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Transient and lineage-restricted requirement of Ebf3 for sternum ossification

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Abstract

Osteoblasts arise from bone-surrounding connective tissue containing tenocytes and fibroblasts. Lineages of these cell populations and mechanisms of their differentiation are not elucidated well. Screening enhancer-trap lines of zebrafish allowed us to identify Ebf3 as a transcription factor marking tenocytes and connective tissue cells in skeletal muscle of embryos. Ebf3 knockout (KO) mice had sternum ossification defects, without affecting chondrogenesis, due to the defective generation of Runx2⁺ pre-osteoblasts. Conditional and temporal Ebf3 KO mice indicated requirements of Ebf3 in the lateral plate mesenchyme cells (LPMs), especially in tendon/muscle connective tissue (MCT) cell-specific manner and a stage-specific Ebf3 requirement at embryonic day 9.5-10.5. Upregulated expressions of connective tissue markers such as Egr1/2 and Osr1, increased number of Islet1⁺ mesenchymes, and downregulation of Runx2 regulator Shox2-gene expression in Ebf3-deleted thoracic LPMs suggest critical roles of Ebf3 in onset of LPM differentiation towards osteoblasts, forming sternum tissues.

Keywords: pre-osteoblast, sternum, fibroblast, Runx2, Scleraxis, lateral plate mesenchyme cells

Introduction

The sternum constitutes the most ventral part of the thoracic skeleton, protects the heart and lungs in vertebrates. The sternum first arose in vertebrae during the colonization to land; while avians have well-developed sternal tissues, most of fish do not have sternum-like bones. Evolution of the sternum implies its critical roles in fore-limb locomotion as connecting sites for the pectoral-muscle tendon [1,2].

In general, evidence suggests that osteoblasts differentiate from mesenchymal stem cells (MSCs) that possess differentiation potentials into osteoblasts, chondrocytes, tenocytes, and perhaps even muscle connective tissue (MCT) cells, fibroblastic cells and adipocytes [3]. A recent study involving single-cell analyses of embryos from E9.5-E13.5 enabled to roughly visualize developmental trajectories of mesenchymal cells into chondrocytes, osteoblasts and MCT cells, in which the close proximity of the latter two has been suggested [4]. However, cellular hierarchies of mesenchymal differentiation into osteoblasts and MCT cells are not delineated very well.

Compared to the somitic mesenchymal differentiation into axial bones, the differentiation of mesenchymes into the sternum bone is relatively simple. All mesenchymal cells in the sternum including chondrocytes, osteoblasts and proximal connective tissue cells originate from the lateral plate mesoderm in common. Existence of osteo-chondro progenitors in the lateral plate mesoderm has been shown by the lineage trace of reporter gene expression activated by paired related homeobox 1(Prx1)-Cre [5-7].

Runx-related transcription factor 2 (Runx2) is an osteoblast master regulator during development and regeneration [8,9]. The essential roles of Runx2 in the osteo-lineage cells have been well studied, and its activation is defined as the most important indicator of pre-osteoblasts [10,11]. Expression of the Runx2 gene begins in a portion of lateral plate mesenchymal cells (LPMs) at approximately E10.5, which is preceded by Prx1-Cre expression [12,13]. Thus, the use of Prx1-Cre to study LPM morphogenesis provides an ideal experimental system to elucidate the molecular/cellular mechanism of the Runx2⁺ pre-osteoblasts generation from LPMs.

In contrast to mesenchymal differentiation of skeletal components, cell lineages of skeletal muscle cells are elucidated very well, with the identification of the MyoD family and Pax3/Pax7-encoding myogenic transcription factors [14]. All the skeletal muscle in the trunk including pectoral muscles are derived from somites. However, skeletal muscle tissues are complex because different types of MCT cells intercalate into muscle fibers, and these MCT-cell lineages are heterogenous [15]. Recent studies on skeletal myogenesis are focusing on MCT cells that play critical roles in muscle development and maintenance [16]. Skeletal muscle cells should colonize a region near the

skeletogenic cell components including osteogenic and chondrogenic cells together with tenocytes and MCT cells to build the functional locomotorium during development. Thus, the embryonic tenogenic/MCT cells represent a missing link to design a proof-of-concept experiment to integrate mechanisms underlying the incorporation of skeletogenic and myogenic cell into the unified locomotorium.

From this idea, we screened enhancer-trap lines of zebrafish and identified the early B-cell factor3 (Ebf3) as a transcription factor marking scleraxis (Scx)-expressing tenocytes and MCTs of zebrafish and mouse embryos in common. Ebf3 is required for the sternum ossification. Ebf3 is required in Scx+ cell lineage of LPMs transiently during early embryonic stage. Analyses of embryonic LPMs at that stage suggest roles of Ebf3 in the differentiation of osteo-fibroblastic progenitors towards Runx2+ pre-osteoblasts at the boundary of lateral and somitic mesoderms.

Results

Ebf3 is expressed in tendon/muscle connective-tissue progenitors of zebrafish embryos

We screened Gal4-UAS enhancer trap lines of zebrafish generated by Dr. Kawakami et al., in which UAS:GFP-expression was found in mesenchymal cells surrounding or adherent to skeletal muscle fibers. One line, SAGFF(LF)19A, showed activated UAS:GFP in a group of cells aligned at the segmental boundary of somites at approximately 48 h post-fertilization (Figure S1A and S1B, Figure 1A). In this line, gal4 of SAGFF(LF)19A is trapped upstream of the gene encoding Ebf3. The UAS:GFP signals of SAGFF(LF)19A were detected in Ebf3-positive cells by immunofluorescence staining with an antibody against Ebf3 (Figure S1C). Further, the UAS:GFP⁺ cells were observed in Tenascin⁺ segmental boundaries between myosin heavy chain (MF20)⁺ muscle bundles (Figure 1B) and developed extremely long filopodia that grew through the boundary of muscle fibers (Figure 1C). Moreover, in situ hybridization revealed that some of these cells co-expressed mRNA for zScleraxisa (zScxa), encoding a tendon-specific transcription factor, showing that UAS:GFP⁺ cells are tendon progenitors (Figure 1C). The UAS:GFP⁺ cells were also found in the muscle connective tissue (MCT) cells in close proximity to skeletal muscles, termed MCT cells (Figure 1B). Ebf3⁺ mesenchymes are composed of progenitors of tenocytes and MCT cells in zebrafish embryos.

Ebf3 is dominantly expressed in the thoracic lateral plate mesenchymal tissues

MCT cells play critical roles in skeletal development. Scx is one of the best studied key factors that function in tendon development [17-20], yet our understanding of MCT and tendon development is still limited. We therefore investigated whether Ebf3 is expressed similarly in the non-myogenic mesenchyme of mice. Whole-mount immunofluorescence staining of mouse embryos showed that Ebf3 was expressed in the syndetomes, the regions between Pax7⁺ dermomyotomes and Sox9⁺

sclerotomes, and in the thoracic Lateral Plate Mesoderm (LPM) (Figure 2A). We crossed Prx1-Cre mice with R26R^{Tdtomato/Tdtomato} mice, as the crossed line marks LPMs in embryos. Analyses of immunofluorescence staining on transverse sections indicated that Ebf3 is expressed in Prx1-Cre R26R^{Tdtomato}-activated thoracic LPMs, in addition to the syndetome adjacent to Pax7⁺ dermomyotome (Figure 2B). Moreover, whole-mount in situ hybridization (WISH) revealed the highest expression of Ebf3 in the thoracic lateral plate mesoderm and sternal primordial bars at E10.5 and E11.5 (Figure 2C). In contrast, other Ebf transcription factors, Ebf1 and Ebf2, were transcribed in the axial mesenchyme and limb buds but not in the thoracic lateral plate mesoderm at E10.5 (Figure 2C). Ebf2 starts to be expressed in primordial bars at E11.5 (Figure 2C). Ebf4 was not expressed in mouse embryos at E10.5 and E11.5 (data not shown).

The regulation of Ebf3 for the sternal osteoblast differentiation in the lateral plate mesoderm

To investigate roles of Ebf3 in development, we prepared conditional Ebf3-knockout (KO) mice (Ebf3^{flx/flx}). The targeting vector harbored loxP sites and an FRT-flanked neomycin resistance cassette, in which exon5 and exon6 were flanked by a loxP site. These exons encoded the DNA-binding domain of Ebf3 (Figure S2). We generated whole-body Ebf3-KO mice by crossing Ebf3^{flx/flx} mice with Ebf3^{flx/+} CAG-Cre mice. Alizarin red and Alcian blue staining revealed that Ebf3-KO embryos had defects in sternal-bone formation, while sternal chondrogenesis occurs normally in these mice, with the exception of deformations in the first segment of the sternum (5/5 embryos) (Figure 2E). Sternal-ossification and chondrogenesis defects were not observed in CAG-Cre Ebf3 heterozygous mice (4/4 embryos). Ossification defects in the third and fourth metatarsal bones were also observed (Figure S3), whereas ossification of the ribs and vertebrae bones was normal (Figure 2D). The sternum is derived from the thoracic lateral plate mesenchymal cells (LPMs), whereas ribs and vertebral bones are derived from the somatic mesenchymal cells [21] (Figure 2C and 3A). To evaluate whether Ebf3 is required for the sternum ossification in osteo-fibro lineage cells, we used Prx1-Cre mice which marks osteo-fibro progenitors originate from the lateral plate mesoderm. We crossed Ebf3^{flx/flx} R26^{Tdtomato/Tdtomato} mice with Ebf3^{flx/+} Prx1-Cre mice and generated LPMs-specific conditional Ebf3-KO mice (Figure 3A). Prx1-Cre Ebf3-KO embryos showed defects in sternum ossification (6/6 embryos), similar to that observed in CAG-Cre general Ebf3-KO embryos (Figure 3B), while no defects were observed in Prx1-Cre Ebf3 heterozygous mice (10/10 embryos), indicating that the Ebf3 expressed in LPMs is required for sternum ossification. We also evaluated LPM-derived osteoblasts and pre-osteoblasts of the sternum in the Prx1-Cre Ebf3-KO embryos (Ebf3^{flx/flx} R26^{Tdtomato/+} Prx1-Cre) by immunofluorescence staining with antibodies against Osterix (Osx) and Runx2, respectively. In the ossifying sternum tissue, most Prx1-Cre R26R^{Tdtomato}-activated sternum periosteum tissues were occupied by Runx2⁺ pre-osteoblasts in the wild-type and Prx1-Cre Ebf3

heterozygous embryos. Osx^+ osteoblasts were also present in the sternum periosteum of these mice. In contrast, the numbers of Osx^+ cells and $Runx2^+$ cells in the sternum periosteum decreased prominently in Prx1-Cre Ebf3-KO embryos (Figure 3C).

A previous study reported the defect in muscle differentiation of the diaphragm in the Ebf3 Pan-KO mouse embryo [22]. Since Ebf3 is expressed in Prx1-Cre R26R^{TdTomato}-activated connective tissue cells of the diaphragm and intercostal muscles adjacent the sternum (Figure S4C and S7C), we examined whether the diaphragm and intercostal skeletal tissues are affected in Prx1-Cre Ebf3-KO mice. The diaphragm and intercostal muscles and tendon/connective tissues adjacent to these muscles were substantially normal in Prx1-Cre Ebf3-KO embryos, implying that Ebf3 expressed in LPM-derived MCT are not involved in myogenic defects in these tissues (Figure S7A, S7B and S7D).

Ebf3 regulates the generation of Runx2⁺ pre-osteoblasts in the thoracic LPMs before the onset of the sternal ossification

Ebf3 is expressed in the periosteum and Prx1-Cre R26R^{TdTomato}-activated tendon/connective tissue cells surrounding the sternum, and connective tissue cells interspersed among intercostal myofibers at the ossification stage (E18.5) (Figure S4A and S4C). Ebf3 is undetectable in Osx^+ or $Runx2^+$ osteoblast-lineage cells and Tenascin⁺ cells, suggesting that Ebf3 regulates earlier stages of the osteoblast differentiation than E18.5 (Figure S4B). There was no significant difference in the number of mesenchymes marked with Prx1-Cre R26R^{TdTomato} between Prx1-Cre Ebf3 KO and heterozygous mice at E12.5 (Figure S5C). Runx2 is known as the master regulator of lineage commitment from mesenchymal stem cells to osteoblasts [8,9]. We found that a large population of the thoracic LPMs were Ebf3/Runx2 double positive (Figure 4A). To determine the roles of Ebf3 in pre-osteoblast differentiation, we investigated expression of Runx2 in the Prx1-Cre R26R^{TdTomato}-activated chest wall of Prx1-Cre Ebf3 KO mice. Compared to Prx1-Cre Ebf3 heterozygous mice, the number of $Runx2^+$ cells significantly decreased in Prx1-Cre Ebf3 KO mice (Figure 4B and 4C) while the numbers of $Sox9^+$ cells, the mesenchymal-cell population including progenitors of chondrocytes, were similar between these mice (Figure S5A, S5B and 4D). Thus, Ebf3 regulates the number of $Runx2^+$ pre-osteoblasts in the thoracic lateral plate mesoderm.

The Ebf3 expression at E9.5-E10.5 is critical for the sternal ossification

To define the functional timing of Ebf3 expression required for sternum ossification, we generated tamoxifen-induced temporal Ebf3-KO mice by crossing Ebf3^{flox/flox} mice with Ebf3^{flox/+} Ubc-CreER^{T2} mice (Figure 5A). We then injected tamoxifen into these pregnant dams at different developmental stages (E9.5/E10.5/E11.5/E12.5) and evaluated sternal ossification (Figure 5B). Sternal phenotypes were reproducible only when tamoxifen was injected into these mice at E9.5 (5/5 embryos) or E10.5 (4/5 embryos), but not at E8.5 (6/7 embryos) E11.5 (3/3 embryos), E12.5 (7/7 embryos) or in control

(Ubc-CreER^{T2} Ebf3 heterozygous and wild-type) mice, suggesting that Ebf3 functions in sternum ossification mainly at approximately E9.5–E10.5 (Figure 5C).

Ebf3 regulates the differentiation of osteo-fibrogenic progenitors

To elucidate how Ebf3 regulates the differentiation of thoracic LPMs at E10.5, we compared transcripts in LPMs isolated from Prx1-Cre Ebf3-KO mice with those from Prx1-Cre Ebf3 heterozygous mice. Trunks without limb buds, heart and other organs in thoracic /abdominal cavity were isolated, and then thoracic wall tissues were excised. Prx1-Cre R26R^{TdTomato}-activated LPMs were isolated from these thoracic wall tissues by fluorescence-activated cell sorting (FACS). RNA-seq analysis was performed for comparison (Figure 6A).

By using IPA software (Ingenuity Pathway Analysis), downstream analysis was performed for this RNA-seq data. In the IPA Knowledge Base derived from omics data, annotated genes were categorized by their biological functions. In the category of “Cellular Development”, we focused on three groups associated with the differentiation of (1) connective tissue (CT) cells, (2) bone cells/osteoblasts, and (3) mesenchymal cells (Figure 6B). The Venn diagram in Figure 6C shows the differentially expressed genes annotated in these three groups, in which Red and Blue-colored genes were those significantly up- and down-regulated, respectively, in Prx1-Cre Ebf3 KO mice (Figure 6C). Most of differentially expressed genes were found in (1) CT cells and the merged region of (1) and (2) (Figure 6C).

These data provide insight into genes associated with the phenotype of the sternum ossification. Increased transcript levels of genes that regulate the formation of tendon and MCTs, such as early growth response protein1 (Egr1), early growth response protein2 (Egr2), and odd skipped related gene 1 (Osr1), were found in Prx1-Cre Ebf3-KO embryos (Figure 6D). The expression of biglycan (Bgn), a proteoglycan involved in maintaining tendon-progenitor pools and connective tissue growth factor (Ctgf) was also upregulated in Prx1-Cre Ebf3-KO embryos (Figure 6D). We also confirmed an increase in expression of the Jun B proto-oncogene (Junb), fibroblast growth factor 9 (Fgf9), and epidermal growth factor receptor (Egfr), which regulate proliferation and maintain undifferentiated states during the initial steps of osteoblast differentiation (Figure 6E). However, transcripts of the short stature homeobox2 (Shox2) gene, encoding a site-specific regulator of Runx2 during long-bone formation, were decreased in Prx1-Cre Ebf3-KO mice (Figure 6E).

In contrast, genes encoding tendon-associated transcription factors such as Scx and its upstream factor homeobox protein mohawk (Mkx) were expressed similarly in Prx1-Cre Ebf3-KO and control embryos (Figure 6D). The expression levels of collagen genes, such as those encoding collagen types I and III, did not differ between Prx1-Cre Ebf3-KO and heterozygous embryos (Figure S6A). The expression of regulatory genes involved in chondrocyte differentiation, such as the Sry-related HMG box (Sox)-family genes (Sox9, Sox5, Sox6) were unchanged in Prx1-Cre Ebf3-KO embryos (Figure S6B). These results suggest that progenitors of pre-osteoblasts remain undifferentiated and express

MCT markers in the absence of Ebf3.

Ebf3 regulates the differentiation of Scx-Cre lineage cell

Previous studies proposed the term “primaxial” to define the domain of the uniform somitic cell population and “abaxial” to define the domain where somite-derived cells differentiate in the context of lateral plate-derived MCT. The boundary region between these two domains is termed “the lateral somitic frontier” [21,23]. One upregulated gene, insulin gene enhancer protein 1 (Islet1) (Figure 6F), encodes a LIM-homeodomain protein and was expressed in the lateral somitic frontier; further, its expression overlapped with that of Ebf3 (Figure 6G). Moreover, the number of Islet1⁺ / Prx1-Cre: TdTomato⁺ cells were significantly higher in Prx1-Cre Ebf3-KO than in Prx1-Cre Ebf3 heterozygous embryos (Figure 6H). In contrast, no change in Scx expression was observed in Prx1-Cre Ebf3-KO mice (data not shown). During mouse embryogenesis, Scx transcripts are first detected between E9.5 and E10.5 in the sclerotome, mesenchymes of the body wall, and limb buds. As development proceeds, the expression of Scx is gradually restricted to regions where tendon and ligament tissues are formed [17]. When Scx:GFP R26R^{TdTomato/TdTomato} mice were crossed with Prx1-Cre mice, Scx: GFP was co-expressed in Ebf3⁺ cells in some TdTomato⁺ LPMs (Figure 7A). To determine whether Ebf3 is required in Scx⁺ LPMs, Ebf3^{flx/flx} mice were crossed with Ebf3^{flx/+} Scx-Cre mice and found defective sternum ossification in Ebf3^{flx/flx} Scx-Cre mice (6/6 embryos) but not in the heterozygous mice (7/7 embryos). The result suggests that Ebf3 is required in Scx-Cre lineage cells, located at the lateral somitic frontier in E9.5-10.5 mice to ossify the sternum (Figure 7B).

Discussion

In this study, we demonstrate that Ebf3 plays a pivotal role in sternum ossification. Runx1 and Runx2 cooperatively regulate sternal morphogenesis and the commitment of LPMs differentiation into sternal chondrocytes specifically, the sternum tissue has been found to be completely lacking in Prx1-Cre-induced Runx1/Runx2-double KO mice [24]. In contrast, CAG-Cre-, Prx1-Cre- or Scx-Cre-driven Ebf3 deletion resulted in defective ossification of the sternum without affecting its chondrogenesis. Thus, specific roles in sternum ossification, but not in chondrogenesis, comprise a unique feature of Ebf3 compared to many other genes that regulate sternum formation.

The effects of Ubc-CreER^{T2}-driven deletion were extremely informative because they clearly indicated that the timing of Ebf3 function in sternum ossification is E9.5–E10.5. E9.5 approximately corresponds to the earliest stage of limb bud formation, whereas E10.5 is the stage when Runx2 starts to be expressed in a part of the LPMs along the rib cage. This raises the question of what types of mesenchymal cell differentiation are regulated by Ebf3 to produce pre-osteoblasts. A comparison of RNA-seq results between thoracic LPMs isolated from Prx1-Cre-driven Ebf3-KOs and those from

control embryos at E10.5 demonstrated increases in muscle connective tissue (MCT) cell/tenocyte-associated transcripts such as *Egr1*, *Egr2*, and *Osr1* [25,26], as well as a set of proliferating mesenchyme markers including *Islet1*, *Junb*, *Fgf9*, and *Egfr* in the *Ebf3*-KO LPMs [27-31]. In contrast, the expression of *Shox2*, which regulates the formation of proximal appendicular bones and is found as a factor upstream of *Runx2*, was significantly lower in *Ebf3*-KO than in control LPMs. Moreover, an increased number of *Islet1*⁺ cells was observed in *Ebf3*-KO embryos. Thus, mesenchymal differentiation before the activation of *Runx2* is affected by the lack of *Ebf3*, suggesting that *Ebf3* is critical for generating *Runx2*⁺ pre-osteoblasts from their progenitors in the thoracic lateral plate mesoderm. Figure 7C shows our working hypothesis on the roles of *Ebf3* in sternum ossification. Because the sternal chondrogenesis was not affected in *Ebf3* KO mice, osteo-chondrogenic progenitors is located upstream of osteo-fibrogenic progenitors in the hierarchy of LPM differentiation in this hypothesis.

However, the exact identity of osteo-fibrogenic progenitors awaits the further characterization of LPMs. *Islet1*⁺ LPMs can mark mesenchymal stem cells in the lateral plate mesoderm rather than osteo-fibrogenic progenitors. Lineage and single-cell analyses of LPMs will elucidate more precise roles for *Ebf3* in their differentiation and determine whether *Ebf3* promotes the differentiation of LPMs into pre-osteoblasts by inhibiting the self-renewal of *Islet1*⁺ LPMs or whether these cells acquire alternate fates towards cells other than pre-osteoblasts in the absence of *Ebf3*. The elucidation of how *Ebf3* mediates the differentiation of LPMs towards pre-osteoblasts as a transcription factor is also required. Recent studies on the roles of *Ebf1* in B cell development suggest roles for *Ebf1* in epigenetic events, in addition to transcriptional regulation, suggesting that members of the *Ebf* family can dynamically modulate the chromatin structure [32,33].

Notably, *Ebf3*⁺ *Islet1*⁺ cells are found in the lateral somitic frontier. *Pax3*⁺ muscle progenitors begin to cross this boundary at E9.5, differentiate into *MyoD*⁺ myoblasts from E10.5 [14], and form abaxial muscles in the LPM microenvironment, which may differ from that of the somitic mesoderm [21,23]. The requirement of *Ebf3* at E9.5–E10.5, which was determined using *Ubc-CreER*^{T2} mice, indicates that the differentiation of LPMs towards pre-osteoblasts starts at the lateral somitic frontier. Recent studies have indicated the heterogeneity of fibroblastic cells in skeletal muscle, including some *Scx*⁺ or *Osr1*⁺ cells [20,26]. These *Scx*⁺ cells might include the lateral plate mesoderm-derived cells observed in this study. Further characterization of MCT cells is essential to verify that *Ebf3* is required for the differentiation of some types of MCT cells.

Vertebrates have developed the sternum during evolution, providing attachment sites for pectoral muscles that generate the force used for walking or flying [1,2]. In contrast, the sternum is lacking in

most of fish, including zebrafish. None the less, Ebf3 is expressed in progenitors of tenocytes and MCT cells in zebrafish and mice in common. Taken the fact that Scx genes are required for ossification of ventrally elongated precaudal vertebrae in zebrafish into consideration [34], it would be interesting to investigate whether Ebf3 is required in fish also for the abaxial bone formation or other morphogenesis of the LPMs.

In this study, we found critical roles of Ebf3 in the sternum ossification. Whereas members of the Ebf family are expressed redundantly in the somitic mesoderm and limb buds, expression of Ebf3 dominates the others in thoracic LPMs, which should lead to the defective sternum ossification by the lack of Ebf3 in LPMs. In contrast to a previous observation of thinner diaphragm muscles in Ebf3 Pan-KO mice [22], the myogenesis of diaphragm and intercostal muscle was minimally affected in Prx1-Cre LPM-specific Ebf3 KO mice, suggesting that Ebf3 expressed in somitic MCTs is required for the myogenesis or other members of the Ebf family expressed redundantly in somitic and lateral plate mesoderm compensate roles of Ebf3 for myogenesis.

In summary, Ebf3 expressed in Prx-Cre and Scx-Cre-activated lineage cells mediates sternum ossification by regulating differentiation of LPMs towards pre-osteoblasts at the lateral somitic frontier (Figure 7C). The number of Islet1⁺ mesenchymal cells and expressions of connective tissue cell/tenocyte-associated genes such as Egr1, Egr2, and Osrl were increased in Ebf3-deleted thoracic LPMs. How Ebf3 regulates production of Islet1⁺ cells and the fate decision of LPMs into osteoblasts and fibroblasts remain open questions. Identifications of target genes for Ebf3, for example by CHIP analyses, together with single cell analyses of LPMs at the early embryonic stages of wild type and Ebf3 KO mice are essential for more detailed understanding the function of Ebf3 in the LPMs differentiation.

Material and Methods

Analysis of zebrafish embryos

Embryos were fixed in 4% paraformaldehyde (PFA)/PBS overnight at 4°C, rinsed in PBS-T (PBS with 0.05% tween), and exposed to 0.2% triton-X100/PBS. For whole-mount immunostaining, embryos were incubated in Blocking One solution (Nacalai Tesque, Kyoto, Japan), washed with 0.2 % triton-X100/PBS, and stained with the following primary antibodies: anti-Ebf3 (1:1000, R&D Systems, Minneapolis, MN, USA), anti-Tenascin (1:1000, AB19013, Millipore, Billerica, MA, USA), anti-MF20 (1:200, Hybridoma Bank); Alexa Fluor antibodies (Thermo Fisher Scientific, Waltham, MA, USA) were used as secondary antibodies. For whole-mount in situ hybridization (WISH), GFP-

marked RNA probes were generated from the cDNA of Scxa amplified by reverse transcription (RT)-PCR with the following PCR primers.

zScxa Forward Primer: 5'-ACCGGCTCCGAGCCGATACGTGTA-3'

zScxa Reverse Primer: 5'-GGTTGCTGAGGCAGAATGTGCAGA-3'

Generation of Ebf3-conditional knockout mice

To generate Ebf3-floxed mice (Accession no. CDB1070K: <http://www2.clst.riken.jp/arg/mutant%20mice%20list.html>), a targeting vector harboring loxP sites as well as an FRT- flanked neomycin resistance cassette, in which exons 5 and 6 were flanked by a loxP site, were electroporated into TT2 embryonic stem (ES) cells [35]. After cell colony isolation by G418 and Southern blot analysis, ES cells containing the floxed allele were injected into 8-cell stage embryos to generate chimeric mice. The FRT-flanked Neo cassette was removed by mating with Flpe mice. Ebf3^{flox/flox} mice were backcrossed at least 10 times with a C57BL/6NcrSlc background before analysis (Figure S2). These mice were crossed with CAG-Cre, Prx1-Cre (Prx1-Mouse Genome Informatics, Jackson Laboratory, Bar Harbor, ME, USA), Ubc-CreER^{T2}, or Scx-Cre mice to generate CAG-Cre Ebf3^{flox/+} mice, Prx1-Cre Ebf3^{flox/+} mice, Ubc-CreER^{T2} Ebf3^{flox/+} mice, or Scx-Cre Ebf3^{flox/+} mice, and their progeny were inter-crossed with Ebf3^{flox/flox}:Rosa26R^{TdTomato} mice to obtain CAG-Cre or Prx1-Cre or Ubc-CreER^{T2}, Ebf3^{flox/flox} Rosa26^{TdTomato/+} mice.

Other mouse strains

Mouse strains including CAG-Cre, Prx1-Cre, Ubc-CreER^{T2}, and Rosa26R:TdTomato were previously described [13,36-38]. These mice were obtained from the Jackson Laboratory. Scx-Cre mice and Scx:GFP mice [39] were generous gifts from Dr. Syukunami and Dr. Sugimoto (University of Hiroshima, Institute of Biomedical and Health Sciences). All mouse strains were maintained in a C57BL/6NcrSlc background and their genotypes were determined by PCR (Figure S2). All experiments involving animals were performed according to the guidelines of the animal care committee of Kyoto University and the Institutional Animal Care and Use Committee (IACUC) of RIKEN Kobe Branch, and were conformed to relevant guidelines and laws.

Tamoxifen injection

To induce CreER^{T2}-mediated recombination in Ubc-CreER^{T2} Ebf3^{flox/flox} embryos, pregnant dams were treated with 0.1 mL tamoxifen solution (10 mg/mL in peanut oil; Sigma, St. Louis, MO, USA) by gavage on each embryonic day, and embryos were analyzed at E18.5.

Analysis of mouse embryos

For whole-mount in situ hybridization (WISH), digoxigenin-labeled antisense riboprobes were transcribed from cloned gene-specific probes (DIG RNA Labeling Kit, Roche, Basel, Switzerland). The WISH protocol was as follows. Whole embryos were fixed overnight in 4% PFA/PBS. Embryos were fixed washed in PBT (PBS containing 0.1% tween), and dehydrated stepwise in 25%, 50%, and 75% ethanol/PBT and finally sorted at 4 °C in 100% ethanol. The embryos were rehydrated in reverse ethanol/PBT steps and washed with PBT. After treating the embryos with 10 mg/mL Protein K in PBT at 37 °C for 20 min, they were incubated in 2 mg/mL Glycine in PBT at RT, washed in PBT, re-fixed for 20 min with 4% PFA/PBS and 0.2% glutaraldehyde in PBT, washed in PBT, and finally bleached in 6% H₂O₂ in PBT. After further washing with PBT, the embryos were incubated in pre-hybridization solution (50% formamide, 5× SSC, 1% SDS, 50 mg/mL yeast tRNA, 50 mg/mL heparin) at 65 °C for 2 h. Subsequently, the embryos were incubated in probe solution/pre-hybridization solution (pre-hybridization solution with 1:1000 digoxigenin) overnight at 65 °C. Unbound probes were removed through a series of washing steps with Solution 1 (50% formamide, 5× SSC, 1% SDS), Solution 2 (5× SSC, 0.1% tween 20), and Solution 3 (50% formamide, 2× SSC, 1% SDS) at 70 °C for 30 min. After washing with TBST (150 mM NaCl, 100 mM tris-HCl, 1% tween 20), embryos were blocked for 90 min at RT in 1.5% Blocking-Reagent/TBST (Roche), followed by incubation at 4 °C overnight in 1.5% Blocking-Reagent/TBST with digoxigenin antibody conjugated with alkaline phosphatase (1:3000; Roche). Unbound antibody was removed by washing at RT with TBST, and the samples were stained with BM Purple AP Substrate (Roche). For immunostaining, whole embryos were harvested at E9.5–E18.5 in PBS and fixed in 4 % PFA/PBS. Embryos at E9.5–E14.5 were fixed for 1–3 h at room temperature. Embryos at E15.5–E18.5 were fixed overnight at 4 °C. The embryos were then rinsed with PBS and immersed in 5% sucrose/PBS for 30 min, in 15% sucrose/PBS overnight, and in 30% sucrose/PBS overnight at 4 °C prior to embedding in OCT media (Leica Microsystems). Embedded tissues were sliced at intervals of 10 µL. After washing with PBS-T (PBS with 0.05% tween), the embryo tissues were blocked for 60 min at RT in Blocking One (Nacalai Tesque) and stained with primary antibodies for Ebf3 (1:1000, R&D Systems, AF5166), Sox9 (1:1000, Millipore AB5535), Tenascin (1:1000, Millipore, AB19013), GFP (1:500, Nacalai Tesque 04404-84), Osterix (1:1000, ab22552, Abcam, Cambridge, UK), Runx2 (1:500, Abcam, ab23981), Islet1 (1:1000, Abcam, ab109517), MF20-c (1:200, DSHB, AB_214781) and Laminin (1:1000, Sigma, L9393). Followed by washing in PBS- T, secondary antibodies (Alexa Fluor, Thermo Fisher) were used. Tissue samples were imaged using a Leica SP8 confocal microscope (Wetzlar, Germany). Evaluation of the cell number was carried out with ImageJ software. Skeletal preparation of embryos was performed as previously described [40].

RNA-seq analysis

To comprehensively evaluate the developmental function of Ebf3 in the thoracic lateral plate

mesoderm, we analyzed global gene expression changes by RNA-seq analyses in thoracic LPMs samples collected from Prx1-Cre homozygous and heterozygous Ebf3-KO mice at E10.5. Prx1-Cre R26RTdTomato-activated cells were isolated from each tissue by FACS. Total RNA was extracted from the cells isolated by FACS in cell lysis buffer (TRIzol LS Reagent, Life Technologies, Carlsbad, CA, USA). RNA samples were analyzed with an Agilent Bioanalyzer RNA 6000 Pico Kit (5067-1513, Santa Clara, CA, USA) and subjected to library preparation according to the Takara SMARTer kit and DNA High-sensitivity kit (5067-4626, Shiga, Japan). Sequencing was performed with the Illumina NextSeq-500 platform (San Diego, CA, USA), which generated raw sequencing reads. Reads were mapped to the reference genome and transcript annotation was performed (Mouse, mm 10,49,585 genes, 103,982 transcripts) (ENSEMBLE, provided by CLC). Using IPA software (Ingenuity Pathway Analysis, QIAGEN, Hilden, Germany), downstream effects analysis was performed for this RNA-seq data.

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Author Contributions

M. Kuriki, F. Sato, H. Arai, and M. Sogabe performed experiments with mice. F. Sato and K. Kawakami performed experiments with zebrafish. Y. Yoshimoto and C. Shukunami provided Scx:GFP and Scx-Cre mice. M. Kaneko and H. Kiyonari were involved in the generation of Ebf3^{flx/flx} mice. M. Kuriki, F. Sato, and A. Sehara-Fujisawa designed experiments and M. Kuriki and A. Sehara-Fujisawa wrote the manuscript, with comments provided by F. Sato.

Declaration of Interests

The authors declare no conflict of interest.

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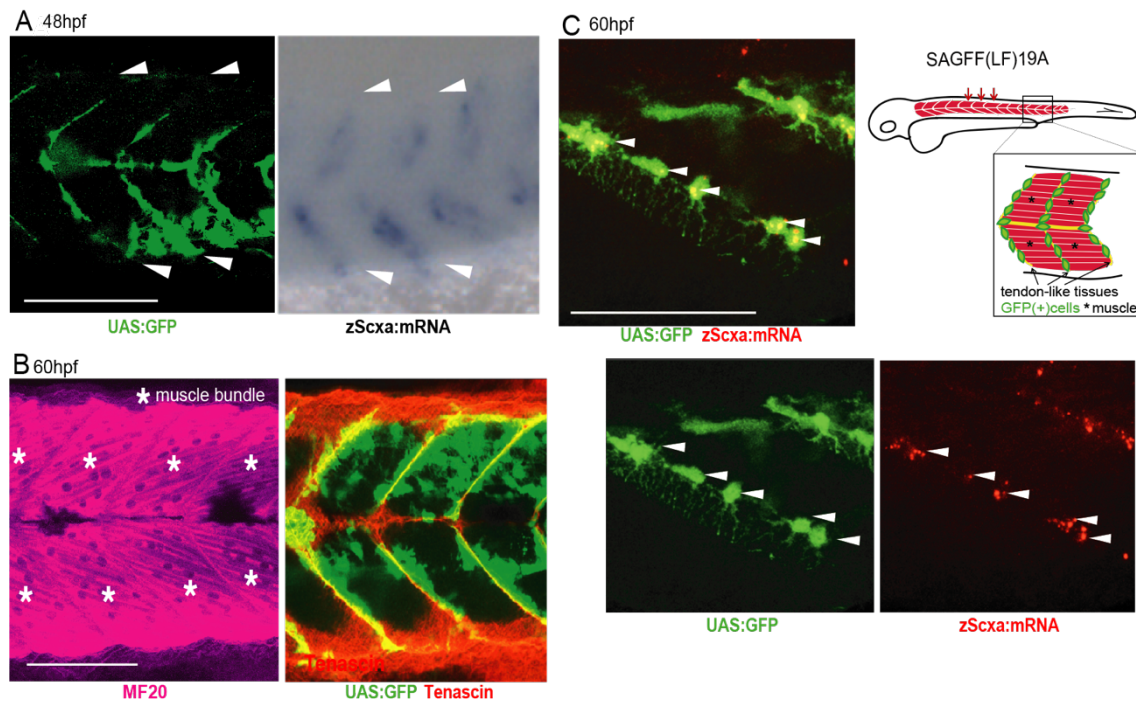


Figure 1.

Identification of an enhancer trap-line marking progenitors of tenocytes and interstitial cells in zebrafish embryos

- A) Expression of UAS:GFP in Gal4-UAS enhancer trap zebrafish line SAGFF(LF)19A and signals for zScxa mRNA transcripts detected by whole-mount in situ hybridization (WISH) at 48 hpf. White arrows show segmental boundaries of somites (scale bar, 100μm).
- B) Whole-mount immunostaining of 60 hpf SAGFF(LF)19A embryos for myogenic (magenta, MF20 detecting myosin heavy chain) and tenogenic (red, tenascin) markers together with UAS:GFP signals at 60 hpf (scale bar, 100μm).
- C) Tenocyte progenitors marked with UAS:GFP and fluorescence in situ hybridization for scleraxis (Red) at 60 hpf. Note that these cells extended long filopodia through the boundaries of skeletal muscle fibers (scale bar, 50μm).

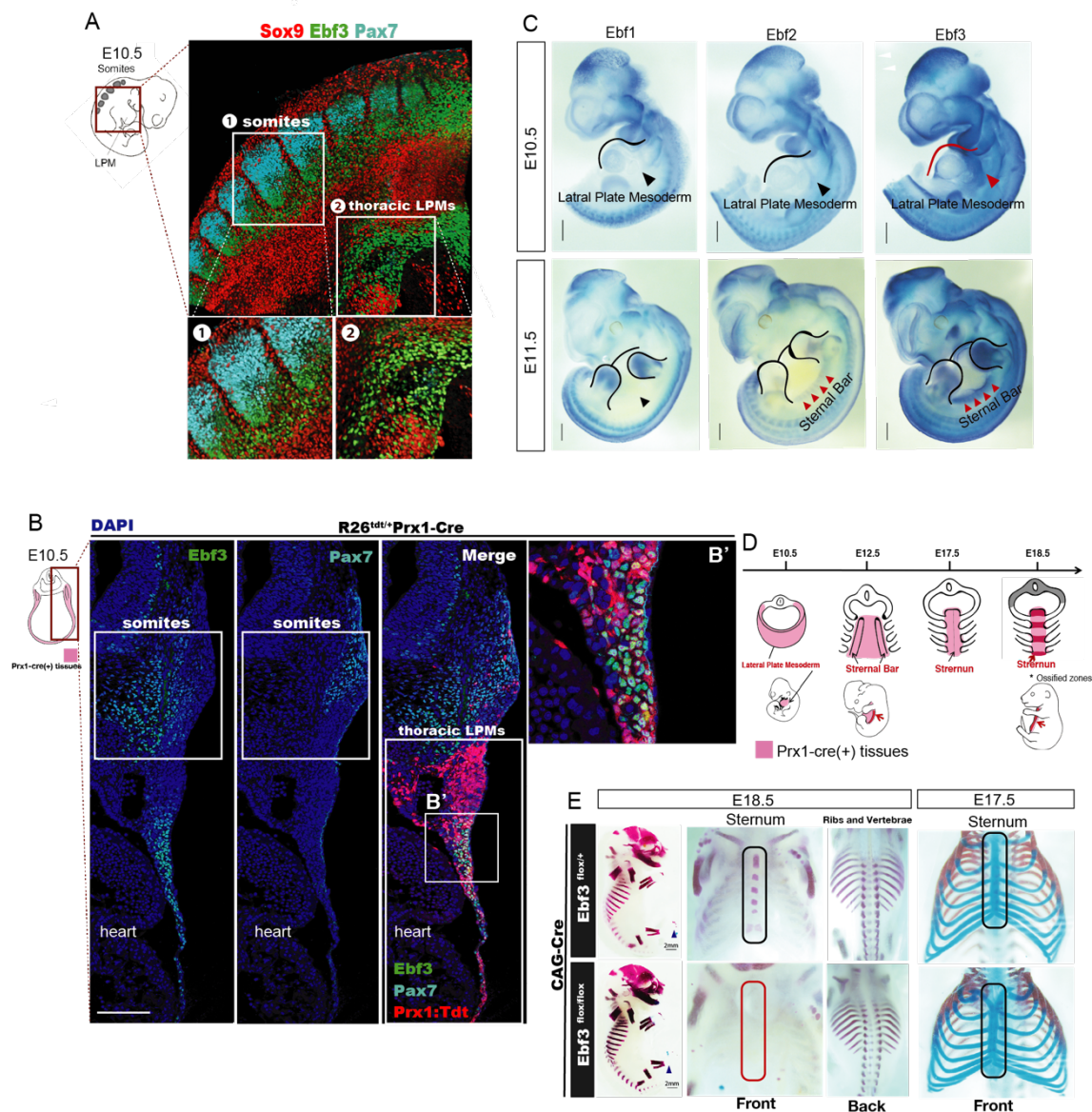


Figure 2.

Essential roles of Ebf3 in sternum ossification

- A) Whole-mount immunostaining of E10.5 mouse embryo with antibodies against Ebf3 (green), Pax7 (cyan), and Sox9 (red). Ebf3 was expressed in the lateral plate mesoderm, as well as in syndetomes located between Pax7⁺ dermomyotomes and Sox9⁺ sclerotomes.
- B) Ebf3 (green) was expressed in Prx1:tdTomato⁺ cells (Red) at the thoracic lateral plate mesoderm and in syndetomes beside Pax7⁺ dermomyotomes (cyan) at E10.5 (scale bar, 100μm). B': enlarged view.
- C) Whole-mount in situ hybridization (WISH) for Ebf1, Ebf2, and Ebf3 in E10.5 and E11.5 mouse embryos (scale bar, 1mm). Red arrows indicate that Ebf3, but not other Ebfs, was expressed in the thoracic lateral plate mesoderm tissue at E10.5 and in the sternal bar at E11.5.

606 D) Schema of sternum development during murine embryogenesis.
607 E) Ossification and chondrogenesis analyses of Ebf3^{flox/flox} CAG-Cre and Ebf3^{flox/+} CAG-Cre embryos
608 at E18.5 and E17.5 based on skeletal preparations (scale bar, 2mm). Ebf3-KO mice exhibited
609 defective sternum ossification without any defects in chondrogenesis.

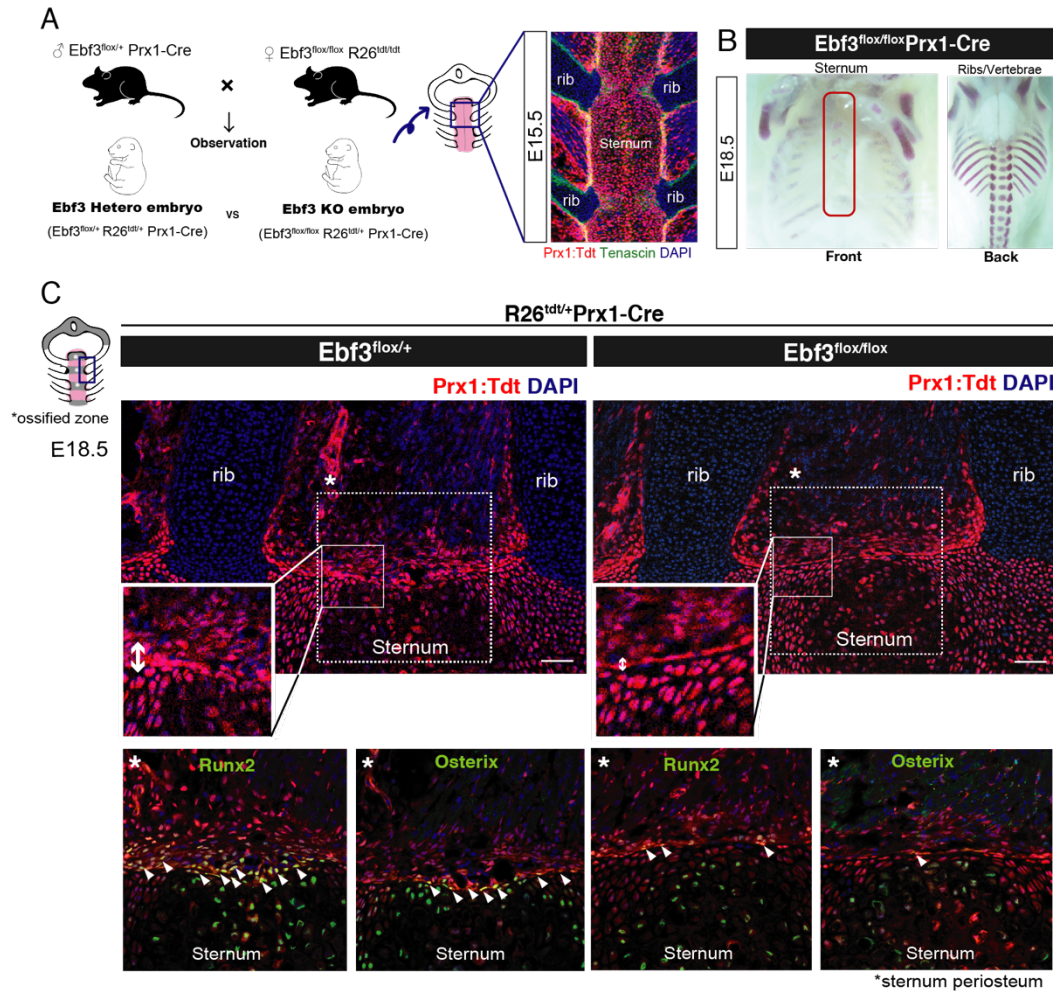


Figure 3.

Ebf3 is required in lateral plate mesoderm derived cells for sternum-osteoblast differentiation

- A) The generation of lateral plate mesoderm specific Ebf3-KO mice embryos by crossing $\text{Ebf3}^{\text{lox/+}} \text{Prx1-Cre}$ line with $\text{Ebf3}^{\text{lox/lox}} \text{Rosa26}^{\text{Tdtomato/Tdtomato}}$ mice. Expression pattern of Prx1-Cre-activated Tdtomato in the sternum tissue and connecting muscles based on longitudinal sections of E15.5 embryos together with immune-staining for Tenascin (green).
- B) Ossification analysis based on Alizarin-red skeletal preparations of thoracic bones in $\text{Ebf3}^{\text{lox/lox}} \text{Prx1-Cre}$ embryos at E18.5.
- C) Immuno-stained sternal longitudinal sections of the sternum of $\text{Ebf3}^{\text{lox/lox}} \text{Prx1-Cre}$ and $\text{Ebf3}^{\text{lox/+}} \text{Prx1-Cre}$ embryos at E18.5, for osteoblast (green, Osterix) and pre-osteoblast (green, Runx2) markers. Upper panels show distribution of lateral plate mesoderm derived cells labeled with Prx1-Cre-activated TdTomato (Red) in interstitial cells surrounding the sternum, which was decreased in Ebf3-KO embryos ($n=7$) compared to that in heterozygous mice ($n=7$) (scale bar, 100 μm). Lower-enlarged panels show much fewer Osterix/Runx2-positive lateral plate mesoderm derived cells in the periosteum of Ebf3-KO mice compared to that in heterozygous mice (white arrows).

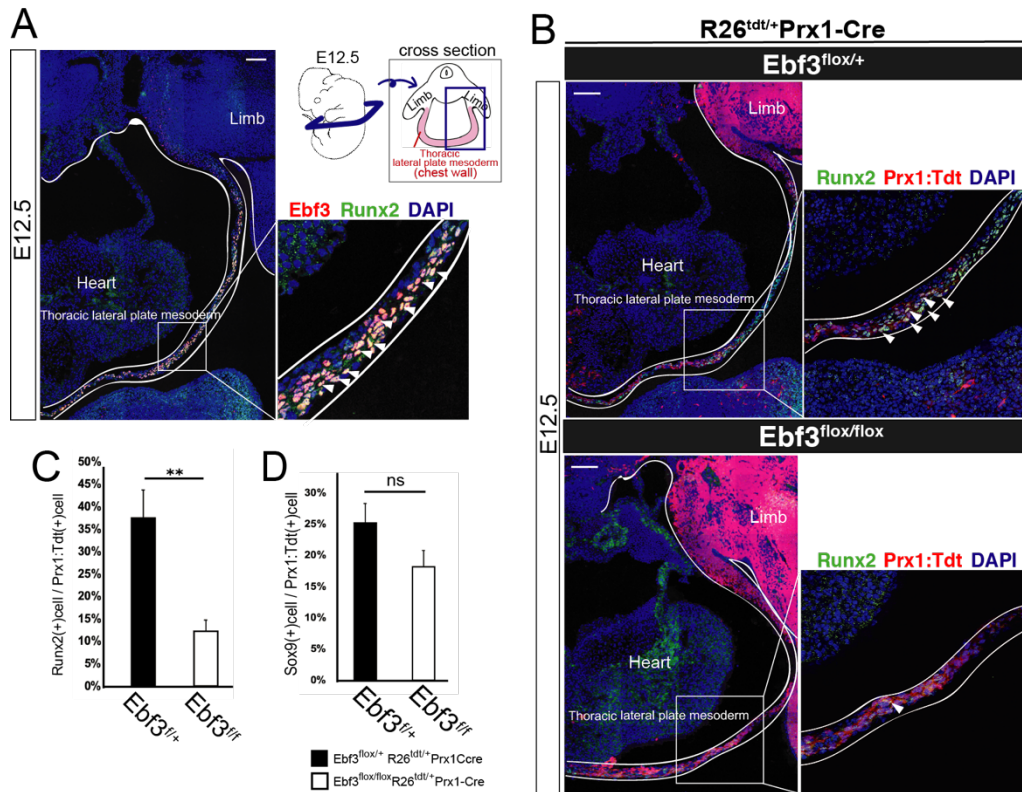


Figure 4.

Decreased number of Runx2⁺ pre-osteoblasts in Ebf3-deficient thoracic lateral plate mesoderm at E12.5

- A) Transverse section of thoracic lateral plate mesoderm at E12.5 immune-stained with anti-Ebf3 and anti-Runx2 antibodies. Most but not all Ebf3-expressing cells were Runx2-positive. White arrows show Ebf3/Runx2-double-positive cells (scale bar, 100μm).
- B) Decrease in the number of Prx1: Tdtomato⁺ Runx2⁺ cells in Ebf3^{flox/flox}Prx1-Cre compared to that in heterozygous embryos. Immuno-stained transverse sections of the thoracic lateral plate mesoderm at E12.5 are shown (white arrows) (scale bar, 100μm).
- C) The ratio of Runx2⁺ cells in Prx1: Tdtomato⁺ thoracic LPMs was lower in Ebf3^{flox/flox}Prx1-Cre KO mice than in Ebf3 heterozygous mice at E12.5. Error bars represent SEM. **P < 0.01 (n = 4).
- D) Ratio of Sox9⁺ cells in Prx1: Tdtomato⁺ thoracic LPMs at E12.5. Error bars represent SEM, ns: non-significant (n = 4).

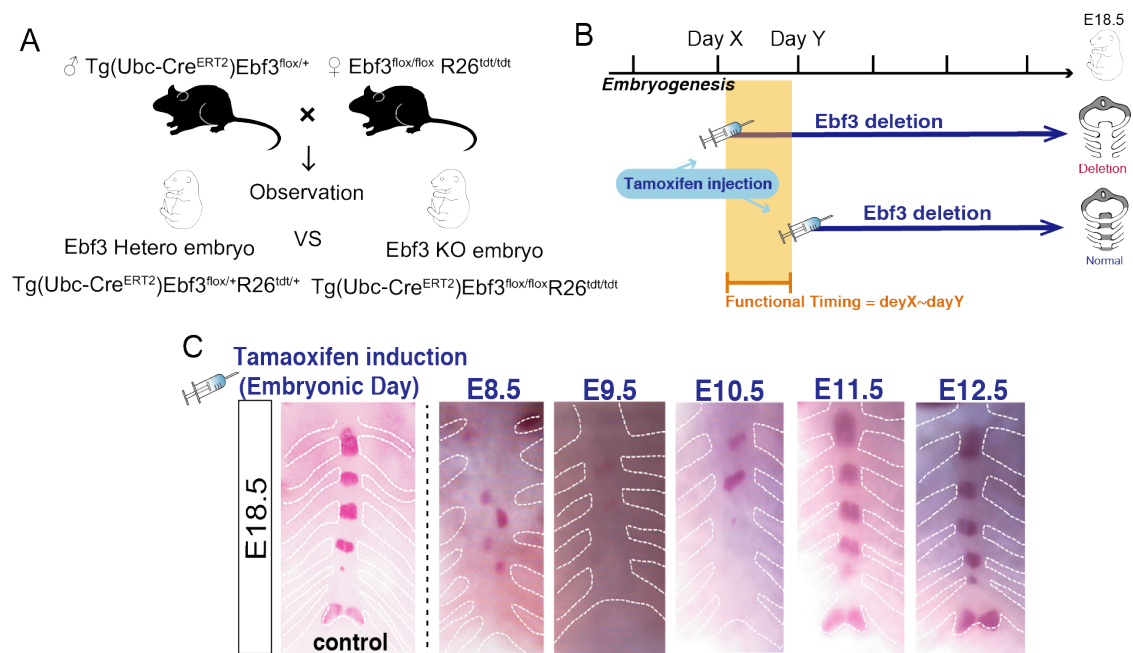


Figure 5.

Ebf3 plays a critical role in sternum ossification at approximately E9.5–E10.5

- A) Generation of tamoxifen-induced temporal Ebf3-KO mouse embryos by crossing the Ebf3^{flox/+} Ubc-CreER^{T2} line with Ebf3^{flox/flox} mice.
- B) Strategy to determine Ebf3-functional timing in sternal ossification in Ubc-CreER^{T2} Ebf3-KO embryos.
- C) Evaluation of sternum phenotypes in Ebf3^{flox/flox} Ubc-CreER^{T2} embryos upon tamoxifen injections at different developmental stages (E8.5–E12.5) based on Alizarin-red skeletal preparations.

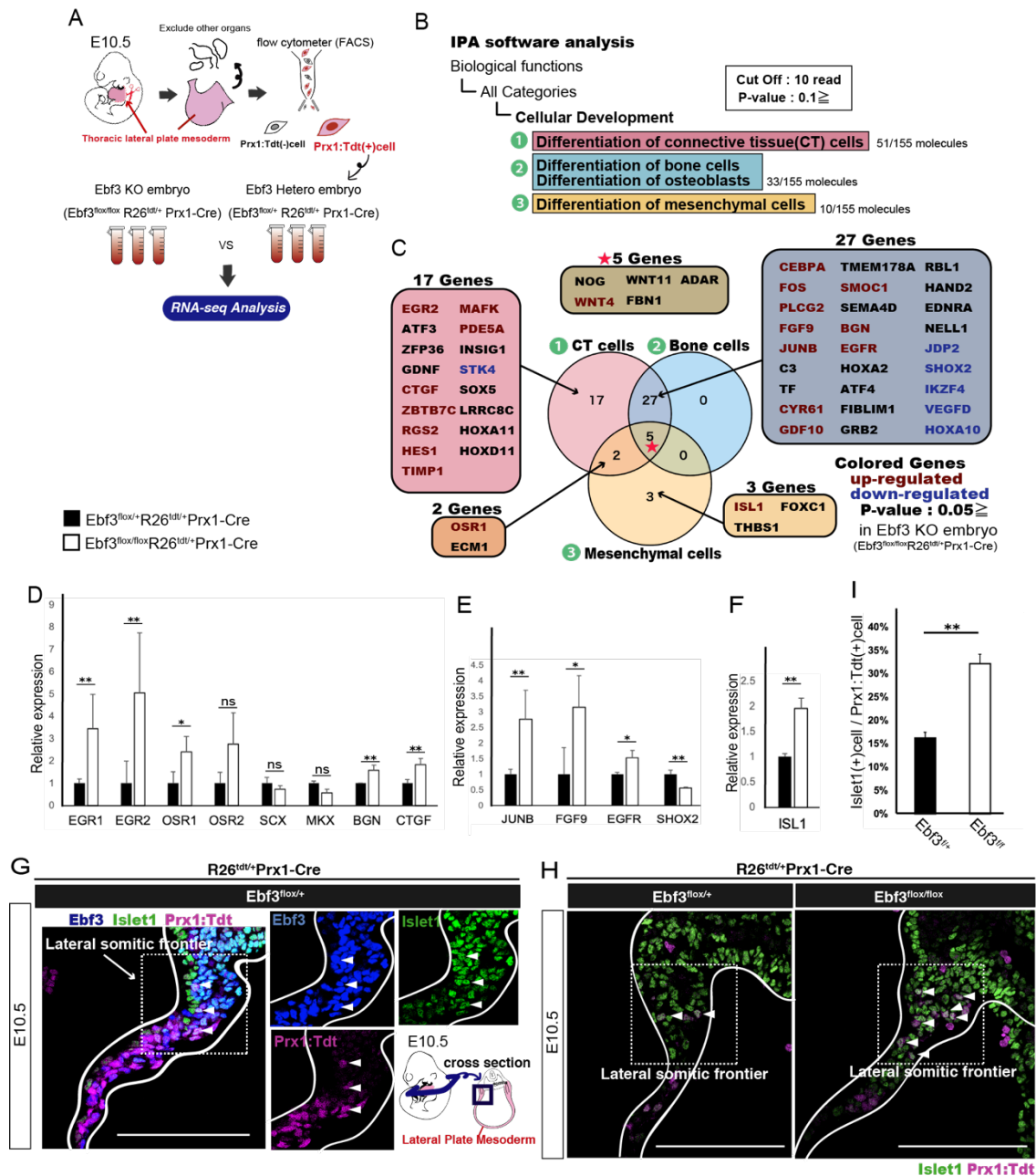


Figure 6.

Ebf3-downstream analysis in mesenchymes of thoracic lateral plate mesoderm by RNA-seq

- A) Thoracic LPMs were excised from Ebf3^{lox/lox}Prx1-Cre and Ebf3^{lox/+} Prx1-Cre embryos at E10.5, and Prx1: Tdtomato⁺ cells were isolated by fluorescence-activated cell sorting (FACS) (n = 3).
- B) Differentially-expressed genes based on RNA-sequence data between Ebf3^{lox/lox}Prx1-Cre and Ebf3^{lox/+} Prx1-Cre embryos (Cut off:10 read, P $\leq$ 0.1) were categorized by their biological functions using ingenuity pathway analysis (IPA) software into three groups: (1) genes required for the differentiation of connective-tissue (CT) cells, (2) Genes required for the differentiation of bone cells and osteoblasts, and (3) genes required for the differentiation of mesenchymal cells.

- C) Venn diagram of these three groups. Red-colored genes were significantly upregulated and blue-colored genes were significantly downregulated in Ebf3^{flox/flox}Prx1-Cre embryos compared to expression in Ebf3^{flox/+} Prx1-Cre embryos ($P < 0.05$) (from RNA-seq data).
- D) Relative expression levels of muscle connective tissue (MCT) cell/tenocyte-associated genes in Ebf3^{flox/flox}Prx1-Cre embryos compared to those in Ebf3^{flox/+} Prx1-Cre embryos (from RNA-seq data).
- E) Relative expression levels of osteoblast-associated genes in Ebf3^{flox/flox}Prx1-Cre embryos compared to those in Ebf3^{flox/+} Prx1-Cre embryos, $**P < 0.01$, $*P < 0.05$ (from RNA-seq data).
- F) Relative expression level of Islet1 in Ebf3^{flox/flox}Prx1-Cre embryos was increased significantly compared to that in Ebf3^{flox/+} Prx1-Cre embryos, $**P < 0.01$ (from RNA-seq data).
- G) Islet1 was expressed in Prx1:Tdtomato⁺ Ebf3⁺ cells at the lateral somitic frontier of the thoracic lateral plate mesoderm at E10.5 (white arrows) (scale bar, 100 μ m).
- H) Increase in the number of Prx1:Tdtomato⁺ Islet1⁺ cells in Ebf3^{flox/flox}Prx1-Cre (n=3) compared to that in heterozygous embryos (n=3). Immuno-stained transverse sections of the lateral somitic frontier at E10.5 are shown (white arrows) (scale bar, 100 μ m).
- I) The ratio of islet1⁺ cells in Prx1:Tdtomato⁺ LPMs increased in Ebf3^{flox/flox}Prx1-Cre compared to that in heterozygous mice at E10.5. Error bars represent SEM. $**P < 0.01$ (n = 3).

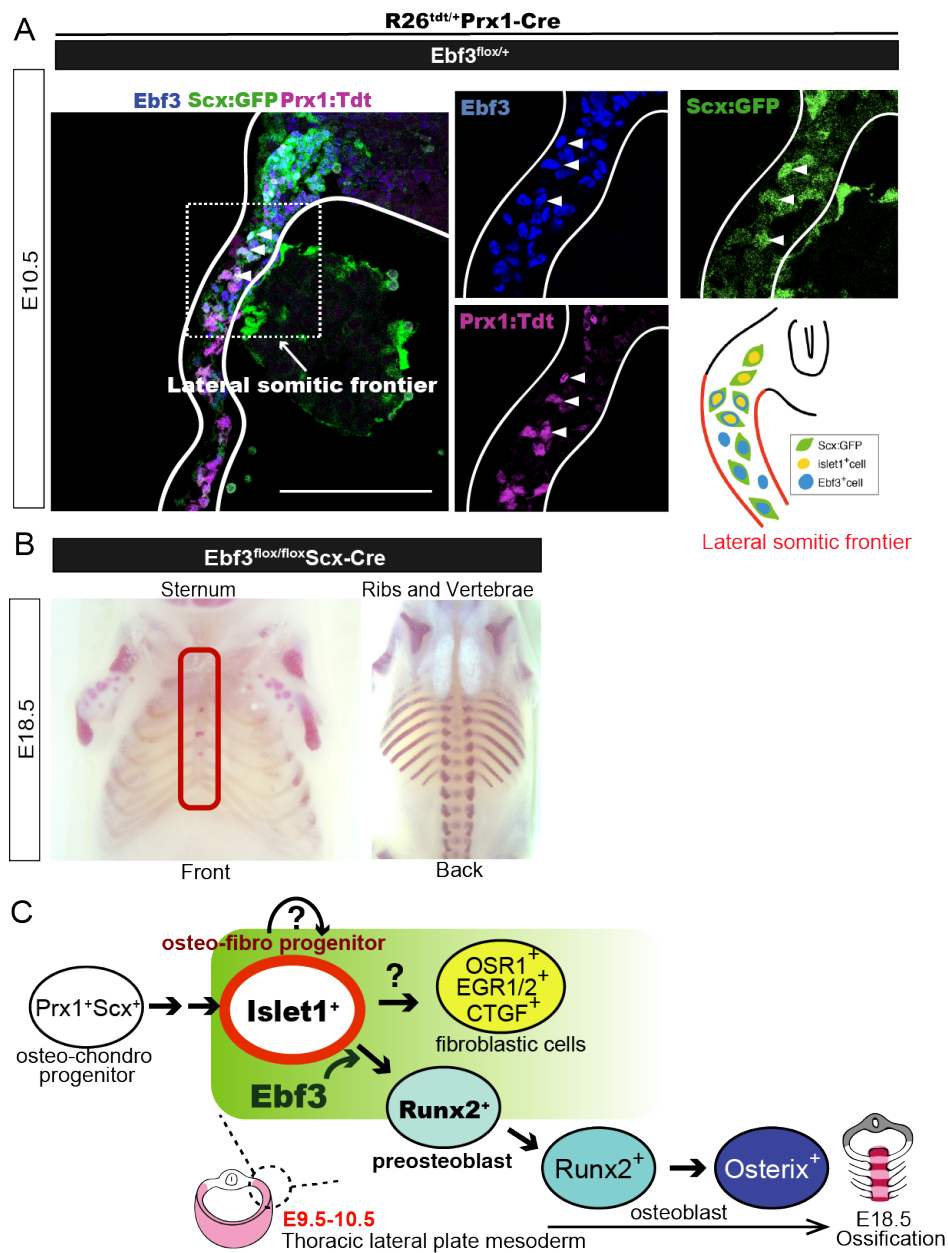


Figure 7.

Ebf3 regulates mesenchymal progenitors in Scx-Cre lineage cells of the thoracic lateral plate mesoderm

A) Ebf3 was expressed in Prx1:Tdt⁺ Scx:GFP⁺ cells at the lateral somitic frontier of the thoracic lateral plate mesoderm at E10.5 (white arrows) (scale bar, 100μm).

B) Ossification analysis based on Alizarin-red skeletal preparations of thoracic bones in Ebf3^{flox/flox} Scx-Cre embryos at E18.5.

C) Roles of Ebf3 in sternum osteoblast differentiation.