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The Role of Tgif1 in Bone Anabolic Signal Transduction

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1 Introduction

1.1 Osteoporosis

Osteoporosis is the most common reason for fragility fractures in the elderly. Affecting approximately 10 million individuals in the United States, nearly 30 million citizens of the European Union, close to 50 million in industrialized countries and estimated 200 million people worldwide, osteoporosis represents a growing medical and socioeconomic burden in the aging society (Wade *et al.*, 2014). About 70% of the population over the age of 80 years are afflicted with osteoporosis, with Caucasian and Asian ethnicities being at greatest risk. In 2010, 3.5 million new fragility fractures were sustained in the EU, and over 9 million worldwide. In Europe, osteoporotic fractures accounted for 2 million disability adjusted life years (DALYs) annually, somewhat more than are accounted for by hypertensive heart disease or rheumatoid arthritis. The current economic burden of incident and prior debilitating fractures was estimated at 37 billion € and the costs are expected to increase by 25% in 2025 in the EU (Hernlund *et al.*, 2013). One in two women, and one in five men, at the age of 50 years is at risk for an osteoporosis related fragility fracture in their remaining lifetime (Harvey, Dennison and Cooper, 2010). Moreover, adults who had a fragility fracture are at considerably greater risk of sustaining a subsequent fragility fracture (Kanis *et al.*, 2004). Hip and vertebral fractures are the two most severe fracture types, often implicating substantial pain, disability and even death. These fractures are associated with an impaired quality of life and up to 20% mortality within the first year, either as a direct cause of trauma and hospitalization or secondary to other complications such as pneumonia or thromboembolic incidents (Center *et al.*, 1999).

Osteoporosis is characterized by a decrease in bone mineral density (BMD) and an increased propensity of fragility fractures. The BMD is a measure of mineral content, prominently calcium, serving as a surrogate for bone strength. In 1994 the World Health Organization (WHO) defined osteoporosis as a BMD of 2.5 standard deviations or more below the sex-matched mean peak bone mass of a young adult (T-score) in dual-energy X-ray absorptiometry (DXA scan). Due to weakened bone

density and microarchitectural deterioration, fractures happen after minimal or no trauma. The most common sites for osteoporotic fractures are the hip, the spine and the forearm (Sambrook and Cooper, 2006).

1.2 The Skeletal System

Bone is a highly specialized connective tissue and a versatile organ. Mechanically, the mineralized skeleton serves as the main support for the body of vertebrates. The musculoskeletal system, comprising bones, joints, tendons and ligaments as well as cartilage allows for locomotion and provides protection for vital organs such as the heart inside the rib cage or the brain inside the skull. Many bones serve elaborate functions, such as the ossicles in the middle ear, the smallest bones of the body, which facilitate hearing.

Metabolically, the skeleton constitutes the major storage site of minerals in the body, most notably calcium and phosphorous. The mineralized bone matrix stores important growth factors such as transforming growth factors (TGFs), insulin-like growth factors (IGFs), and bone morphogenetic proteins (BMPs). In addition, bone serves as an endocrine organ. Via the release of fibroblast growth factor-23 (FGF-23), bone regulates renal phosphate and vitamin D homeostasis (Wöhrle, Bonny and Beluch, 2011)(Perwad and Portale, 2011). Fatty acids, an essential source of energy, are stored in large amount in the bone marrow cavity. In children, the marrow of the long bones is the principal site of hematopoiesis, whereas in adults this occurs mainly in the pelvis, vertebrae, sternum and cranium (Fernández and de Alarcón, 2013). By housing multi-potent stem cells, the skeleton substantially conduces to cellular regeneration of multiple lineages.

With regards to their outer appearance, five types of bone can be distinguished. Long bones are found in the limbs and are characterized by a shaft, the diaphysis, and two epiphyses at the ends, e.g. femur. Short bones can be found in the wrist, e.g. carpal. Flat bones such as the sternum are comparatively thin. Sesamoid bones are embedded in tendons, e.g. patella. Irregular bones do not fit to any of the above-mentioned and have a peculiar shape, e.g. the pelvis, vertebrae or some of the skull bones.

1.3 Bone Structure

Bone can be divided into cortical and cancellous bone. Cortical bone forms the outer shell, is compact and strong and accounts for about 80% of the mass of the human skeleton. The primary anatomical and functional unit of compact bone is the osteon. It consists of columns of layered osteoblasts and osteocytes around central canals, the Haversian canals, which are connected by the perpendicularly oriented Volkmann's canals. Cortical bone is covered by periosteum on its outer, and by endosteum on its inner surface. Skeletal progenitor cells reside on these surfaces and become active during bone remodeling. The periosteum has nociceptive nerve endings and also provides blood supply and therefore nourishment for the cortex. As precursor cells enter developing and fractured bones along with sprouting blood vessels (Maes *et al.*, 2010), the periosteum largely accounts for the regenerative potential of bone (Roberts *et al.*, 2014).

Cancellous bone is located on the inside of the bone and has a trabecular structure. This is formed by the lacunar spaces and canalicular network of osteocytes, which exceeds the surface area of all Haversian and Volkmann systems by 400-fold and is particularly vulnerable in conditions such as osteoporosis (Marotti *et al.*, 1995). It can be found in the epiphyses of long bones where its spongy architecture serves hematopoiesis. The primary anatomical and functional unit of cancellous bone is the trabecula (Fig. 1.1). Predilection to fragility fractures is ultimately depending on bone architecture and resistance. Non-vertebral fracture susceptibility is mainly determined by cortical thickness and durability, whereas trabecular bone architecture and mass are major determinants of vertebral fracture risk (Zebaze *et al.*, 2010)(Holzer *et al.*, 2009).

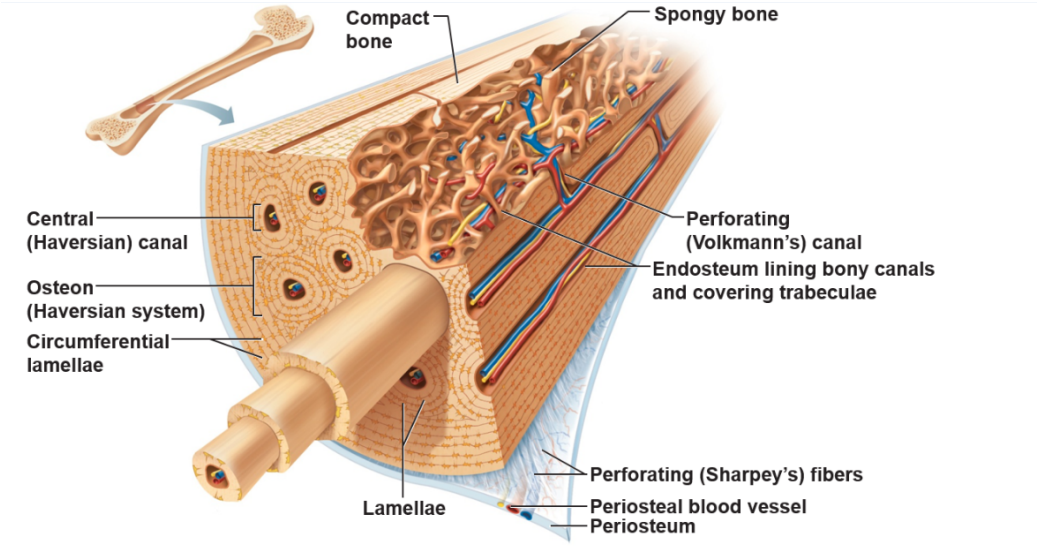

Figure 1.1: Schematic of cortical and cancellous bone structure. The primary anatomical unit of compact bone is the osteon and that of spongy bone is the trabecula. Modified from (Marieb and Hoehn, 2013).

Bone develops via intramembranous or endochondral ossification. Intramembranous ossification occurs during formation of the flat bones of the skull, and in this process bones are directly formed from the mesenchyme. During endochondral ossification, bone is formed from a hyaline cartilage template. Parathyroid hormone-related protein (PTHrP) and Indian Hedgehog (IHH) acting on their respective receptors PTH1R and PTCH1 exert a tightly coupled signaling relay, which is critical for the regulation of endochondral ossification (Silve and Jüppner, 2006). During fetal development, this process begins at primary ossification centers located in the diaphysis of long bones. Secondary ossification occurs after birth until skeletal maturity is reached, and takes place in the epiphyses (Kronenberg, 2003).

1.4 Bone Composition

Bone is composed of different cell types embedded in a mineralized matrix. This extracellular matrix (ECM) consists of an organic and an inorganic part. The former consists primarily of type I collagen fibers and secondarily of proteoglycans

such as chondroitin sulfate and glycosaminoglycans such as hyaluronic acid. Depending on the arrangement of collagen, two sub-divisions can be made: woven bone and lamellar bone. Woven bone is characterized by randomly oriented collagen fibers and is quickly built but mechanically weak. It can be found in fetal bone during development or in callus formation after fractures in the adult. Lamellar bone is highly organized in concentric sheets and columns, called osteons. This takes longer to be formed and is mechanically strong. The inorganic part of the ECM is mostly composed of calcium and phosphate in a form of $Ca_{10}(PO_4)_6(OH)_2$, i.e. hydroxyl apatite. Together, these components allow for both tensile and compressional strength of bone.

Osteoblasts (OBs) are cuboidal shaped cells that reside on bone surfaces and descend from mesenchymal stem cells (MSCs) (Pittenger *et al.*, 1999). During ossification, MSCs condense and differentiate into osteoprogenitor cells, which give rise to osteoblasts, the mature matrix forming cells (Raggatt and Partridge, 2010). Among others, this process is regulated through expression of runt-related transcription factor 2 (RUNX2) and osterix (OSX), both of which are key transcription factors and indispensable for osteoblast differentiation (Long, 2012). This becomes evident as deletions of Runx2 or Osx in mice result in complete absence of osteoblasts (Otto *et al.*, 1997)(Nakashima *et al.*, 2002). Haploinsufficiency of RUNX2 in humans leads to cleidocranial dysplasia (CCD), which is characterized by hypoplastic collar bones and delayed closure of the fontanelles, emphasizing the importance of Runx2 in bone formation (Mundlos *et al.*, 1997). Osteoblasts form bone by deposition of a layer of osteoid on the surface, which is the organic portion of the bone matrix before mineralization. Osteoid consists of fibers, mainly type I collagen, proteoglycans and osteocalcin. Within days to weeks it becomes mineralized, i.e. newly formed bone. Precipitation of calcium and phosphate is catalyzed by alkaline phosphatase (ALP), an enzyme secreted by osteoblasts. Defective mineralization of osteoid results in softening and deviation of bones, seen in rickets in children and osteomalacia in adults. ALP can be helpful in the diagnosis of impeded bone turnover and may reveal vitamin D deficiency. After the bone formation phase, osteoblasts become embedded within the matrix as osteocytes, quiescent on the surface as lining cells or die by apoptosis (Long, 2012).

Osteocytes (OCYs) are terminally differentiated osteoblasts embedded within the bone matrix and constitute 90 to 95% of bone cells (Buenzli and Sims, 2015).

They occupy lacunar spaces and are connected in a fine canalicular network to detect mechanical stimuli and communicate. In contrast to former perception of osteocytes merely being buried, inactive osteoblasts, recent studies have shown that they can orchestrate bone remodeling through regulation of both osteoclast and osteoblast activity and also function as endocrine cells, acting on distant organs such as the kidney (Bonewald, 2011). They also act on cells in immediate proximity in an autocrine/paracrine fashion as they represent the major source of FGF-23, which is crucial for calcium and phosphorous homeostasis, and sclerostin, a potent inhibitor of bone formation.

Lining cells (LCs), which are also derived from osteoblasts, are quiescent and coat bone surfaces. Several studies indicate a possible reversal and propose reactivation of lining cells, upon PTH and other stimuli, as a source of active osteoblasts (Dobnig and Turner, 1995)(Kim *et al.*, 2012).

Osteoclasts (OCs) are giant multinucleated cells that resorb bone matrix. Like monocytes and macrophages, OCs derive from the hematopoietic lineage (Teitelbaum, 2000). Differentiation from osteoclast precursor to active osteoclast depends essentially on the receptor activator of NF-κB ligand (RANKL) (Lacey *et al.*, 1998), a member of the tumor necrosis factor (TNF) family, and the permissive role of macrophage-colony-stimulating factor (M-CSF) (Post *et al.*, 1976). Binding of RANKL to its receptor RANK is the primary mediator of osteoclast differentiation, activation, and survival. Deficiencies in RANKL result in aberrant osteoclastogenesis and are associated with an osteopetrotic phenotype and excess bone mass (Sobacchi *et al.*, 2013). Osteoprotegerin (OPG) is a naturally occurring, estrogen dependent decoy receptor and antagonist of RANKL (Simonet *et al.*, 1997)(Bord *et al.*, 2003). Immunological and malignant disorders that destroy bone are associated with high RANKL activity, including rheumatoid arthritis (Kong *et al.*, 1999), periodontal disease (Teng *et al.*, 2000), multiple myeloma (Pearse *et al.*, 2001), and osteolytic bone metastasis (Morony *et al.*, 2001). In order to resorb bone, osteoclasts form sealing zones via adaptor proteins such as integrins on the surface. Inside these Howship's lacunae, proton pumps on the basal side of the osteoclast, the ruffled border, create a highly acidic microenvironment. Tartrate-resistant acid phosphatase (TRAP) and lysosomal proteolytic enzymes such as cathepsin K, or matrix metalloproteinases, e.g. MMP-9, then degrade bone (Teitelbaum, 2000). Cathepsin K is a key determinant of resorptive

activity of osteoclasts. Individuals with dysfunctional cathepsin K display pycnodysostosis, a condition characterized by osteosclerosis, a dense, but brittle bone phenotype, short stature, and lytic lesions of the distal phalanges because of poorly functioning osteoclasts (Gelb *et al.*, 1996).

1.5 Bone Remodeling

The maintenance of bones depends on the coordinated action of matrix resorbing osteoclasts and matrix forming osteoblasts in a process happening throughout life called bone remodeling. In adults, approximately 10% of the skeletal mass is constantly renewed each year (Manolagas, 2000). The purpose of this turnover is to repair (micro-) fractures, regulate calcium homeostasis or shape the skeleton during development. This occurs on both endosteal and periosteal surfaces, mostly at the interface with the hematopoietic bone marrow, in basic multicellular units (BMUs). BMUs comprise cells of both lineages, and under physiological conditions, the activities of osteoblasts and osteoclasts are coupled and balanced within a BMU. They are coupled, as stimulation of osteoblasts, e.g. via PTH, leads to activation of osteoclasts, e.g. via RANKL/OPG. Recent studies have shown, that both anabolic and catabolic skeletal responses to PTH are mediated by osteocytes (Saini *et al.*, 2013), and that osteocytes are the essential source of RANKL in adult bone remodeling (Nakashima *et al.*, 2011)(Xiong *et al.*, 2011). The activities are balanced, as the net amount of bone resorbed equals the amount of bone formed afterwards. During a remodeling cycle, the resorption phase, which takes one to two weeks, is followed by a formation phase of two to three months in humans (Fig. 1.2). Bone resorption can be evaluated by measuring the metabolites or cleavage products such as tartrate-resistant acid phosphatase 5b (bone TRAP). Bone-specific ALP or osteocalcin are frequently used bone formation markers (Ducy, Schinke and Karsenty, 2000). The amount of osteoid being laid down by the osteoblasts is the mineral apposition rate (MAR). The bone formation rate (BFR) is the MAR multiplied with the surface area undergoing bone formation. These can be evaluated as histomorphometric parameters on bone biopsies. Bone modeling differs from remodeling in that resorption and formation happen inde-

pendently and on different surfaces. Bone modeling shapes skeletal elements during development and growth, happens at a low rate throughout life and is required for repair and adaptation to changes in mechanical loading (Baron and Kneissel, 2013).

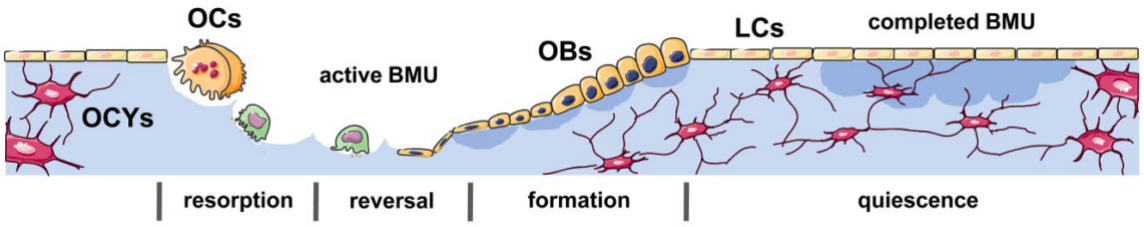

Figure 1.2: Schematic of cellular components within bone. Matrix-embedded osteocytes (OCYs) in large amount, osteoclasts (OCs) active during bone resorption, bone forming osteoblasts (OBs) and quiescent lining cells (LCs), altogether composing a basic multicellular unit (BMU). Physiologically, osteoclast mediated resorption and osteoblast mediated bone formation are coupled and balanced. In osteoporosis, this equilibrium is disturbed. Modified from (Baron and Hesse, 2012).

1.6 Imbalances and Clinical Significance

Disturbances of this tightly regulated turnover, for instance excess in bone resorption or insufficiency of bone formation can lead to conditions associated with low bone mass, most prominent osteoporosis. High bone mass on the other hand can be either due to osteoclast dysfunction, as in osteopetrosis, or caused by excess osteoblast activity, namely osteosclerosis. As osteoblasts and osteoclasts reside on surfaces, trabecular bone with its larger surface area is more prone to imbalances in turnover than cortical bone. The most common osteoporotic fracture sites have a relatively high trabecular to cortical bone ratio and are therefore susceptible if remodeling is disturbed (Fig. 1.3).

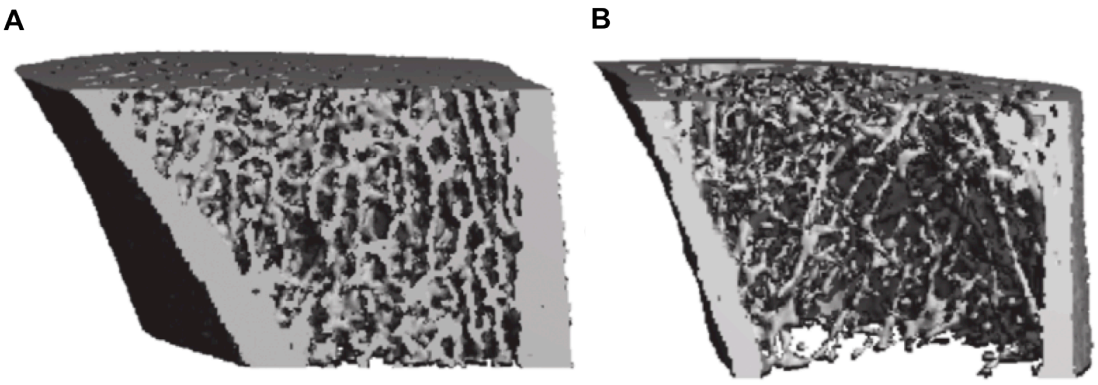

Figure 1.3: Microcomputed tomography (μCT) showing (A) normal and (B) osteoporotic vertebra with trabecular thinning. Trabecular bone is particularly exposed to bone remodeling, as this is happening on surfaces. Modified from (Rachner, Khosla and Hofbauer, 2011).

Individual ten-year fracture probability can be inferred from BMD, typically measured at the hip, biochemical indices of bone resorption and clinical risk factors and evaluated with the WHO fracture risk assessment tool (FRAX) (Kanis *et al.*, 2008). Risk factors for osteoporotic fractures include but are not limited to advanced age, female sex, premature menopause, low body-mass-index, lack of exercise, personal or family history of fragility fractures, poor vision or balance, neuromuscular disorders, renal insufficiency, vitamin D deficiency or low dietary calcium intake, treatment with glucocorticoids, antiepileptic drugs, selective serotonin reuptake inhibitors (SSRIs), proton pump inhibitors (PPIs) or chemotherapy, as well as alcohol consumption and cigarette smoking (Kanis, 2002).

1.7 Therapeutic Options

As osteoporotic and pathological fractures impose a significant burden on both the individual and the society, preventive measures are essential. In addition to lifestyle modifications (cessation of smoking, escalation of physical activity), vitamin D and calcium supplementation is recommended as baseline treatment in every patient at risk for osteoporosis (Rachner, Khosla and Hofbauer, 2011).

Furthermore, specific pharmacological osteoporosis therapies are available. Generally they fall into two classes: anti-resorptive drugs, which slow down bone resorption, and anabolic drugs, which stimulate bone formation (Kawai *et al.*, 2011). Among anti-resorptive drugs, bisphosphonates such as alendronate or zoledronic acid are currently the most widely used substances. They have a long safety record, are inexpensive and can be administered orally or intravenously. In addition, they can be used across a broad spectrum of osteoporosis types, including postmenopausal, male and steroid-induced osteoporosis, as well as other skeletal disorders such as Paget's disease or bone metastases (Rachner, Khosla and Hofbauer, 2011)(Coleman and McCloskey, 2011). Bisphosphonates are embedded within the bone matrix as substitutes for pyrophosphate and upon osteoclastic ingestion inhibit farnesyl pyrophosphate (FPP) synthase, leading to decreased osteoclast activity and increased osteoclast apoptosis (Fisher, Rodan and Reszka, 2000). Although bisphosphonates have proven to be useful in preventing recurrent osteoporotic fractures, they were less effective in primary prevention in patients with osteoporosis without history of prior fractures (Wells *et al.*, 2008). Rare but characteristic adverse effects of treatment include bisphosphonate related osteonecrosis of the jaws (BRONJ) (Ruggiero *et al.*, 2004) and "atypical" subtrochanteric fractures (Hollick and Reid, 2011).

A more recent approach to inhibiting osteoclastogenesis has led to the development of denosumab, a monoclonal antibody that blocks the binding of RANKL to its osteoclast-derived receptor RANK, similar to OPG. This interaction is required for osteoclast formation, activation, and survival (Lacey *et al.*, 1998). By blocking this signaling pathway, denosumab potently inhibits osteoclast-mediated bone resorption (McClung *et al.*, 2006)(Cummings *et al.*, 2009).

Despite beneficial effects on BMD and fracture risk, limitations of anti-resorptive agents remain as they merely impede bone degradation (Eriksen, Díez-Pérez and Boonen, 2014). However, in cases of severe fracture risk in advanced osteoporosis, therapeutics strengthening bone formation are necessary.

1.7.1 Parathyroid Hormone

Teriparatide, the bioactive N-terminal amino acid fragment 1-34 of recombinant human parathyroid hormone (rhPTH) and abaloparatide, an analog to PTHrP, are the sole anabolic agents approved by the FDA for clinical treatment of osteoporosis (Saag *et al.*, 2007)(Miller *et al.*, 2016). Intermittently applied, PTH promotes commitment of mesenchymal progenitors to the osteoblast lineage, osteogenic differentiation and maturation, stimulates osteoblast proliferation, minimizes apoptosis and reactivates lining cells, overall increasing osteoblast number and function (Frolik *et al.*, 2003)(Bellido *et al.*, 2003)(Fig. 1.4). PTHrP is a distinct polypeptide, that shows limited homology to PTH (Datta and Abou-Samra, 2009). As this is clustered in the N-terminus, it binds to and activates the common receptor PTH1R, thus sharing overlapping functions in bone metabolism (Jüppner *et al.*, 1991).

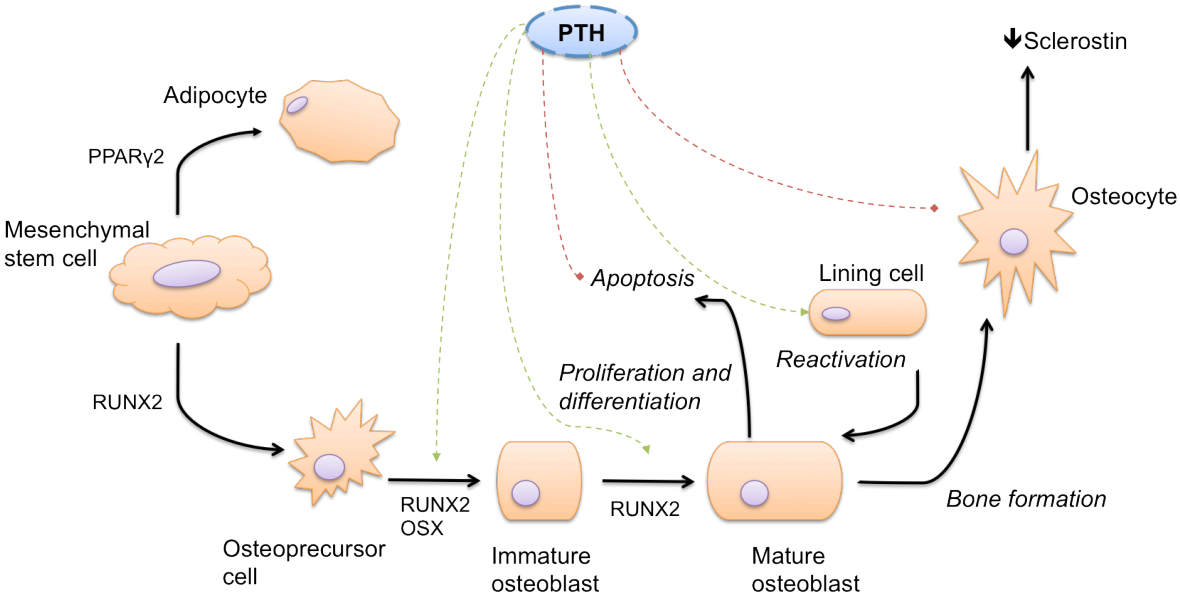

Figure 1.4: Actions of intermittent PTH promoting osteoblastogenesis and bone formation. PTH reduces adipogenesis of mesenchymal progenitors via downregulation of PPARγ and enhances osteogenesis via upregulation of RUNX2 and OSX, thereby supporting proliferation, differentiation and osteoblast maturation. Furthermore, PTH favors reactivation of quiescent lining cells and attenuates osteoblast apoptosis. In osteocytes, PTH decreases secretion of sclerostin, an antagonist of canonical Wnt signaling. Adapted from (Silver and Bushinsky, 2004)(Kraenzlin and Meier, 2011).

Physiologically, PTH is a pivotal regulator of calcium, which is stored in large part in the human skeleton. Precise control of the serum level of ionized calcium, from 1.1 mmol/L to 1.3 mmol/L, is needed to ensure optimum function of physiological processes, particularly cell signaling, neural or muscular function, and bone metabolism (Fraser, 2009). In hypocalcemic states, PTH is released from the parathyroid glands as a polypeptide containing 84 amino acids, with a molecular mass of 9.4 kDa (Prahalad *et al.*, 2006), and a physiological half-life time of approximately four minutes (Bieglmayer, Prager and Niederle, 2002). The parathyroid cells respond to changes in circulating ionized calcium via the calcium-sensing receptor (CaSR) (Tfelt-Hansen and Brown, 2005)(Brown, 2013). Other factors maintaining calcium homeostasis are 1,25-dihydroxyvitamin D_3, which facilitates intestinal calcium and phosphate absorption, and calcitonin, produced by the C cells of the thyroid, which acts on osteoclasts, inhibiting their activity and reducing the release of calcium and phosphate from bone.

PTH increases Ca^{2+} levels in principle by three mechanisms: (i) stimulation of osteoclastic release of skeletal calcium, (ii) enhancing tubular resorption of calcium in the kidney and (iii) increasing activity of renal 1α-hydroxylase, resulting in production of 1,25-dihyroxyvitamin D_3, thereby increasing calcium absorption in the bowel (Fraser, 2009). From the skeleton Ca^{2+} is mobilized indirectly as PTH binds to the parathyroid hormone/parathyroid hormone-related peptide receptor (PTH1R), expressed on bone forming osteoblasts, but not on bone resorbing osteoclasts (McSheehy and Chambers, 1986)(Gardella and Jüppner, 2001). The PTH1R is a G protein-coupled receptor and in bone essentially functions through activation of adenylate cyclase / PKA and phospholipase C / PKC target genes (Abou-Samra *et al.*, 1992)(Mannstadt, Jüppner and Gardella, 1999)(Fig. 1.5). Among other mechanisms, osteoblasts in response to PTH secrete RANKL and suppress synthesis of OPG, which promotes osteoclast maturation, thus ultimately enhancing bone resorption. Sustained elevation of PTH, as seen in hyperparathyroidism, results in osteoporosis and kidney stones composed of calcium oxalate. Pulsatile elevations of PTH however dissociate this coupling sequence by a yet incompletely understood mechanism, and favor osteoblastic bone formation. Stimulation of bone remodeling by PTH occurs on bone surfaces. Therefore, treatment with PTH results in increase in trabecular bone volume and increases cortical porosity (Calvi *et al.*, 2001). As vertebral fracture susceptibility is mainly determined

by trabecular bone architecture, while non-vertebral fracture risk largely depends on cortical bone strength, rhPTH is more protective of vertebral, than of non-vertebral fractures (Zebaze *et al.*, 2010)(Holzer *et al.*, 2009). Intermittent administration of rhPTH (iPTH) has been shown to reduce the risk of vertebral fractures by 65% and that of non-vertebral fractures by 40% (Neer *et al.*, 2001).

Despite benefits in BMD, several limitations with iPTH therapy confine its clinical use, such as the loss of efficacy over time, known as the “anabolic window” (Rubin and Bilezikian, 2003). After an initial gain in bone formation, the net effect is leveled after approximately 24 months of treatment (Kraenzlin and Meier, 2011). Furthermore, as administration of PTH enables bone formation on the basis of remodeling, i.e. stimulating osteoblasts and subsequently osteoclasts, the benefit in terms of BMD seems to fade after discontinuation unless followed by an antiresorptive agent. Studies in which rhPTH was combined with bisphosphonates have generated inconsistent results, but it was recently reported that postmenopausal women with osteoporosis who were treated with both teriparatide (PTH 1-34) and denosumab (RANKL-antibody) for one year had considerably increased BMD at the spine and the hip compared to women who had received either monotherapy alone (Tsai *et al.*, 2013). After the second year of treatment, the increase in spine and hip BMD did not differ among the regimen (Leder *et al.*, 2014). This led to the suggestion that the most cost-effective way to achieve increases in BMD might be to use the combination therapy for one year, followed by an antiresorptive agent alone in the second year (Papapoulos, 2015). Further limitations of intermittent rhPTH are the inconvenient route of daily subcutaneous administration, possibly leading to poor adherence, the costs and adverse reactions such as hypercalcemia, nausea, hypotension or flushing (Kawai *et al.*, 2011). To circumvent necessary subcutaneous injections, alternative forms of delivery (oral, inhalative, transdermal) are currently under study (Cosman *et al.*, 2010). Hence, intermittent administration of rhPTH, which is clinically approved since 2002, is not the be-all and end-all of bone anabolic treatment. A different approach to this potent bone anabolic is abaloparatide, an analog to PTHrP that selectively activates the PTH1R. Recent results from phase III clinical trials for the treatment of postmenopausal osteoporosis have revealed a decrease in fracture risk by 86% and an increase in BMD (Miller *et al.*, 2016). It was approved by the American Food and Drug Administration (FDA) for clinical treatment of osteoporosis in 2017.

1.7.2 Canonical Wnt

Another strong bone anabolic stimulus is canonical Wnt signaling. Wingless (Wg), which was discovered in Drosophila, displays a high degree of conservation across species, including the mammalian integration1 (Int1). Members were subsequently embraced as the Wnt family, a portmanteau of Wingless-related integration sites (Nusse and Varmus, 2012). Non-canonical transduction comprises the Wnt-planar cell polarity pathway, which is involved in regulation of the cytoskeleton (Jenny, 2010), and the Wnt-calcium pathway, controlling intracellular Ca^{2+} (Kohn and Moon, 2005). Canonical Wnt works through the central mediator β-Catenin and is a prominent modulator of bone homeostasis (Cadigan and Peifer, 2009). In mesenchymal progenitor cells, canonical Wnt signaling enhances commitment to (Day *et al.*, 2005) and differentiation along the osteoblastic lineage (Bennett *et al.*, 2007). Moreover, it represses chondrogenesis (Hill *et al.*, 2005) and adipogenesis (Kennell and MacDougald, 2005)(Song *et al.*, 2012). In the hematopoietic lineage, osteoclast differentiation is impeded (Glass *et al.*, 2005). In mature osteoblasts, Wnt/β-Catenin promotes proliferation (Baron and Rawadi, 2007).

Soluble canonical Wnt ligands such as the glycoprotein Wnt3a bind to a receptor complex composed of frizzled (FZD) and LDL receptor-related protein (LRP), e.g. LRP5, on the cellular surface. As a consequence, intracellular dishevelled (DSH) is activated and the “destructosome”, a complex of axin2, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), casein kinase 1a and protein phosphatase 2A, is disassembled. Under idle conditions, β-catenin is constitutively phosphorylated by GSK-3β and thereby marked for degradation by the proteasome. Upon binding of Wnt ligands, the destructosome is dismantled, leading to cytoplasmic accumulation of β-catenin and translocation into the nucleus, where it associates with members of the T cell factor/lymphoid enhancer factor (Tcf/Lef) family to control target gene transcription and endorse bone formation (Baron and Kneissel, 2013).

Human genetic diseases affecting bone illustrate the clinical significance of canonical Wnt signaling. Gain-of-function mutations of Wnt co-receptor LDL receptor-related protein 5 (LRP5) result in high bone mass (Boyden, Mao and Belsky, 2002)(Little *et al.*, 2002), whereas loss-of-function mutations lead to reduced bone

mass, found in osteoporosis-pseudoglioma syndrome (OPPG) (Gong *et al.*, 2001). Further skeletal disorders arise from mutations in sclerostin, an antagonist of Wnt signaling, secreted primarily by osteocytes, which binds to LRP5, and the related LRP4 and 6, and inhibits the binding of canonical Wnt ligands. Mutations in the SOST gene were found in the human diseases sclerosteosis (Brunkow *et al.*, 2001) and Van Buchem disease, both associated with high bone mass (Balemans *et al.*, 2002)(Loots *et al.*, 2005). Notably, the mutations in LRP5 that account for high bone mass decrease the binding of sclerostin and dickkopf 1 (Dkk1), another soluble Wnt inhibitor (Balemans *et al.*, 2008).

Investigations of the canonical Wnt signaling pathway have already been translated into therapeutic progress. Modulation of this cascade has been the goal of ongoing development of bone anabolic pharmaceuticals. Targeting the relatively bone-specific extracellular Wnt antagonist sclerostin, a suppressor of osteoblast differentiation and bone formation, has emerged as one of the most promising approaches thus far (Poole *et al.*, 2005). The sclerostin monoclonal antibody romosozumab has been evaluated in phase III clinical trials (Padhi *et al.*, 2011)(Harrison, 2014). In the treatment of postmenopausal women, it has revealed an 11.3% increase of BMD at the lumbar spine (McClung *et al.*, 2014). The risk of vertebral fractures was reduced by 73% (Cosman *et al.*, 2016). Preclinical data of enhanced Wnt/ β-Catenin signaling by sclerostin disruption further indicate positive effects on fracture repair and implant osseointegration (Agholme *et al.*, 2010)(Ominsky *et al.*, 2011). The “modeling” anabolic actions of sclerostin antibody are distinct from those “remodeling based” of iPTH, as they do not imply bone resorption (Ominsky *et al.*, 2010).

Despite progress in the development of novel bone anabolic drugs, the standard of care in osteoporosis therapy to date are antiresorptive drugs such as bisphosphonates. A great need for additional and affordable anabolic treatments remains in situations of severe osteoporotic bone loss. Insights into the biology of bone remodeling precede innovative therapeutic concepts and could largely benefit public health (Rachner, Khosla and Hofbauer, 2011). PTH- and canonical Wnt signaling constitute two major osteoanabolic pathways that considerably synergize to promote bone formation (Fig. 1.5). As anabolic options for the treatment of extensive bone loss are still limited, further elucidating their mode of action and interaction may contribute to enhancing current or give rise to novel therapeutic approaches.

Miscellaneous regulatory factors are involved in physiologic osteoblast function, including homeodomain proteins (Lian *et al.*, 2006)(Marie, 2015). To identify new molecules that control bone formation, our laboratory performed an unbiased screening assay in murine bone marrow stromal cells. Therein, TG-interacting factor 1 (Tgif1) was revealed as the most abundantly expressed homeodomain protein of unknown function in bone.

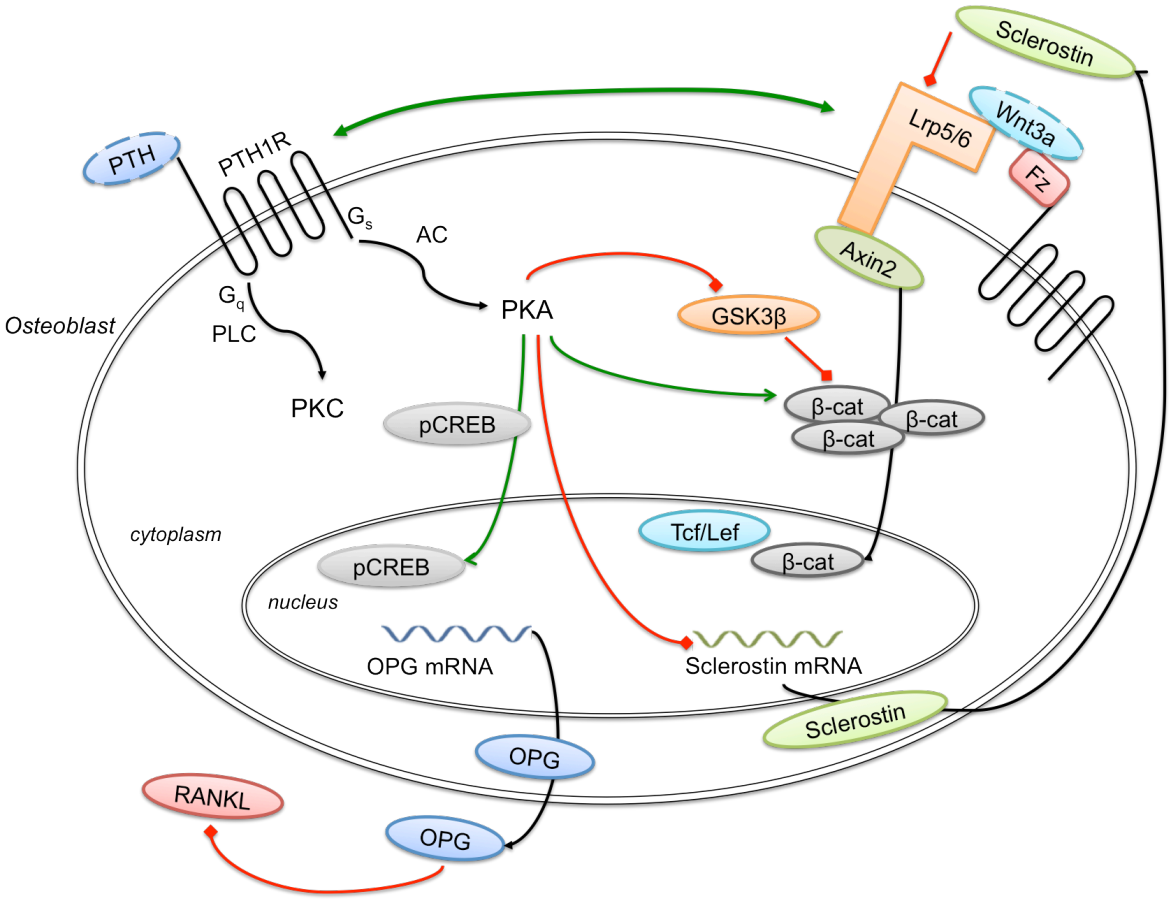

Figure 1.5: Interplay of the PTH and Wnt signaling cascades. Binding of PTH to its receptor PTH1R expressed on osteoblasts activates $G_s\alpha$ / adenylate cyclase (AC) / PKA and $G_q\alpha$ / phospholipase Cβ (PLC) / PKC signaling. AC catalyzes the formation of cAMP, induction of PKA and phospho-CREB dependent activation of target genes. Secretion of RANKL and suppression of OPG synthesis promotes osteoclast maturation in a coupling form. Simultaneous binding of Wnt3a to the receptor frizzled (Fz) and the coreceptors Lrp 5/6 causes the recruitment of Axin2 and prevents the phosphorylation of β-Catenin by GSK3β and its proteasomal degradation. β-Catenin accumulates in the cytosol and translocates into the nucleus, thereby stimulating the expression of Lrp5/6 antagonist sclerostin and the RANKL inhibitor OPG, via the T-cell factor/lymphoid enhancer factor (Tcf/Lef). Binding of PTH cross-activates the Wnt co-receptor Lrp6, inhibits GSK-3β, stabilizes β-Catenin and reduces the expression of Wnt antagonist sclerostin, adding to the bone anabolic effects. Adapted from (Baron and Hesse, 2012).

1.8 Tgif1

TG-interacting factor 1 (Tgif1) is a member of the three-amino-acid loop extension (TALE) subfamily of homeodomain proteins, which regulate various biological processes, including development. Tgif1 had been identified through its ability to compete with the retinoid X receptor (RXRα) for binding to its cognate promoters, thereby suppressing retinoic acid (RA) signaling (Bertolino *et al.*, 1995). Furthermore, Tgif1 interacts with the ligand binding domain of RXRα and recruits the co-repressor carboxy terminal binding protein (CtBP) to RXRα, resulting in the inhibition of retinoid X receptor-dependent transcription (Bartholin *et al.*, 2006). Tgif1 also plays a role in sonic hedgehog (SHH) signaling. A loss of function mutation in the Tgif1 gene in humans can cause holoprosencephaly (HPE), a malformation of the forebrain and craniofacial skeleton, due to aberrant SHH signaling (Nanni *et al.*, 1999). In early embryogenesis, defective gastrulation and neural axis development due to malfunctional Tgif1 have been linked to NODAL signaling, a member of the transforming growth factor-β (TGF-β) family (Gripp *et al.*, 2000)(Powers *et al.*, 2010). Tgif1 mutations can cause HPE by disrupting interplay of the Nodal/Smad2 and SHH pathways (Taniguchi *et al.*, 2012). Smad2 is a key substrate of receptors for the TGF-β family of growth and differentiation factors (Massagué, 2012). Herein, Tgif1 can act as a transcriptional repressor by either binding to the DNA directly or by interfering with TGF-β activated Smads (Wotton, Lo, Lee, *et al.*, 1999)(Lo, Wotton and Massagué, 2001). Tgif1 interacts with the Sin3/Histone Deacetylase (HDAC) co-repressor complex, or recruits CtBP independent of HDAC, to regulate transcription (Wotton, Lo, Swaby, *et al.*, 1999)(Melhuish and Wotton, 2000)(Fig. 1.6). These two modes of repression may play a role at different locations, or act sequentially at the same genes (Melhuish, Gallo and Wotton, 2001)(Wotton *et al.*, 2001). Tgif1 has been further shown to interact with c-Jun, a member of the AP-1 family of transcription factors, which control various cellular processes including proliferation, differentiation or apoptosis, thereby suppressing Smad2 transcriptional activity (Pessah *et al.*, 2001).

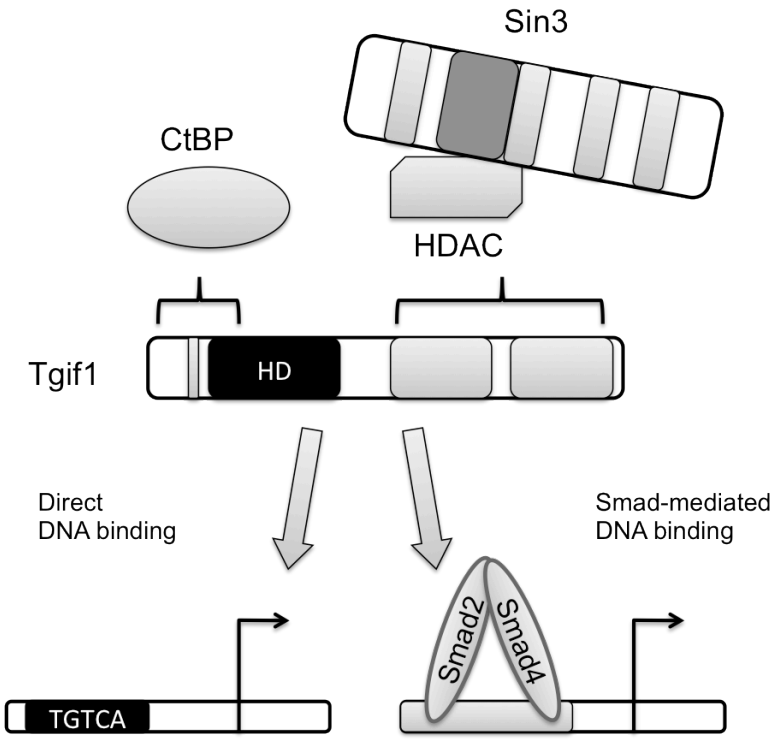

Figure 1.6: Schematic of Tgif1, showing functional domains with roles in recruitment to DNA and transcriptional repression. Interaction with a corepressor complex leads to direct DNA binding to its cognate site or indirect recruitment mediated by a TGF-β-activated Smad2/4 complex. Its role in bone homeostasis and anabolic signal transduction has remained elusive. Adapted from (Wotton *et al.*, 2001).

Tgif1 had been determined as the most abundantly expressed homeodomain protein during osteoblast differentiation in a screening assay. As opposed to the already well-characterized role of Tgif1 in the above-mentioned cascades, its implication in adult bone homeostasis is not determined. Particularly its function in anabolic signal transduction has yet to be resolved. Preliminary work of our group has suggested Tgif1 to be involved in central bone anabolic pathways, including PTH- and canonical Wnt signaling. The overall aim of our current studies is to explore how Tgif1 is implicated in these stimuli affecting osteoblast activity and bone formation.

2 Hypothesis and Objectives

2.1 Hypothesis

Osteoporosis, the most common cause of fragility fractures due to low bone mass, occurs when bone remodeling is disturbed. Current therapeutic regimens predominantly comprise antiresorptive agents aimed at impeding osteoclast function. PTH (1-34) and an analog to PTHrP constitute the only clinically approved drugs for anabolic treatment of extensive bone loss. Antagonizing sclerostin, an inhibitor of canonical Wnt signaling, is emerging as one of the most promising approaches to sustaining bone formation in the near future. Both pathways synergize and deciphering the molecular mechanism by which they increase bone mass is crucial to uncover novel stimulators of osteoblast activity. In an unbiased screening assay during osteoblast differentiation, TG-interacting factor 1 (Tgif1) emerged as the most abundantly expressed homeodomain proteins of unknown function in bone and is therefore a subject of ongoing investigations in our laboratory. We hypothesize that Tgif1 plays a central role in mediating bone anabolic signal transduction, which is a crucial stimulus to osteoblast activity and bone formation. By elucidating the components of anabolic signaling cascades, we aim to expand the knowledge about bone homeostasis and contribute to improving comprehensive therapeutic approaches to diseases such as osteoporosis.

2.2 Objectives

i) To investigate the effect of Tgif1 on osteogenesis *in vitro*, with particular regards to osteoblast differentiation, proliferation and activity.

ii) To explore the involvement of Tgif1 in PTH and canonical Wnt signaling in primary osteoblasts and its regulation.

iii) To determine the impact of global genetic deletion of Tgif1 on anabolic signal transduction.

iv) To evaluate the necessity of Tgif1 for physiological response to PTH and canonical Wnt in osteoblasts.

3 Methods and Material

3.1 Methods

3.1.1 Cell Culture

All primary osteoblasts were cultured in complete Minimum Essential Medium Alpha (alpha-MEM) with nucleosides, including 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S), if not otherwise specified. Cells were serum starved in αMEM containing 1% FBS for four hours before stimulation experiments. All components were obtained from Gibco® by Life Technologies™, Invitrogen. Cells were kept in Petri dishes or culture flasks manufactured by BD Falcon™ at 37.0° C, 5% CO_2 and 95 % relative humidity in a Thermo Scientific Heraeus BBD6220 incubator. Experiments under sterile conditions were conducted in a HERAsafe HS biological safety cabinet by Thermo Scientific, Heraeus Instruments.

3.1.2 Mouse Model

Primary osteoblasts from mice (Mus musculus) were cultured as detailed below, to study the influence of Tgif1 on bone metabolism *in vitro*. For this purpose, we used a mouse strain with germline deletion of the *Tgif1* gene on a C57BL/6J background, i.e. a global knock-out: B6.129S-Tgif1^{tm1Caw}/J (Shen and Walsh, 2005). To compare genotypes, we obtained littermates of the genotypes *Tgif1*$^{+/+}$ and *Tgif1*$^{-/-}$ from heterozygous breeder pairs (*Tgif1*$^{+/-}$ x *Tgif1*$^{+/-}$). Mice were subjected to a twelve-hour light/dark cycle and provided with rodent food and water ad libitum. For the assessment of bone marrow stromal cells (BMSCs) and long bone osteoblasts (LOBs), littermates were sacrificed at ages of ten to twelve weeks via cervical dislocation after deep anesthesia with intraperitoneal ketamine/xylazine. Calvarial osteoblasts (COBs) were acquired from mouse pups on postnatal days P1-3 via decapitation. All experiments were conducted according to protocols approved by the local authority for animal welfare.

3.1.3 Calvarial Osteoblasts

Calvaria of newborn mice are a source of homogenous, easily accessible primary osteoblasts (calvarial osteoblasts, COBs) that are well established in bone research. To isolate these cells, six to twelve littermate mouse pups of the strain C57BL/6J were sacrificed on postnatal days P1-3 via decapitation in the sterile laminar flow hood. After disinfection with 70% ethanol, the soft tissue was removed and the calvaria exposed and isolated. In a first digestion with collagenase A (1 mg/ml) and dispase II (2 mg/ml) in αMEM at 37° C with shaking (750 rpm) for ten minutes, the surface was cleared of fibroblastic cells. Four consecutive digestions, each 25 minutes, then released the osteoblastic cell population. After each digestion the supernatants were collected, added to complete medium (αMEM supplemented with 10% FBS and 1% Pen/Strep) to stop the digestion and pooled per animal. The cells were then centrifuged (five minutes, 900 rpm, room temperature), resuspended in complete medium and cultured for approximately four days in a 10cm-culture dish to sub-confluence until the use for experiments (Fig. 3.1). Adapted from “Osteoblast Isolation from Murine Calvaria and Long Bones” from *Bone Research Protocols* (Bakker and Klein-Nulend, 2012).

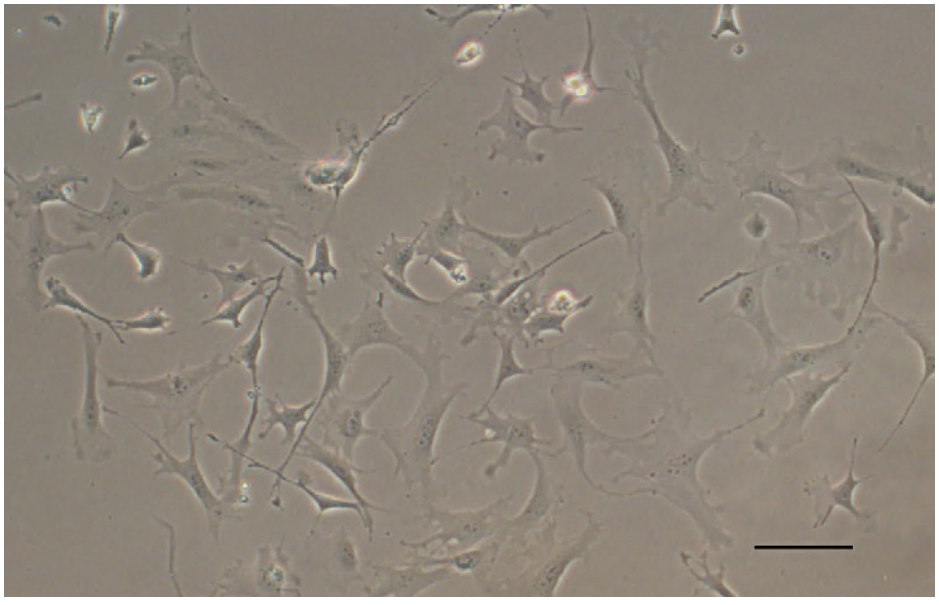

Figure 3.1: Microscopic image (phase contrast) of cultivated COBs. Scale bar represents 200 µm.

3.1.4 Genotyping

Tgif1 genotypes of these pups were determined in parallel by PCR after tail biopsy DNAse digestion. To this end, tail biopsies were incubated in 200 µl of solution A (25 mM NaOH, 0.2 mM EDTA, pH 12) at 90° C in a dry block heater for two hours, with occasional manual shaking. Addition of 200 µl of solution B (40 mM Tris-HCl, pH 5) and vortexing resulted in preliminary DNA solution. Ready-to-use PCR solution was prepared by adding 10x PrimeTaq DNA-polymerase buffer, 2% DMSO, 10 µM Tgif1 forward and reverse primers, 10 mM dNTPs, 5 U/µl PrimeTaq DNA-polymerase and adjusting the final volume with water. Two µl of this genomic DNA were then amplified via polymerase chain reaction (PCR), consisting of denaturation, primer annealing and elongation (Saiki *et al.*, 1988). The protocol was: initial denaturation at 94° C for five minutes, followed by 35 cycles of (i) 94° C for 30 seconds, (ii) 60° C for 45 seconds and (iii) 72° C for one minute, and final extension at 72° C for ten minutes with terminal holding at 12° C. Electrophoretic separation of the Tgif1 amplicons according to band size, i.e. genotype, on a 2% TAE agarose gel with 0.5 µg/ml ethidium bromide was visualized with the ChemiDoc^{TM} MP system and Image Lab software (Fig. 3.2).

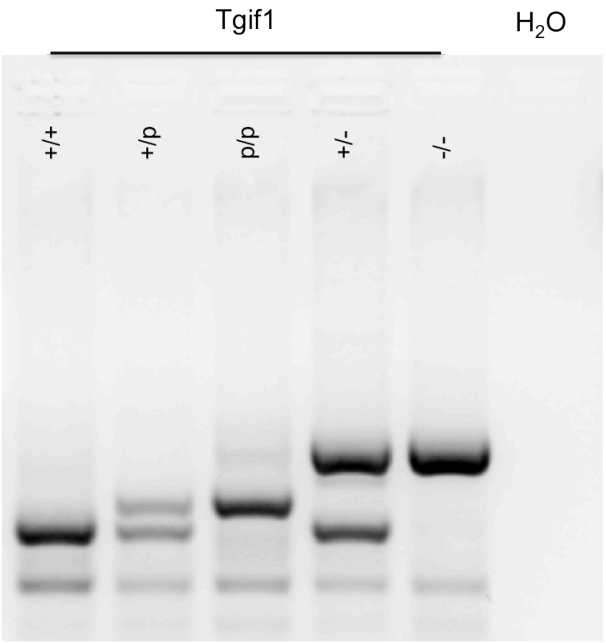

Figure 3.2: Tgif1 genotype after agarose gel separation from newborn mice. + indicates the wild type allel, - the deleted allel, and p indicates a loxP-flanked Tgif1 locus for conditional knock-out (cKO), which under control of bone-specific genes, is used to ascertain organ-specific effects. Water is used as empty control.

3.1.5 Bone Marrow Stromal Cells

Bone marrow stromal cells (BMSCs) are progenitors of the mesenchymal lineage, and capable of differentiating into bone, cartilage, adipose and hematopoietic supporting tissues. They can be separated from hematopoietic cells by their differential adhesion to tissue culture plastic and their prolonged proliferative potential (Krebsbach *et al.*, 1999). To isolate BMSCs, mice were sacrificed at ten to twelve weeks of age via cervical dislocation after deep anesthesia with intraperitoneal ketamine/xylazine. The hind limbs of the animals were aseptically dissected, cleared of soft tissue using surgical scissors and moved to the sterile work bench in Hank's Balanced Salt Solution (HBSS) containing 5% Penicillin/Streptomycin. In a 10cm-dish with fresh HBSS the remaining connective tissue, muscle and periosteum were removed using a scalpel, and the cleaned bones were transferred to a new dish. After cutting off the epiphyses, the bone marrow was flushed out with plain αMEM using a 27gauge-needle and syringe and collected in 15ml-Falcon tubes. This cell population was then centrifuged at 150xg for five minutes and resuspended in αMEM containing 20% FBS and seeded in 10cm-dishes. The cells were supplied with fresh medium every other day for approximately one week until sub-confluence, and then used for experiments (Fig. 3.3).

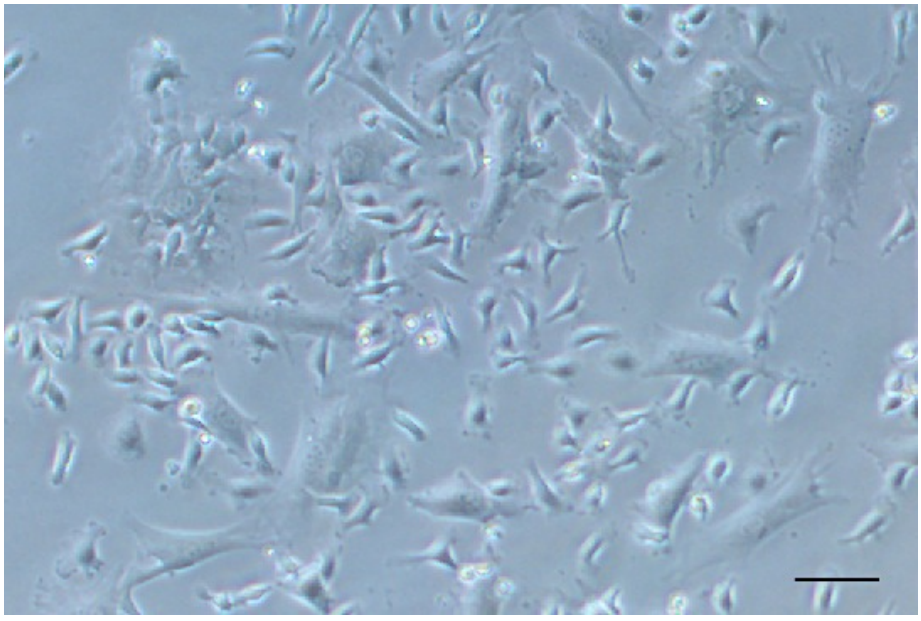

Figure 3.3: Microscopic image (phase contrast) of cultivated BMSCs. Scale bar represents 200 µm.

3.1.6 Long Bone Osteoblasts

Outgrowth cultures from mouse long bones represent a different, well-established source of primary osteoblasts (long bone osteoblasts, LOBs). After flushing of the bone marrow as described above, the hollowed femur and tibia were transferred to a fresh plate with HBSS and chopped into fragments of approximately 1-2 mm before an enzymatic digestion and clearance of surface cells. This was achieved by incubation with collagenase (2 mg/ml in αMEM) solution, at 37° C with shaking (750 rpm) for two hours. The digestion solution was discarded and the bone fragments transferred to a new 6cm-petri dish containing HBSS where they were minced into fine pieces using a scalpel. These bone chips were then transferred to a new 6cm-dish and put into culture with the objective of osteoblast outgrowth and culture. The cells were supplied with fresh medium twice a week for one week, and further expanded in a 10cm-dish for another week until sub-confluence and then used for experiments (Fig. 3.4). Adapted from “Osteoblast Isolation from Murine Calvaria and Long Bones” from *Bone Research Protocols* (Bakker and Klein-Nulend, 2012).

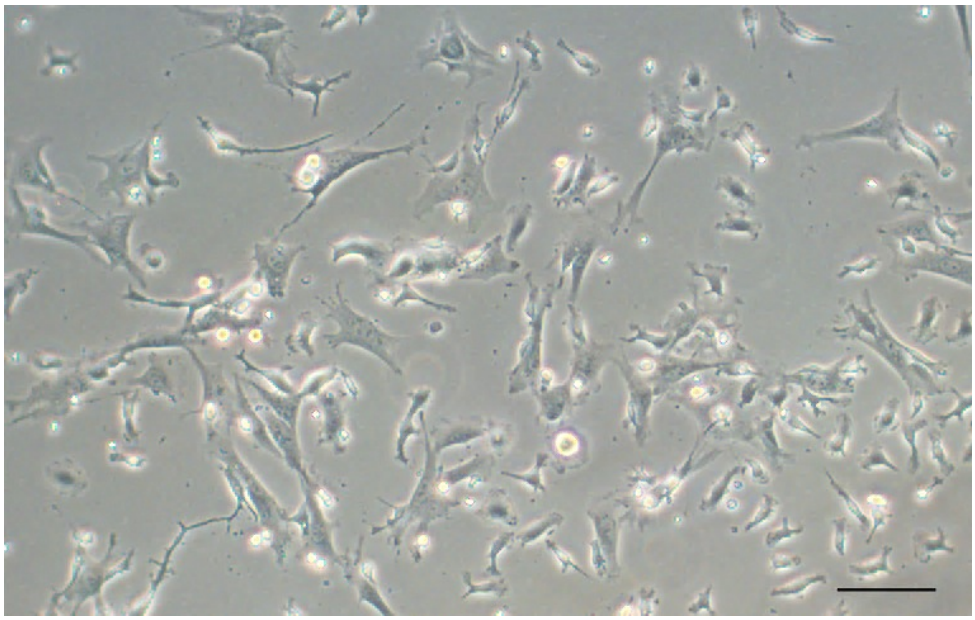

Figure 3.4: Microscopic image (phase contrast) of cultivated LOBs. Scale bar represents 200 µm.

3.1.7 Analysis of mRNA Expression

To investigate gene expression, quantitative real-time polymerase chain reaction (qPCR) is used to amplify and simultaneously quantify genes of interest employing specific primers. The products are detected using fluorescent dyes that intercalate with double-stranded DNA, in our case SYBR Green (Heid *et al.*, 1996). Gene-of-interest expression changes under stimulatory conditions according to experimental design, whereas housekeeping genes remain unaffected by treatment. These changes are put into relation and quantified.

3.1.7.1 RNA isolation

After stimulation, cells were washed with phosphate buffered saline (PBS) and immediately put on ice. Cells were lysed with Buffer RLT Plus and RNA was isolated using the RNeasy® Plus Mini Kit. Preparation was done according to manufacturer's instructions, with the exception of elution in a final volume of 25 µl RNase-free water to obtain higher yield concentrations. This total RNA content was measured using the Nanodrop™ 2000 spectrophotometer. Samples were processed for cDNA synthesis.

3.1.7.2 cDNA synthesis

The cDNA was reverse transcribed using the ProtoScript® M-MuLV First Strand cDNA Synthesis Kit. To up to 6 µl of RNA solution (maximum 1 µg of total RNA), isolated as detailed above, 2 µl of random mix primers were added, including an oligo dT primer, and the volume was adjusted with water to 8 µl. This solution was denatured in the Mastercycler® pro S for five minutes at 70° C. After addition of 10 µl reaction mix and 2 µl enzyme mix, to give a total volume of 20 µl, the samples were retransferred to the PCR cycler for the following program: 5 minutes at 25° C, 60 minutes at 42° C, 5 minutes at 80° C. The reaction product was diluted to reach a concentration of the template RNA of 2.5 ng/µl and stored at -20° C. All preparation was done on ice.

3.1.7.3 Quantitative real-time PCR (qPCR)

Three µl of the cDNA templates were mixed with 0.75 µl forward and reverse primers of the genes of interest, 7.5 µl iQ™ SYBR® Green Supermix and 3 µl H_2O to give a total volume of 15 µl per reaction. All sample preparation was done on ice. Amplification was conducted using the CFX Connect™ Real-Time PCR Detection System. Quantification was done using the ΔCt method according to the MIQE guidelines to determine relative expression and regulation (Bustin *et al.*, 2009). Therein the threshold cycle Ct indicates the replication cycle at which the sample fluorescence exceeds the threshold. The ΔCt between the unregulated housekeeping gene, if not otherwise specified, TATA-binding protein (Tbp) and gene of interest were then quantified. Ct values in the range of 21-26 indicate detectable quantities of mRNA in the samples, ruling out artifacts due to low expression levels. The qPCR primers were custom designed and obtained from Eurofins MWG or Invitrogen (Tab. 3.1). Sequences were as follows:

Table 3.1: Custom designed primer sequences of genes of interest for qPCR analyses

Name	Sequence (5' → 3')	Supplier
Tgif1 F	GCAGACACACCTGTCCACACTA	MWG eurofins
Tgif1 R	GGAATGAAATGGGCTCTCTTCT	
Ramp3 F	TGCACCTTCTTCCACTGTTG	
Ramp3 R	AGGTTGCACCACTTCCAAC	
Axin2 F	GCAGCAGATCCGGGAGGATGAA	
Axin2 R	GATTGACAGCCGGGGGTCTTGA	
Tbp F	GCTCTGGAATTGTACCGCAGC	Invitrogen
Tbp R	CTCTTGGCTCCTGTGCACAC	

3.1.8 Western Blot

Western blot analysis is an established method to detect target proteins of total cell lysates or subcellular protein fractions using specific antibodies. Denatured proteins are separated according to length of polypeptides via gel electrophoresis and transferred to a nitrocellulose membrane (Towbin, Staehelin and Gordon, 1979). Cells were washed with PBS after experiments. Immediate addition of modified RIPA buffer, including protease- and phosphatase inhibitors, gave total cell lysates after 15 minutes of incubation and homogenization on ice. After centrifugation, supernatants were collected and protein concentration was determined with the Bio-Rad protein assay and a microplate reader using the Bradford method. This is a colorimetric assay based on the absorbance shift of dye depending on protein content (Bradford, 1976). If not otherwise specified, 30 µg of protein per sample were mixed with 4x Laemmli sample buffer containing 2-Mercaptoethanol (β-ME) and boiled for ten minutes at 95° C using an Eppendorf Thermostat plus hot plate. Protein standard ladder and samples were loaded onto previously prepared sodium dodecyl sulfate (SDS) gels. These consisted of two parts, the lower separation gel and the upper stacking gel, and contained either separation gel buffer (1.5 M Tris/HCl, pH 8.8) or stacking gel buffer (0.5 M Tris/HCl, pH 6.8) and 10% SDS, 10% ammonium persulfate (APS), 30% Acrylamide/Bisacrylamide and TEMED (Tab. 3.2). Composition was as follows:

Table 3.2: SDS gels for electrophoretic protein separation and Western blot analysis

Separation gel (12%)		Stacking gel (4%)	
Water	3.5 ml	Water	3.0 ml
Separation gel buffer	2.5 ml	Stacking gel buffer	1.25 ml
10% SDS solution	100 µl	10% SDS solution	50 µl
Acrylamide/Bisacrylamide	4.0 ml	Acrylamide/Bisacrylamide	650 µl
TEMED	5.0 µl	TEMED	5.0 µl
10% APS solution	50 µl	10% APS solution	25 µl

The gels were then mounted in Mini-PROTEAN Tetra Cell chambers filled with Tris/Glycine/SDS running buffer. To separate protein bands according to size, gels were run at 180 V for approximately 75 minutes connected to a Powerpac 3000 power supply. Subsequently, the gels were blotted onto nitrocellulose membranes using Tris/Glycine transfer buffer with 20% Methanol at 25 V and up to 1.0 A for 30 minutes in the Trans-Blot® Turbo™ Transfer Starter System. To ascertain successful protein transfer, membranes were stained with Ponceau S. The membranes were briefly washed in Tris-Buffered Saline with Tween 20 (TBS-T) to remove the staining and blocked against non-specific binding with 5% skim milk powder in TBS-T for one hour at room temperature during gentle orbital shaking. The membranes were then incubated with primary antibodies at concentrations of 1:500 to 1:5,000 in 5% milk in TBS-T for two hours at room temperature or for twelve hours at 4° C with gentle shaking. Membranes were washed three times for ten minutes with fresh TBS-T, and subsequently incubated with secondary antibodies and horseradish peroxidase (HRP) conjugates at concentrations of 1:10,000 for one hour at room temperature with gentle shaking. Membranes were washed three times for ten minutes with fresh TBS-T under gentle shaking. Afterwards they were exposed to enhanced chemiluminescence (ECL) reagent or, in case of faint signals, femto chemiluminescent substrate for five minutes. Exposure was done in the ChemiDoc™ MP System for 2 to 300 seconds, depending on signal intensity. Analysis and normalization relative to corresponding loading control was performed with the Image Lab 4.1 software.

3.1.9 Xgal Staining

The BAT-GAL mouse contains an inducible recombinant β-Galactosidase, expressing the LacZ gene from *E. coli*. This can be used as a reporter gene in eukaryotic transfection and visualized with Xgal staining. As it is under the control of T cell factor/lymphoid enhancer factor (Tcf/Lef) responsive elements, this mouse model constitutes a readout for activation of canonical Wnt/β-catenin signaling (Maretto *et al.*, 2003). These mice were crossed with Tgif1$^{+/+}$ and Tgif1$^{-/-}$ animals, resulting in double transgenic mice with the genotypes BAT-GAL^{+}:Tgif1$^{+/+}$ and BAT-GAL^{+}:Tgif1$^{-/-}$. Stimulation of osteoblasts derived from these animals allowed us to study the influence of Tgif1 on PTH-induced Wnt signaling *in vitro.*

After treatment, primary osteoblasts were washed with PBS, mildly fixed in 2% formaldehyde in PBS supplemented with 2 mM $MgCl_2$ for twenty minutes and washed with PBS again. Staining was achieved by incubation with a PBS solution containing the β-Galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal), Potassium ferricyanide ($K_3Fe(CN)_6$), Potassium ferrocyanide ($K_4Fe(CN)_6$), EGTA, NP-40 and $MgCl_2$ at 30° C, pH 7.5 for 24 hours. The indolyl moiety of Xgal is oxidized to an indoxyl in a reaction catalyzed by the ferricyanide/ferrocyanide mixture (Horwitz *et al.*, 1964). The indoxyl moiety underwent dimerization and formed an indigo blue derivate that was detected visually with the Olympus BX 50 light microscope and the cellSens Entry software. Five images per condition were taken randomly and quantified with ImageJ as Xgal positive cells / total cell number, leading to a total of 200-300 cells per condition and experiment.

3.1.10 cAMP ELISA

A pivotal second messenger in the $G_s\alpha$-protein-coupled receptor adenylate cyclase (AC) pathway is 3'-5'-cyclic adenosine monophosphate (cAMP). AC converts ATP to cAMP. To assess activation of this cascade, cAMP levels were measured with a competitive cAMP enzyme-linked immunosorbent assay (ELISA). Isobutylmethylxanthine (IBMX) is a non-specific inhibitor of phosphodiesterase (PDE), used here to prevent degradation of cAMP to AMP by PDE (Essayan, 1999). In a 96-well plate covered with GxR IgG, containing either cAMP conjugated to ALP or endogenous cAMP from samples, an added rabbit polyclonal antibody against cAMP binds in a competitive manner. To this end, cell culture supernatants and total cell lysates obtained as detailed above were collected after experiments and cooled on ice. Samples were diluted in plain αMEM or PBS respectively at a ratio of one to four. Further handling was adherent to the kit's protocol. After the above-delineated incubation the 96-well plate was washed, leaving only bound cAMP. Addition of another solution containing *para*-Nitrophenylphosphate (pNpp) as a substrate for ALP on the cAMP conjugate generated a yellow color upon catalysis. This was detected photometrically with a microplate reader at a wavelength of 405 nm. The amount of signal was inversely proportional to the amount of endogenous cAMP in the sample. With regards to the blank OD, total activity, non-specific binding and given standards, concentra-

tion of cAMP in the samples was calculated with 4-parameter logistic (4PL) curve fitting. Total cell lysates were normalized for protein content.

3.1.11 Osteogenic Differentiation

Physiologically, mature osteoblasts lay down osteoid and subsequently secrete ALP to catalyze matrix mineralization, which can be visualized by staining. To induce osteoblast differentiation *in vitro*, cells were seeded at high density two days before experiments to allow formation of a confluent cell layer and subsequently favor differentiation over proliferation. Complete αMEM was supplemented with mineralization additives, consisting of 50 µg/ml ascorbic acid, 5 mM β-Glycerophosphate and 10 nM dexamethasone and replaced daily.

3.1.11.1 Alkaline phosphatase activity staining

Bone ALP (BALP) is the bone-specific isoform of alkaline phosphatase and has been shown to be a sensitive indicator of bone metabolism, altered in states of high turnover such as Paget's disease (Eastell, 1999). ALP can also be used as a parameter for early osteoblast maturity after differentiation *in vitro*. After osteogenic culture, differentiated cells were washed with PBS, fixed with 3.7% formaldehyde in PBS for 15 minutes and stained for ALP activity. To prepare the staining solution, 5 mg Naphthol AS-MX phosphate disodium salt were dissolved in 200 µl *N,N*-Dimethylformamide and transferred to 50 ml of 100 mM Tris/HCl, pH 8.4. Then, 30 mg Fast Blue RR salt were added and the solution was thoroughly vortexed and filtered. After washing with PBS, the cells were stained with the staining solution for 15 minutes in the dark. Washing with distilled water under gentle shaking reduced background staining before analysis. All reagents were obtained from Sigma-Aldrich.

3.1.11.2 Alkaline phosphatase activity quantification

In addition to staining, ALP enzyme activity can be quantified with the LabAssay^{TM} ALP kit (Yamamoto, Takahashi and Tabata, 2003). This kit uses *para*-Nitrophenylphosphate (pNpp) as a substrate, which is hydrolyzed proportional to

the presence of ALP activity in the sample. To this end, differentiated cells were washed with PBS and lysed with mRIPA buffer on ice. After homogenization, total cell lysates were processed according to manufacturer instructions. The released *p*-Nitrophenol showing yellow color was photometrically measured at a wavelength of 405 nm as the enzyme activity [A.U.]. The values were normalized for protein content in the sample.

3.1.11.3 Alizarin Red staining

Alizarin Red is used in bone biology to determine the presence of calcific deposition by cells of the osteogenic lineage. The peak in staining intensity is at days 10 to 16 in culture of matrix mineralization. This is a crucial step towards the formation of calcified ECM associated with true bone (Puchtler, Meloan and Terry, 1969)(Lievremont, Potus and Guillou, 1982). Calvarial osteoblasts are capable of matrix mineralization in culture (Ecarot-Charrier *et al.*, 1983). The staining solution was prepared by dissolving 2 g of Alizarin Red S in distilled water, adjusting the pH to 4.2 with Hydrochloric acid, adding volume until 100 ml and paper filtration. Differentiated osteoblasts after osteogenic culture for 15 days were washed with PBS and then fixed with 3.7% formaldehyde in PBS for 15 minutes. After washing with PBS, the cells were stained with the above-described solution for 20 minutes. Washing with distilled water under gentle shaking reduced background staining before analysis.

3.1.12 MTS Assay

The MTS assay is a quantitative colorimetric method for determining proliferation and cellular viability as it measures the metabolic activity of cells, which can be used as a surrogate for the cell number (Mosmann, 1983). Primary osteoblasts were seeded in 96-well plates at sub-confluence and left to attach for two days before the start of experiments. At given time points they were incubated with the CellTiter 96® AQ_{ueous} One Solution for four hours. The solution added to the cells contained the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). NAD(P)H-dependent cellular oxidoreductases are capable of reducing this substrate, MTS

tetrazolium, to its colored formazan product, that is soluble in tissue culture medium (Barltrop *et al.*, 1991). The quantity of this product is directly proportional to the number of living cells (Berridge and Tan, 1993) and was detected photometrically with a 96-well plate reader at a wavelength of 490 nm (Cory *et al.*, 1991).

3.1.13 BrdU Assay

Cell proliferation can be quantified by incorporation of 5-bromo-2'-deoxyuridine (BrdU), a synthetic thymidine analog, during the S-phase of the cell cycle (Ellwart and Dörmer, 1985). This is detected with a biotinylated monoclonal anti-BrdU antibody and revealed using a streptavidin-biotin staining system. Cells were seeded onto cover slides with four chambers at sub-confluence and left to attach for two days. At given time points they were incubated with the labeling reagent for twelve hours, washed with PBS, fixed in 70% ethanol for twenty minutes and washed with distilled water. Immunocytochemical staining was carried out as instructed by the manufacturer, using the BrdU staining kit. Brown staining, indicating active DNA replication of cells during the labeling period, was visualized by light microscopy. Three images per genotype and condition were taken randomly and quantified with ImageJ as BrdU positive cells / total cell number, resulting in 300-800 cells per condition and experiment.

3.1.14 Transient Overexpression of Tgif1

To test whether the abundance of Tgif1 protein enhances osteogenic differentiation, Tgif1 was transiently overexpressed using a CMV-driven expression vector encoding mouse Tgif1 with an N-terminal FLAG tag. The plasmid contains resistance genes for Ampicillin and Neomycin/Kanamycin for prokaryotic or eukaryotic selection, respectively. To this end, primary osteoblasts were seeded at confluence in 24-well dishes and left to attach for two days. Per well, 750 ng of plasmid DNA were diluted in 100 µl of serum-free medium. Two µl of polymer-based GeneCellin™ Transfection Reagent were added and incubated for 15 minutes at room temperature. Transient overexpression was achieved by incubation with 100 µl/well of this mixture for twelve hours at 37° C before the start of

differentiation experiments. Transfection success rate was estimated by fluorescence microscopy from control cells transfected with an expression plasmid encoding EGFP.

3.1.15 Statistical Analysis

Independent experiments were completed and reproduced at least three times. Within one experiment biological and technical duplicates were conducted, if not otherwise specified. Images or graphs from representative replicates are shown. Error bars indicate ± standard deviation.

For statistical significance a two-tailed student's t-test for unpaired samples with equal variances was applied. Level of significance was *$p \leq 0.05$, **$p \leq 0.01$ or ***$p \leq 0.001$.

3.2 Material

Acetic acid, Sigma-Aldrich (#A6283)

Acrylamide/Bisacrylamide, Rotiphorese Gel 30, 30 % Acrylamide/Bisacrylamide 37.5:1, Roth (#3029.1)

Alizarin Red S, Sigma-Aldrich (#A5533)

Amersham Protran nitrocellulose membrane, 0.45 µm pore size, GE Healthcare Life Sciences (#10600002)

Ammonium persulfate (APS), Sigma-Aldrich (#A3678)

Anti-ACTIN (42 kDa), mouse monoclonal antibody, clone C4, EMD Merck Millipore (#mab1501)

Anti-active-β-CATENIN (Anti-ABC) (92 kDa), mouse monoclonal antibody, clone 8E7, EMD Merck Millipore (#05-665)

Anti-CREB (43 kDa), mouse monoclonal antibody, clone 86B10, Cell Signaling (#9104)

Anti-phospho-CREB (43 kDa), rabbit monoclonal antibody, clone 87G3, Cell Signaling (#9198)

Anti-Mouse IgG HRP Conjugate, Promega (#W402B)

Anti-Rabbit IgG HRP Conjugate, Promega (#W401B)

Anti-RAMP3 (40 kDa), mouse monoclonal antibody, abcam (#ab56684)

Anti-TGIF1 (37 kDa), rabbit monoclonal antibody, clone EP637Y, abcam (#ab52955)

BrdU Labeling Reagent, life technologies (#00-0103)

BrdU Staining Kit, Invitrogen (#93-3943)

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal), Sigma-Aldrich (#B6024)

CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, MTS assay, Promega (#TB245)

CFX Connect™ Real-Time PCR Detection System, Bio-Rad (#185-5200)

ChemiDoc™ MP System, Bio-Rad (#170-8280). Software: Image Lab 4.1

Clarity™ Western ECL substrate, Bio-Rad (#170-5061)

Collagenase A, Roche (#11088793001)

cOmplete, EDTA-free, Protease Inhibitor Cocktail Tablets, Roche (#05056489001)

Cyclic AMP ELISA Kit, abcam (#ab133039)

N,N-Dimethylformamide, Sigma-Aldrich (#D158550)

Dimethylsulfoxide (DMSO), Sigma-Aldrich (#D8418)

Dispase® II (neutral protease, grade II), Roche (#04942078001)

dNTP Mix, 10 mM, Fermentas (#R0193)

Ethidium Bromide, 10 mg/ml, Carl Roth (#2218.4)

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), Sigma-Aldrich (#E5134)

Fast Blue RR salt, Sigma-Aldrich (#F0500)

Fetal Bovine Serum (FBS), gibco® by life technologies™, Invitrogen (#10270-106)

Forskolin (FSK), Sigma-Aldrich (#F3917)

GeneCellin™ Transfection Reagent, BioCellChallenge (#GC500)

Glycine, ReagentPlus®, Sigma-Aldrich (#G7126)

HERAsafe HS biological safety cabinet by Thermo Scientific, Heraeus Instruments

Hydrochloric acid (HCl), 6 mol/L, Merck EMD Millipore (#110164)

3-Isobutyl-1-methylxanthine (IBMX), Sigma-Aldrich (#I5879)

KT5720, Santa Cruz Biotechnology (#sc-3538)

LabAssay™ ALP kit, Wako chemicals (#291-58601)

4x Laemmli sample buffer, Bio-Rad (#161-0747)

Light microscope BX50, Olympus

Light microscope Axiovert 25, Zeiss

Mastercycler® pro S, Eppendorf (#6325000510)

2-Mercaptoethanol (β-ME), Sigma-Aldrich (#M3148)

Methanol, J.T. Barker (#8045)

Minimum Essential Medium Alpha (αMEM) with nucleosides, L-glutamine and Phenol Red, gibco® by life technologies™, Invitrogen (#22571-020)

Mini-PROTEAN Tetra Cell chamber for Western Blotting, Bio-Rad(#165-8000EDU)

Modified radioimmunoprecipitation assay (mRIPA) buffer, containing 50 mM Tris (#T6066), 150 nM NaCl (#S9888), 0.25% Sodium deoxycholate (#D6750) and 0.5% NP-40 (#74385) at pH 7.5. All reagents by Sigma-Aldrich. If not otherwise specified, protease- and phosphatase inhibitors cOmplete (#05056489001) and PhosSTOP (#04906837001), both Roche, were added.

Nanodrop™ 2000 spectrophotometer, Thermo scientific (#ND-2000)

Naphthol AS-MX phosphate disodium salt, Sigma-Aldrich (#N5000)

Nonidet® NP-40, Sigma-Aldrich (#74385)

Orbital Shaker 3017 by GFL

Phosphate Buffered Saline (PBS), pH 7.4, life technologies, Invitrogen (#10010-056)

PhosSTOP, Phosphatase Inhibitor Cocktail Tablets, Roche (#04906837001)

Penicillin/Streptomycin, 5,000 U/ml Penicillin, 5,000 µg/ml Streptomycin, gibco® by life technologies™, Invitrogen (#15070-063)

Ponceau S 0.1% in 5% acetic acid, Sigma-Aldrich (#P3504)

Powdered milk, Roth (#T145.3). Usually 5% in TBS-T

Powerpac 3000, power supply Bio-Rad (#165-5056)

Precision Plus Protein™ WesternC™ Standards, Bio-Rad (#161-0376)

Precision Protein™ Strep Tactin-HRP Conjugate, 5000x, Bio-Rad (#161-0380)

PrimeTaq DNA-polymerase, 5 U/µl, Primetech (#1800.4)

PrimeTaq DNA-polymerase buffer 10x, containing 650 mM Tris-HCl, 166 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 0.2% Tween20, pH 8.8, Primetech (#0012.4)

Protein assay dye reagent concentrate and protein standard II, Bio-Rad (#500-0006 and -0007)

ProtoScript® M-MuLV First Strand cDNA Synthesis Kit, New England BioLabs (#E6300S)

QB series dry block heater, Grant instruments

qPCR Primers by mwg | operon, eurofins or invitrogen

Reax top shaker, Heidolph instruments (#541-10000-00)

Recombinant Human PTH (1-34), Bachem (#H-4835)

Recombinant Human Wnt-3a, R&D Systems (#5036-WN)

RNeasy® Plus Mini Kit, Qiagen (#74136)

Sartorius analytical balance BP 221 s, Sartorius

Sodium Deoxycholate, Sigma-Aldrich (#D6750)

Sodium Dodecyl Sulfate (SDS) solution, Fluka, Sigma-Aldrich (#05030)

Software CellSens Entry 1.6, Olympus Corp.

Software CFX Manager 3.1, Bio-Rad Laboratories

Software Microsoft Office Professional Plus 2010, Microsoft Corp.

Software Nanodrop 1.4.2, Thermo Fisher Scientific

Software ImageJ 1.48, public domain, National Institutes of Health

Software Image Lab™ 4.1, Bio-Rad Laboratories

Software InkScape 0.48, Free Software Foundation

Software Mendeley Desktop 1.12.3, Mendeley Ltd.

Software Serial Cloner 2.6.1, Serial Basics

Software WorkOut 2.5, Dazdaq Solutions Ltd.

Software ZEN 2012 (blue edition), Zeiss

SuperSignal West Femto Chemiluminescent Substrate, Pierce, Thermo Fisher Scientific (#34095)

iQ™ SYBR® Green Supermix, Bio-Rad (#170-8880)

Thermomixer® comfort, Eppendorf

ThermoStat™ plus, Eppendorf (#5352 000.010)

Tris Acetic Acid EDTA (TAE) buffer, containing 40 mM Tris (#T6066), 20 mM acetic acid (#A6283) and 1 mM EDTA (#E5134) at pH 8.5. All reagents by Sigma-Aldrich.

Trans-Blot® Turbo™ Transfer Starter System, Bio-Rad (#170-4155)

Tris-Buffered Saline and Tween20 (TBS-T), containing 50 mM Tris (#T6066), 150 mM NaCl (#S9888) and 0.05% Tween20 (#P7949) at pH 7.6. All reagents by Sigma-Aldrich.

10x Tris/Glycine/SDS running buffer, Bio-Rad (#161-0732)

Trizma® Base, Sigma-Aldrich (#T6066)

Trizma® hydrochloride, Sigma-Aldrich (#T5941)

Trypsin-EDTA (0.05%), phenol red, gibco® by life technologies (#25300-054)

Tween 20, Sigma-Aldrich (#P7949)

N,N,N′,N′-Tetramethylethylenediamine (TEMED), Sigma-Aldrich (#T9281)

VICTOR™ *X*5 Multilabel Plate Reader, Perkin Elmer (#2030-0050)

4 Results

4.1 Lack of Tgif1 Reduces Osteoblast Differentiation

To determine the role of Tgif1 in osteogenesis, we first tested whether lack affects osteoblast differentiation, which can be visualized by staining for ALP activity. To study this, we used primary osteoblasts from a mouse strain with germline deletion of the *Tgif1* gene, i.e. a global knock-out. BMSCs were seeded at confluence and left to attach for two days before the use for differentiation experiments. They were then either cultured in αMEM (Fig. 4.1A), or supplemented with mineralization additives, i.e. ascorbic acid, β-Glycerophosphate and dexamethasone (Fig. 4.1B), provided fresh daily. On day five, the cells were fixed and stained for ALP activity. The highest intensity in the left wells reveals that $Tgif1^{+/+}$ derived osteoblasts are most positive for ALP staining, compared to heterozygous $Tgif1^{+/-}$ and knock-out $Tgif1^{-/-}$, indicating compromised differentiation capacity in the absence of Tgif1. This was consistent in a Tgif1 genotype “dose-dependent” manner in both differentiation media (Fig. 4.1).

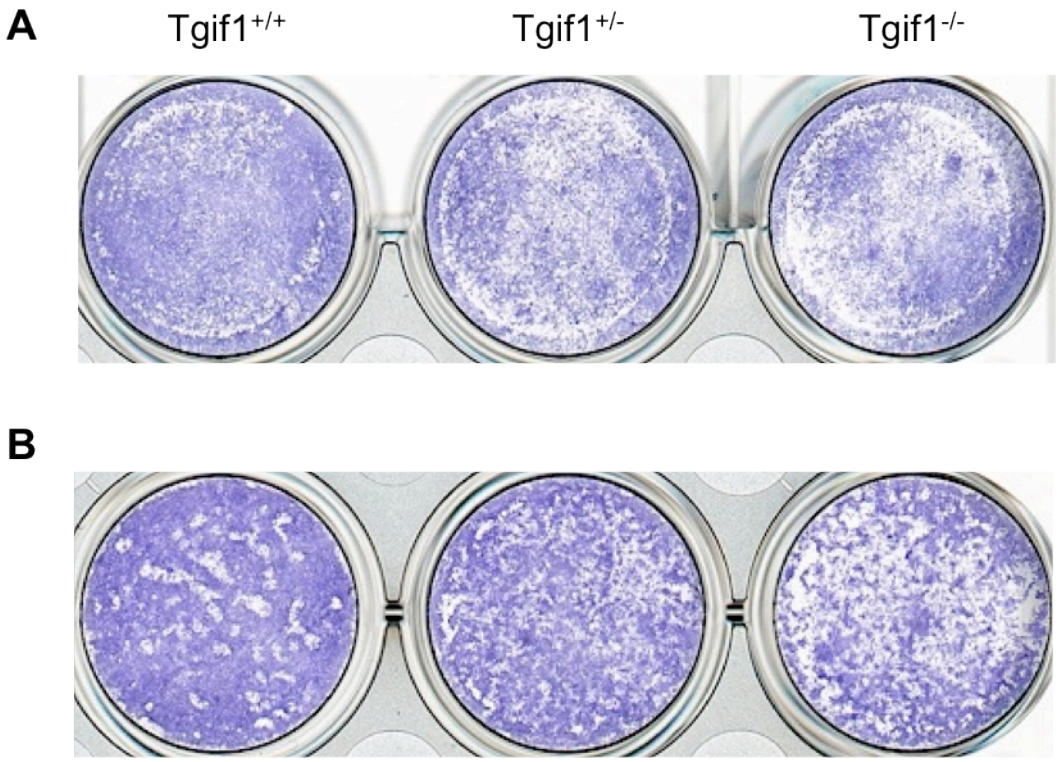

Figure 4.1: Absence of Tgif1 impairs early osteogenic differentiation. Bone marrow stromal cells (BMSCs) were cultured in αMEM (A) without or (B) with mineralization additives for five days. Mineralization additives consisted of 50 µg/ml ascorbic acid, 5 mM β-Glycerophosphate and 10 nM dexamethasone. Fresh medium was added every day. After fixation, cells were stained for alkaline phosphatase (ALP) activity to evaluate early osteogenic differentiation. The intensity was affected by Tgif1 genotype. It was highest in Tgif1$^{+/+}$, reduced in Tgif1$^{+/-}$ and lowest in Tgif1$^{-/-}$, littermates.

At advanced stages of osteoblastic differentiation, cells start to mineralize their extracellular matrix, which can be detected by alizarin red staining *in vitro*. COBs were seeded at confluence and left to attach for one day. After 15 days of osteogenic culture, with fresh medium provided daily, long-term differentiated Tgif1$^{-/-}$ osteoblasts displayed diminished staining for alizarin red compared to littermate controls. This indicates a compromised mineralization in the absence of Tgif1. Consistent with ALP staining, the effect appears to be determined by Tgif1 genotype. The mineralization intensity was highest in Tgif1$^{+/+}$, reduced in Tgif1$^{+/-}$ and lowest in Tgif1$^{-/-}$, comparing littermates (Fig. 4.2). Both methods, staining for ALP activity and alizarin red, visualize physiologic and essential osteoblasts functions *in vitro*. These appear to be affected by Tgif1 genotype and compromised when Tgif1 is absent.

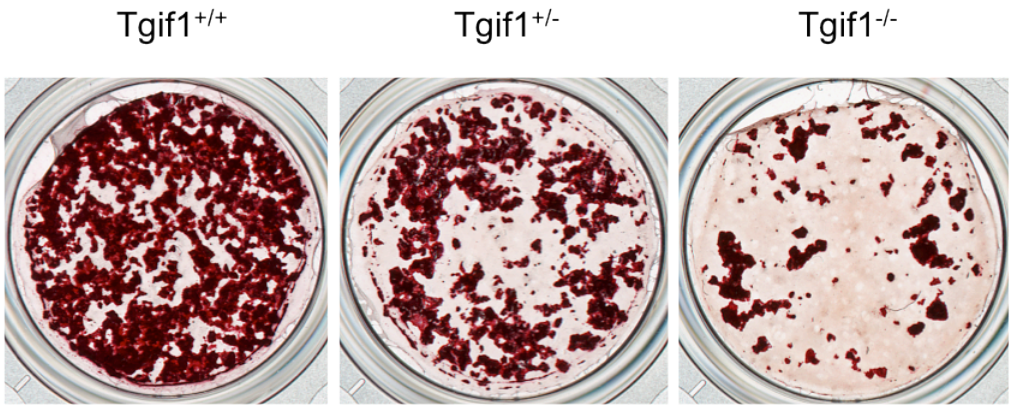

Figure 4.2: Absence of Tgif1 impairs mineralization. Calvarial osteoblasts (COBs) were cultured in osteogenic medium for 15 days. Mineralization additives consisted of 50 µg/ml ascorbic acid, 5 mM β-Glycerophosphate and 10 nM dexamethasone. Fresh medium was added every day. After fixation, cells were stained with Alizarin Red to visualize advanced osteogenic differentiation. The mineralization intensity was affected by Tgif1 genotype. It was highest in Tgif1$^{+/+}$, reduced in Tgif1$^{+/-}$ and lowest in Tgif1$^{-/-}$ littermates.

4.2 Tgif1-Deficiency Results in a Decreased Proliferation

Bone formation is maintained by differentiation and proliferation of osteoblast precursor cells. To explore whether the observed phenotype was solely due to impaired differentiation in osteoblasts deficient of Tgif1 or also a diminished proliferation and metabolic activity, we conducted a colorimetric MTS assay to determine cellular viability.

BMSCs derived from Tgif1$^{+/+}$ and Tgif1$^{-/-}$ mice were seeded and left to attach for two days. At a given a time point, the cells were incubated with MTS solution for four hours. The optical density (OD), directly proportional to cellular viability, was measured photometrically on days 0, 2 and 5. At the beginning of the experiment, no difference in proliferative activity between the Tgif1 genotypes was detected (day 0), as cells were seeded in equal quantity (OD 0.56 vs. 0.56). On day two, a modest but significantly increased activity was detected in the Tgif1$^{+/+}$ cells, which became more pronounced over time (OD 0.68 vs. 0.63). On day five, Tgif1$^{+/+}$ cells displayed a 1.3-fold greater absorbance, hence viability, compared to cells from Tgif1$^{-/-}$ littermates (OD 0.85 vs. 0.64) (Fig. 4.3).

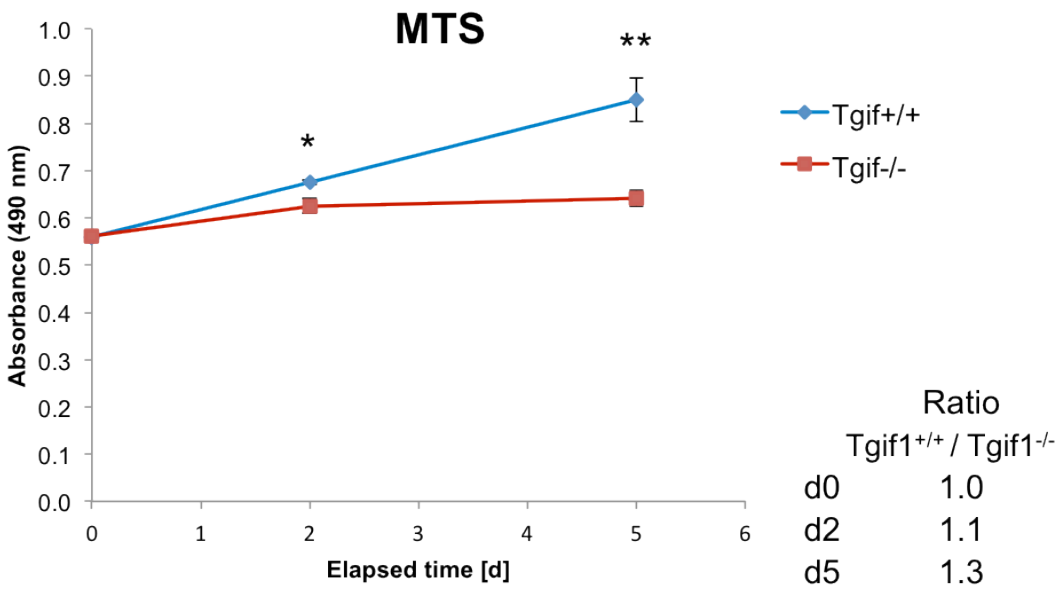

Figure 4.3: Lack of Tgif1 results in a decreased proliferative activity. BMSCs were incubated with MTS solution for four hours at different time points after seeding. NAD(P)H-dependent cellular oxidoreductases convert this substrate. The product was detected photometrically at 490 nm and optical density was directly proportional to cellular viability. At the beginning of the experiment (d0), there was no detectable difference in absorbance, as cells were equal in number. After two days (d2) a slight but significant difference was apparent which became more pronounced until the last time point (d5). Ratio of optical density in $Tgif1^{+/+}$ compared to $Tgif1^{-/-}$ cells. *p ≤ 0.05, **p ≤ 0.01 $Tgif1^{+/+}$ vs. $Tgif1^{-/-}$.

To further assess osteoblast proliferation, we performed a BrdU assay. This visualizes replication during the S-phase of the cell cycle. LOBs from $Tgif1^{+/+}$ and $Tgif1^{-/-}$ mice were used. Either directly (day 0), or after having reached confluence (day 4), the cells were labeled with a BrdU solution in order to allow incorporation of proliferating cells. The following day (1 or 5), they were processed with a BrdU staining kit. Immunocytochemical labeling was detected by light microscopy. Proliferative cells displayed brown nuclear staining and were quantified by BrdU positive cells / total number of cells. On day 1, $Tgif1^{+/+}$ osteoblasts were more proliferative than those from $Tgif1^{-/-}$ littermates (56.6% vs. 27.4%). After cells were kept in culture for five days, and had formed a confluent layer in the well, similar to the previous differentiation assays, the osteoblasts only faintly incorporated BrdU. This suggests ceased proliferation due to contact inhibition by day five and was seen in both Tgif1 genotypes (6.1% vs. 5.7%) (Fig. 4.4). Taken together, these assays indicate a compromised proliferative activity in osteoblasts lacking Tgif1.

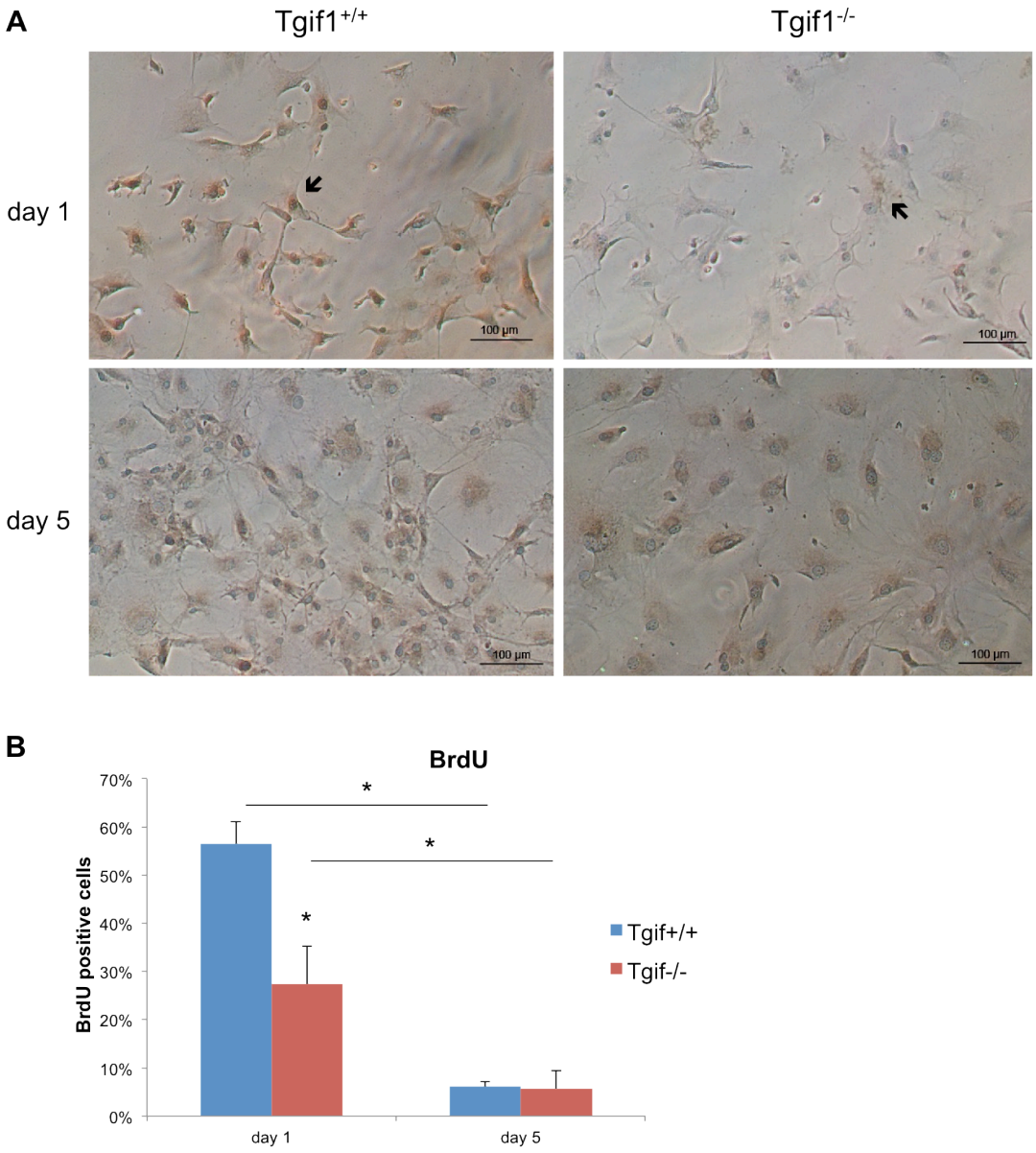

Figure 4.4: Lack of Tgif1 leads to a decreased proliferation. (A) LOBs were seeded at sub-confluence and cultured for either one or five days. They were incubated with a BrdU labeling reagent for twelve hours. Cells in the S-phase of the cell cycle incorporated this synthetic thymidine analog. After fixation, cells were incubated with a biotinylated monoclonal anti-BrdU antibody, which was detected using a streptavidin-HRP conjugate and DAB detection. Immunocytochemical staining was visualized by light microscopy. Brown nuclear staining for BrdU indicated proliferation (arrows). On day 1, Tgif1$^{-/-}$ LOBs stained significantly less positive than Tgif1$^{+/+}$. On day 5, this difference between Tgif1 genotypes was abolished as the cells had reached a confluent layer and terminated proliferation. Scale bars represent 100 µm. (B) Three images per condition were quantified by BrdU positive cells / total cell number. *p ≤ 0.05 Tgif1$^{+/+}$ vs. Tgif1$^{-/-}$ and day 1 vs. day 5 respectively.

4.3 Tgif1 Promotes Osteogenic Differentiation

Based on the finding that Tgif1-deficiency in osteoblasts decreased differentiation and proliferation, we tested whether increasing Tgif1 protein levels would enhance it. To this end, recombinant Tgif1 was transiently overexpressed using a CMV-driven expression vector, encoding mouse Tgif1 tagged with an N-terminal FLAG tag.

BMSCs were seeded at confluence and left to attach for two days. They were then transfected with either an empty Flag vector (control) or a plasmid encoding Flag-tagged Tgif1 using GeneCellin™ Transfection Reagent for twelve hours. Differentiation was induced the next day with fresh osteogenic medium daily and assessed on day five by ALP staining. The slightly higher intensity of ALP staining in cells that were transfected with recombinant Tgif1, compared to cells transfected with the control vector, suggests a supportive effect of Tgif1 on osteoblast differentiation (Fig. 4.5).

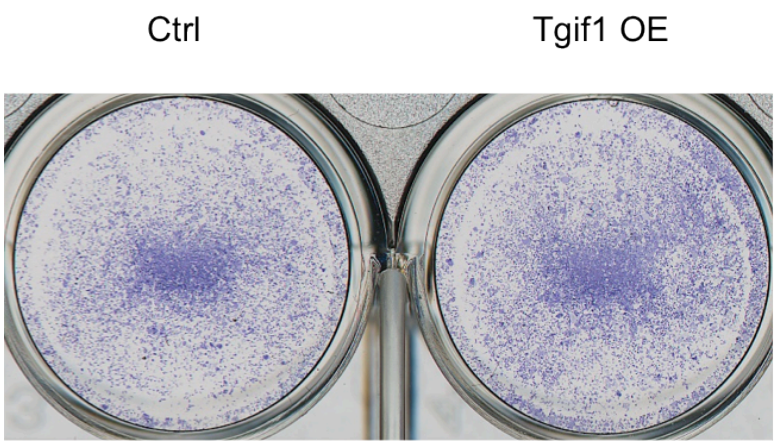

Figure 4.5: Overexpression (OE) of Tgif1 enhances osteogenic differentiation. BMSCs were transfected with either an empty Flag vector (control), or a vector encoding Flag-tagged mouse Tgif1. Transient overexpression was achieved by incubation of 750 ng of plasmid DNA. BMSCs were then cultured in osteogenic medium for four days. Fresh medium was added every day. After fixation, cells were stained for ALP activity to ascertain early osteogenic differentiation. A modest increase in staining intensity is noted in cells overexpressing Tgif1.

4.4 PTH Induces Osteoblast Differentiation

Intermittent administration of recombinant human PTH 1-34 (iPTH) is a strong stimulus to bone formation and favors differentiation of osteoblast progenitors. COBs were isolated and cultured as detailed above and seeded at confluence two days before the experiment. To induce differentiation, the cells were supplied with osteogenic medium alone (Ctrl) or additional 100 nM PTH for four hours daily. After five days of culture, the cells were washed, fixed and stained for ALP activity. As expected, intermittent administration of PTH increased ALP activity in COBs (Fig. 4.6).

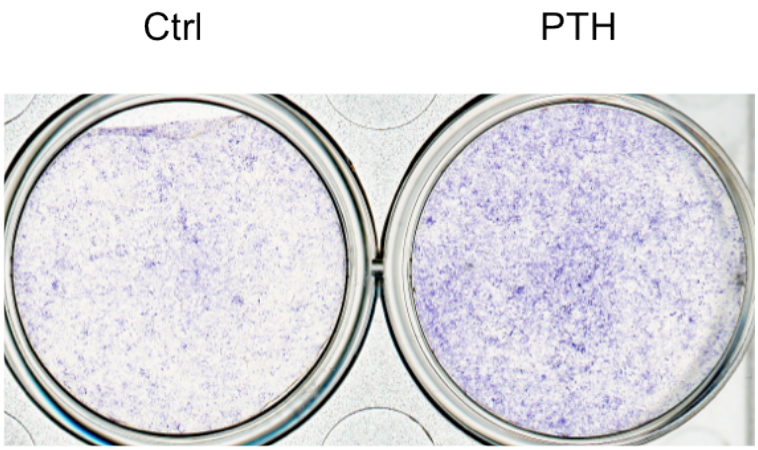

Figure 4.6: Induction of osteogenic differentiation by PTH. COBs were cultured in osteogenic medium for five days. 100 nM PTH in fresh medium was applied intermittently for four hours per day. After fixation, cells were stained for ALP activity to determine early osteogenic differentiation. The staining intensity was higher in the PTH stimulated wells compared to untreated cells.

4.5 Tgif1 Represents a PTH and Canonical Wnt Target Gene

PTH and canonical Wnt signaling are key anabolic pathways and noted for targeting a variety of growth factors and cytokines that affect osteoblasts and their precursors during bone development, remodeling or repair. Having demonstrated the cell-autonomous significance of Tgif1 for osteoblast differentiation and proliferation under basal conditions, we aimed to investigate whether Tgif1 is regulated by the influential bone anabolic stimuli.

In order to resemble such physiological stimuli during bone formation, we treated primary osteoblasts *in vitro* with recombinant proteins to activate the respective signal transduction pathways. The cells used in these experiments were isolated from either the calvariae of newborn mice (age postnatal days 1-3) or from the long bones of 10-12 week old wild type mice, cultured as detailed above. Both cell types were seeded at sub-confluence two days before the experiment. Before stimulation, the cells were serum starved for four hours. They were then incubated with either 100 nM PTH or 100 ng/ml Wnt3a for four hours. Subsequently, the cells were washed, lysed and homogenized to obtain total cell lysates. Samples were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Protein detection with specific antibodies is shown by Western blot analysis. In order to verify the conditions used for stimulation with PTH and Wnt3a, the following known pathway components were analyzed: phospho-CREB and active β-Catenin as stimulation controls for PTH and canonical Wnt signaling respectively. In comparison to the vehicle treated control (left), the stimulation (right) increased protein abundance of Tgif1. The amounts of the known target proteins were increased concomitantly, confirming the effectiveness of stimulatory conditions (Fig. 4.7).

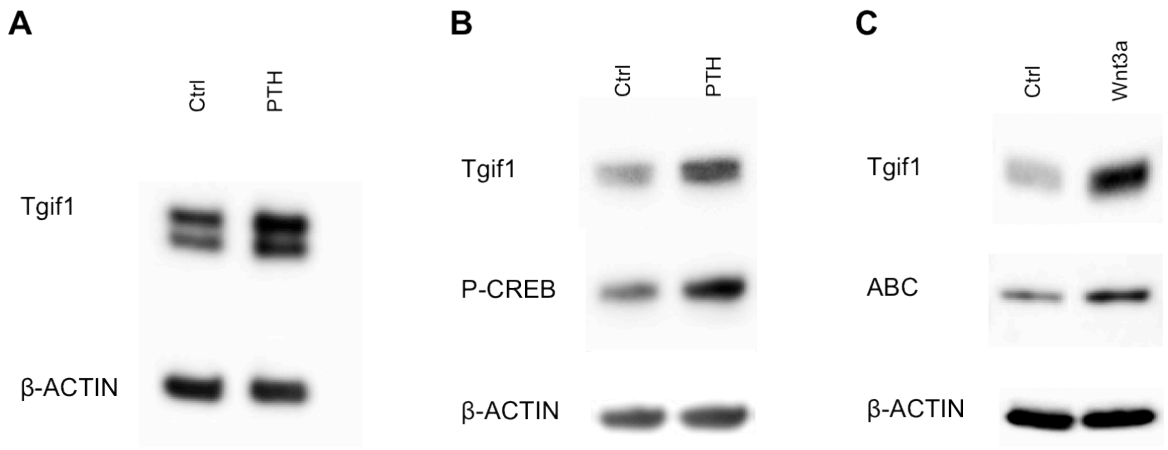

Figure 4.7: Induction of Tgif1 protein expression by different stimuli in primary osteoblasts. Primary osteoblasts were treated for four hours with the indicated stimuli. Total cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and proteins were detected with specific antibodies. For all blots β-Actin was used as loading control. (A) COBs or (B) LOBs were stimulated with PTH (100 nM). (C) LOBs were treated with Wnt3a (100 ng/ml). All stimulations resulted in the induction of Tgif1 protein. Known effectors P-CREB, phospho-cAMP response element-binding protein; and ABC, activated β-catenin; served as respective stimulation controls and displayed concomitant increase.

To investigate whether abundance of Tgif1 protein was paralleled by elevated mRNA levels, RNA was isolated from osteoblasts after stimulation with PTH or Wnt3a, reversely transcribed into cDNA and analyzed by quantitative real-time PCR (qPCR). The resultant mRNA was assessed by qPCR analysis and relative expression was calculated according to the MIQE guidelines.

The known target genes of the PTH and canonical Wnt pathways Ramp3 and Axin2 were used as controls to confirm effective treatment. Stimulation increased mRNA levels of Ramp3 and Axin2 by 3.8- and 9.7-fold in LOBs, and by 44.4- and 8.7-fold in BMSCs compared to vehicle treated control, demonstrating that the stimulatory conditions activated the pathways. Interestingly, Tgif1 mRNA levels were significantly elevated by 1.5-fold in LOBs and by 2.1-fold in BMSCs after treatment with Wnt3a, whereas a tendency was observed after incubation with PTH (Fig. 4.8).

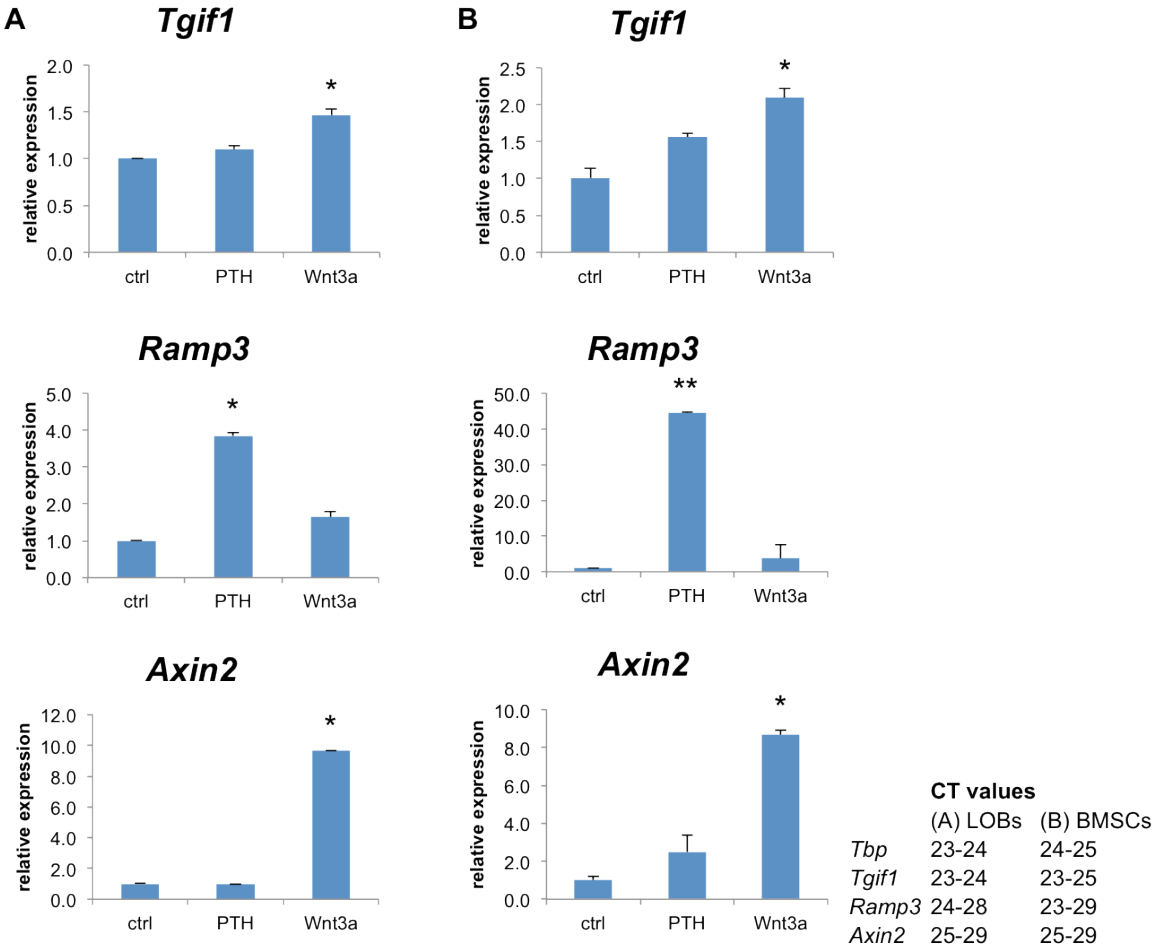

Figure 4.8: Induction of Tgif1 mRNA by different stimuli in primary osteoblasts. (A) LOBs or (B) BMSCs were stimulated with 100 nM PTH or 100 ng/ml Wnt3a for four hours. The cells were lysed, RNA was isolated and reverse-transcribed into cDNA before qPCR analysis with specific primers. Tgif1 mRNA levels were significantly increased upon stimulation with Wnt3a, whereas a tendency was observed after treatment with PTH. The target genes Ramp3 and Axin2 served as controls for activation of PTH and canonical Wnt signaling. Expression was enhanced after the specific stimuli. *p ≤ 0.05, **p ≤ 0.01 respective stimulation vs. vehicle treated control. C_t value as qPCR threshold cycle.

4.6 PTH Activates Tgif1 via the AC/PKA Pathway

PTH exerts its anabolic effects on bone by binding to its receptor PTH1R. The subsequent activation of $G_s\alpha$ and adenylate cyclase (AC) leads to an activation of protein kinase A (PKA), phosphorylating CREB to p-CREB, which can then translocate to the nucleus and bind to transcription factors such as AP-1. Although it

has been shown that PTH physiologically activates several other signal transduction pathways, including $G_q\alpha$ / phospholipase C / PKC, the anabolic effects of PTH on bone are in large part being attributed to PKA signaling.

In order to elucidate whether PTH regulates Tgif1 expression via the dominant PKA cascade, we activated this pathway pharmacologically by adding Forskolin, a cell-permeable activator of adenylate cyclase. Forskolin increases intracellular levels of 3',5'-cyclic adenosine monophosphate (cAMP), a pivotal second messenger. Thus, it endorses one major branch of PTH signaling, partly mimicking its physiological effects. LOBs were serum starved before stimulation with 100 nM PTH or 1 µM Forskolin (FSK) for two hours. Total cell lysates were processed for Western blot analysis. FSK was able to resemble the effects of PTH as both increased Tgif1 and phospho-CREB protein levels, compared to cells treated with a vehicle control (Fig. 4.9A).

Consecutively, the membrane permeable, competitive PKA inhibitor KT5720 was used to block the PTH-PKA pathway. After serum starvation, LOBs were incubated with different concentrations of KT5720 for one hour prior to stimulation with 100 nM PTH for two hours. While PTH augmented Tgif1 protein levels in the presence of low levels (1 µM) of KT5720, higher concentrations (5 µM) abolished this effect. Note that as expected, phosphorylation of CREB was also inhibited by 5 µM KT5720, indicating that blocking PKA activity was effective at this concentration (Fig. 4.9B).

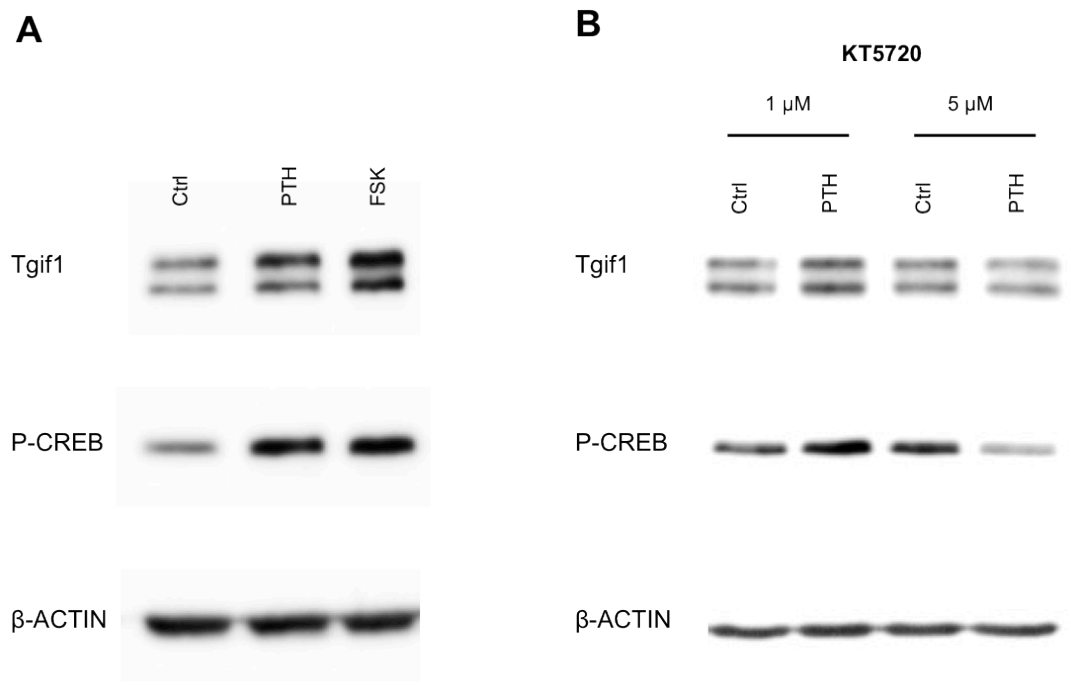

Figure 4.9: Induction of Tgif1 protein via the AC/PKA pathway. After stimulation, total cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and proteins were detected with specific antibodies. For all blots β-Actin was used as loading control. (A) LOBs were treated with 100 nM PTH or 1 µM of the AC activator Forskolin (FSK) for two hours. This resulted in the concomitant increase of Tgif1 and phospho-CREB compared to vehicle treated control. (B) LOBs were pre-incubated with 1 or 5 µM of the PKA inhibitor KT5720 for one hour before stimulation with 100 nM PTH for two hours. KT5720 dose-dependently abolished Tgif1 and phospho-CREB protein induction.

Primary osteoblasts deficient of Tgif1 are compromised in their ability of maturation. PTH represents a compelling stimulus for osteogenic differentiation. PTH exerts bone anabolic effects via the $G_s\alpha$/AC/PKA branch in large part, and Tgif1 is involved in this cascade. Adenylate cyclase (AC) catalyzes the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic adenosine monophosphate (cAMP). To assess whether lack of Tgif1 affects the formation of this early second messenger, we conducted a competitive cAMP enzyme-linked immunosorbent assay (ELISA) to quantify intracellular cAMP concentrations.

LOBs were serum starved before treatment with 100 nM PTH or 1 µM Forskolin (FSK) for 20 minutes, together with 2 mM 3-isobutyl-1-methylxanthine (IBMX). As

before, FSK was utilized as an activator of AC, to mimic the effects of PTH signaling via the AC-PKA branch. IBMX is a non-specific inhibitor of phosphodiesterase (PDE), used here to prevent degradation of cAMP to AMP by PDE. cAMP accumulation was assessed in cell culture supernatants from LOBs using a kit. In this competitive ELISA, endogenous cAMP from samples and cAMP conjugated to ALP compete for binding to a specific antibody. The amount of added substrate, converted by ALP on the cAMP conjugate, was detected photometrically. Concentration of endogenous cAMP, inversely proportional to this colorimetric signal, was calculated with 4-parameter logistic (4PL) curve fitting. Independent of Tgif1 genotype, both PTH and FSK markedly increased the abundance of cAMP in the samples compared to vehicle treated controls. Although the cAMP ELISA did not reveal differences between Tgif1 genotypes under basal conditions or after induction with PTH (188 vs. 202 pg/ml) levels of cAMP were reduced after incubation with FSK in Tgif1$^{-/-}$ (137 vs. 40 pg/ml) (Fig. 4.10A).

Total cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and proteins were detected with specific antibodies. Consistent with previous results (Fig. 4.9A), administration of both PTH and FSK for 20 minutes amplified levels of Tgif1 protein in Tgif1$^{+/+}$ osteoblasts to a similar extent. Note that Western blot analysis revealed markedly reduced levels of phospho-CREB in Tgif1$^{-/-}$ compared to Tgif1$^{+/+}$ littermate controls. As opposed to previous observations, the known target and central mediator of AC/PKA signaling is not induced by PTH or FSK in both Tgif1 genotypes. The lack of induction may be due to a shorter period of stimulation: 20 minutes vs. 120 minutes (Fig. 4.10B).

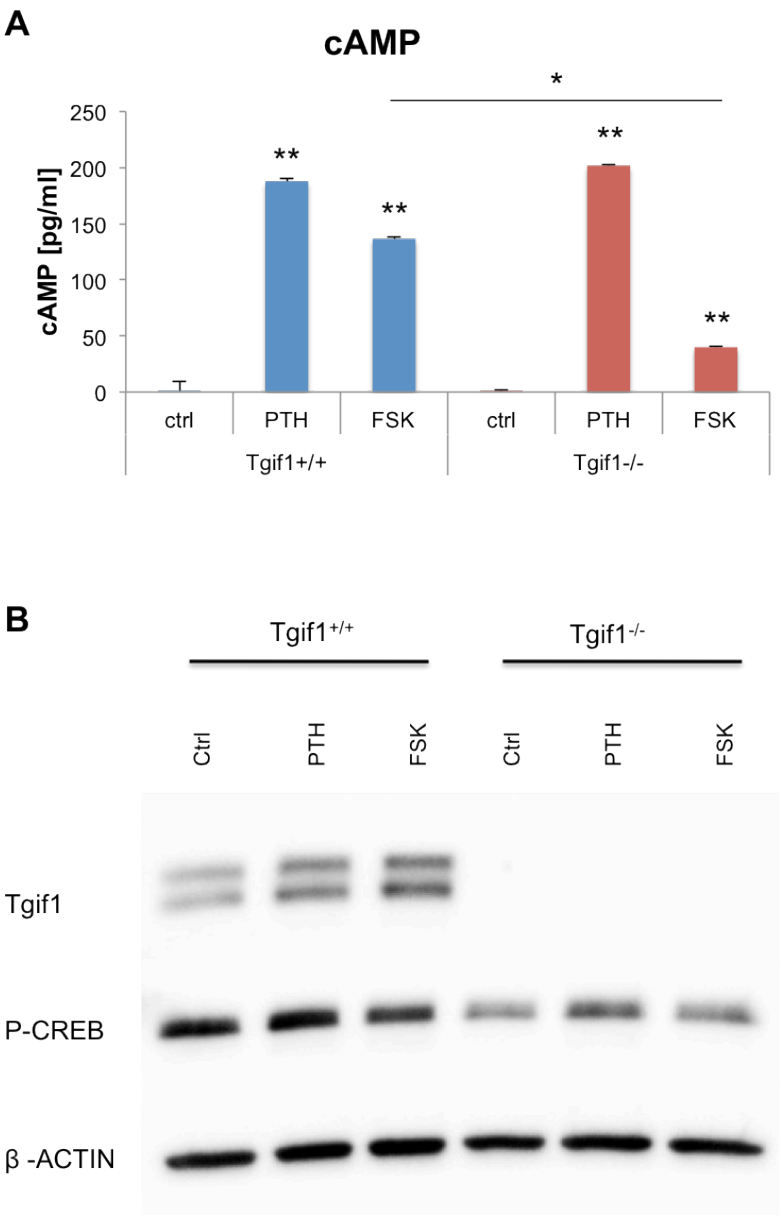

Figure 4.10: Activation of AC/PKA/p-CREB is impaired in Tgif1-deficiency. LOBs were incubated with 2 mM IBMX (vehicle control) and 100 nM PTH or 1 µM FSK for 20 minutes. (A) A competitive cAMP ELISA was conducted to quantify intracellular concentrations of cAMP. Levels of cAMP were not altered between Tgif1 genotypes under basal conditions or after stimulation with PTH, but lower in Tgif1$^{-/-}$ after incubation with FSK. *p ≤ 0.05 FSK treated Tgif1$^{+/+}$ vs. Tgif1$^{-/-}$ and **p ≤ 0.01 stimulation vs. vehicle treated control. (B) Total cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and proteins were detected with specific antibodies. β-Actin was used as loading control. Stimulation with PTH and FSK resulted in augmented Tgif1 protein levels in Tgif1$^{+/+}$. Levels of phospho-CREB were reduced in Tgif1$^{-/-}$.

4.7 Tgif1-Deficiency Impairs the Response to PTH

Osteoblasts lacking Tgif1 displayed diminished levels of PTH-induced phospho-CREB, a crucial mediator of PKA signaling. Although levels of cAMP were not altered, apparently cells deficient of Tgif1 are not fully responsive to PTH.

LOBs were seeded at sub-confluence and left to attach for two days. After serum starvation and two hours of treatment with 100 nM PTH, 100 ng/ml Wnt3a or vehicle, the cells were washed, lysed and analyzed for protein and mRNA content. Tgif1$^{+/+}$ cells responded with a concomitant elevation of Tgif1 and the known effector proteins phospho-CREB and active β-Catenin for PTH and canonical Wnt signaling respectively. In Tgif1$^{-/-}$ cells, stimulation with Wnt3a led to comparable activation of β-Catenin. Administration of PTH, however, did not increase phospho-CREB protein levels (Fig. 4.11A).

Observations from qPCR analysis from BMSCs revealed that after similar treatment, Tgif1$^{-/-}$ cells displayed impaired response of Ramp3, a PTH target gene, compared to Tgif1$^{+/+}$ cells (11.2- vs. 22.0-fold). Axin2, a canonical Wnt target gene, remained unaffected by loss of Tgif1 (induction upon Wnt3a stimulation 7.5- vs. 7.4-fold, Fig. 4.11B). This lack of PTH target gene expression indicates malfunctional activation of PTH mediated AC/PKA signaling in Tgif1-deficiency in distinct experiments and different sources of primary osteoblasts. Canonical Wnt target genes responded to stimulation irrespective of Tgif1 genotype.

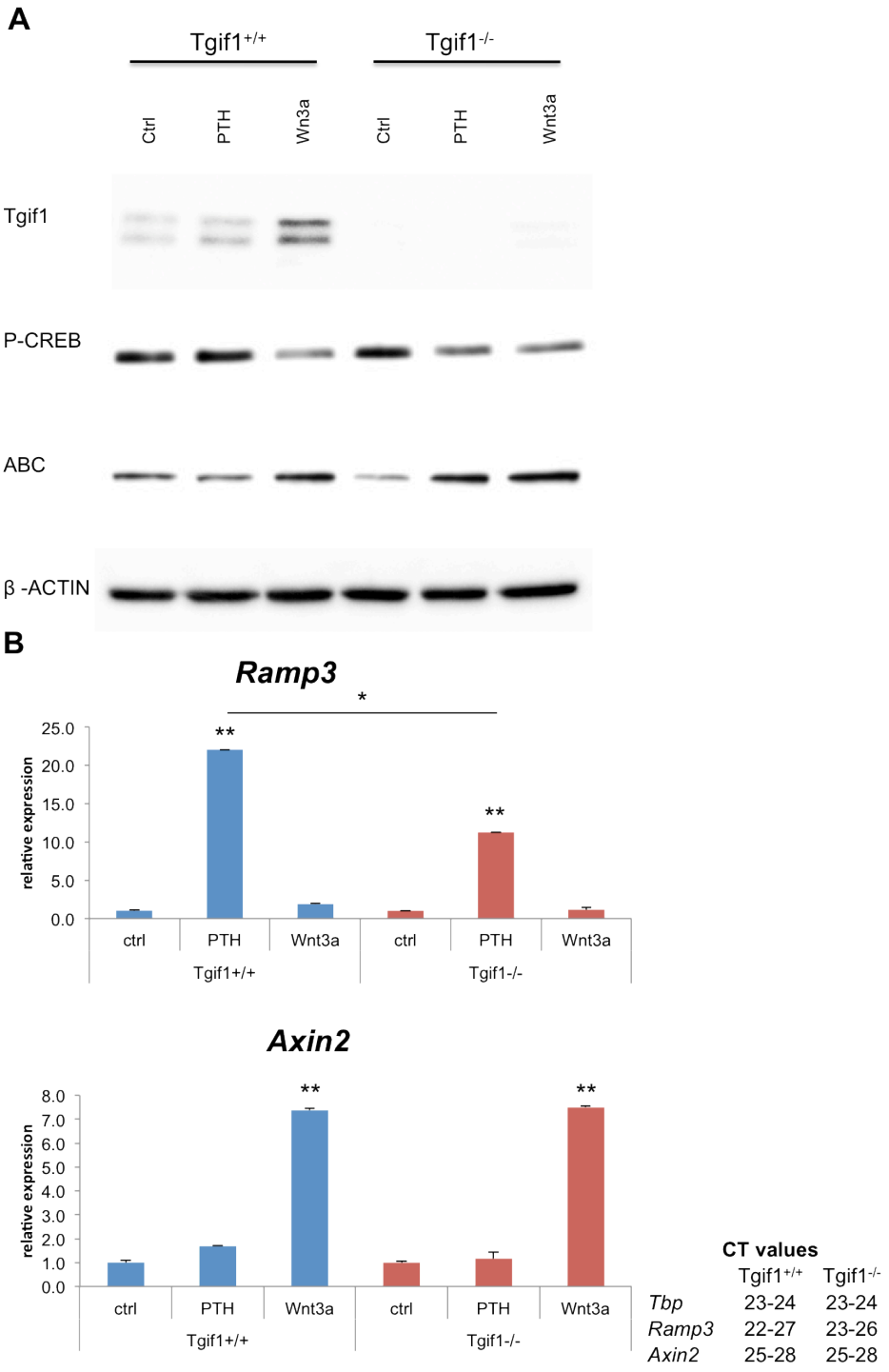

Figure 4.11: Lack of Tgif1 results in an impaired response to PTH. (A) LOBs were treated with PTH or Wnt3a for two hours. Both stimulation with PTH and Wnt3a increased abundance of Tgif1 protein. Abundance of phospho-CREB after treatment with PTH is seen in Tgif1$^{+/+}$ only. Stimulation with Wnt3a resulted in comparable elevation of active β-catenin (ABC) in both Tgif1 genotypes. (B) BMSCs were stimulated correspondingly and processed for qPCR analysis. PTH enhanced the expression of Ramp3 mRNA in both Tgif1 genotypes, although to a significantly lesser extent in cells deficient of Tgif1. Wnt3a increased Axin2 mRNA to a similar extent in both Tgif1 genotypes. *p ≤ 0.05 PTH treated Tgif1$^{+/+}$ vs. Tgif1$^{-/-}$ and **p ≤ 0.01 respective stimulation vs. vehicle treated control.

4.8 Tgif1 Connects PTH and Canonical Wnt Signaling

PTH and canonical Wnt signaling synergize to favor bone formation, and both induce Tgif1 expression. The effects of iPTH on bone result, in part, from cross-activation of canonical Wnt signaling, by that constituting a potent bone anabolic stimulus. Thus, we aimed to investigate whether Tgif1 is involved in this cross-talk. The double transgenic BAT-GAL^{+}:Tgif1$^{+/+}$ and BAT-GAL^{+}:Tgif1$^{-/-}$ mouse gives insight into the activity of Wnt/β-Catenin signaling in the presence or absence of Tgif1. When stimulated with PTH, these animals allow evaluating the cross-activation of canonical Wnt signaling as a function of Tgif1.

BMSCs derived from BAT-GAL^{+}:Tgif1$^{+/+}$ and BAT-GAL^{+}:Tgif1$^{-/-}$ mice were seeded at sub-confluence and left to attach for two days. After serum starvation, they were treated with 100 nM PTH for four hours, washed and mildly fixed. Staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal), a substrate for inducible β-Galactosidase, was done to assess activation of Tcf/Lef responsive elements. Under basal conditions, cells derived from Tgif1$^{+/+}$ and Tgif1$^{-/-}$ displayed comparable Xgal staining (13.2% vs. 15.8%). Stimulation with PTH led to an increased Xgal staining, indicating cross-activation of the canonical Wnt pathway, as assayed by activity of Tcf/Lef transcription factors. Interestingly, this cross-talk was compromised in osteoblasts isolated from Tgif1$^{-/-}$ animals, as indicated by the reduced intensity of Xgal staining and lower density of Xgal positive cells . This was quantified by Xgal positive cells / total number of cells. When normalized to vehicle treated controls of the according genotype, stimulation with PTH increased the number of Xgal-positive cells by 3.2-fold in Tgif1$^{+/+}$ osteoblasts (41.8%), compared to 1.8-fold in Tgif1$^{-/-}$ osteoblasts (29.1%) (Fig. 4.12). This suggests that cross-activation of the canonical Wnt pathway is impaired in the absence of Tgif1.

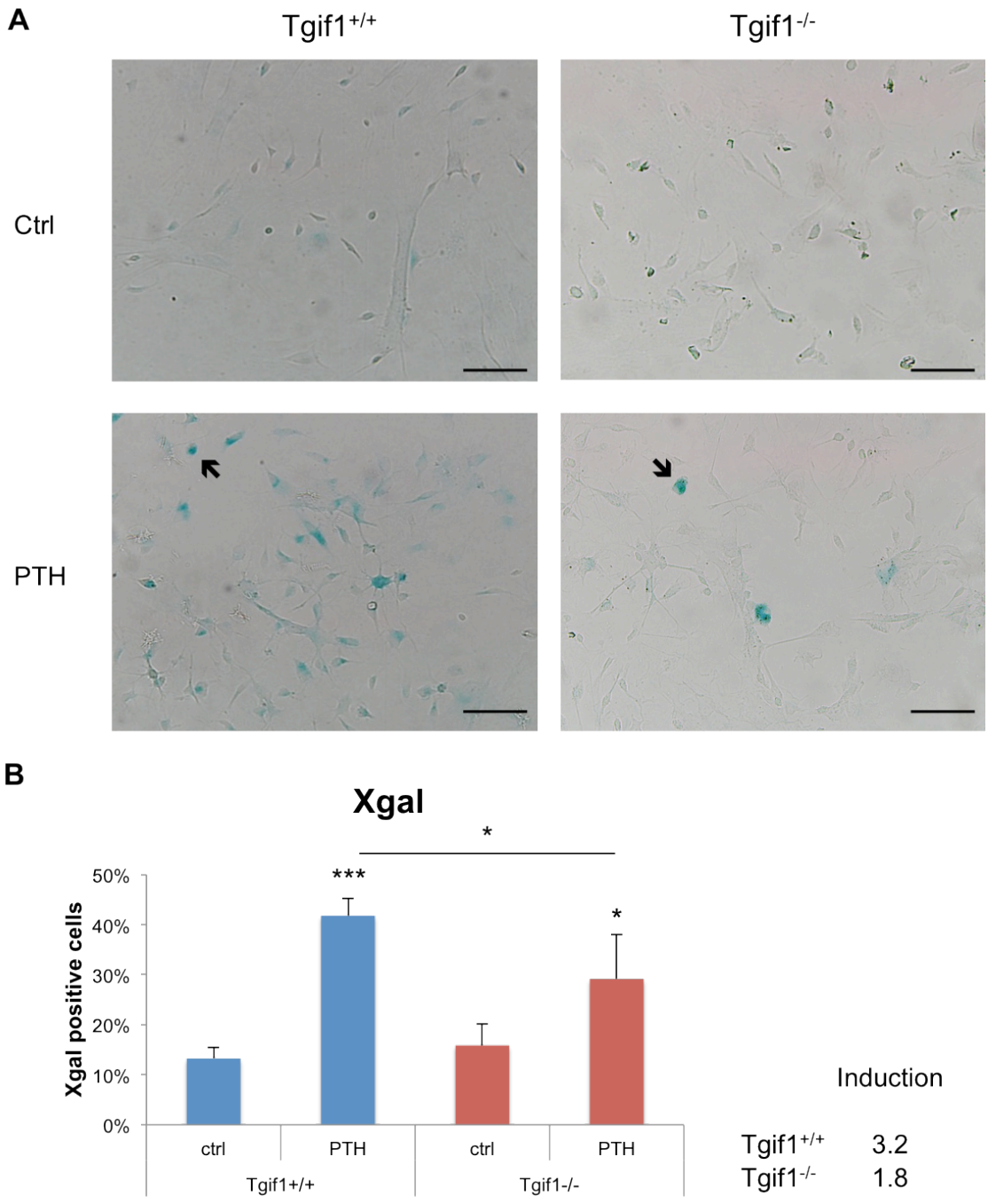

Figure 4.12: Absence of Tgif1 reduces the activation of the canonical Wnt pathway upon PTH stimulation. (A) BMSCs from BAT-GAL^{+}:Tgif1$^{+/+}$ and BAT-GAL^{+}:Tgif1$^{-/-}$ mice were treated with PTH 100 nM for four hours. After fixation, cells were incubated with Xgal staining solution for 24 hours. The resulting indigo blue staining, indicating active β-Galactosidase under control of Tcf/Lef, was detected by light microscopy (arrows). Scale bars represent 100 µm. (B) Quantification from five images per condition by Xgal positive cells / total cell number. Induction as stimulation over vehicle treated control. *p ≤ 0.05 Tgif1$^{-/-}$ PTH vs. control and PTH treated Tgif1$^{+/+}$ vs. Tgif1$^{-/-}$. ***p ≤ 0.001 Tgif1$^{+/+}$ PTH vs. vehicle treated control.

4.9 Lack of Tgif1 Blunts PTH-induced Osteoblast Differentiation

In light of a compromised response to bone anabolic stimuli and the observed phenotype under basal conditions, we hypothesized that Tgif1 is required for PTH-induced osteoblast differentiation. To test this hypothesis, we cultured osteoblasts from mice lacking Tgif1 and control littermates under osteogenic conditions. PTH was applied intermittently to elicit osteoblast differentiation.

LOBs were seeded at confluence and left to attach for two days. The cells were then cultured in complete αMEM, supplemented with mineralization additives (vehicle control) and treated with PTH 100 nM for four hours per day for five days. Differentiation was assessed by staining for alkaline phosphatase (ALP) activity (Fig. 4.13A) and quantified. Total cell lysates from corresponding wells were obtained with modified RIPA buffer. After processing of the samples according to the manufacturer's instructions, optical density was measured at 405 nm and normalized for protein content (Fig. 4.13B). Both ALP activity staining and quantification revealed that lack of Tgif1 led to a diminished differentiation capability. Interestingly, intermittent administration of PTH augmented ALP activity by 2.1-fold in $Tgif1^{+/+}$ LOBs (0.28 vs. 0.59 AU/mg). ALP activity was overall lower in $Tgif1^{-/-}$ LOBs. This became more pronounced after PTH treatment, as relative induction by PTH was only 1.2-fold (0.18 vs. 0.25 AU/mg) (Fig. 4.13B).

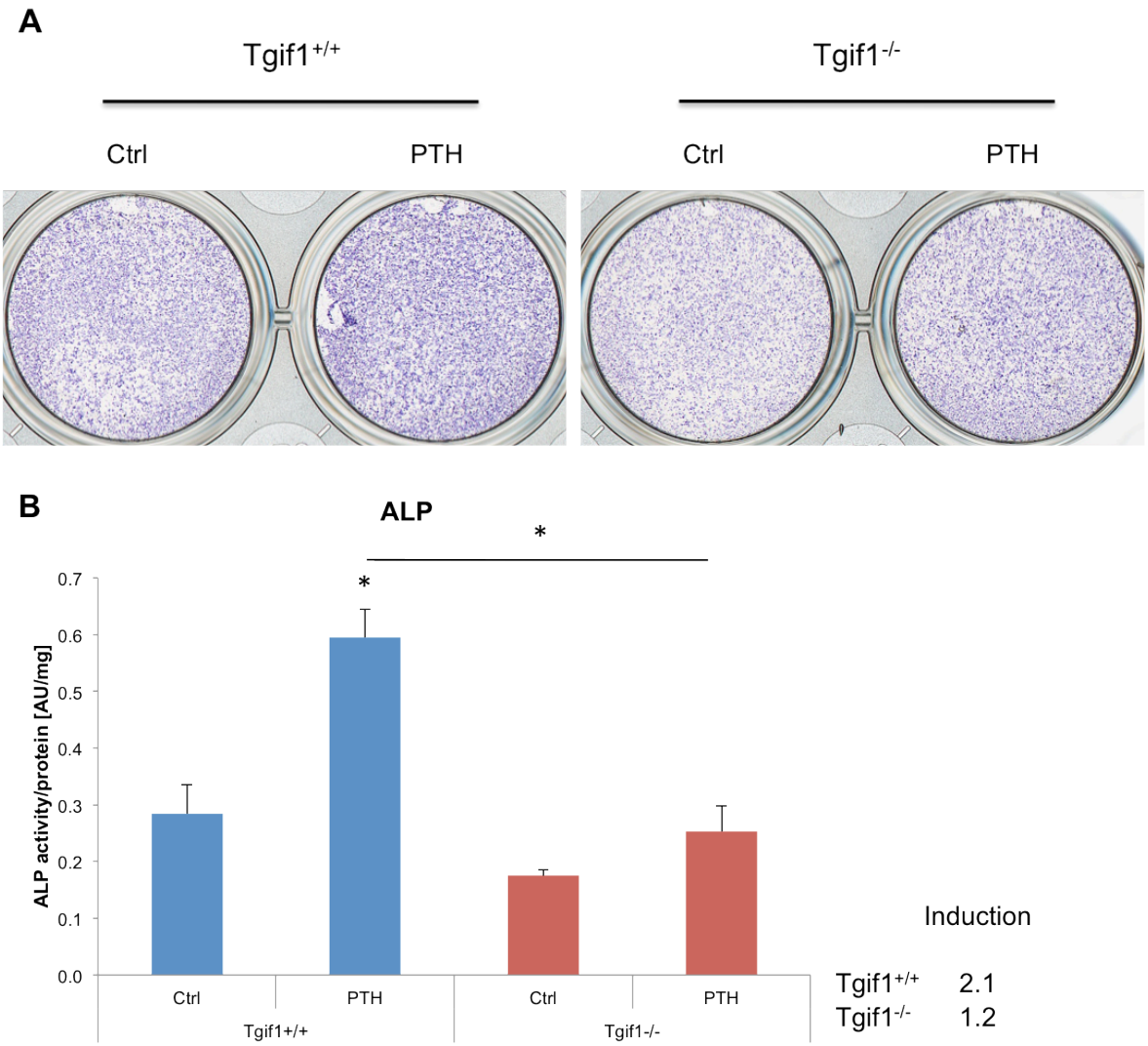

Figure 4.13: PTH-induced osteoblast differentiation is impaired in the absence of Tgif1. LOBs were cultured in osteogenic medium. 100 nM PTH was applied intermittently. (A) After fixation, LOBs were stained for ALP activity to determine osteoblast differentiation. (B) Cells from corresponding wells were lysed and ALP enzyme activity was determined. The optical density was proportional to the activity of ALP in the sample. ALP staining and enzyme activity was lower in Tgif1$^{-/-}$ LOBs compared to Tgif1$^{+/+}$ littermate controls. Relative induction of ALP by PTH was more pronounced in Tgif1$^{+/+}$ compared to Tgif1$^{-/-}$ LOBs and treatment with PTH revealed a significant difference between genotypes. *p ≤ 0.05 PTH vs. vehicle control in Tgif1$^{+/+}$ and PTH treated Tgif1$^{+/+}$ vs. Tgif1$^{-/-}$.

5 Discussion

Here, we report Tgif1 as a novel regulator of osteoblast differentiation. We found that less mature cells of the osteoblast lineage, BMSCs, as well as mature calvarial and long bone osteoblasts have a compromised osteogenic differentiation when Tgif1 is absent. This was true for both early and late stages of osteoblast differentiation. Tgif1$^{-/-}$ osteoblasts also exhibited reduced expression of marker genes for advanced differentiation. Functionally, osteogenic differentiation was affected by Tgif1 genotype in a consistent "dose-dependent" pattern. It was highest in Tgif1$^{+/+}$, reduced in Tgif1$^{+/-}$ and lowest in Tgif1$^{-/-}$ osteoblasts. In a screening assay performed during osteoblast differentiation, Tgif1 was identified as the most abundantly expressed homeodomain protein of unknown function in bone. In other tissues, Tgif1 has been implicated in the differentiation of the anterior neural system (Jin *et al.*, 2006), retinal progenitor cells (Satoh and Watanabe, 2008), the trophoblast (Pathirage *et al.*, 2013), preadipocytes (Horie *et al.*, 2008) and myeloid cells (Yan *et al.*, 2013)(Hamid, Patterson and Brandt, 2008), but also in cartilage and tendon differentiation (Lorda-Diez *et al.*, 2009). The latter arise from the same mesenchymal progenitors that osteoblasts descend from.

Beyond its role in differentiation, Tgif1 has been connected to energy metabolism, as it is involved in NADPH oxidase 2 activation and reactive oxygen species production, required for cellular activity (Huang *et al.*, 2012). We therefore explored whether compromised ability to differentiate in the absence of Tgif1 was associated with decreased proliferative potential. An assay conducted to verify whether Tgif1 impacts metabolic activity and proliferation revealed that in the absence of Tgif1, cells were less viable. This is consistent with reports of other cell types, but new in bone (Yeh *et al.*, 2012)(Huang *et al.*, 2012). To date, studies have linked Tgif1 to proliferation in developing murine embryonic fibroblasts (Mar and Hoodless, 2006)(Zerlanko *et al.*, 2012), murine hematopoietic stem cells (Yan *et al.*, 2013), as well as human myeloid leukemia cells (Hamid and Brandt, 2009) and the development of human upper urinary tract urothelial carcinoma (Yeh *et al.*, 2012). To further explore its role in osteoblast proliferation, we performed *in vitro* BrdU assays. To our presumption, the Tgif1 genotype as well as the elapsed time after seeding, and consequently the cellular density, are critical determinants of

proliferation. Therefore, a sub-confluent seeding density with day one as proliferative and day five as post-proliferative were chosen. Observations revealed that osteoblasts lacking Tgif1 were less proliferative on day one, which is in line with above-mentioned reports of different tissues. After several days in culture, the cells had formed a confluent layer and ceased to proliferate due to contact inhibition, irrespective of Tgif1 genotype. Thus, by day five the difference between $Tgif1^{+/+}$ and $Tgif1^{-/-}$ in proliferative activity was blunted. In light of our previous findings, the Tgif1 genotype can therefore not account for differences observed during osteogenic differentiation, as for those assays cells were seeded at confluent density at the beginning of experiments, and kept in culture to differentiate from then onward. In brief, the formerly observed phenotype, less ALP activity or mineralization staining in $Tgif1^{-/-}$ compared to $Tgif1^{+/+}$ osteoblasts, cannot be explained merely through a defect in their proliferative potential, but rather by a particularly pronounced defect in their osteogenic differentiation capability when Tgif1 is absent.

Conversely, transient overexpression of Tgif1 moderately increased staining for ALP activity after osteogenic culture. However, due to low transfection efficiency of about 10%, as determined by fluorescent light microscopy of enhanced green fluorescent protein (EGFP) plasmid transfected controls, the effect of Tgif1 overexpression in primary cells, although detectable, remained moderate. The effects were more pronounced in a bone marrow derived stromal cell line, ST2 (Ogawa *et al.*, 1988), which were more robust to transfection, resulting in higher transfection rates and ALP activity staining. This underlines the significance of Tgif1 for osteogenic differentiation, a hallmark in osteoblast maturation and bone formation.

We were able to confirm that iPTH is conducive to osteogenic differentiation in primary osteoblasts from different sources. This can be seen as a proof of principle in our hands, consistent with the reports in the literature, to further study the effects of Tgif1 in the context of bone anabolic PTH signaling. Intermittent PTH (iPTH) mediates bone growth through endorsement of osteoblast proliferation and differentiation (Pettway *et al.*, 2008), attenuation of osteoblast apoptosis (Jilka *et al.*, 1999), transformation of lining cells into active osteoblasts (Dobnig and Turner, 1995)(Kim *et al.*, 2012) and reduction of sclerostin, a bone formation inhibitor (O'Brien *et al.*, 2008). PTH has been shown to act less on early stage progenitors,

as it does not increase CFU-OBs (Jilka *et al.*, 1999), but favors osteoblastic differentiation of mesenchymal stem cells (Méndez-Ferrer *et al.*, 2010), as well as maturing osteoblasts, for instance by increasing levels of Runx2 or Osx (Krishnan *et al.*, 2003)(Qin *et al.*, 2003). Both are key transcription factors driving differentiation of mesenchymal progenitors towards the osteogenic lineage (Vaes *et al.*, 2006)(Fig. 1.4). This may serve as an explanation, why in our hands, iPTH did not promote differentiation of BMSCs, but evidently did in mature COBs and LOBs. PTH supports exit from the cell cycle by decreasing expression of Cyclin D1 and histone H4 (Onyia *et al.*, 1995), and increasing cyclin-dependent kinase inhibitors, thereby favoring differentiation over proliferation at later stages (Qin *et al.*, 2005)(Datta *et al.*, 2005). These actions are affected by osteoblast maturity, as PTH has been shown to induce Cyclin D1 in a PKA-dependent mechanism, enhancing proliferation in early osteoblastic cells (Datta, Pettway and Chen, 2007). PTH activates ALP and enhances production of osteocalcin, both of which can be detected and used as bone formation markers (Jilka *et al.*, 2010). PTH also accelerates the development of ossicles and matrix mineralization (Pettway *et al.*, 2005). Moreover, PTH attenuates adipogenic differentiation of mesenchymal progenitor cells by inactivating peroxisome proliferator activated receptor γ (PPARγ), a crucial regulator of adipogenesis, and reducing the number of adipocytes in the bone marrow (Rickard *et al.*, 2006). Forskolin has been shown to favor osteogenesis at the expense of adipogenesis in mesenchymal stem cells via activation of AC/PKA signaling, similar to PTH (Kao *et al.*, 2012). As opposed to canonical Wnt signaling, differentiation into chondrocytes is not blocked by PTH to privilege osteogenesis. In contrary, studies from a mutant form of PTH1R (DSEL), which stimulates AC normally, but not PLC, revealed that hypertrophic differentiation of chondrocytes was delayed (Guo *et al.*, 2002). Hence, PTH signaling via the PLC pathway is required for normal chondrogenesis and PTHrP-signaling via the common receptor PTH1R is essential for endochondral ossification (Kronenberg, 2003). However, deletion of $G_s\alpha$ in the osteoblastic lineage ($G_s\alpha^{OsxKO}$ mice) results in profound osteoporosis, underlining the importance of the AC pathway for normal osteogenesis (Wu *et al.*, 2008)(Kronenberg, 2010).

PTH and canonical Wnt signaling are influential modulators of bone formation. Our findings demonstrate for the first time that Tgif1 is a target gene of these anabolic stimuli *in vitro* as protein levels were elevated by both PTH and Wnt3a, consistent-

ly in different primary osteoblasts. This was apparent as early as 20 minutes, reaching maximum induction at 120 to 240 minutes of stimulation. Concomitantly increased protein levels of the known effectors phospho-CREB and active β-Catenin for PTH and Wnt respectively, indicate effective induction of Tgif1 through these pathways. QPCR analyses revealed significant elevation of Tgif1 mRNA upon administration of rhWnt3a, but not with rhPTH. The observed tendency is likely depending on osteoblast maturity, as the cells used here were in culture for approximately seven days at experimental use. We found that stimulation with PTH induced Tgif1 mRNA significantly at 14 days and more pronounced after 28 days in culture. Taken together, these data suggest positive translational regulation and increase of Tgif1 protein beyond *de novo* synthesis, or reduced degradation and slower turnover after stimulation with PTH. Treatment with Wnt3a led to an induction of both Tgif1 protein and mRNA levels, suggesting positive transcriptional regulation.

In PTH signaling, activation of the G-protein coupled receptor (GPCR) leads to activation of both the $G_s\alpha$ / adenylate cyclase (AC) and $G_q\alpha$ / phospholipase Cβ (PLC) signaling branches (Swarthout *et al.*, 2002)(Jilka, 2007)(Fig. 1.5). Relative intensity of AC and/or PLC signaling via the PTH1R may strongly be regulated by changes in its surface expression (Takasu, Guo and Bringhurst, 1999). Na^+/H^+ exchanger regulatory factors (NHERFs) bind to PTH1R and may also determine the activation of PKC (Mahon *et al.*, 2002). Undoubtedly, PLC signaling through the PTH1R is essential for skeletal homeostasis. PLC enhances formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG), which in conjunction with Ca^{2+} activates PKC and AP-1 target genes, such as c-Jun to promote osteoblast proliferation. Above-mentioned mutant PTH1R (DSEL) that stimulates AC normally, but fails to activate PLC, displays attenuated new bone formation when infused with PTH in mice (Guo, Liu, Yang, Bouxsein, Thomas, *et al.*, 2010). Furthermore, distinct mechanisms of PKC-δ activation, independent of PLC, play a role in promoting osteoblast differentiation (Yang *et al.*, 2006). However, $G_s\alpha$ not only mediates the effects of PTH, but also facilitates the commitment of mesenchymal progenitors to the osteoblast lineage in association with enhanced canonical Wnt signaling (Wu *et al.*, 2011). As the AC/cAMP/PKA/pCREB cascade is perceived as the dominant mechanism for the anabolic actions of PTH on trabecular bone, and

that PLC signaling is dispensable for an anabolic effect of intermittent PTH (1–34) (Yang *et al.*, 2007), we investigated regulation of Tgif1 via the PKA pathway.

Forskolin, a cell-permeable activator of adenylate cyclase, resembled the effect of PTH-induced increase of Tgif1 and phospho-CREB protein. In a complementary approach, this branch was blocked prior to stimulation. KT5720, a membrane-permeable competitive inhibitor of PKA, abolished the PTH-mediated increase of Tgif1 protein levels. This was exclusively the case at a higher dose, whereas a lower dose of KT5720 was ineffective. Phosphorylation of CREB was also inhibited by the higher concentration only, indicating that obstruction of PKA activity by KT5720 was effective. Both compounds are well established tools to modulate AC / PKA signaling (Huang, Martin and Kandel, 2000). Altogether, these results strongly suggest that induction of Tgif1 by PTH considerably depends on activation of the $G_s\alpha$ / AC / cAMP / PKA / phospho-CREB pathway.

We investigated whether balance of the pivotal second messenger cAMP is disturbed if Tgif1 is absent. A competitive cAMP ELISA assay revealed no distinctive difference between Tgif1 genotypes. In fact, cell culture supernatants displayed levels of cAMP below the limit of detection (LOD) if samples were unstimulated, and above the LOD after treatment with PTH, indicating strong activation of AC irrespective of the Tgif1 genotype. Forskolin induced less cAMP formation in $Tgif1^{-/-}$ compared to $Tgif1^{+/+}$. However, these data are suggestive of a potential defect in the PTH1R / $G_s\alpha$ / AC relay downstream of cAMP formation. Phospho-CREB is the active form of cAMP response element binding protein (CREB) and modulates the effects of protein kinase A (PKA) on protein synthesis. Indeed, Western blot analyses showed decreased protein levels of phospho-CREB in $Tgif1^{-/-}$ osteoblasts under basal conditions and after stimulation with both PTH and Forskolin. These findings demonstrate an impaired PKA signaling in the absence of Tgif1.

$Tgif1^{+/+}$ and $Tgif1^{-/-}$ LOBs were stimulated with PTH and Wnt3a. Protein analyses showed decreased levels of PTH-induced phospho-CREB, whereas Wnt-induced active β-Catenin (ABC) was not compromised in $Tgif1^{-/-}$. This was consistent through different primary cell types and confirmed by qPCR analyses. Herein, expression of known target genes Ramp3, for PTH, and Axin2, for canonical Wnt signaling, served as respective controls for effective stimulation (Yang *et al.*,

2008). The induction of PTH target genes was markedly reduced in Tgif1$^{-/-}$ osteoblasts, while canonical Wnt target genes responded to a similar extent independent of Tgif1 genotype. These findings illustrate the role of Tgif1 in ensuring activation of PTH signaling, while it is not necessary for the activation of the canonical Wnt pathway. A recent study has revealed Tgif1 as a canonical Wnt target gene, involved in a positive feed-forward loop, consistent with our findings. In a model of Tgif1 ablation, nucleocytoplasmic transit of β-Catenin was impeded (Zhang *et al.*, 2015). These observations may be due to experimental design or an organ specific mechanism, as Wnt-induced mammary tumorigenesis was investigated in *MMTV-Wnt1* mice.

Synergism with canonical Wnt signaling downstream of the PTH1R contributes to the osteoanabolic effects of PTH in large part. After binding of PTH, the receptor PTH1R associates with LPR6, leading to recruitment of Axin2, disassembly of the destructosome and stabilization of β-Catenin, thereby enhancing canonical Wnt signaling (Wan *et al.*, 2008). Conversely, disruption of LRP6 in osteoblasts blunts the bone anabolic response to PTH (Li *et al.*, 2013). PTH also directly augments β-Catenin levels, independent of LRP6, involving both PKA and PKC pathways (Tobimatsu *et al.*, 2006). Furthermore, PTH-induced signaling downstream of the PTH1R inactivates GSK-3β, preventing phosphorylation and proteasomal degradation of β-Catenin (Suzuki *et al.*, 2008). Ultimately, PTH reduces expression of the osteocyte-derived Wnt antagonists sclerostin (Keller and Kneissel, 2005)(O'Brien *et al.*, 2008) and Dkk1 (Guo, Liu, Yang, Bouxsein, Saito, *et al.*, 2010). In summary, these mechanisms lead to enhanced translocation of β-Catenin into the nucleus where it forms a complex with T-cell factor/Lymphoid enhancer factor (Tcf/Lef), enabling the transcription of Wnt target genes (Fig. 1.5). Thus, activation of the canonical Wnt signaling cascade substantially contributes to the bone anabolic effects of PTH (Baron and Hesse, 2012).

Primary osteoblasts deficient of Tgif1 did not fully respond to PTH, as shown by impaired target gene expression. To investigate whether this was due to malfunctional cross-activation of β-Catenin, we used osteoblasts from double transgenic BAT-GAL^{+}:Tgif1$^{+/+}$ and BAT-GAL^{+}:Tgif1$^{-/-}$ mice, establishing a readout for activation of canonical Wnt/β-Catenin signaling in the presence or absence of Tgif1 (Maretto *et al.*, 2003). Stimulation with PTH and staining for Xgal, an artificial substrate of inducible β-Galactosidase, allowed us to study the influence of Tgif1 on

this signaling cross-talk *in vitro*. While little differences between Tgif1 genotypes were observed under basal conditions, treatment with PTH resulted in stronger Tcf/Lef activation in Tgif1$^{+/+}$ osteoblasts. Quantified intensity of Xgal staining was reduced in Tgif1$^{-/-}$. These findings suggest a supportive role of Tgif1 for PTH-induced β-Catenin signaling in this model. As depicted above, cross-activation of canonical Wnt considerably accounts for the bone anabolic effects of PTH. An influential mechanism to endorse bone formation is downregulation of sclerostin. However, as these experiments were conducted in *in vitro* osteoblasts cultures, and the source of sclerostin is primarily osteocytes, this mechanism is unlikely.

In Tgif1-deficiency, osteoblasts fail to properly activate AC/PKA-signaling and cross-activate β-Catenin. Presence of Tgif1 and intermittent stimulation with parathyroid hormone are conducive to osteogenic differentiation. Hence, we explored the effect of Tgif1-deficiency on PTH-induced osteogenic differentiation. Intriguingly, in addition to the initial observations under basal conditions, where Tgif1-deficiency decreased osteoblast differentiation, the induction of ALP activity by iPTH seen in Tgif1$^{+/+}$, was blunted in Tgif1$^{-/-}$ LOBs. This underlines the relevance of Tgif1 in mediating the effects of PTH on promoting osteoblast differentiation.

Taken together, these data demonstrate the importance of Tgif1 for physiologic bone anabolic response *in vitro*. PTH and canonical Wnt signaling considerably synergize to favor bone formation and converge at multiple layers. While Tgif1 is essential in eliciting bone anabolic response to PTH, it appears dispensable for canonical Wnt signaling. Our ongoing aim within this project is to unravel the components of these pathways, proposing Tgif1 as a novel, essential mediator (Fig. 5.1). To elucidate physical interaction, coimmunoprecipitation assays using transfected cells are pending. Further experiments imply a constitutively active mutant of PTH1R to endorse PTH signaling and clarify how bone anabolic response and cross-activation of β-Catenin are affected in the presence or absence of Tgif1. At this point, data gathered in our laboratory indicate that PTH induces Tgif1 via AC/PKA/p-CREB/AP-1. Complementary results have reinforced the significance of Tgif1 mediating bone anabolic effects of iPTH *in vivo*, partly by contributing to the downregulation of sclerostin, an antagonist to canonical Wnt signaling. Genetic deletion of Tgif1 and subsequent treatment with sclerostin-antibody or activation of

LRP6 revealed that Tgif1 is subordinate for the bone anabolic response to canonical Wnt signaling. With regards to the limitations of current treatment options, a desirable long-term goal is to potentially target Tgif1 to support bone formation. Directly enhancing Tgif1 however will prove difficult, as this would require viral transduction. An indirect approach, utilizing components inhibiting miRNAs, which are targeting Tgif1, may be more promising in preventing extensive bone loss and is forthwith being investigated (Krzeszinski *et al.*, 2014). In conclusion, our data establish Tgif1 as a novel stimulator of osteoblast function, positively regulated by two major osteoanabolic signaling pathways. Pharmacological modulation of PTH and canonical Wnt signaling are effective in sustaining bone formation. Expanded knowledge on pathway components and their function may contribute to improving current or opening novel therapeutic concepts in diseases such as osteoporosis.

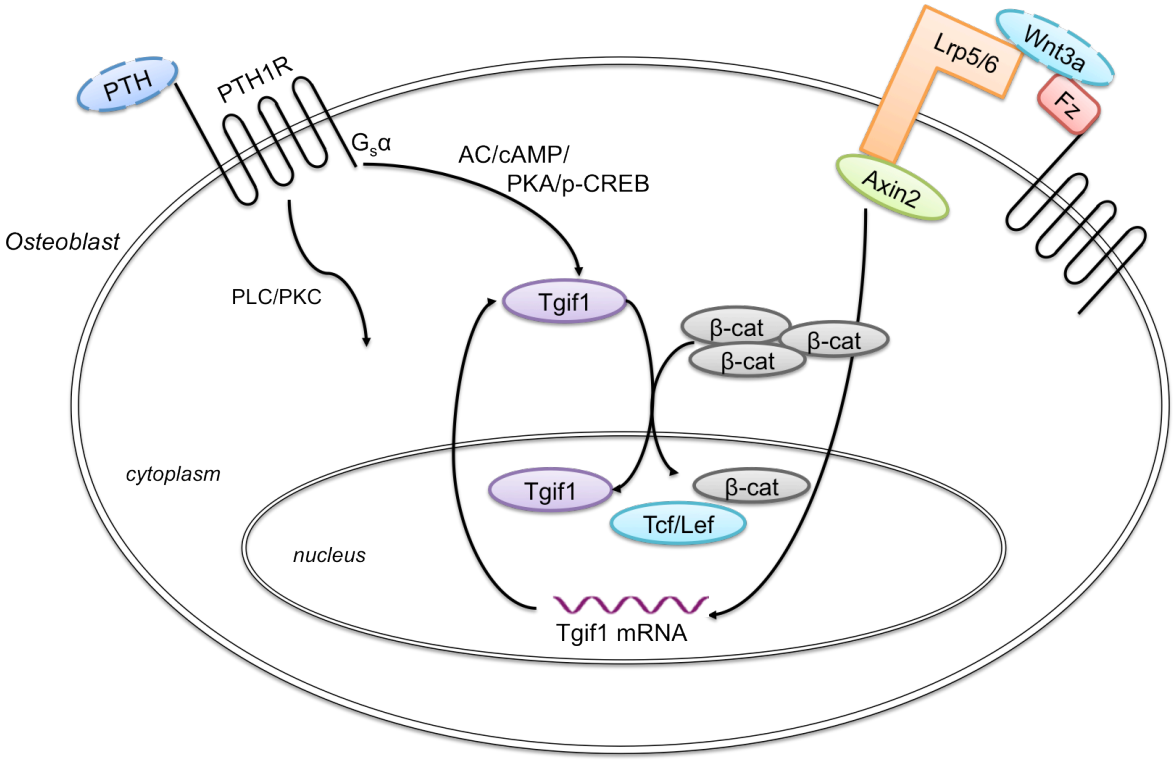

Figure 5.1: Interaction of PTH and canonical Wnt pathways mediated by Tgif1. Binding of PTH to its receptor PTH1R activates Tgif1 through $G_s\alpha$ / adenylate cyclase (AC) / PKA / p-CREB to promote osteoblast differentiation and function. This is perceived as the dominant mechanism for the bone anabolic effects of PTH and is impaired in the absence of Tgif1. Canonical Wnt signaling, another strong bone anabolic stimulus, enhances Tgif1 protein levels and gene expression via β-Catenin. While Tgif1 is subordinate for the effect of canonical Wnt alone, it does enhance cross-activation by PTH. Both signaling cascades converge at multiple layers and synergize to enable osteoblast function and bone formation.

6 Summary

6.1 English

Osteoporosis is the most common cause of fragility fractures in the elderly. Disturbed bone remodeling, which depends on coupled and balanced osteoclastic bone resorption and osteoblastic bone formation, results in low bone mineral density. While antiresorptive drugs are still the standard of care, remedies augmenting bone formation are required to countervail extensive bone loss. Parathyroid hormone (1-34), and an analog to PTHrP in the United States, serve as the sole bone anabolic agents approved in clinical practice, while antagonizing sclerostin, an inhibitor of canonical Wnt signaling, has emerged as a promising concept in the near future. Osteoblast activity is enabled by bone anabolic signaling and regulatory factors, including homeodomain proteins. In an unbiased screening performed during osteoblast differentiation, TG-interacting factor-1 (Tgif1) emerged as the most abundantly expressed homeodomain protein of unknown function in bone and is a subject of ongoing investigations in our laboratory.

Genetic deletion of Tgif1 diminished differentiation and proliferation, whereas transient overexpression enhanced differentiation in primary osteoblasts. We determined induction of Tgif1 by PTH and canonical Wnt signaling, both potent bone anabolic pathways known to amplify osteoblast differentiation and function. Modification of signal transduction downstream of PTH1R revealed regulation via $G_s\alpha$/AC/cAMP/PKA/p-CREB, a central mechanism for the bone anabolic effects of PTH. Induction of PTH target genes was attenuated in Tgif1$^{-/-}$ osteoblasts, whereas Wnt target genes responded to stimulation irrespective of Tgif1 genotype. Cross-activation of Wnt/β-Catenin, an influential aspect of bone anabolic PTH, was reduced as determined by double transgenic BAT-GAL^{+}:Tgif1$^{-/-}$ mice. Ultimately, PTH-induced osteoblast differentiation was blunted in Tgif1$^{-/-}$ osteoblasts.

In conclusion, we introduce Tgif1 as an essential mediator of two major anabolic signaling cascades that control osteoblast function and bone formation. Expanded knowledge on their pathway components may contribute to improving current or unveiling novel therapeutic approaches to diseases such as osteoporosis.

6.2 Deutsch

Osteoporose ist die häufigste Ursache pathologischer Frakturen nach minimalem Trauma im Alter. Gestörter Knochenumbau – ausgewogener Abbau durch Osteoklasten und Aufbau durch Osteoblasten – führt zu Abnahme der Knochendichte. Antiresorptive Substanzen stellen die gängige Form der Therapie dar. Bei ausgedehntem Knochenverlust allerdings sind anabole Substanzen erforderlich. Zur Förderung des Knochenaufbaus sind Parathormon (1-34), sowie ein Analogon von PTHrP in den USA, die einzigen klinisch zugelassenen Pharmaka, während Antikörper gegen Sclerostin, ein Inhibitor des kanonischen Wnt-Signalwegs, vielversprechende Ansätze für die nahe Zukunft bieten. Die Aktivität von Osteoblasten wird durch anabole Signalwege stimuliert und durch eine Reihe von Faktoren reguliert, darunter Homöodomäne Proteine. Um solche Faktoren zu ermitteln führte unsere Arbeitsgruppe ein Screening während der Differenzierung von Osteoblasten durch und identifizierte mit TG-interacting factor-1 (Tgif1) ein stark exprimiertes Homöodomäne Protein, dessen Rolle im Knochenstoffwechsel bislang unbekannt war.

Das Fehlen des Tgif1 Gens führte zu verminderter Differenzierung und Proliferation, wohingegen Überexprimierung förderlich auf die Differenzierung primärer Osteoblasten wirkte. Tgif1 wurde durch Gabe von PTH und Wnt3a, welche beide die Differenzierung und Funktion von Osteoblasten fördern, stimuliert. Modifikation der Signalkaskade offenbarte die Regulation von Tgif1 über $G_s\alpha$/AC/cAMP/PKA/p-CREB, eine zentrale Achse über welche PTH seine osteoanabole Wirkung entfaltet. Die Induktion von PTH Zielgenen war bei $Tgif1^{-/-}$ Osteoblasten reduziert, wohingegen Wnt Zielgene auf Stimulation in beiden Genotypen gleichermaßen anstiegen. Ein substanzieller Anteil der anabolen Wirkung von PTH verläuft über Aktivierung von Wnt/β-Catenin. Dies war in doppelt transgenen BAT-GAL^{+}:$Tgif1^{-/-}$ Mäusen eingeschränkt. Schließlich war die Induktion osteogener Differenzierung durch PTH in $Tgif1^{-/-}$ Osteoblasten deutlich reduziert.

Anhand dieser Daten stellen wir Tgif1 als essentiellen Mediator zweier zentraler anaboler Signalwege vor, welche Osteoblastenaktivität und Knochenaufbau fördern. Der Wissenszuwachs über diese Signalkaskaden mag bei der Osteoporose zur Verbesserung der bestehenden oder Erschließung neuartiger Therapiekonzepte beitragen.

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8 Declaration

I hereby declare that this doctoral thesis has been written independently only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated herein.

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Levi Matthies

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