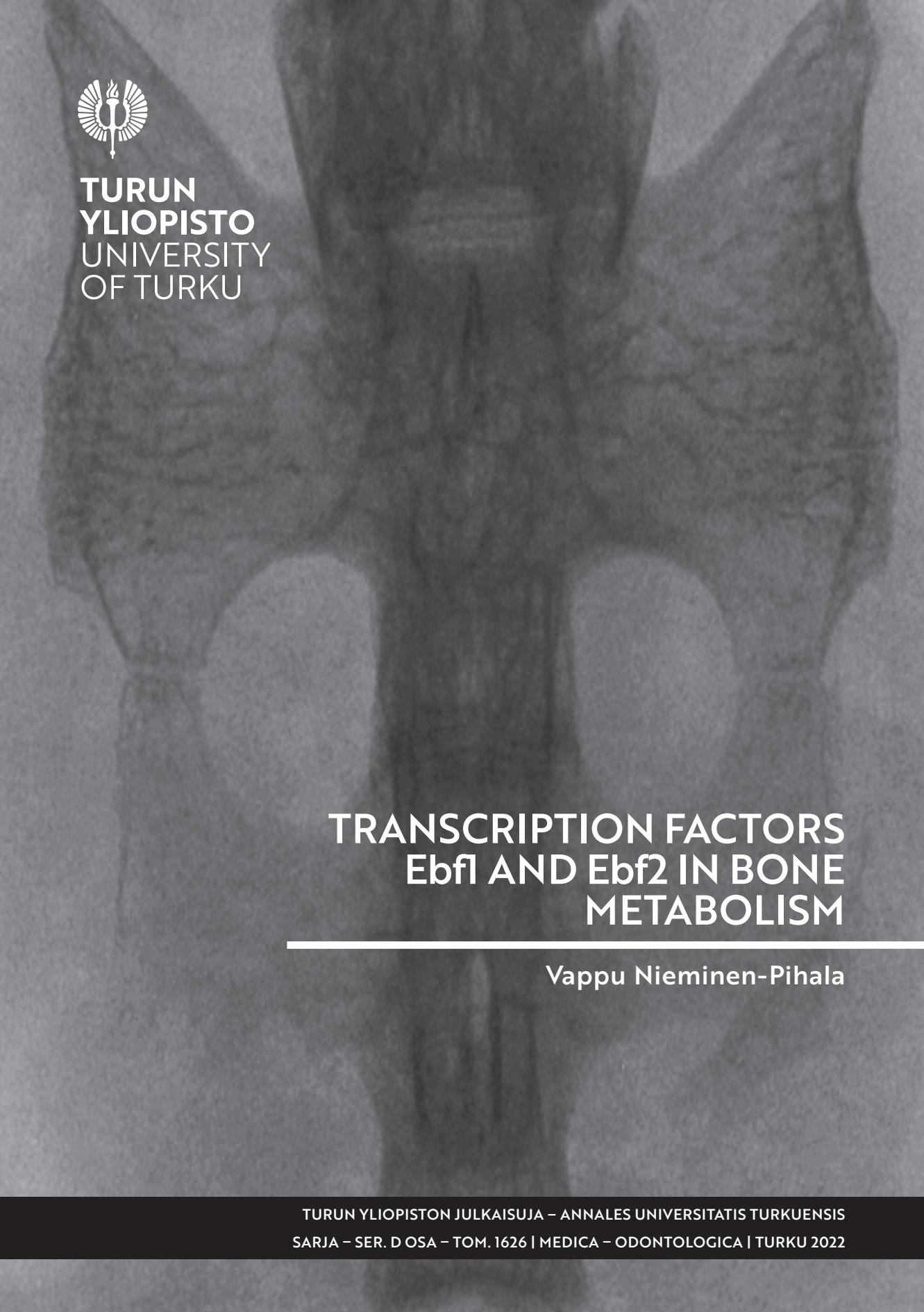

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TRANSCRIPTION FACTORS Ebf1 AND Ebf2 IN BONE METABOLISM

Vappu Nieminen-Pihala

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To my family

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ABSTRACT

Bone formation and bone metabolism are controlled by several different factors and signaling pathways. Transcription factors participate in this cascade by regulating the differentiation of osteoblasts, osteoclasts and chondrocytes. One of the additions to the group of bone-related transcription factors are early B-cell factors. Early B-cell factor 1 (Ebf1) was first identified as a key transcription factor regulating B-cell differentiation. Further work has found that it also regulates neuronal and adipocyte differentiation, as does its family member Ebf2.

We and others have shown that *Ebf1* is expressed in osteoblasts and that genome-wide deletion of *Ebf1* results in increased bone formation *in vivo*. In contrast, global *Ebf2* knock out mice have osteopenia in cancellous and cortical bone due to enhanced bone resorption and increased number of osteoclasts. However, interpreting the effects of global *Ebf1* and *Ebf2* deletion on bone is challenging, as both factors are expressed in multiple tissues. This results in failure to thrive and could therefore affect the skeletal development. Therefore, the functions and molecular mechanisms of action at specific stages of osteoblasts differentiation remained to be elucidated in detail.

This thesis investigates the role of Ebf1 and Ebf2 in osteoblast differentiation and function, at different stages of bone development. To achieve this, we analysed several conditional, osteoblast targeted *Ebf1* and *Ebf2* knockout mouse models. Results of our work show that Ebf1 promotes early osteoblast differentiation by regulating Osterix expression. We also concluded that Ebf1 inhibits bone accrual in the Osterix-expressing osteoblasts *in vivo,* but it is redundant in the function of mature, osteocalcin-expressing osteoblasts. Deletion of both *Ebf1* and *Ebf2* in mesenchymal lineage cells led to significant, age progressive increase in bone volume. The phenotype was to some extent gender dependent.

Results presented in this thesis have established that Ebf1 and Ebf2 have prominent effect on bone formation. By understanding the specific roles of Ebf transcription factors in osteoblast differentiation, we are one step closer to guiding MSC differentiation to facilitate bone repair and developing future therapies.

KEYWORDS: Ebf1, Ebf2, bone, osteoblast, transcription factor

TURUN YLIOPISTO
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TIIVISTELMÄ

Luunmuodostusta ja luun aineenvaihduntaa säätelevät useat eri tekijät sekä signalointireitit. Tähän prosessiin osallistuvat transkriptiotekijät säätelevät osteoblastien, osteoklastien sekä kondrosyyttien erilaistumista. Ebf-ryhmän (engl. Early B-cell factor) transkriptiotekijät kuuluvat luunmuodostuksen säätelijöihin. Ebf1 (Early B-cell factor 1) tunnistettiin ensimmäistä kertaa B-solujen erilaistumisen säätelyssä. Myöhemmin sen on todettu osallistuvan myös adiposyyttien sekä hermosolujen erilaistumisen säätelyyn, yhdessä saman säätelyperheen toisen tekijän, Ebf2:n kanssa.

Aiemmat tutkimukset osoittavat *Ebf1*:n ilmenevän osteoblasteissa. Globaaleissa poistogeenisissä hiirimalleissa *Ebf1*:n puuttuminen johti kasvaneeseen luumassaan. *Ebf2*:n globaali poistogeenisyys taas johtaa hiirimalleissa osteopeniaan lisääntyneen luun resorption ja kasvaneen osteoklastimäärän myötä. Luustovaikutusten tulkinta globaalisti poistogeenisissä hiirimalleissa on kuitenkin haastavaa, sillä *Ebf1* ja *Ebf2* ilmenevät useissa eri kudostyypeissä. Tämä voi johtaa sekundäärisiin muutoksiin, jotka osaltaan vaikuttavat luunmuodostukseen. Näin ollen spesifit vaikutukset luusolujen erilaistumiseen ja molekyylitason mekanismeihin ovat yhä epäselviä.

Tutkimuksemme selvitti Ebf1- ja Ebf2- transkriptiotekijöiden merkitystä osteoblastien erilaistumisessa ja toiminnassa luunmuodostuksen eri vaiheissa. Analysoimme useita *Ebf1*- ja *Ebf2*- hiirimalleja, joissa poistogeenisyys oli kohdennettu osteoblasteihin. Tutkimuksen tulokset osoittavat, että Ebf1 edistää varhaista osteoblastien erilaistumista. Totesimme kuinka osteoblastien erilaistumisen edetessä Ebf1 heikentää luun kertymistä *in vivo*, mutta ei enää osallistu kypsien osteoblastien toimintaan. *Ebf1*:n ja *Ebf2*:n samanaikainen puute mesenkymaalisissa esiastesoluissa johti iän myötä etenevään luumassan lisääntymiseen. Ilmiasu oli joiltain osin riippuvainen sukupuolesta.

Tämän tutkimuksen tulokset vahvistavat Ebf-transkriptiotekijöiden merkitystä luunmuodostuksessa. Ymmärtämällä paremmin Ebf-transkriptiotekijöiden spesifejä vaikutuksia osteoblastien erilaistumisessa, olemme askeleen lähempänä mesenkymaalisten stroomasolujen hyödyntämistä luuvaurioiden korjaamisessa sekä tulevaisuuden lääkekehityskohteena.

AVAINSANAT: Ebf1, Ebf2, luu, osteoblasti, transkriptiotekijä

Table of Contents

Abbreviations

ALP	Alkaline phosphatase
B.Ar	Bone area
BAT	Brown adipose tissue
BLC	Bone lining cell
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BRC	Bone remodeling compartment
BSP	Bone sialoprotein
BV/TV	Bone volume/tissue volume
CCD	Cleidocranial dysplasia
ChIP	Chromatin immunoprecipitation
Col1a1	Collagen type 1
Cort.Ar/T.Ar	Cortical area fraction
DBD	DNA binding domain
EBF	Early B-cell factor
Ec.Pm	Endocortical perimeter
hOC	Human osteocalcin
HSC	Hematopoietic stem cell
IGF-1	Insulin like growth factor 1
IL-7	Interleukin-7
LSC	Lacuna-canalicular system
M.Ar/T.Ar	Marrow area fraction
M-CSF	Macrophage colony stimulating factor
MMI	Mean moment of inertia
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal cell
OC	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
Osx	Osterix

Prrx1	Paired related homeobox 1
Ps.Pm	Periosteal perimeter
RANKL	Receptor activator of nuclear kappa-B ligand
ROI	Region of intrest
Runx2	Runt family transcription factor 2
Tb.N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TGF-β	Transforming growth factor beta
TRAP	Tartrate-resistant acid phosphatase
SOC	Secondary ossification centre
Sox9	SRY-box transcription factor 9
SSC	Skeletal stem cell
STAT5	Signal transducer and activator of transcription 5
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
Wnt	Wingless-type MMTV integration site family
μCT	Microcomputed tomography

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Nieminen-Pihala V**, Tarkkonen K, Laine J, Rummukainen P, Saastamoinen L, Nagano K, Baron R and Kiviranta R (2021). Early B-cell Factor1 (Ebf1) promotes early osteoblast differentiation but suppresses osteoblast function. *Bone 146:115884: DOI:10.1016/j.bone.2021.115884*

II **Nieminen-Pihala V***, Rummukainen P*, Wang F, Tarkkonen K, Ivaska K and Kiviranta R (2022). Age-progressive and gender-dependent bone phenotype in mice lacking both Ebf1 and Ebf2 in Prrx1-expressing mesenchymal cells. *Calcified Tissue International 2022: DOI:10.1007/s00223-022-00951-7*
(*equal contribution)

1 Introduction

Bone formation and bone metabolism are controlled by several different factors and signaling pathways. Transcription factors participate in this cascade by regulating the differentiation of osteoblasts, osteoclasts and chondrocytes. The most studied transcription factors of osteoblast differentiation and function are probably Runt family transcription factor 2 (Runx2) and Osterix (Osx). The importance of these two factors for normal bone development has been demonstrated in several studies in which the lack of either Runx2 or Osterix resulted in total absence of osteoblasts and bone.

One of the additions to the group of bone-related transcription factors are early B-cell factors. Early B-cell factor 1 (Ebf1) was first identified as a key transcription factor regulating B-cell differentiation. Further studies have demonstrated that it also regulates differentiation of neuronal cells and adipocytes, as does its family member Ebf2. We and others have shown that *Ebf1* is expressed in osteoblasts and that genome-wide deletion of *Ebf1* results in increased bone formation *in vivo*, implicating Ebf1 as a negative regulator of osteoblast function. In contrast, global *Ebf2* knockout mice have osteopenia in cancellous and cortical bone due to enhanced bone resorption and increased number of osteoclasts.

Based on these data, Ebfs are capable of modulating bone homeostasis. However, interpreting the effects of global Ebf1 and Ebf2 deletion on bone is challenging, as both transcription factors are expressed in multiple tissues. This results in failure to thrive and could therefore affect the skeletal development. Therefore, the functions and molecular mechanisms of action at specific stages of osteoblast differentiation remained to be elucidated in detail. Ebf1 and Ebf2 are likely to be able to compensate for the lack of each other, but the unique and redundant functions remain to be discovered.

The focus of this thesis was to study the role of Ebf1 and Ebf2 in osteoblast differentiation and function, at different stages of bone development. To achieve this, we analysed several conditional, osteoblast-targeted *Ebf1* and *Ebf2* knockout mouse models. To further investigate the redundant and non-redundant effects of Ebf1 and Ebf2 on bone formation we created Ebf1xEbf2$_{Prrx1}$ mice, in which both *Ebf1* and *Ebf2* have been simultaneously deleted in the mesenchymal cell lineage.

2 Review of the Literature

2.1 Bone metabolism

Bones are formed through two distinct processes, intramembranous and endochondral ossification. Intramembranous ossification is at times also referred to as direct ossification, as bone is formed directly, without cartilaginous intermediate. In humans, intramembranous bone formation is restricted to the formation of flat bones, such as facial and cranial bones. The majority of the human skeleton is formed by endochondral ossification **(Figure 1)**. Condensated mesenchymal stromal cells (MSCs) form the initial scaffold during the embryological phase, followed by chondrocyte anlagen, which is finally replaced by bone. Bone growth continues into adolescence and ends at the time of growth plate fusion (Shim, 2015).

The adult skeleton is composed of approximately 80% cortical bone and 20% cancellous bone. Constant remodeling maintains the structural integrity of the bone tissue and participates in calcium and phosphate homeostasis. During childhood, the overall rate of bone turnover is positive, as newly formed bone exceeds the amount of resorbed bone. Once peak bone mass is reached in early adulthood, the balance remains in equilibrium until age-related bone loss begins. The loss of trabecular bone due to menopause has long been the traditional representation of age-related changes in bone (Khosla et al., 2005). Despite the known high percentage of cortical bone, age-related increase in cortical porosity has only been recognized during the past decade as another important component of bone quality (Farr et al., 2015; Seeman, 2013). The cellular level process of bone remodeling is discussed in more detail in section 2.1.5.

Whereas bone remodeling is a coupled process of osteoblasts and osteoclasts, bone modeling is a process where bones are shaped by independent actions of osteoblasts and osteoclasts (Langdahl et al., 2016). Bone modeling is predominantly a function of new, actively growing bone, but it also occurs in adult bones as a response to long term muscle activity or increased weight. Anabolic agents that stimulate bone modeling while inhibiting bone resorption are promising new treatment options of bone diseases, such as osteoporosis (Langdahl et al., 2016; Paik et al., 2020).

The following chapters describe the cellular components of bone remodeling; cartilage synthesizing chondrocytes, bone forming osteoblasts, bone resorbing osteoclasts and bone occupying osteocytes in more detail.

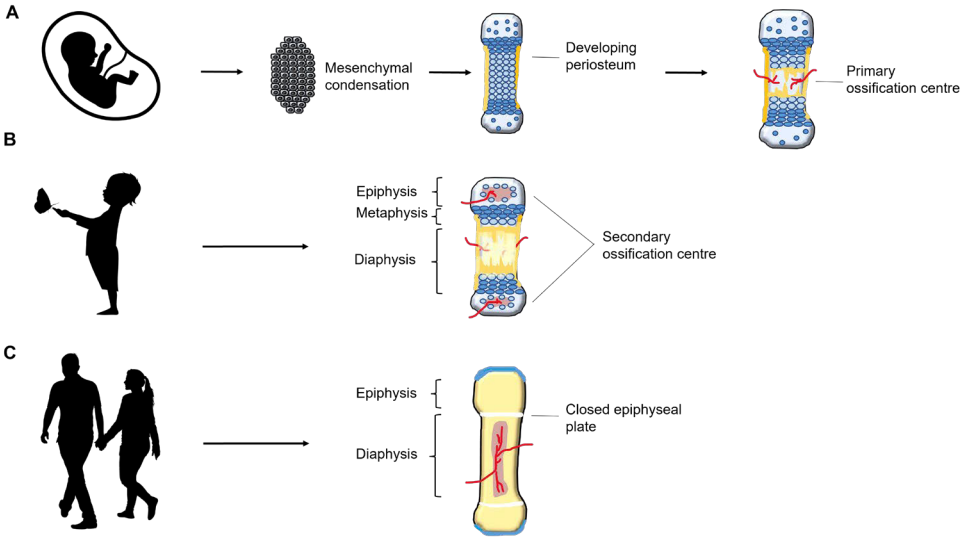

Figure 1. The majority of the human skeleton is formed by endochondral ossification. **A)** Endochondral ossification starts during the fetal stage through the formation of a mesenchymal cell condensation. The cells at the centre of the condensed area differentiate into chondrocytes and begin to secrete cartilage matrix. The surrounding cells form the perichondrium and subsequently turn into the bone collar and periosteum. Chondrocyte hypertrophy and blood vessel infiltration trigger osteoblast differentiation and the subsequent mineralization of the cartilage matrix. This defines the formation of the primary ossification centre in the diaphysis. **B)** By the end of the fetal stage, the majority of the cartilage template has been replaced by bone, and epiphyseal growth plates have formed at the metaphysis. The secondary ossification centres form postnatally to the epiphyses. Longitudinal bone growth continues into adolescence, until the growth plate fuses. **C)** At the time of sexual maturation, epiphyseal growth plates fuse and longitudinal growth ends. Estrogen plays an important role in this process for both males and females. Functions of bone cells now focus on the maintenance of bone remodeling instead of bone growth.

2.1.1 Chondrocytes

Chondrocytes originate from multipotent MSCs, common precursors for osteoblasts, myoblasts, and adipocytes. The main function of chondrocytes is the formation of articular cartilage that protects underlying structures and facilitates movement in the joints, and the formation of the cartilaginous model during endochondral ossification and longitudinal growth of bones. Unlike the endochondral anlagen, articular cartilage is a permanent structure which in normal homeostasis persists through life (Heinemeier et al., 2016).

In articular cartilage chondrocytes are sparse in the surrounding extracellular matrix that is rich in collagen II and proteoglycans, such as aggrecan. Endochondral ossification anlagen contains significantly higher quantities of chondrocytes. During differentiation, chondrocytes undergo maturation from MSCs into proliferating chondrocytes and hypertrophic chondrocytes. The main transcription factor in the induction of chondrocyte differentiation is SRY-box transcription factor 9 (Sox9) (Bi et al., 1999), followed by Runt-related transcription factor 2 (Runx2) (Takeda et al., 2001). Bone morphogenic proteins (BMPs) also regulate multiple aspects in chondrocyte differentiation and maturation (Yoon et al., 2006). These main transcription and growth factors are further regulated by multiple signaling cascades during chondrocyte differentiation.

In endochondral ossification the lifecycle of chondrocytes end once the hypertrophic chondrocytes have produced the final anlagen for mineralization. There has been a long debate on the mechanism of chondrocyte-to-osteoblast conversion. The more traditional model is based on the notion of the replacement of apoptotic chondrocytes by MSCs, which subsequently differentiate into osteoblasts and mineralize (Mackie et al., 2008). Increasing evidence has suggested that the chondrocyte-to-osteoblast conversion might also proceed through transdifferentiation of chondrocytes. Transdifferentiating chondrocytes mature and hypertrophy, but instead of apoptosis, they directly differentiate into osteoblasts (Aghajanian et al., 2018). In cell tracking studies, murine hypertrophic chondrocytes have been shown to transdifferentiate into cells expressing osteogenic genes such as type I collagen (*Col1a1*), osteocalcin (*OC*), and *Runx2* (Park et al., 2015). Transdifferentiation also plays a role in fracture healing (Zhou et al., 2014).

2.1.2 Osteoblasts

Osteoblasts are mainly known for their bone forming function. They regulate bone matrix mineralization and secrete extracellular matrix proteins such as Col1A1, osteopontin (OPN), osteocalcin, and express alkaline phosphatase (ALP) (Blair et al., 2017). Osteoblasts also originate from the multipotent MSCs. There is ongoing debate whether these MSCs should and could be defined more specifically based on their temporospatial properties and differentiation fate. Studies have shown different populations of MSCs to be responsible for the perinatal osteoblastogenesis (Mizoguchi et al., 2014) and the postnatal osteoblastogenesis (Zhou et al., 2014a). A subgroup of MSCs, skeletal stem cells (SSCs), has also been defined in mice (Chan et al., 2015) and in humans (Chan et al., 2018). SSCs have been described as progenitors of bone and cartilage, but not muscle or adipose tissue. Different lineage tracing techniques have been used to characterize and delineate SSCs further. However, defining the criteria of SSCs is still a work in progress, and the currently

suggested SSC population is still considered very heterogenic (Ambrosi et al., 2019). In addition to MSCs, osteoblasts may also potentially be derived from chondrocytes (Yang et al., 2014), bone lining cells (BLC) (Matic et al., 2016) and pericytes (Xu et al., 2020).

Osteoblast differentiation and maturation is a multistep process that requires specific regulators expressed in a timely manner. The primary commitment towards osteoblast lineage in endochondral ossification is guided by Sox9, which drives the common multipotent stem cells to form the precartilaginous condensation (Liu et al., 2018). The subsequent expression of *Runx2* is the first step in the actual preosteoblast lineage differentiation (Ducy et al., 1997). In the following stages preosteoblasts proliferate, mature to osteoblasts, and begin to synthesize a matrix that will subsequently mineralize. The transcriptional regulation of bone formation is further discussed in the section 2.2.

Osteoblasts are histologically described as cuboidal cells with prominent Golgi apparatuses and rough endoplasmic reticulum due to active protein synthesis. Bone lining osteoblastic cells are described as quiescent flat shaped osteoblasts that cover inactive bone surfaces (Miller et al., 1989). Mature osteoblasts have three fates: becoming bone lining cells, becoming osteocytes (discussed further in section 2.1.4), or undergoing apoptosis (Manolagas, 2000).

2.1.3 Osteoclasts

Osteoclasts are large multinucleated cells. Their main function is to take part in the maintenance of bone homeostasis by resorbing bone. Osteoclasts have traditionally been described as originating from mononuclear cells of the hematopoietic stem cell (HSC) lineage (Udagawa et al., 1990). Recent studies have indicated that in addition to HSCs, fetal osteoclasts may also originate from embryonic erythro-myeloid progenitors (Jacome-Galarza et al., 2019; Yahara et al., 2020). Whether there are specific roles and functions for osteoclasts derived from different developmental origins remains to be discovered.

Formation of osteoclasts is regulated by several factors and pathways. The macrophage colony-stimulating factor (M-CSF) initiates osteoclast differentiation (Udagawa et al., 1990) and induces proliferation of monocyte/macrophage derived precursor cells. These precursors are then further activated by the receptor activator of nuclear factor kappa-B/ligand (RANK/RANKL) to form preosteoclasts (Kong et al., 1999; Li et al., 2000). The bone resorption ability of osteoclasts is dependent on their ability to fuse and become multinucleated. This fusion is regulated by factors such as the nuclear factor of activated T cells 1 (NFATc1) (Kim et al., 2008) and dendritic cell-specific transmembrane protein (DC-STAMP) (Yagi et al., 2005).

For efficient bone resorption, osteoclasts prepare a sealed microenvironment over the resorbed area. By creating a sealing zone, osteoclasts prevent leakage of proteolytic enzymes during resorption (Vaananen et al., 1995). Resorbed bone is degraded via secretion of hydrochloric acid and proteolytic enzymes to the contacting area between the osteoclast and bone surface. Bone mineral components are dissolved in acidic pH and exposed organic material is further broken down by proteolytic enzymes such as cathepsin K (CatK) (Bossard et al., 1996) and tartrate-resistant acid phosphatase (TRAP) (Minkin, 1982). The degradation products are then removed by the vesicular process of transcytosis (Salo et al., 1997).

As with osteoblasts, apoptosis has been suggested as the end point of osteoclast lifecycle (Hughes et al., 1996). Recently McDonald et al. presented interesting evidence of *in vivo* osteoclast recycling as an alternative to the traditional apoptotic cell fate (McDonald et al., 2021). Their data indicated osteoclasts to have ability to fission into smaller, motile daughter cells, osteomorphs. These cells had the ability to reassemble into osteoclasts in a new a location, outside the original resorption pit. Future research will show what kind of role these osteomorphs have in normal and pathophysiological bone homeostasis.

2.1.4 Osteocytes

Osteocytes are derived from the MSC lineage, representing terminally differentiated osteoblasts. They constitute over 90% of the cells in the adult skeleton. During osteocyte differentiation, the cells bury themselves into the collagen matrix while building dendritic processes. The lacuna-canalicular system (LCS) forms between these dendritic processes, connecting osteocytes in their residing lacunae with other bone cells. The lifespan of osteocytes can vary from years to decades and is significantly longer compared to osteoblasts and osteoclasts, which only live from days to a few weeks (Parfitt, 1994). With old age and in bone diseases, such as osteoporosis, the number of osteocyte lacunae (Busse et al., 2010) and the connecting canaliculi tunnels (Milovanovic et al., 2013) are reduced, leading to bone fragility.

Osteocytes have long been known as mechanosensing cells, but the specific mechanisms are still not completely understood. Mechanical loading of bones results in interstitial fluid flow in the LCS stimulating the mechanotransduction process. Gap junction proteins, such as Connexin43, in the dendritic processes of osteocytes regulate osteocyte viability and modulate mechanical stimulation-induced responses (Li et al., 2013; Xu et al., 2015). The physical deformation of the bone matrix under mechanical stimulation can also function as a trigger for mechanotransduction (Wu et al., 2017). Osteocytes respond to mechanical stimulus by intermediate signaling transmission, including calcium oscillations (Jing et al., 2014), the release of nitric oxide (Santos et al., 2010), and adenosine triphosphate (Kringelbach et al., 2015).

This signaling cascade leads to several bone formation enhancing events, such as the activation of Wnt (Wingless-type MMTV integration site family)-signaling by reducing the levels of Wnt-signaling antagonist sclerostin (Morrell et al., 2018), which is also secreted by osteocytes.

In regards to osteoclast regulation, osteocytes are an important source of RANKL (Nakashima et al., 2011; Xiong et al., 2015) and osteoprotegerin (OPG) (Kramer et al., 2010). The dendritic processes of osteocytes provide a new method of transport for RANKL to reach osteoclast precursors (Honma et al., 2013). Recently, osteocytic RANKL has been identified as an essential factor in age-related cortical bone loss (Kim et al., 2020). Osteocytes take part in phosphate metabolism by producing phosphaturic hormone, fibroblast growth factor 23, that regulates phosphate homeostasis in the kidneys (Feng et al., 2006).

Osteocytes are also able to regulate their perilacunar and pericanalicular matrix and thereby release calcium. During lactation, skeletal resorption is temporarily increased both in rodents (Gonen et al., 2005) and in humans (Costa et al., 2012). In rodents, part of this osteolysis arises from the enlarged osteocyte lacunae during the lactation period (Kaya et al., 2017). In pathological conditions, osteocytic osteolysis is present in disuse osteopenia (Blaber et al., 2013) and in glucocorticoid induced secondary osteoporosis (Lane et al., 2006). Unlike the earlier view of osteocytes as isolated cells within the inert bone matrix, recent research has in fact presented osteocytes as active endocrine cells (Dallas et al., 2013; Delgado-Calle et al., 2022).

2.1.5 Bone remodeling

The cycle between replacement of old and damaged bone with new bone is the basis of bone remodeling and maintenance of mineral homeostasis in the adult skeleton. This cycle takes place in cavities, in a temporary structure called the basic multicellular unit (BMU). In trabecular bone BMU consist of osteoclasts, osteoblasts, and capillary blood supply. The concept of the trabecular BMU has been updated with the inclusion of a canopy of cells. The canopy is formed by a thin layer of BLCs. The mesenchymal bone marrow envelope has been suggested to be the cellular origin of BLCs in human cancellous bone (Kristensen et al., 2014). Together, the BMU and the canopy forming cells create the bone remodeling compartment (BRC) (Hauge et al., 2001) **(Figure 2)**. Intracortical BMUs within Haversian canals adopt a more cylindrical shape, including the cutting cone of osteoclasts followed by reversal zone and closing cone lined with osteoblasts (Parfitt, 1994). The model of the bone remodeling cycle within the BMU is described as consecutive steps of resorption, reversal, and formation (Eriksen et al., 1984b). In adult human bones, approximately one million BMUs are actively participating in bone remodeling at any time (Manolagas, 2000).

Osteocyte apoptosis has been stated to be one of the activators (initiators) of the bone remodeling cascade and osteoclast recruitment (Gu et al., 2005; Noble et al., 1997). At the beginning of trabecular bone remodelling, BLCs are released from the cancellous bone surface by a disruption of the junctions between lining cells and embedded osteocytes. Subsequently, the bone lining cells form a raised canopy over the resorption site. (Hauge et al., 2001) Osteoclastogenic factors such as osteocyte-derived RANKL then recruit hematopoietic stem cells from the circulation to the site for osteoclast differentiation and maturation (Nakashima et al., 2011). Mature osteoclasts then proceed to resorb the bone matrix, creating eroded pits (called Howship's lacunae) in trabecular bone and cutting cones in cortical bone.

To avoid excess resorption, osteoclast activity is terminated at the end of the resorption period. A so-called reversal period then occurs that is characterized by a careful balance of osteoclast inhibition and osteoblast activation. During this period, the eroded surface becomes filled with reversal cells. The early reversal cells resemble osteoblast precursors and have close interactions with adjacent osteoclasts (Abdelgawad et al., 2016). In contrast, late reversal cells next to the osteoid represent more cuboidal, collagen-depositing osteoblasts (Andersen et al., 2013). During the reversal period, osteoblast activity is induced in response to bone resorption in a coupling process (Howard et al., 1981). Suggested coupling factors can be divided into matrix-derived factors, secreted factors, and membrane-bound mediators.

Matrix-derived coupling factors are stored in the bone matrix as latent growth factors that are released upon bone resorption (Hauschka et al., 1986). The insulin like growth factor (IGF-1) has been reported as the most abundant growth factor in the bone matrix, and has been stated to be capable of stimulating osteoblast differentiation (Xian et al., 2012). The transforming growth factor β (TGF-β) is also one of the most abundant proteins in the bone matrix. It remains in an inactive state within the bone matrix until being released in response to bone resorption (Tang et al., 2009). In its active state, TGF-β recruits precursor cells for osteoblast differentiation. Of the TGF-β-family, BMPs constitute the largest subdivision; and within the BMP-family, BMP2 is an important activator of osteogenic genes (Halloran et al., 2020).

Both osteoblasts and osteoclasts secrete soluble coupling factors. To activate bone formation within the BMU, osteoclasts secrete products to promote osteoblast precursor recruitment and differentiation. These include osteoblastogenesis promoting clastokine sphingosine 1 phosphate (S1P) (Ishii et al., 2009), WNT10b (Bennett et al., 2007) and complement factor 3a (Matsuoka et al., 2014). By secretion of OPN to the resorption lacunae, osteoclasts are able to regulate both osteoclastogenesis and bone formation (Luukkonen et al., 2019). As the function of the BMU is to balance bone formation and resorption, osteoclasts also produce

factors to inhibit osteoblast differentiation. One of such factors is semaphoring 4D, which inhibits IGF-1 pathway (Negishi-Koga et al., 2011).

Osteoblasts secrete osteoclastogenesis promoting and inhibiting coupling factors. Probably the most known are M-CSF (Lacey et al., 1994) – which promotes osteoclast precursor proliferation, RANKL – which promotes osteoclast differentiation and activation (Li et al., 2000), and WNT5A – a promotor of osteoclastogenesis (Maeda et al., 2012). Osteoblasts also secrete OPG, which is an inhibitor of RANKL-dependent stimulation. Notably, a recent study has indicated how OPG production by mature osteoblasts is an important regulating signal in the termination of the resorption phase in the BMU (Cawley et al., 2020).

Membrane-bound coupling factors act in a cell contact-dependent manner. Bidirectional signaling of the Ephrin family is transduced via osteoclast-bound Ephrin B2 (EFNB2) and osteoblast-bound Ephrin B4 (EFNB4). In reverse signaling, from osteoblast to osteoclast, EFNB4 activates EFNB2 to suppress osteoclast differentiation. Accordingly, forward signaling from osteoclast to osteoblast promotes osteoblast differentiation and suppresses apoptosis (Tonna et al., 2014). Other suggested osteoblast membrane-bound coupling factors include FAS ligand, which induces osteoclast apoptosis (Krum et al., 2008), and semaphoring 3A, which inhibits osteoclastogenesis (Hayashi et al., 2012).

Recent studies have provided more insight into the diversity and spatiotemporal organization of the cells and activities of the human BMU. Unlike the previously suggested model of separate resorption and reversal (Eriksen et al., 1984b), it is possible that several resorption episodes occur consecutively and overlap with reversal periods. The duration of the resorption-reversal period is important to the expansion of osteoprogenitor cells and initiation of bone formation (Lassen et al., 2017).

In the formation stage of bone remodeling, osteoblasts form the unmineralized organic matrix, the osteoid. Around 90% of the organic matrix consists of type 1 collagen, and the remaining 10% is composed of noncollagenous proteins, including osteocalcin and OPN. During mineralization, hydroxyapatite crystals are deposited into the collagen matrix, stabilizing and fortifying bone structure. (Boskey et al., 2013) Following mineralization, osteoblasts either undergo apoptosis, turn into bone lining cells, or become embedded within the mineralized bone matrix and differentiate into osteocytes. This can also be described as the terminal phase of remodeling, a steady state before the next wave of remodeling. In humans, one remodeling period is estimated to take approximately 200 days (Eriksen et al., 1984a).

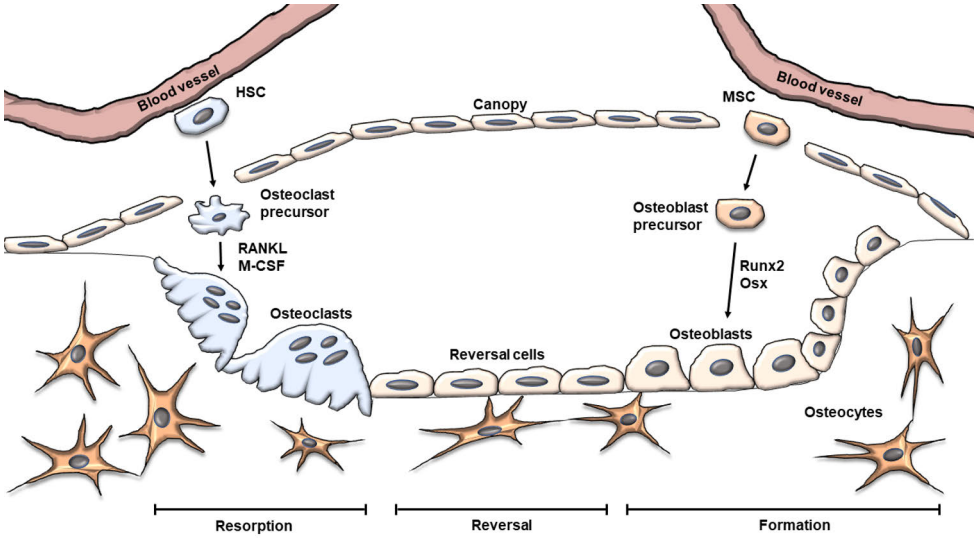

Figure 2. A simplified representation of the bone remodeling compartment (BRC) in trabecular bone. At the beginning of the resorption cycle, bone lining cells form a canopy over the BRC. Factors secreted by osteocytes attract hematopoietic stem cells (HSC) to the resorption site and induce their maturation into bone resorbing osteoclasts. The transition from active resorption to new bone matrix formation is connected by a reversal period. During this period, coupling factors inhibit osteoclast function to avoid excess resorption, and to induce osteoblast differentiation and function, which drives bone formation. By the end of the bone formation and mineralization phase, osteoblasts either undergo apoptosis, turn into bone lining cells, or differentiate into osteocytes. After this terminal stage, a new cycle of resorption-formation is initiated. Modified from (Sims et al., 2015).

2.2 Transcriptional regulation of bone formation

The differentiation fate of mesenchymal stromal cells into mature osteoblasts is determined by lineage-specific transcription factors (Komori, 2006). Runt family transcription factor 2 (Runx2) (Ducy et al., 1997) and Osterix (Osx) (Nakashima et al., 2002) are key determinants of osteoblast differentiation and bone development. As studies have shown, the loss of either *Runx2* or Osterix results in the total absence of osteoblasts and bone in mice (Boyle et al., 2003; Otto et al., 1997). Runx2 and Osterix not only induce early osteoblast differentiation, but they also inhibit bone accrual, depending on the stage of the differentiation cascade (**Figure 3**).

Activating transcription factor 4 (ATF4) regulates the later stages of osteoblast differentiation. It is able to bind osteoblast-specific elements in the promoter region of the osteocalcin gene and transactivate its expression (Yang et al., 2004) in co-operation with Runx2 (Xiao et al., 2005). *Atf4*-deficiency leads to decreased bone mass and increased brittleness of bones in mice (Makowski et al., 2014).

Different signaling pathways work in collaboration with transcription factors to regulate osteogenic lineage commitment and differentiation. These signaling pathways include Hedgehog (HH) signaling, Wnt/β –catenin signaling, TGF-β, BMP-signaling, and fibroblast growth factor (FGF) signaling (Chan et al., 2021; Liu et al., 2020). As an example, Runx2 and FGF, HH, Wnt and parathyroid like hormone (Pthlh) reciprocally regulate each other in cranial suture mesenchymal cell proliferation (Qin et al., 2019). Whereas Wnt-signaling is a known regulator of both Osterix and Runx2 in commitment, differentiation, and maintenance of osteoprogenitors (Rodda et al., 2006). Accordingly, Osterix and Runx2 have been recognized as regulators of sclerostin, a known Wnt antagonist, in mice (Sevetson et al., 2004; Yang et al., 2010) and in humans (Pérez-Campo et al., 2016).

The following chapters discuss the two key transcription factors, Osterix and Runx2, in more detail.

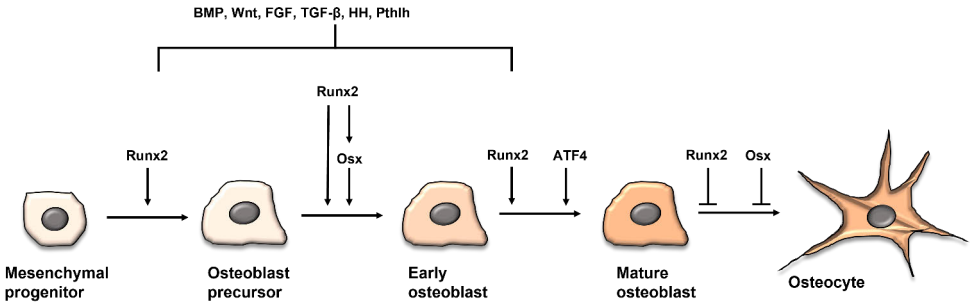

Figure 3. A schematic representation of Runx2 and Osterix function in different stages of mesenchymal cell differentiation into osteoblasts and subsequently into osteocytes.

2.2.1 Runx2

Runx2 belongs to the family of Runt-related transcription factors, also known as core-binding factor α (Cbfa) (Wang & Speck, 1992) and polyomavirus enhancer binding protein 2 (PEBP2) (Ogawa et al., 1993). However, the family is generally referred to as the Runt-related transcription factor or Runx (Van Wijnen et al., 2004). The Runx family of transcription factors includes three members, Runx1, Runx2, and Runx3. They all have a common DNA-binding Runt-domain and require heterodimerization with cotranscription factor core-binding factor β (Cbfb) to increase their DNA-binding capacity (Ogawa et al., 1993).

Runx2 was first identified in a study searching for regulators of osteocalcin (Ducy et al., 1997). Expression of *Runx2* was restricted to cells of the osteoblast lineage and was detected as early as in the embryonal mesenchymal condensations. Forced expression of *Runx2* in non-osteoblastic cells has been shown to induce the

expression of principal osteoblast-specific genes such as osteocalcin and bone sialoprotein (*BSP*). Runx2 was considered an osteoblast-specific transcription factor and a regulator of osteoblast differentiation (Ducy et al., 1997). Subsequent studies on disruption of *Runx2* in mice have presented a complete absence of osteoblasts and bone. Both intramembranous and endochondral ossification were also affected, and the mice were unviable (Komori et al., 1997; Otto et al., 1997). *Runx2*-deficient calvaria-derived mesenchymal stromal cells were unable to differentiate into osteoblasts (Kobayashi et al., 2000). More recently, proliferation of osteoblast progenitors was shown to be dependent on Runx2 (Kawane et al., 2018). Taken together, Runx2 is an essential factor in promoting early osteoblast differentiation.

Overexpression of *Runx2* in mice has provided evidence for the inhibitory role of Runx2 in the late stages of osteoblast development. Overexpression of *Runx2* using Col1a1 promoter has been found to inhibit terminal maturation of osteoblasts and lead to osteopenia and fragility (Liu et al., 2001). The expression pattern of *Runx2* also varies during osteoblast maturation. *Runx2* is strongly expressed in immature osteoblasts, but is downregulated during osteoblast maturation (Maruyama et al., 2007).

The role of Runx2 in chondrocyte maturation was verified by severely disturbed structure of the cartilage and lack of hypertrophic chondrocytes observed in *Runx2*-deficient mice (Inada et al., 1999). *Runx2* expression increases gradually during chondrocyte maturation, and is needed for the induction of chondrocyte hypertrophy (Enomoto et al., 2000). Runx2 has also been found to be essential for the survival and transdifferentiation of terminal hypertrophic chondrocytes (Qin et al., 2020). A recent study suggested a novel role for Runx2, arguing that it regulates apoptosis of hypertrophic chondrocytes during endochondral ossification (Rashid et al., 2021). Several studies have tried to elucidate the possible temporospatial role of Runx2 in chondrocytes, but results have been contradictory (Takeda et al., 2001; Ueta et al., 2001). However, there is an agreement that Runx2 is able to induce several factors regulating chondrocyte differentiation, such as Indian hedgehog, matrix metalloproteinase 13 (MMP13), and vascular endothelial growth factor (VEGF) (Komori, 2018).

In humans, loss-of-function mutations in *RUNX2* lead to cleidocranial dysplasia (CCD) (Otto et al., 2002). Patients with CCD have various skeletal changes such as incompletely formed clavicles, increased width of cranial fontanelles, Wormian bones (intrasutural bones caused by abnormal ossification of the calvaria), excess teeth, and short stature (Mundlos et al., 1997). At the moment, there is no treatment for CCD. Treatment of *Runx2* haploinsufficiency in mice with histone deacetylase inhibitor MS-275 was able to prevent delayed cranial suture closure, but more studies are still needed (Bae et al., 2017).

RUNX2 overexpression has been discovered in several cancer types when compared to normal tissues. Bone metastases associated with prostate cancer have high *RUNX2* expression (Brubaker et al., 2003). *RUNX2* is also overexpressed in osteosarcoma, a malignant bone cancer (Yang et al., 2013), and could potentially serve as a prognostic biomarker of tumor metastasis and survival in osteosarcoma patients (Liang et al., 2021). In breast cancer, elevated RUNX2 expression is associated with the clinical stage, histological grade of the tumor and overall poor prognosis (Onodera et al., 2010). Runx2 promotes the metastatic potential of bone-derived tumor cells by facilitating autophagy, and it could therefore be useful in evaluating the progression of breast cancer (Tandon et al., 2018). In search for early diagnostic biomarkers, *RUNX2* has been suggested as a potential candidate also in pancreatic cancer (Jin et al., 2021a) and gastric cancer (Guo et al., 2021).

2.2.2 Osterix

The discovery of Runx2 raised interest in finding more osteoblast-specific transcription factors. Osterix was first identified in a screen of osteoblast-specific cDNAs (Nakashima et al., 2002). Based on amino acid sequence, Osterix was categorized as a zinc finger transcription factor with a strong transcription activation domain. It resembles the specificity protein (SP) family of transcription factors, and is thereby also called Sp7 (Nakashima et al., 2002). Notably, mouse and human Osterix proteins share 95% sequence similarity (Gao et al., 2004).

Osterix transcripts were initially found in differentiating chondrocytes of early mouse embryos, and in trabecular bone cells at a later embryonal stage (Nakashima et al., 2002). Later studies have verified Osterix expression in adult mouse osteoblasts, hypertrophic chondrocytes, and osteocytes (Zhou et al., 2010). In embryonic human tissues, Osterix expression was first discovered in osteoblastic cells of the appendicular and craniofacial skeleton (Gao et al., 2004).

The significance of Osterix for bone formation *in vivo* was first presented by analysis of Osterix-null mice which had severely defected bone formation. In the Osterix-null mice endochondral and intramembranous bone formation was completely absent. Despite cartilage matrix formation, mesenchymal cells were unable to deposit bone matrix. All osteoblast-specific differentiation markers were also absent, except for Runx2. This indicated that Osterix acts downstream of Runx2. (Nakashima et al., 2002) As the Osterix-null mice died shortly after birth, only newborn bone formation status was initially analysed. To gain more information on the role of Osterix in adult skeleton, Baek et al. inactivated Osterix under the control of the Col1a1 promoter (Baek et al., 2009). These mice had a progressive osteopenic phenotype consisting of morphological changes in the trabecular and cortical bone parameters of long bones. Osteoclasts were unaffected. Postnatal deletion of Osterix

with tamoxifen-inducible Cre (CAG-CreER) led to a complete arrest of osteoblast differentiation and bone formation, abnormal cartilage accumulation, and impaired osteocyte maturation (Zhou et al., 2010). These studies confirmed the role of Osterix also in the postnatal bone growth and homeostasis. Yoshida et al. have presented data on the stage-dependent regulation of Osterix mRNA expression and protein activity during osteoblast differentiation (Yoshida et al., 2012). In the late stage of bone maturation, Osterix inhibited osteoblast differentiation similarly to Runx2.

Osterix exerts its effect on osteogenesis by regulating several downstream factors in the osteogenic cascade. For example, Osterix is able to activate the osteocalcin promoter (Niger et al., 2011), and the induction of *Col1a1* in mesenchymal and osteoblastic cells is dependent on Osterix expression (Ortuño et al., 2013). Osterix is also able to directly regulate *BSP* expression (Yang et al., 2016).

Osterix-null mice had defective cartilage mineralization (Nakashima et al., 2002), and Osterix was also shown to upregulate chondrocyte-specific *Sox9* expression in mesenchymal cells (Fu et al., 2007). In chondroprogenitor cell line ATD5, silencing of Osterix by shRNA led to significant downregulation of chondrogenic transcription factors *in vitro* (Omoteyama et al., 2010), whereas chondrocyte-specific deletion of Osterix using the Col2a1 promoter or the paired related homeobox 1 (Prrx1) promoter arrested endochondral ossification at the hypertrophic stage in mice (Nishimura et al., 2012). The same study was the first to strongly indicate *MMP13* as the target of Osterix during endochondral ossification. Cheng et al. have presented similar data on haploinsufficient Osx_{Col2a1} knockout mice, emphasizing the effect of endochondral bone formation on the length of long bones (Cheng et al., 2013). The same group later presented data on the regulation of chondrocytes by Osterix in secondary ossification centre (SOC) formation (Xing et al., 2019).

In humans, mutations in the Osterix gene have been linked to rare connective tissue disorders. Two studies have associated Osterix gene variants with recessive osteogenesis imperfecta (OI). The first study reported a case with clinical manifestations of recurrent fractures, mild bone deformities, and delayed tooth eruption (Lapunzina et al., 2010). The second study described three siblings with a novel homozygous Osterix variant. Their symptoms included bone fragility and impaired growth. One of the affected siblings also had significant hearing loss, which is commonly observed in OI. (Fiscaletti et al., 2018) More recently a heterozygous missense mutation within Osterix has been identified as a cause of juvenile Paget's disease (Whyte et al., 2020).

There is also growing evidence on the involvement of Osterix expression in breast cancer tumor metastasis. Osterix has been found to be highly expressed in metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-361. However, Osterix is undetectable in the non-metastatic breast cancer cell line MCF7. (Dai et

al., 2015) The same study was the first to identify Osterix in connection with several genes associated with breast cancer metastasis, such as *VEGF*, matrix metalloproteinase 9 (*MMP-9*), β-catenin and E-cadherin. Further studies have demonstrated that Osterix overexpression decreases the chemosensitivity of breast cancer cells and that downregulation of Osterix accordingly increases chemosensitivity (Wu et al., 2017). Ability of Osterix to potentiate breast cancer cell migration and tumor angiogenesis both *in vitro* and *in vivo* was recently reported (Qu et al., 2019). In a tissue array of 154 patient samples, Osterix was highly expressed in 57.1% of the breast cancer tissues, and weakly expressed in only 9.1 % of the samples. Osterix correlated significantly with lymph node metastasis and poor survival rate. The same study also confirmed previous findings of *VEGF* and *MMP-9* upregulation by Osterix. (Yao et al., 2019) Osterix expression has also been reported in cancer-associated fibroblasts (CAF), supporting the role of Osterix in bone metastases. Notably, Osterix positive CAF-cells expressed hematopoietic marker CD45 and Osterix protein expression was present during early hematopoiesis. This data challenges the earlier notion of Osterix expression being limited to cells of mesenchymal origin (Ricci et al., 2020). Together, these studies present Osterix as an important regulator of bone metastases in breast cancer.

2.3 Early B-cell factors (Ebfs)

2.3.1 Classification and structure

Early B-cell factor was first identified as a novel nuclear factor regulating the *Cd79a* (*mb-1*) gene in B-cell differentiation (Hagman et al., 1991). Same factor was also found almost simultaneously by another group (Wang et al., 1993). They described a transcriptional activator for rat olfactory-specific genes and named it accordingly as Olf-1. In this thesis, the abbreviation ‘Ebf’ will be used.

Functional characterization of Ebf has revealed that it has a DNA-binding domain (DBD) (Travis et al., 1993) and an ability to act as a dimer through an atypical helix-loop-helix-loop-helix (HLHLH) domain (Hagman et al., 1993). Further analysis on the Ebf’s DBD has proven that it contains a novel zinc-coordination motif (Hagman et al., 1995). This ‘zinc knuckle’ motif was later shown to be required for the transcriptional activation of B-cell-specific genes (Fields et al., 2008). These two motifs are connected by an IPT/TIG (immunoglobulin, plexins, transcription factors-like/transcription factor immunoglobulin) domain. (**Figure 4)** Based on the novel and unique structural features, Ebfs were classified as a new family of HLHLH-proteins.

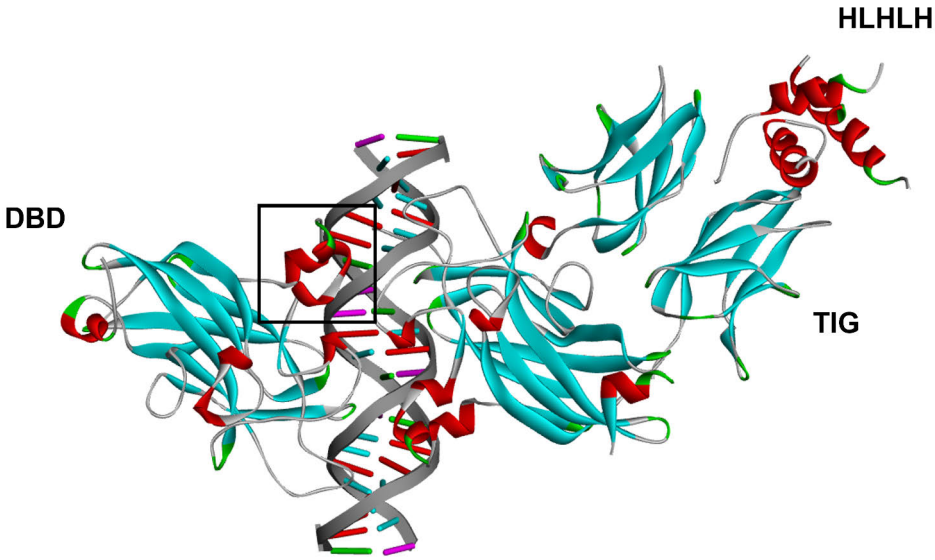

Figure 4. Schematic representation of dimeric Ebf1 bound to DNA. The DNA-binding domain (DBD) and atypical helix-loop-helix-loop-helix (HLHLH) are connected by an IPT/TIG (immunoglobulin, plexins, transcription factors-like/transcription factor immunoglobulin) domain. The zinc knuckle is highlighted by a black box. Structure was obtained from Protein Data Bank in Europe PDBe (accession code: 3MLP) and visualized using Discovery Studio software.

In mammals, the Ebf family consists of four known members. The first identified member of the Ebf family was named Ebf1. Two more Ebfs were later identified in mice and labelled Ebf2 and Ebf3 (Garel et al., 1997; Wang et al., 1997). Finally, it was Wang et al. who identified the fourth member, Ebf4 (Wang et al., 2002). A high degree of sequence conservation has been observed between *Ebf* transcripts of mice and humans (Hagman et al., 1993). Ebf1, Ebf2, and Ebf3 share more than 75% of overall identity, and more than 90% in the DBD domain (Wang et al., 1997). Structural determination has indicated that the DBD of Ebf shares similar features with the Rel homology superfamily of transcription factors (Siponen et al., 2010), and Ebf proteins have been suggested to represent a branch of this Rel superfamily (Treiber et al., 2010a).

After initial discovery in B-cell and neuronal cell differentiation, the Ebf family is now known to play a role in multiple cell types as both an activator and repressor of transcription. In the subsequent chapters, Ebf1, Ebf2 and Ebf3, which are the most widely studied members of Ebf family, are discussed further.

2.3.2 Early B-cell factor 1 (Ebf1)

2.3.2.1 Regulation of *Ebf1* expression

Ebf1 expression is a crucial factor in the establishment of the B-cell differentiation. The regulation of *Ebf1* gene expression, however, has not yet been fully elucidated, and most of the data arises from studies related to B-cell differentiation. The currently proposed mechanisms form a complex network including upstream control, reciprocal activation, and autoregulation. Ebf1 has been described as part of a global network of transcription factors orchestrating B-cell fate (Lin et al., 2010).

Depletion of either transcription factor E12 (Bain et al., 1997) or Ebf1 (Lin et al., 1995) is known to be crucial for B-cell development. Kee and Murre were the first to report the Ebf-related hierarchy on B-cell development (Kee et al., 1998). They showed that upstream expression of the E2A protein E12 induces the expression of *Ebf1*. Transcription factor PU.1 also participates in the upregulation of *Ebf1* along E2A (Medina et al., 2004). Interleukin-7 (IL-7) and interleukin receptor (IL-7R) also work by upregulating *Ebf1* in the stage transition of adult B-cell development (Kikuchi et al., 2005). Development of B-cells is also dependent on interaction with osteoblasts (Grčević et al., 2021), as bone marrow osteoprogenitors are an important source of IL-7 (Panaroni et al., 2015; Wu et al., 2008).

Regulation of *Ebf1* gene expression involves two differentially regulated promoters. The distal α-promoter contains binding sites for the E2A encoded protein E47 (Smith et al., 2002), IL-7, and the signal transducer and activator of transcription 5 (STAT5). The proximal β-promoter is regulated by PU.1 and Pax5. Transcription from these two promoters generates two distinct isoforms of Ebf1 that are functionally equivalent. The distal α-promoter is more involved in the initiation of *Ebf1* gene expression, whereas the proximal β-promoter is needed for the maintenance of *Ebf1* gene expression (Roessler et al., 2007).

The complex regulatory network of *Ebf1* expression also includes regulatory loops. Initial characterization of the *Ebf* promoter has indicated that it is a direct target for transactivation by Ebf1 (Smith et al., 2002). Autoregulation was later confirmed in analysis of the distal *Ebf1* α-promoter (Roessler et al., 2007). Pax5 and Ebf1 are both critical in initiating and maintaining the B-cell identity, and they form a self-reinforcing regulatory loop where Pax5 upregulates expression of *Ebf1* and Ebf1 interacts with enhancer elements in the *Pax5* gene (Decker et al., 2009; Roessler et al., 2007). In the context of a negative feedback circuit, *Ebf1* expression inhibits Id2, an inhibitor of E2A (Pongubala et al., 2008). In the common lymphoid progenitor compartment, Ebf1 and FoxO1 form a positive feedback circuit for the pro-B-cell-specific program of gene expression (Mansson et al., 2012).

Direct inhibitors of Ebf1 are e.g. multi-zinc finger transcription cofactors ZNF423 and ZNF521 (Mesuraca et al., 2015). The inhibitory effects of these two factors reach beyond B-cell differentiation, as they also regulate Ebf1 expression in bone homeostasis (Kiviranta et al., 2013) and olfactory neurons (Roby et al., 2012). As with other factors, Ebf1 also forms regulatory loops with these two inhibitors. In several different cancers, *EBF1* is downregulated through different epigenetic mechanisms (Armartmuntree et al., 2021; Xing et al., 2020). These mechanisms are further discussed in section 2.3.2.3.

2.3.2.2 Regulation by Ebf1

Treiber et al. initially defined the distinct events involved in gene regulation by Ebf1. They stated that the 'binding of Ebf1 is specific to hematopoietic tissue, it induces changes in chromatin structure and in collaboration with other transcription factors and cofactors, directs the ultimate transcription state of target genes' (Treiber et al., 2010b).

The first evidence of Ebf1 as an epigenetic regulator was obtained in association to the *mb-1* promoter, where Ebf1 mediated changes in chromatin accessibility (Maier et al., 2004). Ebf1 has been described as a pioneering factor in controlling the epigenetic status and accessibility of target genes during B-cell development (Hagman et al., 2005). Ebf1 not only facilitates the specification and B-cell program of gene expression, but it also suppresses the alternative cell-fate determinants in B-cell commitment. Lymphoid progenitors lacking Ebf1 have the potential to differentiate into T-cells, natural killer cells, and myeloid cells (Pongubala et al., 2008). Restoration of Ebf1 suppresses genes encoding alternative cell-fate determinants such as *C/EBPα* (myeloid lineage) and *Id2* (E2A inhibitor) (Pongubala et al., 2008). T-lineage specification is attenuated by Ebf1-driven repression of *Tcf7* (Nechanitzky et al., 2013) and Gata3 (Banerjee et al., 2013).

Recently, Li et al. verified that Ebf1 occupancy precedes the formation of accessible chromatin. Many of the downregulated genes are only transiently bound by Ebf1, whereas the upregulated genes in the B-cell pathway mainly show persistent Ebf1 binding (Li et al., 2018). Direct binding of Ebf1 to the regulatory regions of target genes has also been shown in osteoblast differentiation (Zee et al., 2013), and during glomerular development, Ebf1 has been observed to act through secondary mechanisms (Nelson et al., 2019).

The known complexity of gene regulation by transcription factors in normal homeostasis and dysregulated transcription in diseases (Henley et al., 2021; Spitz et al., 2012) applies also to Ebfs. The pioneering role, dimer properties and high similarity between members of the Ebf family indicates gene regulation by Ebfs to be a complex process which needs to be elucidated further.

2.3.2.3 Roles in normal physiology and pathophysiology

Ebf1 expression was first detected in mouse B-cells (Hagman et al., 1991) and rat olfactory neurons (Wang et al., 1993). A targeted mutation in the *Ebf1* gene led to failure in B-cell differentiation, confirming the importance of Ebf1 in B-cells (Lin et al., 1995). Notably, low *EBF1* expression has been connected to acute (Mullighan et al., 2007) and chronic lymphocytic leukemia in humans (Seifert et al., 2012; Wang et al., 2021). In murine models, haploinsufficiency of *Ebf1* combined with activation of STAT5, a known factor in hematological diseases (Tolomeo et al., 2019), is sufficient to rapidly initiate acute lymphoblastic leukemia (Heltemes-Harris et al., 2011). Normal expression of *Ebf1* is required to safeguard early stage B-cells from leukemic transformation in mice by limiting STAT5, which activates IL-7 and Myc expression signaling and folate metabolism (Ramamoorthy et al., 2020; Somasundaram et al., 2021). In patients with chronic lymphocytic leukemia, expression levels of *EBF1* were reduced, whereas expression of STAT5 was increased, compared to healthy controls (Wang et al., 2021).

Ebf1 mRNA expression is also detected in adipocytes. In addition to mouse adipose tissue (Hagman et al., 1993), *Ebf1* expression has been observed in the 3T3-L1 preadipocytic cell line. mRNA expression of *Ebf1* has been found to increase when adipocyte differentiation progresses. Retroviral transduction of *Ebf1* expression vector further augmented adipogenesis in 3T3-L1 cells, mouse embryonic fibroblasts and NIH 3T3 fibroblasts. (Åkerblad et al., 2002) Further studies have suggested that Ebf1 could induce the expression of *C/EBPα* and *PPARγ*. Ebf2 has also been found to be involved in mouse adipogenesis, (Jimenez et al., 2007) which is discussed in section 2.3.3.

The first global knockout mouse model of Ebf1 (Ebf1KO) had lipodystrophy and provided insight into the *in vivo* effects of *Ebf1* deletion in adipogenesis (Hesslein et al., 2009). Ebf1KO mice had significantly reduced subcutaneous fat storages, but significantly increased adiposity in the liver and bone marrow (Fretz et al., 2010). Interestingly, heterozygous deletion in mice did not lead to changes in body composition nor in the amount of white adipose tissue (WAT) or brown adipose tissue (BAT). This global knockout model also had a pronounced bone phenotype, which is discussed in section 2.4.

Griffin et al. have shown that one of the major pathways regulated by Ebf1 in adipocytes is related to inflammation (Griffin et al., 2013). In humans, reduced *EBF1* levels have been linked to increased lipolysis and adipocyte hypertrophy (Gao et al., 2014). Petrus et al. have suggested that EBF1 could be used as a potential BMI-independent marker for metabolic syndrome (Petrus et al., 2014). As obesity is known to be associated with inflammation, the controversial results from mice and human studies highlight that the specific roles of Ebf1 on adipogenesis and inflammation are still unresolved (Griffin, 2021).

Ebf1 has also been studied in the context of glomerular development. The Ebf1KO mouse model presented significantly increased levels of serum osteocalcin despite markedly low bone mineral density (Hesslein et al., 2009). As the major route of clearance for osteocalcin is through the kidneys, the group hypothesized whether the kidney function of the Ebf1KO mice was also affected. Ebf1KO mice indeed presented a reduction of more than 66% in glomerular filtration rate and reduced glomerular maturation. *Ebf1* mRNA was expressed at different time points during kidney development, but the defect in glomerular function nevertheless developed at the last stages of glomerular maturation. (Fretz et al., 2014) Conditional deletion of *Ebf1* in mesangium/pericytes (Foxd1-cre) suggested that Ebf1 regulates formation of the glomerular capillary network. Notably, the progression of chronic kidney disease was faster in the conditional model than in the global knockout model, suggesting that the lack of Ebf1 in some Ebf1 expressing cell types of the global knockout model had a protective effect (Nelson et al., 2019).

EBF1 is recognized as a susceptibility loci for triple-negative breast cancer in humans (Purrington et al., 2014) and a possible regulator of methylation in specific cancer subtypes (Fernandez-Jimenez et al., 2017). In gastric cancers, *EBF1* function is suppressed by various mechanisms, including epigenetic inactivation and mutations. In patient samples of gastric carcinoma, the expression levels of *EBF1* negatively correlate with the expression levels of telomerase reverse transcriptase (*TERT*), a known contributor to cancer formation and progression (Xing et al., 2020). In cholangiocarcinoma patients, oxidative stress (Armartmuntree et al., 2018) and hypermethylation (Armartmuntree et al., 2021) have been linked to suppressed *EBF1* expression and advanced cancer progression. A recent study presented data on EBF1 expression in human tumor pericytes and contribution to angiogenesis (Pagani et al., 2021).

2.3.3 Early B-cell factor 2 (Ebf2)

Ebf2 was initially found in the embryonic olfactory epithelium (Wang et al., 1997) and in the developing central nervous system (Garel et al., 1997) of mice. Global *Ebf2* knockout mice (Ebf2KO) are viable but have progressive defects. The phenotype of the mice is runted, with ‘hunchbacked’ posture and unsteady gait. Due to defective migration of gonadotropin releasing hormone-synthetizing neurons, Ebf2KO mice have secondary hypogonadism. Peripheral nerves of Ebf2KO mice also feature defective axon sorting, signs of segmental dysmyelination, and hypomyelination. (Corradi et al., 2003) More specifically, the onset of myelination was found to be delayed in Ebf2KO mice (Giacomini et al., 2011). In addition, defects in Schwann cell function and myelination were later found to be influenced by the expression levels of *Ebf1* and *Ebf2* genes (Moruzzo et al., 2017). Motor

deficits were connected to the role of Ebf2 in the maintenance of cerebellar Purkinje cells (Hoxha et al., 2013) and axial motor neuron development (Catela et al., 2019). Ebf2 was also identified as a novel regulator of dopamine neuron development in the midbrain (Yang et al., 2015).

Whereas Ebf1 is important in WAT formation and metabolism, Ebf2 plays a role in beige and brown adipocytes. In a comparative study aiming to identify PPARγ binding sites in BAT and WAT, *Ebf2* was found to be highly enriched in the BAT-specific binding regions, and *Ebf2* expression was able to stimulate differentiation of BAT *in vitro* and *in vivo*. In global *Ebf2* knockout mice, BAT depots were reduced in size, and they lacked the usual BAT characteristics, such as expression of uncoupling protein 1. (Rajakumari et al., 2013) Molecular profiling of embryonic brown preadipose cells further verified the role of *Ebf2* as a selective marker of brown and beige adipogenic precursor cells and its ability to reprogram myoblasts into brown adipose precursors (Wang et al., 2014). Accordingly, overexpression of *Ebf2* has been shown to be able to induce browning and beiging of WAT *in vitro* and *in vivo* (Stine et al., 2016).

Inhibition of Ebf2 activity by transcriptional regulator Zfp423 suppresses the thermogenic gene program in the WAT of mice *in vivo* (Shao et al., 2016). Shapira et al. have elucidated the mechanisms behind Ebf2 regulation of brown adipogenesis by demonstrating the factor's direct binding ability and its regulation of lineage-specific enhancers and chromatin remodeling complexes (Shapira et al., 2017). Zfp423 maintains WAT identity by preventing these Ebf2 downstream functions (Shao et al., 2021). Ebf2-regulated adaptive thermogenic functions are reversible in prolonged cold exposure. Targeted deletion of *Ebf2* in mouse adipocytes was observed to ablate BAT function *in vivo*; however, prolonged cold exposure was able to restore the thermogenic profile and function of Ebf2 (Angueira et al., 2020).

In pathological conditions, *EBF2* expression has been suggested as a possible biomarker for prostate cancer (Nikitina et al., 2017) and bladder cancer (Liao et al., 2021). Expression of *EBF2* is also significantly increased in osteosarcoma, a tumor in bone (Patiño-García et al., 2009). Recently, the focus of research related to the interaction between *EBF2* and osteosarcoma has been on identifying specific miRNAs which could downregulate the expression of *EBF2* and inhibit osteosarcoma migration and invasion (Chen et al., 2019; Li et al., 2019).

2.3.4 Early B-cell factor 3 (Ebf3)

Ebf3 was also initially discovered in mouse olfactory tissue (Wang et al., 1997) and in the central nervous system (Garel et al., 1997). Global *Ebf3* knockout mice (Ebf3KO) display reduced viability, surviving only a few hours after birth (Wang et al., 2004). This is mostly due to respiratory failure, as a lack of Ebf3 causes a defect

in the diaphragm and prevents its normal function (Jin et al., 2014). As with Ebf2KO mice, Ebf3KO mice also have issues regarding male mating efficiency (Wang et al., 2004). Ebf3, together with Ebf2, is also responsible for the migration of Cajal-Retzius cells in cortical lamination of the brain (Chiara et al., 2012). Later studies have further presented Ebf3 as a key regulator of migrating neocortical cells by regulation of postmitotic neuronal marker NeuroD1 (Iwai et al., 2018).

In humans, de novo mutations in *EBF3* are connected to neurodevelopmental syndrome. These mutations are located in highly conserved regions and are predicted to affect DNA binding and loss of function. Clinical features of the syndrome include intellectual disability and developmental delay in speech and motor abilities (Chao et al., 2017; Harms et al., 2017; Sleven et al., 2017). The syndrome has been recently defined as 'hypotonia, ataxia, and delayed development syndrome' (HADDS) (Nishi et al., 2021).

There is increasing evidence that Ebf3 acts as a significant tumor suppressor. Initially, a genome wide screen analysis revealed *EBF3* to be expressed in normal adult brain in humans, but to be repressed by methylation and/or deletion in high-grade gliomas. Demethylation by 5-aza-deoxycytidine was able to reactivate *EBF3* expression *in vitro*, suggesting that aberrant methylation was responsible for the silencing of *EBF3*. (Zardo et al., 2002) Epigenetic inactivation of EBF3 has been detected in several cell brain tumor cell lines, colorectal tumor cell lines and breast tumor cell lines. Silencing of *EBF3* in tumor cell lines is however not universal, as same study presented how colon cancer tumor lines SW48 and U2-OS glioblastoma cell lines express normal levels *EBF3*. Transduction of tumor cell lines with recombinant adenovirus for wild-type *Ebf3* restricted growth of the tumor cell lines through cell cycle arrest and apoptosis. (Zhao et al., 2006)

In humans, *EBF3* silencing and methylation have been detected in gliomas (Zardo et al., 2002), head and neck squamous cell carcinoma (Bennett et al., 2008), gastric carcinoma (Kim et al., 2012), and pediatric acute myeloid leukemia (Tao et al., 2015). In a study of gastric carcinoma, promoter methylation of *EBF3* was detected in 40% of the cancer tissues but not in any of the normal tissues analysed. Promoter methylation also correlated significantly with lymphatic invasion and poor survival, suggesting *EBF3* as an independent prognostic marker in gastric carcinoma. (Kim et al., 2012) A recent study further associated changes in *EBF3* methylation with aggressive tumorigenic changes in several cancer types and cancer cell lines (Rodger et al., 2019).

As timely diagnosis of cancer significantly enhances patient survival, finding new markers for early detection is essential. Epigenetic events, such as DNA methylation, occur during the early stages of carcinogenesis and could therefore serve as future biomarkers of cancer (Locke et al., 2019). The ability to use *EBF3* as an independent prognostic marker of gastric carcinoma is therefore promising (Kim

et al., 2012). Future research will tell if *EBF3* methylation status could be used as a potential indicator of tumor progression and metastasis ability.

2.3.5 Co-operation between Ebfs

As all three mammalian Ebfs have structurally highly similar DNA-binding domain, it has been suggested they also share redundant and combinatorial functions. First evidence of this came from a study analysing the phenotype of the global Ebf1KO mouse strain. Although *Ebf1* has been known to be expressed in the olfactory epithelium, disruption of *Ebf1* was not observed to lead to brain abnormalities (Lin et al., 1995). Further studies have hypothesized that the lack of phenotype in the central nervous system was due to functionally identical properties and overlapping expression patterns of other *Ebf* genes that compensated for the loss of *Ebf1* (Garel et al., 1997). The same effect was also noted in the regulation of early migration of Caja-Retzius cells from the cortical hem. Again, Ebf transcription factors were found to be expressed in overlapping forebrain territories. Moreover, loss of *Ebf2 in vivo* resulted in a transient decrease in Caja-Retzius cell numbers, but also in strong upregulation of *Ebf3*, suggesting a compensatory mechanism (Chiara et al., 2012).

Co-operation between different Ebfs has also been suggested to be an important factor in Ebf2 dependent regulation of adipocyte differentiation. Adipocyte-specific deletion of *Ebf2* was shown to ablate BAT function, leading to cold intolerance in mice. In contrast, adipocyte-specific deletion of *Ebf1* had no effect on the expression of *Ebf2* or other BAT markers in BAT, and the effect on BAT tissue was modest. Adipocyte-specific deletion of both *Ebf1* and *Ebf2* simultaneously led to even stronger downregulation of the brown adipocyte markers and ablation of BAT recruitment upon cold exposure. (Angueira et al., 2020) These results suggest Ebfs to have individual but also redundant roles in adipose tissue.

Ebf1, *Ebf2*, and *Ebf3* are all expressed in osteoblasts induced from mouse calvarial cells (Kieslinger et al., 2005) and in bone marrow mesenchymal stromal cells, where they participate in the maintenance of the functional hematopoietic stem cell niche (Derecka et al., 2020; Kieslinger et al., 2010; Seike et al., 2018). Ebfs have redundant and non-redundant functions in osteoblast differentiation, and these aspects are discussed in further chapters of this thesis.

2.4 Ebfs in bone formation

As members of the Ebf family were discovered to be expressed in multiple mammalian tissues, effects on bone formation were also of interest. Analysis of the Ebf2-LacZ reporter mouse (Corradi et al., 2003) revealed expression of *Ebf2* in immature osteoblastic cells. Global *Ebf2* knockout mice developed cancellous and

cortical bone osteopenia as well as decreased bone mass due to enhanced bone resorption and an increased number of osteoclasts (Kieslinger et al., 2005). Even though *Ebf2* is not expressed in osteoclasts, it is able to regulate osteoclast differentiation via osteoblast-dependent mechanisms. In the absence of *Ebf2*, the expression of *OPG*, an inhibitor of osteoclastogenesis, was highly suppressed in the osteoblasts (Kieslinger et al., 2005). More recently, the downregulation of *Ebf2* and the subsequent decrease in *OPG* expression has been linked to spinal cord injury-induced osteoporosis in rats (Liu et al., 2021).

Data on Ebf1 in bone formation is more complex. The global Ebf1KO mouse model resulted in increased bone formation and osteoblast differentiation, suggesting that Ebf1 is a negative regulator of bone formation (Hesslein et al., 2009). The same effect was recapitulated in the haploinsufficient *Ebf1* mouse model. In this case, overexpression of *Ebf1* in osteoblasts under the ColI2.3 promoter led to significantly reduced bone volume and low osteoblast activity in mice. (Kiviranta et al., 2013) However, global *Ebf1* and *Ebf2* knockout mice had severe developmental defects and failure to thrive due to effects in the central nervous system. Therefore, bone phenotype could be secondary to the changes in brain development and other metabolic effects.

Studies using conditional knockout models targeted to bone have tried to overcome these issues. Runx2-Cre was used by Zee et al. to target *Ebf1* deletion to osteoblast lineage (Zee et al., 2013). Body composition of these mice was normal at four weeks of age, without changes in bone formation, bone mass or osteoblast number. Seike et al. targeted *Ebf1* and *Ebf3* deletion to the mesenchymal cell lineage (Seike et al., 2018). They reported no phenotype in the *Ebf1* knockout, whereas deletion of *Ebf3* led to a high bone mass phenotype. Notable in Seike et al.'s study is the reasonably old age of the mice, 18 and 90 weeks. More recently, Derecka et al. studied *Ebf1* deletion in the mesenchymal cell lineage in mice eight to 14 weeks old (Derecka et al., 2020). These mice presented modestly increased trabecular bone volume.

3 Aims

Despite the many important discoveries in this field, the transcriptional control of osteoblast differentiation and function is still not yet fully understood. Global knockout mouse models have demonstrated that Ebf1 and Ebf2, two members of the Ebf family of transcription factors, have a role in bone formation. However, it has remained unclear, which of the effects of Ebfs are autonomous for bone cells and which are secondary due to effects of Ebfs on other cell types. Moreover, the high structural similarity and redundant functions observed in other cell types raise the question of whether Ebf1 and Ebf2 co-operate in bone formation.

The specific objectives of this study were:

1. To study the role of Ebf1 in bone formation at early and late stages of osteoblast differentiation.
2. To study the role of Ebf2 in bone formation and in the differentiation of mesenchymal cell lineage.
3. To investigate the possible interplay between Ebf1 and Ebf2 in bone formation.

4 Materials and Methods

4.1 Ethics statement (I, II)

All mouse studies were approved by the Finnish ethical committee for experimental animals (license numbers KEK/2010-3003-Kiviranta, 5186/04.10.07/2017 and 14044/2020), complying with the international guidelines on the care and use of laboratory animals, and were conducted under the supervision of the trained staff at the Central Animal Laboratory, University of Turku. The protocols of animal experiments followed 3Rs principle (Replace, Reduce and Refine).

4.2 Mouse strains (I, II)

The animals were housed in cages under standard laboratory conditions (temperature of 22°C, light from 6:00AM to 6:00PM). Water and soy-free food pellets were available ad libitum, excluding a four-hour fasting period before euthanasia. The ages and gender of the mice used in this study are summarized in **Table 1**.

Table 1. Knockout mouse models used in this study.

Mouse model	Knockout (Global or targeted Cre)	Age	Analysed gender	Used in
$Ebf1^{+/-}$	Global, heterozygous	Newborn, < 72h	Not specified	Study I, *in vitro*
$Ebf1_{Osx}$	Osx-Cre	12 weeks 24 weeks	Females and males	Study I
$Ebf1_{hOC}$	hOC-Cre	12 weeks 24 weeks	Females and males	Study I
Ebf2KO	Global	3 weeks	Males	Study II
$Ebf2_{Prrx1}$	Prrx1-Cre	6 weeks 12 weeks	Females and males	Study II
Ebf1x$Ebf2_{Prrx1}$	Prrx1-Cre	6 weeks 12 weeks	Females and males	Study II

4.2.1 Global knockout and osteoblast-targeted conditional *Ebf1* knockout mice (I)

$Ebf1^{+/-}$ mice were kindly provided by Dr. J. Hagman (National Jewish Health, Denver, CO) and Dr. R. Grosschedl (Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany; (Lin et al., 1995)), and maintained on C57BL background.

$Ebf1^{fl/fl}$ mice were also provided by Dr. J. Hagman and have been described previously (Treiber et al., 2010b). To create conditional, osteoblast-targeted *Ebf1* knockout mice, $Ebf1^{fl/fl}$ mice were crossed with Osx- or hOC-Cre mouse lines, respectively. Both Osx-Cre and hOC-Cre mice have been described previously (Rodda et al., 2006; Zhang et al., 2002).

Osx-driven Cre expression is known to have an effect on bone phenotype (Huang et al., 2015), thus Cre-positive $Ebf1^{+/+}$ Osx-Cre+ mice were used as controls. The hOC-Cre mice do not have a bone phenotype, so Cre-negative $Ebf1^{fl/fl}$ hOC-Cre- mice could be used as controls. Samples were collected from male and female mice at 12 weeks and 24 weeks of age.

4.2.2 Global *Ebf2* knockout mice (II)

The original targeting vector for *Ebf2* was a kind gift from Soren Warming (Department of Molecular Biology, Genentech Inc., South San Francisco, CA, US). A chimera mouse line was generated by the Turku Center for Disease Modeling, University of Turku, Finland.

To generate global *Ebf2* knockout mice, heterozygous *Ebf2* chimeras were first bred with mice expressing Cre under the control of CMV enhancer - chicken-beta-actin (CAG) promoter. This led to deletion of exons 4 to 6 in all tissues of the $Ebf2KO^{+/-}$ mice. As the breeding of the $Ebf2KO^{+/-}$ males and $Ebf2KO^{+/-}$ females on C57BL/JC background led to unviable offspring, $Ebf2KO^{+/-}$ mice were bred with 129SvJ mice. Breeding the subsequent generation of $Ebf2KO^{+/-}$ males and $Ebf2KO^{+/-}$ females on crossbred C57BL/JC and 129SvJ background led to more viable $Ebf2KO^{-/-}$ offspring. Male mice were analysed at three weeks of age.

4.2.3 Mesenchymal cell-targeted *Ebf2* knockout mice (II)

To create targeted deletion of *Ebf2* in the mesenchymal lineage ($Ebf2_{Prrx1}$ knockout mice), heterozygous *Ebf2* mice from the chimera breeding were crossed with Flippase (Flp) expressing mice to remove the Neo cassette flanked by Flp recombinase target sites (Frt) and to create $Ebf2^{fl/+}$ mice (II, Suppl. Fig. 1, A). Mesenchymal cell-targeted conditional knockout was subsequently achieved by crossing $Ebf2^{fl/+}$ mice with a mouse strain expressing Cre recombinase driven by the

Prrx1 promoter specifically expressed in early limb mesenchymal cells. These Tg(Prrx-cre)1cjt/J mice were acquired from The Jackson Laboratory (Bar Harbor, ME, USA)(Logan et al., 2002). Male and female mice were analysed, at six and 12 weeks of age.

4.2.4 Mesenchymal cell-targeted Ebf1xEbf2 double knockout mice (II)

To achieve a conditional Ebf1xEbf2$_{Prrx1}$ knockout in which both *Ebf1* and *Ebf2* have been deleted in the mesenchymal cell lineage, the Ebf2$_{Prrx1}$Cre+ males were crossed with Ebf1$^{fl/fl}$Cre- females (from Study I). Male and female mice were analysed, at six and 12 weeks of age.

4.2.5 Genotyping (I, II)

Genotyping was carried out from DNA extracted from ear marks two-week-old to three-week-old mice. To verify that the Cre recombination had taken place, whole bone DNA was extracted from femurs (Study I, Suppl. Fig. 1) or humeri (Study II, Suppl. Fig. 1, B-D). Sequences of the PCR primers used for the verification are listed in **Table 2**. For samples collected for Study I, bone marrow was included in the whole bone samples. For Study II, bones were first centrifuged to remove the bone marrow.

Table 2. PCR primers used for verification of successful recombination.

Primers	Sequence	Product size after successful recombination (bp)	Used in
Ebf1del_F Ebf1del_R	AACGAGCGGAACCCTACTTG CTAGAGCCCTTCTGAAGCCG	392 bp	Verifying cre/loxP recombination in Ebf1cKO mouse models
Ebf2del_F Ebf2del_R	CAGGGGTGTAGTTGGTTGGA GCAGGGGATGAAAAGCCCAGA	496 bp	Verifying cre/loxP recombination in Ebf2cKO mouse models

4.3 Analysis of the mouse phenotypes (I, II)

4.3.1 Histology and histomorphometry (I, II)

Following euthanasia of the mice, femurs, tibias and lumbar vertebrae were immediately collected, fixed in 3.7% formaldehyde, dehydrated, and stored in 70% EtOH at +4°C. Bone lengths were measured with a vernier caliper. For histological analyses, tibias were fixed in formaldehyde, decalcified in 10% EDTA, embedded in paraffin, and cut into 4 μm sections. Cut sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E).

For the analysis of adiposity in SOC, the number of adipocyte 'ghosts' in sagittal tibial sections were counted manually using 3DHISTEC's CaseViewer programme. The area of the secondary ossification centre was outlined and measured (in mm^2) using ImageJ software. The number of adipocyte 'ghosts' was normalized to the area and reported as adipocyte number/mm^2. Bone marrow adiposity was analysed using the same tibial samples by defining a 1 mm^2 region of interest inside the marrow cavity.

For the growth plate analysis in Study II, sagittal sections of H&E-stained proximal tibia were used. The average thickness of one medial and two marginal lines of proliferative and hypertrophic zones in the growth plate was measured by ImageJ software. Cell density was analysed by calculating the average cell number in eight regions (each 100x100 μm) of the proliferative zone and in 16 to 17 regions (each 50x50 μm) of the hypertrophic zone.

For the histomorphometric analysis, mice were injected with 20 mg/kg of Calcein (C0875, Sigma) and 40 mg/kg of Demeclocycline (D6140, Sigma) nine and two days prior to sacrifice, respectively. Tibias were harvested and fixed in 3.7% formalin overnight (o/n) and transferred to 70% ethanol for a minimum of three days. Fixed bones were dehydrated with acetone and embedded in methyl methacrylate. Undecalcified, 4-μm-thick sections were cut with hard tissue microtome (RM2255, Leica), deplastified, and stained with Von Kossa to visualize the mineralized bone. For static parameters, toluidine blue staining was used for the osteoblast measurements, and staining for Tartrate-resistant acid phosphatase (TRACP) was used to identify osteoclasts. Unstained slides were utilized for the dynamic parameters. The slides were analysed using Osteomeasure-histomorphometry workstation (OsteomeasureXP 3.1.0.1, Osteometrics).

Structural parameters, such as bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were obtained by taking an average of two measurements from consecutive sections. All parameters were calculated and expressed according to the standardized nomenclature (Dempster et al., 2013).

4.3.2 Microcomputed tomography (I, II)

Bones were scanned and analysed with SkyScan 1072 or SkyScan 1272 X-ray computed tomography (Bruker). A plastic holder was used to ensure immobilization and constant positioning of the sample. Cross-sectional images were reconstructed with NRecon 1.6.4.1 software (Bruker). The regions of interest (ROI) were drawn and analysed with CTan 1.10.10 software (Bruker-microCT).

In Study I, the scanning voxel resolution was 8.4 μm. For the analysis of the trabecular bone in the femur and tibia, ROI excluding the cortical bone was defined 50 layers (420 μm) below the growth plate, for a total of 120 layers (1008 μm). Cortical bone was analysed beginning from 4500 μm below the growth plate, for a total of 50 layers (418 μm). For the analysis of the trabecular bone in the fourth lumbar vertebrae, a ROI excluding the cortical bone was defined 30 layers (252 μm) above the distal growth plate and extending to 100 layers (840 μm), proximally.

In Study II, the scanning voxel resolution was 8.37 μm for the tibia, 5 μm for the femur, and 7 μm for vertebra. For the analysis of the trabecular bone in the tibia, ROI excluding the cortical bone was defined 80 layers (670 μm) distally from the proximal growth plate, for a total of 120 layers (1004 μm). Cortical bone was analysed beginning from 5022 μm distally from the growth plate, for a total of 100 layers (837 μm). To analyse the SOC area of the tibia, a ROI excluding the cortical bone was defined 10 layers (84 μm) proximally from the proximal growth plate and extending a total of 12 layers (100 μm).

For the analysis of the trabecular bone in the femur in Study II, a ROI excluding the cortical bone was defined 200 layers (1000 μm) proximally from the distal growth plate, for a total of 120 layers (600 μm). Cortical bone was analysed beginning from 5500 μm proximally from the growth plate, for a total of 100 layers (500 μm).

As Ebf1xEbf2$_{Prrx1}$ mice had significantly shorter tibia and femur compared to the controls, this was taken into account during the μCT-analysis and the ROIs were accordingly adjusted to bone length.

For the analysis of the trabecular bone in the fourth lumbar vertebrae in Study II, a ROI excluding the cortical bone was defined 30 layers (210 μm) proximally from the distal growth plate and extending a total of 100 layers (700 μm).

4.4 Cell culture experiments (I)

4.4.1 Primary cell osteoblast cultures

Calvarial primary cells from mouse pups less than 72 hours old were collected by enzymatic digestion (0.1% collagenase (Roche) and 0.2% dispase (Sigma) in α-minimal essential medium (α -MEM), at +37°C). Osteoblast differentiation was induced with differentiation media containing αMEM, 10% fetal bovine serum (FBS USA origin, Gibco), 1% penicillin/streptomycin, 100 µg/ml ascorbic acid, 3 mM β-glycerol phosphate, and 10 nM dexamethasone.

4.4.2 Retroviral overexpression cultures

293T Phoenix retroviral packaging cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin/streptomycin and 10 % heat-inactivated FBS (iFBS). Cells were plated 24 hours prior to transfection on T75 bottles at a seeding density of 3*10^6/bottle. At approximately 50% confluency, the cells were transfected with either puromycin-resistant retroviral control vector (pMSCV) or vector containing Ebf1 (pMSCV-Ebf1) (Jimenez et al., 2007). Viral supernatants were harvested at 48 hours and 72 hours post transfection.

Primary mouse calvarial cells (4.4.1) were plated 24 hours prior to infection on T75 bottles at a seeding density of 6.5*10^5/bottle. Cells were incubated with media containing retroviruses and 4 µg/ml polybrene at approximately 50% confluency for a minimum of 3 hours. The infection was repeated 24 hours later and selection by puromycin (3 µg/ml) was started 48 hours after the first infection.

Cells surviving the selection were expanded in culture, maintaining the confluency below 80%. For osteoblast induction, cells were plated on 12-well plates at a seeding density of 4.0*10^4/well. Osteoblast differentiation was induced once the wells had reached confluency, usually three days after the seeding. At the end of culture (day 14), cell lysates were collected for RNA isolation.

4.4.3 Histochemical analysis of primary osteoblast cultures

For ALP and Von Kossa stainings, cells were fixed with 3.7% formalin for 15 min and washed twice with water. Fixed cells were incubated with ALP staining solution (Naphtol AS phosphate, Fast blue RR salt, N,N-dimethylformamide, all from Sigma) for 45 min at room temperature and rinsed with water. For Von Kossa staining, cells were stained with 2.5% silver nitrate (Thermo Scientific) for 30 min at room temperature and rinsed with water.

4.5 Molecular biological methods (I, II)

4.5.1 RNA extraction and RT-PCR (I, II)

Total RNA from the humeri was isolated by first pulverizing the collected bones in liquid nitrogen, followed by isolation using TRIsure reagent (Bioline) and RNeasy Mini Kit (QIAGEN).

Total RNA from cell culture lysates was isolated using either the RNeasy mini kit (QIAGEN) or the NucleoSpin RNA Plus (Macherey-Nagel; Study I).

cDNA was prepared with DyNAmo cDNA Synthesis Kit (Thermo Fisher) (Study I) or Sensifast cDNA synthesis kit (BioLine). Quantitative real-time PCR (qPCR) was performed using either iQ SYBR Green Supermix (Bio-Rad Laboratories; Study I) or Dynamo Flash SYBR Green qPCR Kit (Thermo Fisher). The samples were run with the Bio-Rad CFX96 qPCR system. The data was normalized to beta-actin as an internal control, and the results were analysed by ΔΔCt-method . Primers used for the qRT-PCR are listed in **Table 3**.

Table 3. qRT-PCR primers.

Target gene	Forward	Reverse
ALP	ACTCAGGGCAATGAGGTCAC	CACCCGAGTGGTAGTCACAA
Col1a1	AGACATGTTCAGCTTTGTGGA	GCA GCT GAC TTCAGG GATG
Ebf1	AGATTGAGAGGACGGCCTTTGT	TCTGTCCGTATCCCATTGCTG
Ebf2	AAACCCAAGGGACATGAGG	CACATGTCCATCCACATTTACTG
OC	TGAGGACCATCTTTCTGCTCA	TGGACATGAAGGCTTTGTCA
Osx	GTCCTCTCTGCTTGAGGAAGAA	GGGCTGAAAGGTCAGCGTAT
Runx2	GCCCAGGCGTATTTCAGA	TGCCTGGCTCTTCTTACTGAG
β-actin	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGGGGG
CycB	GAAGCGCTCACCATAGATGC	GGGACCTAAAGTCACAGTCAAGG

4.5.2 Luciferase assay (I)

HEK293T cells were cultured and maintained DMEM with 1% penicillin/streptomycin and 10% iFBS. For the reporter assays, cells were plated on 96-well plates and co-transfected with Osx-Luc reporter plasmid (Nishio et al., 2006), pCDNA3-Flag-Ebf1 (Jimenez et al., 2007), and pRL promoterless Renilla

plasmid using Fugene 6 (Invitrogen) as a transfection reagent. Reporter activity was subsequently measured after 24 to 48 hours with Dual Luciferase Reporter assay (Promega) and normalized to Renilla activity.

4.5.3 Chromatin immunoprecipitation (I)

The protocol for chromatin immunoprecipitation (ChIP) was modified from Östling et al. (Östling et al., 2007). In brief, the murine osteoblastic MC3T3-E1 (ATCC) cell line was cultured and maintained in α-MEM with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS USA origin, Gibco). At subconfluence, the cells were cross-linked with 1% formaldehyde. Quenching was performed with 125 mM of glycine. The cells were scraped, washed in PBS, and resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, and protease inhibitors (Complete, Roche)). Fragmentation of the chromatin samples was performed by sonication with Bioruptor (Diagenode) to an approximate size of 500 bp. After chromatin shearing, the chromatin was diluted with IP buffer (150 mM NaCl, 20 mM Tris-HCl, 1% Triton-X and protease inhibitors). Antibodies against mouse Ebf1 1C12 (Abnova, Everest) or mouse normal IgG (Santa Cruz) were added to samples and incubated overnight. Dynabeads Protein A beads (Invitrogen) were used to harvest protein–chromatin complexes. After washing, cross-links were reversed by incubating the samples overnight at 65°C. DNA was purified and subjected to qPCR analysis using primers specific for Osterix promoter and the Dynamo Flash qPCR kit (Thermo Fischer). Primer sequences were 5’-gcccatattcctgtttcccacccg-3’ and 5’-tgctctgtctgtagggatccacc-3’.

4.6 Statistical analyses (I, II)

All *in vitro* experiments were repeated at least three times.

Results are presented as mean ± SD. Statistical analysis was performed by two-tailed Student’s t test with Bonferroni’s correction or Benjamini-Hochberg Procedure. P-values < 0.05 were considered statistically significant and are indicated with asterisks as * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

5 Results

5.1 Ebf1 in osteoblast differentiation (I)

5.1.1 Ebf1 promotes osteoblast differentiation *in vitro*

Ebf1 has previously been suggested to be a negative regulator of osteoblast differentiation (Hesslein et al., 2009; Kiviranta et al., 2013). To avoid the severe developmental problems of the global *Ebf1* knockout mouse model, haploinsufficient Ebf1$^{+/-}$ newborn mice were used to study the effects of Ebf1 on osteoblast differentiation in calvarial cell cultures. Calvarial cells from Ebf1$^{+/-}$ mice formed less mineralized bone nodules and had lower ALP activity compared to the WT control cells (I, Fig. 2, A–B).

Expression of *Ebf1* mRNA was significantly decreased in the cultured Ebf1$^{+/-}$ osteoblasts, confirming that haploinsufficiency was sufficient to reduce Ebf1 expression. There was also a modest, non-significant increase in the expression of *Ebf2* mRNA (I, Fig. 2, C). mRNA expression of osteoblastic genes Osterix, *Runx2*, *ALP*, *Col1a1*, and osteocalcin was significantly reduced, further verifying impaired osteoblastic differentiation of Ebf1$^{+/-}$ cells (I, Fig. 2, C).

To evaluate whether excess Ebf1 could induce osteoblast differentiation, *Ebf1* was overexpressed in mouse primary calvarial osteoblasts *in vitro*. Overexpression of *Ebf1* in osteoblast progenitors led to enhanced osteoblast differentiation, which was demonstrated by increased *ALP* mRNA expression and ALP activity (I, Fig. 3, B–C). *Ebf1* overexpression stimulated the mRNA expression of Osterix, but had no effect on *Runx2* mRNA expression (I, Fig. 3, C).

These data led us to hypothesize that Ebf1 may directly control osteoblast differentiation by regulating Osterix expression.

5.1.2 Ebf1 regulates Osterix expression

To study the ability of Ebf1 to regulate Osterix, the Osterix promoter sequence was first examined, and a putative Ebf1 binding site was indeed identified in the proximal Osterix promoter (I, Fig. 4, A). To confirm that the binding site was functional, HEK293T cells were transfected with a construct containing the luciferase gene

under the control of 2 kb Osterix promoter (Osx-luc). The promoter site was able to significantly activate luciferase expression, verifying the functionality of the site (I, Fig. 4, B). ChIP assay was used to confirm the interaction between Ebf1 and the putative binding site with two specific Ebf1 antibodies in MC3T3-E1 osteoblasts. Using qPCR with specific primers flanking the Ebf1 consensus site, a robust enrichment of the Ebf1 promoter signal with both antibodies, compared to IgG control, was found (I, Fig. 4, C).

These data supported our hypothesis that Ebf1 is able to regulate Osterix and demonstrated that Ebf1 binds to the proximal Osterix promoter and induces Osterix expression.

5.1.3 Deletion of *Ebf1* in early committed osteoblasts results in increased bone mass

To determine the osteoblast-specific functions of Ebf1 at different stages of osteoblast differentiation, we generated conditional knockout mice, in which Ebf1 deletion was targeted to early committed osteoblasts, using Osx-Cre ($Ebf1_{Osx}^{-/-}$) (Rodda et al., 2006), and to late mature osteoblasts, with hOC-Cre ($Ebf1_{hOC}^{-/-}$) (Zhang et al., 2002).

Male and female $Ebf1_{Osx}$ mice were analysed at 12 and 24 weeks of age. Both male and female $Ebf1_{Osx}^{-/-}$ mice had normal body weight and length of tibia when compared to the controls (I, Suppl. Fig. 2; **Table 4**). Hesslein et al. had previously reported increased adipocyte infiltration of the bone marrow in global Ebf1KO mice (Hesslein et al., 2009), but this phenomenon was not observed in our conditional $Ebf1_{Osx}^{-/-}$ mice (I, Suppl. Fig. 3).

Bone phenotype was first analysed by μCT. The deletion of *Ebf1* in 12-week-old $Ebf1_{Osx}^{-/-}$ male mice resulted in significantly elevated trabecular bone mass, demonstrated by a 30% increase in bone volume (BV/TV) together with increased trabecular thickness (Tb.Th) and trabecular number (Tb.N). Cortical bone thickness (Cortical.Th) and cortical bone area (Cortical B.Ar) were increased. Results were similar in 12-week-old $Ebf1_{Osx}^{-/-}$ females. (I, Fig. 5, E-I; Suppl. Fig. 6-8; **Table 4**) Data from the tibial and vertebral μCT analysis of 12-week-old males were in accordance with the femur data (I, Suppl. Fig. 4 & 5). However, deletion of *Ebf1* in older, 24-week-old male mice had no effect on the analysed bone parameters (I, Suppl. Table 1, Suppl. Fig. 9).

Table 4. Bone length, body weight and femoral µCT results of the 12-week-old $Ebf1_{Osx}$ mice.

	Males Control	$Ebf1_{Osx}^{-/-}$	Females Control	$Ebf1_{Osx}^{-/-}$
Body weight (g)	21.2 ± 3.2	22.1 ± 3.1	16.0 ± 1.8	17.2 ± 2.72
Length of tibia (mm)	17.2 ± 0.8	16.9 ± 0.5	16.6 ± 0.4	17.1 ± 0.7
BV/TV (%)	22.1 ± 2.45	29.5 ± 2.44***	15.9 ± 2.78	21.3 ± 3.94*
Tb.N (1/mm)	3.65 ± 0.34	4.14 ± 0.37*	2.70 ± 0.41	3.27 ± 0.38*
Tb.Th (mm)	0.06 ± 0.00	0.07 ± 0.00***	0.06 ± 0.00	0.06 ± 0.00
Cortical Th (mm)	0.18 ± 0.01	0.22 ± 0.01***	0.18 ± 0.00	0.21 ± 0.00***
Cortical B.Ar (mm^2)	0.83 ± 0.06	1.04 ± 0.05***	0.70 ± 0.01	0.82 ± 0.04***

* P < 0.05; *** P < 0.001

Histomorphometric analysis confirmed an increase in trabecular bone in $Ebf1_{Osx}^{-/-}$ mice, compared to controls (I, Fig. 6, B). The number of osteoblasts (N.Ob/B.Pm) and osteoblast surface (Ob.S/B.Pm) were also significantly increased in the $Ebf1_{Osx}^{-/-}$ mice (I, Fig. 6, B; Table 1). There were no significant differences in the osteoid parameters, mineral apposition rate (MAR), or bone formation rate (BFR/BV) (I, Table 1; Suppl. Fig. 11). Osteoclast parameters were comparable to controls (I, Table 1). Taken together, the osteoblastic cell population was significantly increased, whereas osteoblast activity and bone resorption were unaffected.

Gene expression analysis of the total bone RNA from 12- and 24-week-old $Ebf1_{Osx}^{-/-}$ mice gave no additional information about the underlying mechanism, as no significant differences were observed in the mRNA expression of *Ebf1* or in other osteoblast-related genes analysed (I, Suppl. Fig. 10). This was likely due to the contribution of bone marrow cells to the total RNA pool, as the hematopoietic cells expressing *Ebf1* were not flushed before RNA extraction.

5.1.4 Ebf1 is redundant in the maintenance of mature osteoblast function

To study Ebf1 function in late-stage osteoblasts, hOC-Cre (Zhang et al., 2002) was used to target *Ebf1* deletion in the mature cells. $Ebf1_{hOC}^{-/-}$ male and female mice were analysed at 12 and 24 weeks of age. Although successful deletion of the *Ebf1* allele could be verified (I, Suppl. Fig. 1, D), no changes in any of the parameters analysed were observed.

$Ebf1_{hOC}^{-/-}$ males and females both had normal body weight and tibia length (I, Suppl. Fig. 2; **Table 5**). In µCT analysis, $Ebf1_{hOC}^{-/-}$ mice femurs were comparable to the controls regarding trabecular and cortical bone parameters of males and females at 12-weeks-of-age (I, Fig. 7, E-1; **Table 5**) and at 24-weeks-of-age (I, Suppl. Table 2). Results from the µCT analysis of the tibia and vertebra were in accordance (I, Suppl. Fig. 12–17). The histomorphometric parameters were also examined, and

Ebf1$_{hOC}^{-/-}$ bones were comparable to controls (I, Fig. 6, D; Table 2). No changes were detected in the bone mRNA expression (I, Suppl. Fig. 10).

Taken together, we were able to conclude that Ebf1 regulates Osterix expression in early osteoblast differentiation. As the differentiation progresses, Ebf1 inhibits bone accrual in Osterix-expressing osteoblasts, while being redundant in the maintenance of mature, osteocalcin-expressing osteoblast function.

Table 5. Bone length, body weight and femoral µCT results of the 12-week-old Ebf1$_{hOC}$ mice.

	Males		**Females**	
	Control	**Ebf1$_{hOC}^{-/-}$**	**Control**	**Ebf1$_{hOC}^{-/-}$**
Body weight (g)	26.7 ± 1.9	25.4 ± 1.7	21.1 ± 2.2	21.2 ± 1.8
Length of tibia (mm)	18.0 ± 0.4	17.8 ± 0.4	17.1 ± 0.3	17.4 ± 0.5
BV/TV (%)	30.9 ± 5.06	29.7 ± 3.50	23.6 ± 3.19	21.7 ± 6.53
Tb.N (1/mm)	4.34 ± 0.53	4.41 ± 0.35	3.57 ± 0.41	3.25 ± 0.55
Tb.Th (mm)	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.01
Cortical Th (mm)	0.21 ± 0.00	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
Cortical B.Ar (mm^2)	1.09 ± 0.14	1.08 ± 0.10	0.95 ± 0.06	0.93 ± 0.13

5.2 Ebf2 in osteoblast differentiation (II)

5.2.1 Global deletion of *Ebf2* results in runted structure and decreased bone formation

To further study the effects of Ebfs on bone, we next focused on Ebf2, which is also known to affect bone formation (Kieslinger et al., 2005). We started by generating a global *Ebf2* knockout mouse strain (Ebf2KO). As the global deletion of *Ebf2* has been reported to lead to progressive developmental defects (Corradi et al., 2003), the Ebf2KO mice were analysed at three weeks of age. Only male mice were analysed, which is a limitation to our study.

Ebf2KO mice were runted, represented by significantly reduced body weight and tibial length (II, Suppl. Fig. 2, A). µCT analysis of the tibia was in line with the previously published global Ebf2 knockout phenotype (Kieslinger et al., 2005). Trabecular bone volume was significantly decreased, and trabecular separation increased in Ebf2KO when compared to controls. Cortical bone thickness, periosteal perimeter (Ps.Pm) and endocortical perimeter (Ec.Pm) were also significantly decreased in Ebf2KO male mice compared to controls. (II, Suppl. Fig. 2, B)

As the bone phenotype of Ebf2KO mice was similar to the previously published Ebf2KO mouse model, we decided to proceed with a more specific, bone-targeted *Ebf2* conditional knockout model.

5.2.2 Deletion of *Ebf2* in Prrx1-expressing cells

As the global *Ebf2* knockout model has its limitations (Corradi et al., 2003; Kieslinger et al., 2005), we decided to target the deletion of *Ebf2* more specifically on long bone development by using limb bud mesenchyme specific –driven Cre (Prrx1-Cre). $Ebf2_{Prrx1}$ male and female mice were analysed at six and 12 weeks of age.

At six weeks of age, body weight and tibial length were otherwise comparable between controls and $Ebf2_{Prrx}^{-/-}$, apart from the reduced tibial length observed in $Ebf2_{Prrx}^{-/-}$ females (II, Fig. 1, A). Bone phenotype was modest. Results from μCT analysis were comparable to controls, except for the significantly reduced Ps.Pm, Ec.Pm and mean moment of inertia (MMI) in six-week-old $Ebf2_{Prrx}^{-/-}$ females (II, Fig. 1, B).

At 12 weeks of age, body weight and tibial length were comparable to controls in both $Ebf2_{Prrx}^{-/-}$ males and females (II, Fig. 2, A). In μCT analysis there were no significant differences observed between the knockout and control mice, in males or females (II, Fig. 2, B). We confirmed that the Cre recombination had taken place in the long bones, thereby verifying the validity of the mouse model (II, Suppl. Fig. 1, B–D).

5.3 The interplay between Ebf1 and Ebf2 in bone formation

5.3.1 Age-progressive decrease in the length of long bones in $Ebf1xEbf2_{Prrx1}$

To address the possible interplay between Ebf1 and Ebf2, we proceeded to create a double knockout mouse model in which both *Ebf1* and *Ebf2* were deleted in the mesenchymal cell lineage using Prrx1-Cre. Both the male and female $Ebf1xEbf2_{Prrx1}$ mice were analysed at six and 12 weeks of age.

The body weight of $Ebf1xEbf2_{Prrx1}^{-/-}$ mice was comparable to controls at both six weeks and 12 weeks of age in males and females. Length of tibia was significantly reduced in $Ebf1xEbf2_{Prrx1}^{-/-}$ male and female mice at six weeks of age. Reduction in tibial length was observed to progress with age. At the later time point of 12 weeks, the difference was even more pronounced in $Ebf1xEbf2_{Prrx1}^{-/-}$ males and females when compared to controls. (II, Fig. 3, A-D; **Table 6**)

5.3.2 Increased bone mass in Ebf1xEbf2$_{Prrx1}$ females

The bone phenotype of Ebf1xEbf2$_{Prrx1}$ mice was first studied through µCT. In femurs of the Ebf1xEbf2$_{Prrx1}^{-/-}$ females, BV/TV, Tb.N, and Cort.B.Ar were significantly increased compared to controls at six weeks of age (II, Fig. 4, A; **Table 6**). Differences in the tibial bone parameters were less pronounced (II, Suppl. Fig. 3, A). In males, there were no significant differences in any of the studied parameters in femurs (II, Fig. 4, A; **Table 6**). In tibia, marrow area fraction (M.Ar/T.Ar) and Ec.Pm were decreased in Ebf1xEbf2$_{Prrx1}^{-/-}$ males at six weeks of age (Suppl. Fig. 3, A).

As with bone length, the structural bone phenotype was also more pronounced at the later timepoint of 12 weeks. At 12 weeks there was a nearly 50% increase in the femur BV/TV, and a more than 50% increase in the Tb.N of Ebf1xEbf2$_{Prrx1}^{-/-}$ females compared to controls. Accordingly, Tb.Th was significantly increased, whereas Tb.Sp was decreased by more than 20%. Cort.Th and Cort.B.Ar were also significantly increased in Ebf1xEbf2$_{Prrx1}^{-/-}$ females compared to controls (II, Fig. 5, A; **Table 6**). A similar trend was also detected in the tibia (II, Suppl. Fig. 3, B). Vertebrae of 12-week-old Ebf1xEbf2$_{Prrx1}^{-/-}$ females were comparable to controls, suggesting that *Prrx1* is not expressed in the vertebra (II, Fig. 4).

12-week-old Ebf1xEbf2$_{Prrx1}^{-/-}$ male femurs were comparable to controls. In tibias Tb.Th was significantly lower in 12-week-old Ebf1xEbf2$_{Prrx1}^{-/-}$ males when compared to controls (Suppl. Fig. 3, B).

Table 6. Bone length, body weight and femoral µCT results of the 6&12-wk-old Ebf1xEbf2$_{Prrx1}$ mice.

6-weeks	**Males**		**Females**	
	Control	**Ebf1xEbf2$_{Prrx1}$**	**Control**	**Ebf1xEbf2$_{Prrx1}$**
Body weight (g)	21.7 ± 2.49	21.1 ± 3.39	16.6 ± 3.05	16.3 ± 1.96
Length of tibia (mm)	16.3 ± 0.51	15.6 ± 0.27*	15.9 ± 0.54	14.9 ± 0.46**
BV/TV (%)	38.8 ± 9.73	40.9 ± 8.46	23.2 ± 5.52	41.4 ± 9.43**
Tb.N (1/mm)	6.22 ± 1.58	7.11 ± 1.14	5.24 ± 1.18	8.08 ± 1.45**
Tb.Th (mm)	0.06 ± 0.00	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Cortical Th (mm)	0.18 ± 0.01	0.19 ± 0.02	0.14 ± 0.03	0.15 ± 0.03
Cortical B.Ar (mm^2)	0.95 ± 0.21	1.09 ± 0.28	0.72 ± 0.12	0.88 ± 0.17*

12 weeks	**Males**		**Females**	
	Control	**Ebf1xEbf2$_{Prrx1}$**	**Control**	**Ebf1xEbf2$_{Prrx1}$**
Body weight (g)	28.7 ± 2.76	26.9 ± 2.78	21.9 ± 0.71	21.6 ± 1.12
Length of tibia (mm)	17.8 ± 0.38	16.6 ± 0.22***	17.5 ± 0.22	16.1 ± 0.20***
BV/TV (%)	15.8 ± 2.82	15.6 ± 2.55	9.10 ± 3.27	19.2 ± 3.40***
Tb.N (1/mm)	3.13 ± 0.52	3.31 ± 0.58	1.89 ± 0.53	3.59 ± 0.57***
Tb.Th (mm)	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00*
Cortical Th (mm)	0.20 ± 0.01	0.21 ± 0.02	0.19 ± 0.01	0.22 ± 0.01***
Cortical B.Ar (mm^2)	1.22 ± 0.20	1.15 ± 0.14	0.87 ± 0.05	1.17 ± 0.16***

* P < 0.05; ** P < 0.01; *** P < 0.001.

Histomorphometric analysis of the female Ebf1xEbf2$_{Prrx1}^{-/-}$ tibia further strengthened the observation of the age-progressive phenotype. Structural bone parameters evaluated at six weeks of age were comparable to controls. (II, Fig. 6, A). At 12 weeks, however, the structural parameters (BV/TV, Tb.Th, Tb.N) were significantly increased (II, Fig. 6, B). Dynamic parameters of histomorphometry analysis remained unchanged at six weeks and 12 weeks of age in females. Osteoclast number (N.Oc/T.Ar) was significantly elevated in 12-week-old Ebf1xEbf2$_{Prrx1}^{-/-}$ females. (II, Suppl. Table 3)

In search of the possible mechanism responsible for the bone phenotype, mRNA expression of selected bone-specific genes in Ebf1xEbf2$_{Prrx1}$ mouse long bones were analysed. No statistically significant differences between Ebf1xEbf2$_{Prrx1}^{-/-}$ and controls, in males or females at six weeks (II, Suppl. Fig. 5, A) or at 12 weeks of age were observed (II, Suppl. Fig. 5, B).

5.3.3 Abnormalities in the growth plate and secondary ossification in Ebf1xEbf2$_{Prrx1}$ males

To get more insight into the mechanism underlying the decreased bone length of Ebf1xEbf2$_{Prrx1}^{-/-}$ mice, the growth plate at six weeks of age was analysed. The overall thickness of the growth plate was significantly reduced in males (II, Fig. 7, A), but there were no significant differences in the cellular densities of proliferative or hypertrophic zones (II, Fig.7, B&C). Despite markedly shorter bones in Ebf1xEbf2$_{Prrx1}^{-/-}$ females (II, Fig. 3, B & D; **Table 6**), all growth plate parameters studied were comparable to controls (II, Fig. 7, A–C).

The adiposity and ossification of the SOC in Ebf1xEbf2$_{Prrx1}$ tibias were also analysed. μCT analysis of the Ebf1xEbf2$_{Prrx1}^{-/-}$ males revealed significantly reduced trabecular number in the SOC at six weeks of age (II, Suppl. Table 4), but not at 12 weeks of age. In females, there were no differences in either age group between Ebf1xEbf2$_{Prrx1}^{-/-}$ and controls (II, Suppl. Table 3). Ossification of SOC was further analysed also in Ebf2$_{Prrx1}$ and Ebf1$_{Osx}$ mice, but there were no changes in any of the parameters in males or females (II, Suppl. Table 4).

Bone marrow adiposity of the Ebf1xEbf2$_{Prrx1}$ mice was analysed from the SOC area and the tibial diaphysis bone marrow cavity. The adipocyte number was significantly increased in the SOC of six-week-old Ebf1xEbf2$_{Prrx1}^{-/-}$ males compared to the controls (II, Suppl. Fig. 7, C), but not in 12-week-old mice (II, Suppl. Fig. 7, D). Adiposity of the bone marrow cavity was comparable to controls (II, Suppl. Fig. 7, E). In females, no differences were observed (II, Suppl. Fig. 7, F).

6 Discussion

6.1 Ebf1 in osteoblast differentiation

Transcription factor Ebf1 has been suggested to regulate bone formation. Previous studies have shown that *Ebf1* is expressed in osteoblasts and that global deletion of *Ebf1* results in increased bone formation, both in homozygous (Hesslein et al., 2009) as well as in heterozygous (Kiviranta et al., 2013) knockout mice *in vivo.* Interpretation of the bone phenotype in Ebf1KO mice is challenging, as *Ebf1* is expressed in multiple tissues. This results in failure to thrive and could therefore significantly affect the skeletal development.

Haploinsufficient $Ebf1^{+/-}$ mice provided us a way to overcome some of the challenges of the Ebf1KO. Calvarial cells from $Ebf1^{+/-}$ mice presented significantly impaired osteoblast differentiation *in vitro*, whereas overexpression of *Ebf1* in mouse calvarial cells led to enhanced osteoblast differentiation *in vitro*. Zee et al. had previously made the same observation, as overexpression of *Ebf1* in the osteoblast cell line MC3T3-E1 led to increased extracellular matrix mineralization *in vitro* (Zee et al., 2013). However, previously reported overexpression of *Ebf1 in vivo* had the opposite effect. In ColI2.3-Ebf1 mice, in which *Ebf1* is overexpressed in mature osteoblasts, bone formation was suppressed (Kiviranta et al., 2013). Differentiation capacity of ColI2.3-Ebf1 osteoblasts *in vitro* was also decreased. These results gave us the first indication that Ebf1 might have opposite roles at different stages of osteoblast differentiation and bone formation. Overexpression of *Ebf1* in the early stage would enhance the differentiation, whereas in mature osteoblasts *Ebf1* overexpression would inhibit bone accrual.

Throughout the *in vitro* experiments, we noted that mRNA expression levels of Osterix correlated with levels of *Ebf1* mRNA expression. Upon studying this association further, we were able to show that Ebf1 directly induces Osterix expression and stimulates osteoblast differentiation *in vitro*. Based on an earlier study by Yoshida et al., Osterix is required for osteoblast differentiation, and overexpression of Osterix in mature osteoblasts results in low bone mass and inhibition of normal osteoblast function (Yoshida et al., 2012). These data further verified our hypothesis, and provided a possible mechanism for the negative effect

of *Ebf1* overexpression in mature osteoblasts of the ColI2.3-Ebf1 mice (Kiviranta et al., 2013).

To achieve more detailed information on the effects of osteoblasts, bone-targeted conditional *Ebf1* knockout models have been used. These studies present varying data on the effects of Ebf1 in bone formation and some suggest that Ebf1 has only a very minor role in osteoblast differentiation *in vivo* (Seike et al., 2018; Zee et al., 2013). However, the age of the mice, and therefore bone remodeling status, as well as promoters used to drive Cre recombinase expression are different between studies. In young mice, the peak bone mass might not have been reached at the time of analysis. For example, trabecular bone volume is greatest at six to eight weeks of age in C57BL/6J mice (Glatt et al., 2007). In older mice, the response to mechanical loading and on regulation of bone mass are different when compared to younger, actively growing mice (Chermside-Scabbo et al., 2020). Therefore, comparing the results of different studies is not entirely straightforward.

We decided to study the temporospatial, osteoblast-specific effects further in two conditional knockout mouse models where *Ebf1* deletion was targeted to different stages of osteoblast differentiation. Deletion of *Ebf1* in the early osteoblasts led to increased bone mass in 12-week-old $Ebf1_{Osx}^{-/-}$ mice, while deletion in mature osteoblasts in $Ebf1_{hOC}^{-/-}$ mice did not result in a bone phenotype. Despite higher bone mass and osteoblast number, bone formation parameters in the histomorphometry analysis remained unchanged in $Ebf1_{Osx}^{-/-}$ mice. This could reflect the age-dependent nature of bone formation activity, as 12-week-old mice are already considered skeletally mature (Ferguson et al., 2003). Our data is in accordance with a previously published mouse model of Prrx1-Cre targeted deletion of *Ebf1* in mesenchymal cell-lineage, where trabecular bone volume and osteoblast numbers were modestly but significantly increased (Derecka et al., 2020). Our *in vivo* experiments supported the hypothesis that Ebf1 promotes early osteoblast differentiation by regulating Osterix expression. We also concluded that Ebf1 inhibits bone accrual in the Osterix-expressing osteoblasts *in vivo,* but it is redundant in the function of mature, osteocalcin-expressing osteoblasts (**Figure 5**).

The discrepancy between *in vivo* and *in vitro* results in *Ebf1* knockout studies needs to be acknowledged and addressed. In the first global *Ebf1* knockout model, osteoblasts were reported to be indistinguishable from the controls *in vitro*, although bone formation was significantly increased *in vivo* (Hesslein et al., 2009). Runx2-Cre targeted deletion of *Ebf1* in osteoblasts led to fewer mineralized nodules and decreased ALP activity *in vitro*, while there was no phenotype *in vivo* (Zee et al., 2013). In contrast, mesenchymal cell-targeted *Ebf1* knockout mice had increased trabecular bone mass *in vivo*, but the osteoblast differentiation was impaired *in vitro* (Derecka et al., 2020). As different Ebfs have been suggested to have both redundant and non-redundant functions, we hypothesized whether the discrepancy between the

in vivo and *in vitro* results might be due to co-operation or compensation by different Ebfs. Ebf2 was a strong candidate, as the global Ebf2 mouse model has been reported to have a bone phenotype (Kieslinger et al., 2005). We therefore proceeded to study the effect of Ebf2 and the possible interplay between Ebf1 and Ebf2 in bone formation.

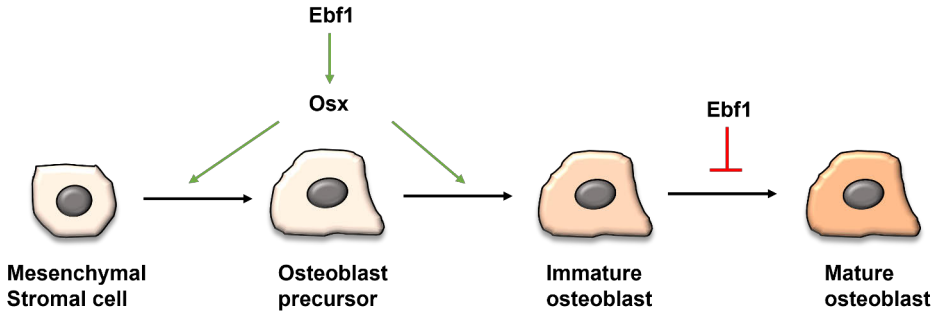

Figure 5. Suggested model of Ebf1 function during osteoblast differentiation. Ebf1 promotes early osteoblast differentiation by regulating Osterix expression. In mature, Osterix-expressing osteoblasts Ebf1 inhibits bone accrual.

6.2 Ebf2 in osteoblast differentiation

The first characterized Ebf family related mouse phenotype was the global Ebf2KO mouse model. In contrast to global Ebf1KO mice, global Ebf2KO mice have osteopenia, decreased bone mass, and an increased number of osteoclasts (Kieslinger et al., 2005). The same study identified *OPG* as a direct target of Ebf2 and indicated it as being responsible for the increased number of osteoclasts in Ebf2KO mice. We confirmed these results with our global *Ebf2* knockout, which also presented reduced body weight and trabecular and cortical bone parameters (**Table 7**).

As with Ebf1, Ebf2 is also an important regulator of multiple cell types, including neuronal cells (Garel et al., 1997). Therefore, the fact that the global Ebf2KO bone phenotype may not be due to the effects of Ebf2 in bone cells cannot be excluded. To achieve more insight into bone-specific *Ebf2* deletion, we created a mesenchymal cell targeted *Ebf2* knockout mouse model. According to our knowledge, this is the first study to report on a conditional, mesenchymal cell-targeted *Ebf2* knockout.

Unexpectedly, the mesenchymal cell-targeted deletion of *Ebf2* resulted in a very mild phenotype. Tibial length and periosteal perimeter were reduced in six-week-old $Ebf2_{Prrx1}^{-/-}$ females, but otherwise mice were comparable to controls. (**Table 7**) Cre recombination was verified in the long bones, supporting functionality of the knockout construct. We concluded that the mild phenotype of the mesenchymal cell-targeted *Ebf2* knockout mice indicated that the bone phenotype of the global Ebf2KO model was caused by deletion in non-skeletal cells.

6.3 The interplay between Ebf1 and Ebf2 in bone formation

Previous studies have suggested that various Ebfs co-operate in different regulatory processes. This co-operation was already suggested in the first studies on Ebfs in the development of the central nervous system (Garel et al., 1997). Ebf1, Ebf2, and Ebf3 all act redundantly in the stromal support of immature hematopoietic cells (Kieslinger et al., 2010). Ebf1 and Ebf3 also co-operate in the maintenance of bone marrow cavities and HSC niche formation (Derecka et al., 2020; Seike et al., 2018). It is therefore possible that the lack of one Ebf could be compensated for by the other members of Ebf family *in vivo*.

To study this interplay in more detail, we created a conditional double knockout mouse model by deleting both *Ebf1* and *Ebf2* in the mesenchymal cell lineage. Analysis of the Ebf1xEbf2$_{Prrx1}^{-/-}$ mice revealed a significant, age-progressive decrease in the length of long bones when compared to controls (**Table 7**). Altered chondrocyte biology and decreased bone length are frequently observed in mouse models with a decreased bone mass phenotype (Maeda et al., 2007; Tang et al., 2020). Interestingly, in our study, the effects on bone volume were the opposite. Long bones of Ebf1xEbf2$_{Prrx1}^{-/-}$ females displayed an age-progressive increase in trabecular and cortical bone parameters. Structural parameters of the histomorpohometric analysis further verified the results obtained by μCT. Dynamic histomorphometric parameters, however, remained unaffected. These data could indicate that Ebf1xEbf2$_{Prrx1}^{-/-}$ mice have a somewhat uncoupled phenotype in long bones with decreased bone length but increased trabecular and cortical bone volume, at least in females.

A growth plate defect was detected in the Ebf1xEbf2$_{Prrx1}^{-/-}$ males, where the overall thickness of the growth plate was significantly decreased. Although the bones were also significantly shorter in the Ebf1xEbf2$_{Prrx1}^{-/-}$ females, no abnormalities in the growth plate structure were discovered. At six weeks of age, the growth plate is already well established in mice and longitudinal growth is exponential (Glatt et al., 2007). Therefore, an earlier time point might have been needed to detect changes in the growth plate. Previous studies on Ebfs have reported reduced bone length in the global Ebf1KO (Hesslein et al., 2009) and Ebf2KO (Kieslinger et al., 2005). In conditional, bone-targeted *Ebf1* knockout models, bone lengths have been comparable to controls (Seike et al., 2018; Zee et al., 2013). This could indicate that the defect in bone length in the global *Ebf* knockout models is not specific to Ebf function in cells of mesenchymal lineage. Based on our results, simultaneous ablation of both *Ebf1* and *Ebf2* in mesenchymal lineage is necessary to perturb longitudinal growth of long bones.

The defect in the length of Ebf1xEbf2$_{Prrx1}^{-/-}$ mouse limbs could be speculated as being linked to the regulation of hypertrophic chondrocytes by Osterix. In Study I,

Ebf1 was shown to promote early osteoblast differentiation by regulating Osterix expression. Osterix is also expressed in chondrocytes (Nishimura et al., 2012), and it regulates endochondral bone formation through chondrocyte hypertrophy (Cheng et al., 2013).

We acknowledge, that the detailed molecular mechanisms underlying the age-progressive and gender-dependent phenotype of Ebf1xEbf2$_{Prrx1}$ long bones remains to be further elucidated. Our data raises new questions on the role of Ebfs and the regulation of bone formation, but it also complements observations made in previous studies.

Table 7. Summary of the studied mouse phenotypes.

		Males			Females		
Mouse model	**Age**	**Weight**	**Length of tibia**	**Trab. BV/TV**	**Weight**	**Length of tibia**	**Trab. BV/TV**
Ebf1$_{Osx}$	12 weeks	↔	↔	↑***	↔	↔	↑*
	24 weeks	↔	↔	↔	↔	↔	↔
Ebf1$_{hOC}$	12 weeks	↔	↔	↔	↔	↔	↔
	24 weeks	↔	↔	↔	↔	↔	↔
Ebf2KO	3 weeks	↓**	↓*	↓*	N/A	N/A	N/A
Ebf2$_{Prrx1}$	6 weeks	↔	↔	↔	↔	↓*	↔
	12 weeks	↔	↔	↔	↔	↔	↔
Ebf1xEbf2$_{Prrx1}$	6 weeks	↔	↓*	↔	↔	↓**	↑**
	12 weeks	↔	↓***	↔	↔	↓***	↑***

↔, comparable to (age-matched) controls; ↑, increased (compared to controls); ↓, decreased (compared to controls); N/A, data not available; * P < 0.05; ** P < 0.01; *** P < 0.001

6.4 Limitations of the study

Data presented in this thesis has some limitations. Firstly, we were unable to analyse a similar number of mice in each experiment. In Study II, the analysis of Ebf2$_{Prrx1}$ male mice was unbalanced, with only three mice in the control group. In the study of Ebf2KO mice, only male mice were analysed. Due to the severity of the global knockout phenotype, we made the ethically based decision not to also collect females. To study the mechanisms underlying the phenotype of Ebf1xEbf2$_{Prrx1}$ mice, *in vitro* osteoblast cultures could provide us more detailed information. Mice analysed in these studies were from six weeks to 24 weeks old. With an earlier timepoint, we could possibly detect the effects on growth plate and early bone formation. As with all animal models, extrapolating results of these studies into humans should be made with caution and taking into account the model-specific limitations.

6.5 Future perspectives

From very early on, the Ebf family of transcription factors were known to include a unique and structurally highly similar DNA-binding domain (Siponen et al., 2010) which acts as a dimer (Treiber et al., 2010a). Of the three mammalian Ebfs, Ebf1 and Ebf3 have been described as the most similar in their structural homology (Dubois et al., 2001). The structural similarity and dimer properties could indicate that certain functions of the Ebf family require the presence or absence of different Ebfs in gene regulation. There is strong evidence on the redundant functions of Ebfs in multiple regulatory processes. In the regulation of bone formation, Seike et al. have recently published a study where *Ebf1* and *Ebf3* were deleted in the mesenchymal cell lineage using Prrx1-Cre, separately and simultaneously (Seike et al., 2018). Deletion of *Ebf1* in the mesenchymal lineage did not result in gross bone abnormalities in mice. Deletion of *Ebf3*, however, led to osteosclerotic bone marrow and markedly increased trabecular bone volume. The high bone mass phenotype was further enhanced when both *Ebf1* and *Ebf3* were deleted in the early mesenchymal cells. Whereas deletion of *Ebf1* and *Ebf3* in mature osteoblasts using Col2.3 promoter had no effect on the bone phenotype. These findings support our data that suggests Ebf1 is redundant in the maintenance of mature osteoblast function. The additive effect of the simultaneous deletion of *Ebf1* and *Ebf3* supports the notion of certain regulatory functions requiring the presence of several Ebf family members. The analysis of mesenchymal cell-targeted *Ebf1* knockout by Seike et al. could, however, be challenged. They presented neither μCT-data nor histomorphometric analysis data to support their conclusion that Ebf1 does not play a role in early osteoblast differentiation.

The temporospatial expression patterns between different Ebfs vary in the bone marrow stromal cells. Ebfs have mostly been studied in leptin receptor positive ($LepR^+$) and CXC chemokine ligand 12 (CXCL12) abundant reticular (CAR) cells (Omatsu et al., 2010) and perivascular $CD45^-$ $CD31^-Lin^-PDGFR\alpha^+Sca1^+$ (PαS) cells (Morikawa et al., 2009). These cells participate in the maintenance and function of the hematopoietic stem cell niche and have the ability to differentiate into osteoblasts and adipocytes. From the heterogenic population of mesenchymal stromal cells, CAR cells are defined as the main source of bone formed by adult bone marrow in mice (Zhou et al., 2014a). *Ebf1* and *Ebf3* are most abundantly expressed in the CAR cells, whereas in PαS cells *Ebf2* expression prevails (Derecka et al., 2020; Seike et al., 2018). Osx-Cre and Prrx1-Cre have been shown to effectively target the CAR cell population, yet only 50% PαS cell population was targeted by Prrx1-Cre (Greenbaum et al., 2013).

Seike et al. further specified that *Ebf1*, *Ebf2* and *Ebf3* were expressed in these subpopulations at the newborn stage in mice, while only *Ebf3* was significantly expressed in the CAR cells at the adult stage (Seike et al., 2018). Recently, the human

counterpart of CAR cells was identified (Aoki et al., 2021). These cells were able to differentiate into adipocytes and osteoblasts and, interestingly, expressed high levels of *EBF3*. These data raise the question whether we should be more focused on the role of Ebf3 in bone formation in the future, and to what extent our presented data could be affected by the possible redundant functions of Ebf3 in mesenchymal cells in absence of Ebf1 and/or Ebf2.

Our study is the first to present a gender-specific bone phenotype in a *Ebf* knockout mouse model. Previous studies have not specified whether their data was obtained from only male or female mice, or if both genders were analysed (Hesslein et al., 2009; Kieslinger et al., 2005; Seike et al., 2018). Skeletal dimorphism in mice is known to be established during early puberty, at around three to five weeks of age (Callewaert et al., 2010). The early puberty period is critical for the sexually dimorphic outcome in endochondral ossification (Fairfield et al., 2020; Linz et al., 2015). Genetic background is also one factor, as C57BL/6-strain female mice reportedly have more osteoblasts compared to males (Zanotti et al., 2014).

Gender-dependent observations in the bone phenotype of Ebf1xEbf2$_{Prrx}$ female mice raised the question of whether Ebf1 is linked to estrogen regulation. Currently there are only a few studies indicating connection to estrogen signaling. Le at al. were the first to identify Ebf1 as a regulator of estrogen receptor beta (ERβ) (Le et al., 2013). Their aim was to map binding sites of ERβ in MCF7/C4-12/FlagERβ-breast cancer cell line, which is void of estrogen receptor alpha (ERα). The Ebf1 binding motif was found to be enriched in ERβ binding sites, and Ebf1 was observed to negatively regulate ERβ at protein level. To see if the effect was specific to ERβ, Le et al. transiently transfected Ebf1 and ERα into the cell line and found Ebf1 to be able to also downregulate ERα. They concluded that Ebf1 has antagonistic effects on both estrogen receptors, although ERβ was more affected.

At the time of writing this thesis, a study by Lotesto and Raimondi is the only one to follow up on the regulation of estrogen receptors by EBF1 (Lotesto et al., 2020). By using a bioinformatics approach, they went through *EBF1* expression in relation to estrogen receptor expression and clinical data from five different types of hormone-dependent cancers. The interaction between *EBF1* and *ERα* was inconsistent between cancer types, whereas the crosstalk between *EBF1* and *ERβ* was more consistent. In contrast to previous studies, *EBF1* was not universally downregulated in all studied cancer types. High *EBF1* expression correlated with high expression of *ERβ* in breast cancer, cervical squamous cell carcinoma and uterine carcinosarcoma samples. This evidence would suggest a positive regulation between *EBF1* and *ERβ*, which is opposite from the *in vitro* results presented by Le et al. (Le et al., 2013).

It has been well established that estrogen and ERα and ERβ are essential in bone metabolism (Emmanuelle et al., 2021). ERα and ERβ are known to antagonize each

other's action in bone as well as in other tissues (Khalid et al., 2016). Their expression pattern in bone is also different. In humans, ERα expression is higher in cortical bone, whereas ERβ expression localizes more in trabecular bone (Bord et al., 2001). Out of these two receptors, functions of ERβ are still less characterized in bone, and previous knockout mouse models have struggled with issues of specificity. Nicks et al. have presented data on mesenchymal cell-specific deletion of *ERβ* which led to significant, age-progressive increase in trabecular bone mass in female *ERβ* knockout mice without any detected changes in osteoblast/osteoclast balance (Nicks et al., 2016). Fusion of the epiphyseal growth plate and cessation of longitudinal growth in humans is due to estrogen (Juul, 2001; Shim, 2015). In mice growth plates remain unfused despite sexual maturity (Emons et al., 2011; Kennedy et al., 1999). There is however evidence on the involvement of both ERα and ERβ in the regulation of longitudinal bone growth in mice (Börjesson et al., 2012; Chagin et al., 2004; Jin et al., 2021b).

It would be tempting to speculate on the role and contribution of Ebf1 in regulating ERβ expression in osteoblasts regarding the gender-dependent increase in bone volume and reduced bone length observed in our Ebf1xEbf2$_{Prrx1}^{-/-}$ female mice. However, there is not yet any experimental data supporting an interaction between Ebf1 and ERβ in osteoblasts.

Employing transcription factors as therapeutic targets has long been a topic of interest. Science has made great leaps forward since the initial description of transcription factors as 'undruggable' targets (Bushweller, 2019). Currently the most successful applications of transcription factors as drug targets are in the field of cancer therapeutics (Lambert et al., 2018). In terms of bone, there is a great need for new anabolic therapies (Russow et al., 2019; Seeman et al., 2019). As the literature discussed in this thesis and the novel data from our studies show, the Ebf family of transcription factors have several functions, redundant and non-redundant, in various cell types and tissues. Ebf family members present a strong potential serving as future biomarkers in different cancer types. Moreover, Ebf2 is implicated as an important regulator of BAT and could potentially be part of applications targeting obesity by upregulating thermogenesis in BAT.

Data presented in this thesis has further established that Ebf1 and Ebf2 have a role in bone formation while also acknowledging the questions that still remain to be answered. Identifying Ebf1 as a regulator of Osterix, one of the most important transcription factors in bone metabolism, is an important finding. By understanding the specific roles of Ebf transcription factors in osteoblast differentiation, we are one step closer to guiding MSC differentiation to facilitate bone repair and developing future therapies.

7 Conclusions

Based on the study presented in this thesis, the following conclusions can be drawn:

1. Ebf1 promotes early osteoblast differentiation directly by binding to the Osterix promoter and inducing Osterix expression.
2. Ebf1 suppresses bone formation in committed, Osterix-expressing osteoblasts *in vivo*, but is redundant in the maintenance of mature, Osteocalcin-expressing osteoblasts.
3. Mesenchymal cell lineage-targeted deletion of *Ebf2* results in a very mild bone phenotype.
4. Simultaneous deletion of *Ebf1* and *Ebf2* in mesenchymal lineage cells leads to age-progressive and to some extent gender-dependent bone phenotype.
5. Further studies are required to elucidate the molecular mechanisms underlying the bone phenotype in Ebf1xEbf2$_{Prrx1}$ mice and the possible role of Ebf3 in bone formation.

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