

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**AAV DIO "Cre-On"**

**Cre recombination**

**G**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**J**

**K**

**L**

**M**

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Supplementary Figure S1. BMP-dependent Smad1 transcriptionally represses only full-length *Ppif* promoter activity. Dual luciferase reporter assay was performed on ST2 cells. A) Diagram: distinct *Ppif* promoter mutant constructs containing several SBEs were cloned into the pGL4.10 vector; B) pCMV-Smad1 was co-transfected with the proximal (P), distal (D), middle (M), M+P, or D+M *Ppif* luc-reporter. Plot shows the actual data points (biological replicates), calculated means and *P* value determined by an unpaired *t*-test.
Supplementary Figure S2. Human BMSC osteogenic differentiation increases mitochondrial ROS while downregulating PPIF relative expression. Human BMSCs were cultured in osteogenic media. RNAseq was performed and MitoSox mitochondrial ROS staining done at day 0 and 14; A) D14 differentiated OBs show increased ROS production compared to D0 undifferentiated BMSCs. B) RNAseq data shows downregulation of CypD gene PPIF relative expression in osteoinduced human BMSCs, confirmed by ALP, and BGLAP osteogenic markers, when compared to undifferentiated cells. Plot shows the actual data points from three independent samples/ experiments, calculated means and P value determined by an unpaired t-test.
Supplementary Figure S3. Mouse BMSCs osteogenic differentiation downregulates CypD protein expression. Mouse BMSCs were cultured in osteogenic media and protein lysates collected at day 0 and day 14. Shown is the western blot (representative of 3) and band density quantification demonstrating decreased CypD protein level for osteoinduced vs undifferentiated BMSCs. Plot shows the actual data points, calculated means and P value determined by an unpaired t-test.
Supplementary Figure S4. CypD GOF mice genotyping. F1 generation for OB-specific inducible Col1-CreER; caCypD<sup>Tg</sup> mice were genotyped to confirm the presence of caCypD (K166Q) insert. Cre primer was used to allocate control and experimental groups. Electrophoresed gel shows DNA target amplified from three independent mice per group: Cre<sup>-</sup> control and Cre<sup>+</sup> experimental mice.
Supplementary Figure S5. Bone volumetric parameters and biomechanical properties in mice with osteoblast-specific CypD conditional deletion with or without CypD re-expression. A) Immunofluorescence eGFP signal confirming the successful viral infection and recombination in the tibia of Cre* mice 2mo after intra-bone marrow injection (BM: bone marrow). Bone volumetric and biomechanical properties of Col1-CreERt2; CypDf/f mice with or without CypD re-expression were measured by µCT and a torsion test, respectively. No differences on the following bone morphological and biomechanical parameters were found between groups - non-normalized data: B) Cortical area; C) Bone marrow area; D) Cortical cross-sectional area; E) Trabecular bone mineralization density; F) Trabecular number; G) Trabecular thickness; H) Trabecular space; I) Polar moment of inertia; J) Yield torque; K) Maximum torque. For analysis of bones with AAV-DIO-delivered CypD re-expression, data was normalized to the contralateral PBS-injected limb to account for differences in bone phenotype between animals. No differences on the following bone morphological parameters were found between Cre- and Cre* mice - normalized data: L) Cortical area; M) Bone marrow area; N) Cortical cross-sectional area; O) Trabecular bone mineralization density; P) Trabecular number; Q) Trabecular thickness; R) Trabecular space; S) Polar moment of inertia. Plot shows the actual data points, calculated means and P value determined by an unpaired t-test. Paired t-test was used when left and right tibia from the same mouse were compared. Specimens’ genotype guide: PBS_CypD+/+: wild type; PBS_CypD-/-: CypD conditional KO; AAV-DIO_CypD+/+: wild type caCypD rescue; AAV-DIO_CypD-/-: CypD conditional KO caCypD rescue.