Transcriptional regulation of gilthead seabream bone morphogenetic protein (BMP) 2 gene by bone- and cartilage-related transcription factors

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Abstract

Bone morphogenetic protein (BMP) 2 belongs to the transforming growth factor β (TGFβ) superfamily of cytokines and growth factors. While it plays important roles in embryo morphogenesis and organogenesis, BMP2 is also critical to bone and cartilage formation. Protein structure and function have been remarkably conserved throughout evolution and BMP2 transcription has been proposed to be tightly regulated, although few data is available. In this work we report the cloning and functional analysis of gilthead seabream BMP2 promoter. As in other vertebrates, seabream BMP2 gene has a 5' non-coding exon, a feature already present in DPP gene, the fruit fly ortholog of vertebrate BMP2 gene, and maintained throughout evolution. In silico analysis of seabream BMP2 promoter revealed several binding sites for bone and cartilage related transcription factors (TFs) and their functionality was evaluated using promoter-luciferase constructions and TF-expressing vectors. Runt-related transcription factor 3 (RUNX3) was shown to negatively regulate BMP2 transcription and combination with the core binding factor β (CBFβ) further reduced transcriptional activity of the promoter. Although to a lesser extent, myocyte enhancer factor 2C (MEF2C) had also a negative effect on the regulation of BMP2 gene transcription, when associated with SRY (sex determining region Y)-box 9 (SOX9b). Finally, v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1) was able to slightly enhance BMP2 transcription. Data reported here provides new insights toward the better understanding of the transcriptional regulation of BMP2 gene in a bone and cartilage context.

1. Introduction

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the TGFβ superfamily and form a subfamily with more than 20 members (Bragdon et al., 2011). BMP2 was first identified in bone and later associated with the control of osteogenesis and chondrogenesis through BMP signaling pathway (reviewed by Carreira et al., 2014; Rosen, 2009). Beside its critical role during skeletogenesis, BMP2 is also involved in many other physiological processes, such as embryonic patterning and organogenesis (reviewed in Asahina, 2014; Hogan, 1996). BMP2 gene is flanked by regions classified as gene deserts (long regions without nearby genes) that may contain important regulatory elements, and the presence of long-range elements controlling BMP2 transcription was reported in mammals (Chandler et al., 2007; Dathe et al., 2009). The remarkable conservation of protein structure and function (Carreira et al., 2014) conjugated with its crucial role during development, maintained throughout vertebrate evolution, suggest that BMP2 transcription may be tightly controlled (Sugiura, 1999). The conservation of BMP2 gene, in particular its promoter region, has been reported in mammals (i.e. mouse and human; Abrams et al., 2004; Sugiura, 1999) and binding sites for several bone- and cartilage-related transcription factors (TFs), such as RUNX and SOX9, were predicted. Although the activation of human BMP2 promoter by RUNX2 has not been proved (Helvering et al., 2000), RUNX2 was shown to effectively increase BMP2 gene transcription while BMP2 was also able to regulate RUNX2 transcription in a feedback regulatory mechanism (Choi et al., 2005). Surprisingly, not much more is known about transcriptional regulation of BMP2 by bone- and cartilage-related TFs and thus much remains to be done regarding this question.

By sharing with mammals a number of important characteristics (e.g. gene functions, organ systems and physiological/biochemical mechanisms) fish have been recognized as a suitable alternative to the mammalian systems, in particular for genetic studies (Laizé et al., 2014). During the last years several biochemical, molecular and cellular tools have been developed from the gilthead seabream (Fonseca et al., 2011; Marques et al., 2007; Pombinho et al., 2004; Tiago et al., 2014). This together with the increasing availability of gene and transcripts in public sequence databases validated the gilthead seabream as a suitable model to better understand mechanisms of gene regulation, and this was further confirmed by several genetic and functional studies (Conceição et al.,

2008; Ferraresso et al., 2008; González-Mariscal et al., 2014; Rafael et al., 2006; Rosa et al., 2014).

The aim of this work is to evaluate the activity of gilthead seabream *bmp2* promoter and get insights into its transcriptional regulation by bone- and cartilage-related transcription factors. The presence of *cis*-regulatory elements will be predicted in silico and their functionality will be accessed through luciferase reporter assays.

Materials and methods

Amplification of genomic DNA

5' flanking region and intron I of gilthead seabream *bmp2* were amplified by PCR from a *Sca*I GenomeWalker library (Clontech) using Advantage Polymerase Mix (Clontech), 0.2 µM of Adaptor Primer 1 (AP1; initial PCR) or AP2 (nested PCR) and gene-specific primers SauBMP2_1Rv or SauBMP2_2Rv (initial PCR), and SauBMP2_3Rv or SauBMP2_4Rv (nested PCR), respectively. Nested PCR was performed using a 1:50 dilution of the initial PCR. DNA fragments were separated on agarose gel, purified using GeneJET Gel Extraction kit (Thermo Scientific), cloned into TOPO vector (Life Technologies) and sequenced on both strands. Gene-specific primers were designed according to the sequence available in GenBank (accession no. AY679787) and are listed in Table 1.

Preparation of promoter-luciferase and deletion constructs

Constructs containing 5' flanking region and intron I (construct C1, -1531/+53) or only intron I (construct C2, +301/+1282) of gilthead seabream *bmp2* were amplified using reverse primer SauBMP2_*Hin*dIII_5Rv in combination with forward primers SauBMP2_*Xho*I_1Fw and SauBMP2_*Kpn*I_2Fw, respectively. Deletion constructs of 5' flanking region were amplified using reverse primer SauBMP2_*Hin*dIII_6Rv in combination with forward primers SauBMP2_*Xho*I_1Fw (construct C3, -1531/+53), SauBMP2_*Xho*I_3Fw (construct C4, -842/+53), SauBMP2_*Xho*I_4Fw (construct C5, -656/+53), SauBMP2_*Xho*I_5Fw (construct C6, -367/+53), SauBMP2_*Xho*I_6Fw (construct C7, -294/+53) and SauBMP2_*Xho*I_7Fw (construct C8, -59/+53). DNA fragments were digested with *Hin*dIII and *Xho*I or *Kpn*I endonucleases and directionally cloned into pGL3 vector (Promega) upstream the firefly luciferase gene. All construct

were sequenced on both strands to confirm direction and absence of mutations. Primers used for PCR amplification of these constructs are listed in Table 1.

In silico sequence analysis

Presence of *cis*-regulatory elements, i.e. transcription factor binding sites, in the 5' flanking region and intron I of gilthead seabream *bmp2*, were predicted using MatInspector (V7.1; Cartharius et al., 2005) at www.genomatix.de and PATCH (Vpublic 1.0; Chekmenev et al., 2005) at www.gene-regulation.com. Sites with scores below 0.75 (MatInspector) and 0.85 (PATCH) were not considered. Repetitive sequences were identified using RepeatMasker software (Vopen-4.0.5) at www.repeatmasker.org.

Cell culture and transient transfection assays

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 1% of penicillin-streptomycin (Life Technologies) and 1% of L-glutamine (Life Technologies), and maintained at 37ºC in a 5% CO_2 humidified atmosphere. Sub-confluent cultures were trypsinized every three days and cells seeded in a 10-cm plate at a density of 8.7×10^5 cells/plate. The day before the transfection, cells were seeded in a 24-well plate at a density of 5×10^4 cells/well, then further cultured for 16 h. Cultures at 50-60% confluence were transfected with 250 ng of each of the DNA constructs using 1 µl of X-tremeGENE HP DNA transfection reagent (Roche). When appropriate, expression vectors (50 ng; pCMX backbone) containing the coding sequence of zebrafish *ets1a* (KF774190), *cbfß* (KF709197), *runx3* (MASN isoform; AB043789), *mef2ca* (BC059188), *mef2cb* (EU825718) and *sox9b* (NM_131644), under the control of CMV promoter, were co-transfected with selected constructs of *bmp2* promoter. pRL-null vector (Promega), which express *Renilla* luciferase (Rluc) but lacks promoter and enhancer elements, was used in all the transfections (25 ng) to normalize the firefly luciferase (Fluc) activity. After 48 h, cells were lysed and luciferase activity was measured using Dual-Luciferase Reporter Assay system (Promega) in a BioTek Synergy 4 plate reader. Luciferase activity was determined from the ratio Fluc/Rluc.

Results and Discussion

BMP2 gene structure has been conserved throughout evolution

Sequences of BMP2 gene were collected from several vertebrate species (2 genes in zebrafish) using on-site Blast facilities of Ensembl database and their structure was compared with that of the gilthead seabream gene, recently cloned in our lab (GenBank accession no. AY679787) and with fruit fly *dpp* gene, the ancestor of vertebrate BMP2 genes (Kingsley, 1994; Fig. 1). In zebrafish (and probably in most Ostariophysi), two genes – *bmp2a* and *bmp2b* – were identified, while only one gene has been reported in the genome of other vertebrates, and *bmp2b* would represent the orthologous gene (Marques et al., 2015). BMP2 gene structure has been remarkably conserved throughout evolution and orthologs in vertebrates and fruit fly share the same simple structure: three exons – including a 5´non-coding exon – and two introns inserted within the same phase in all species evaluated. Transcription start site (TSS) was determined in gilthead seabream *bmp2* from the longest RACE-PCR fragment and in other species, if not already available, from the longest cDNA or EST sequence available in public databases. Although its size is variable (from 246 to 1212 nucleotides), BMP2 5' non-coding exon has been conserved throughout evolution and may work as an additional mechanism of regulation (Barrett et al., 2013). The presence of 5' non-coding exons has been observed in genes which transcription is under the control of alternative promoters (Banday et al., 2012; Conceição et al., 2008). In mammals, while some studies report the existence of at least two major TSSs driven by two alternative promoters (Feng et al., 1997; Ghosh-Choudhury et al., 2001; Sugiura, 1999), others defend the occurrence of a single TSS regulated by a single promoter located in the 5' flanking region of the gene (Heller et al., 1999; Helvering et al., 2000). GenBank and Ensembl public databases were thoroughly searched for vertebrate BMP2 transcripts and among the several hundreds of hits collected none indicated transcript variants with alternative 5'UTR and therefore no evidence of BMP2 gene transcription being under the control of alternative promoters was found (results not shown). Thus, both the 5' flanking region and intron I were analyzed in silico for the presence of regulatory elements and further tested, independently or in combination, for their capacity of regulating luciferase gene transcription.

In silico analysis and basal activity of gilthead seabream bmp2 promoter and intron I

RepeatMasker software revealed the presence of several repetitive sequences, namely T-rich regions, tri and tetra nucleotide repeats, in both 5´flanking region and intron I of

gilthead seabream *bmp2* (Fig. 2). Repetitive sequences, also known as DNA satellites, are commonly found in vertebrate genomes (Tomilin, 2008). When discovered, repetitive sequences were described as “junk” or “parasitic” DNA (Doolittle and Sapienza, 1980; Orgel and Crick, 1980), but nowadays they are widely recognized as essential for genome function (Shapiro and von Sternberg, 2005). Tandem repeats can vary both in number and in length of the repeat unit (Gemayel et al., 2010) and certain types of repeats have been associated with protein binding sites, interaction with transcription factors or disease development (Baldi and Baisnee, 2000). T-rich regions have been described as transcriptional activators of both prokaryotic and eukaryotic promoters (Haque et al., 2004; Kube et al., 1999; Nishi and Itoh, 1986; Sohaskey et al., 1999) while some classes of triplet DNA repeats were associated with the development of neurodegenerative disorders, such as Huntington’s disease and fragile X syndrome (Walker, 2007). Tetranucleotide repeats are among the most common repeats found in the genome of vertebrate species, and elevated microsatellite alterations at selected tetranucleotide repeats were observed in several types of cancer (Bacolla et al., 2008; Katti et al., 2001). PATCH software was unable to identify TATA or CAAT consensus sequences upstream the TSS of gilthead seabream *bmp2*. TATA-less promoters, also known as dispersed promoters, are very common in vertebrate genes (Barrett et al., 2013; Gagniuc and Ionescu-Tirgoviste, 2012) and several BMP genes have been reported to have TATA-less promoters (Hino et al., 1996; Kawai and Sugiura, 2001; Shore et al., 1998; Simon et al., 2002; Tamada et al., 1998), including human and mouse BMP2 genes (Ghosh-Choudhury et al., 2001; Sugiura, 1999). A survey of other BMP2 genes (i.e. zebra finch, spotted gar, zebrafish and fruit fly; Fig. 1) failed to identify TATA boxes upstream TSS, suggesting that the presence of TATA-less promoters in BMP2 genes is a common feature. To further evidence the TATA-less nature of gilthead seabream *bmp2* promoter, various SP1 binding sites, GC-rich regions normally present in dispersed promoters (Pugh and Tjian, 1991; Smale and Kadonaga, 2003), were predicted using PATCH software in the 5’ flanking region and intron I (Fig. 2).

In order to evaluate the functionality of these regions in regulating *bmp2* transcription, 5’ flanking region and intron I or only intron I (C1 and C2, respectively; Fig.2) were cloned into pGL3 vector, upstream from the firefly luciferase gene. With the purpose of identifying regulatory regions in the 5’ flanking region, several deletion constructs (C3 to C8; Fig.2) were individually transfected into HEK-293 cells (selected for their

remarkable transfectability, at rates hundred times superior to those in bone cells, in particular of fish origin) and their activity determined from firefly luciferase activity measurements normalized with the activity of the promoter less pGL3 basic vector. While 5’ flanking region (C3) increased 15 times the luciferase activity in HEK-293 cells, intron I (C2) failed to trigger any transcriptional activity, indicating that (1) intron I does not have any transcriptional activity, (2) does not function as a stand-alone and alternative promoter but as an enhancer/silencer of the main promoter (i.e. the 5’ flanking region), or (3) HEK-293 cells are not suitable to study its transcriptional activity (e.g. they do not express the necessary factors). Although this should be further confirmed, the 3-fold decrease in luciferase activity observed when 5’flanking region and intron I were combined (C1) suggest that intron I may serve as a silencer of *bmp2* transcription and in fact, several TF-binding sites were predicted in this region (results not shown).

Transfections of deletion constructs related to the 5’ flanking region (C4-C8) resulted in a gradual decrease of luciferase activity that may be linked to the presence of binding sites for positive regulators in the deleted promoter regions. At least two enhancers may be present in *bmp2* promoter, as suggested by the two drops in luciferase activity observed from C4 to C5 and from C6 to C7. Although additional studies are required to confirm the presence of enhancers in these regions (e.g. site-directed mutagenesis), we propose that the T-rich sequence identified through *in silico* analysis (present in C6 but absent in C7) may be responsible for the halving of the luciferase activity observed in C7.

Runx3 *and* Cbfβ *interact to regulate seabream bmp2 promoter*

The 5´flanking region of gilthead seabream *bmp2* was analyzed using MatInspector and PATCH online tools, to identify *cis*-regulatory elements that may be involved in its regulation. Several putative binding sites for Runt-related transcription factors (Runx) were identified (Fig. 3A), similarly to what has been described in human *BMP2* and *BMP4* gene promoters (Helvering et al., 2000). While the role of RUNX2 in the regulation of BMP2 gene transcription has already been demonstrated in mouse and human (Choi et al., 2005; Javed et al., 2008; Yang et al., 2003), the transcriptional action of RUNX3 remains unknown, although it has been clearly associated with mechanisms of skeletogenesis and chondrogenesis (Soung et al., 2007; Wigner et al., 2013; Yoshida et al., 2004; Zheng et al., 2007). Co-expression of RUNX3 and BMP2

was reported in mouse dental pulp cells and in human osteosarcoma cell line U-2 OS and HEK-293 (Zheng et al., 2007) and up-regulation of *runx3* expression was recently associated with tissue mineralization in the notochord of Atlantic salmon (Uhlén et al., 2015; Wang et al., 2014). For these reasons and in order to evaluate the functionality and the regulatory potential of the predicted sites, HEK-293 cells were co-transfected with *bmp2* promoter constructs and vectors expressing zebrafish Runx3 (MASN isoform) and/or zebrafish Cbfβ, a transcriptional co-regulator of Runx factors (Fig. 3B; Warren et al., 2000). Runx3 expression decreased luciferase activity (1.7 fold in C3 and 1.3 fold in C5), suggesting that it may work as a negative regulator of BMP2 transcription. While CBFβ expression did not significantly change luciferase activity, the co-expression of Runx3 and Cbfβ strongly decreased luciferase expression in C3 (6.7 fold) and in C5, although to a lesser extent (2.3 fold). Cbfβ cannot bind to DNA (Gu et al., 2000) and was therefore not expected to trigger any change in *bmp2* promoter activity. But it is known to efficiently mediate the interaction of Runx family members with the transcription machinery (Blake et al., 2000; Wang et al., 1993) and the potentiation of the transcriptional regulation of *bmp2* gene by Runx3 further confirm the capacity of Cbfβ in co-regulating gene transcription. Data reported by Kundu and co-workers (Kundu et al., 2002) revealed that, in bone and cartilage tissues, CBFβ interacts with RUNX2, enhancing its transactivation capability. Similarly RUNX genes were shown to be susceptible to auto- and cross-regulation by RUNX family members (Drissi et al., 2000; Spender et al., 2005), an effect further enhanced upon addition of its CBFβ partner (Conceição et al., 2013; Simões et al., unpublished data).

A significant decrease in luciferase activity was observed when the region in C3 containing 4 putative Runx binding sites (-1429/-1424; -984/-980; -847/-843; -695/-688), and later on the region in C5 containing 2 possible Runx sites (-387/-380; -299/-295) were removed, indicating the presence of functional responsive elements for Runx3/Cbfβ in the regions -1531/-656 and -656/-294. Expression of Runx3 alone or in combination with CBFβ did not significantly modified luciferase activity in C7 nor in C8 (data not shown), indicating that the binding site located in C7 (-64/-60) is probably not functional. Future studies should aim at identifying the functional Runx sites (e.g. through site-directed mutagenesis of putative DNA binding elements) and at confirming Runx3/Cbfβ interaction (e.g. through electrophoretic mobility shift assay and chromatin immunoprecipitation assay).

Here, we provide evidence for the capacity of Runx3 to regulate *bmp2* transcription, suggesting its involvement in bone and cartilage metabolism, an hypothesis that should be explored in future studies.

Mef2c/Sox9b *negatively regulate bmp2 transcription*

The presence of several *cis*-regulatory elements related to Mef2 and Sox9 was also predicted in gilthead seabream *bmp2* promoter (Fig. 4A). To evaluate their functionality, promoter constructs were co-transfected in HEK-293 cells with vectors expressing zebrafish *mef2c* (a mixture of zebrafish *mef2ca* and *mef2cb* were used, since both forms produced similar results) or *sox9b*. Because Mef2c is known to physically interact with members of the Sox family (Agarwal et al., 2011), *mef2c* and *sox9b* expression vectors were also co-transfected in some experiments (Fig. 4B). A mild repression (up to 2 fold) of *bmp2* promoter activity was observed upon co-transfection of C3 promoter construct with *mef2c* or *sox9b* expression vectors, indicating that both factors are negative regulators of *bmp2* gene transcription. Co-transfection of both factors did not significantly change luciferase activity but deletion of the region containing the two predicted binding sites for Sox9 in C4 abolished not only the negative regulation by Sox9b, but also the negative regulation by Mef2c, even though no binding site for Mef2c was removed. Although we cannot exclude that in silico analysis failed to predict Mef2 binding site(s) in this region, we propose that Mef2c regulation of *bmp2* gene transcription is Sox9b-dependent, in a way similar to what has been reported for *Col10a1* in mice (Dy et al., 2012). Inhibition in C3 upon individual expression of Mef2c or Sox9b could be related to the presence of endogenous Sox9, which transcript was detected at basal levels in HEK-293 cells (Blache et al., 2004). The negative regulation by Mef2c/Sox9b was attenuated (1.2 fold) upon deletion of the region containing two predicted MEF2C responsive elements (C5). The decrease on luciferase activity observed in C4 and C5 upon expression of *sox9b* or co-expression of *mef2c* and *sox9b* could be related to the presence of a Sox9 binding site(s) not predicted through *in silico* analysis. Both MEF2C and SOX9 factors have been implicated in the regulation of bone and cartilage formation (Dy et al., 2012; Mackie et al., 2008) and are co-expressed with *BMP2* in several cell lines including the human osteosarcoma U-2 OS and HEK-293 cells (see the Human Protein Atlas database at www.proteinatlas.org). There are also evidences of Mef2c and Sox9 being regulators of BMP signaling pathway mediators (Dalcq et al., 2012; Wu et al., 2010) and vice versa; the expression

of both transcription factors was shown to be regulated by Bmp2 (Kawakami et al., 2006; Zheng et al., 2013). In agreement with data presented here, Liao and co-workers recently demonstrated that *Bmp2* expression is lowered in *Sox9*-enhanced chondrogenesis in mouse cells (Liao et al., 2014). Dy and co-authors have recently reported a cooperation between SOX9 and MEF2C during cartilage formation in mouse (Dy et al., 2012), in a way similar to the cooperation reported here. In this study, the stimulation of *Col10a1* transcription by MEF2C was SOX9-dependent, and MEF2C-enhancing capacity was lost upon SOX9 inactivation, even after *Mef2c* overexpression (Dy et al., 2012).

Ets1 *enhances bmp2 transcription*

Four binding sites for Ets1 were predicted in gilthead seabream *bmp2* promoter and their functionality was tested through co-transfection in HEK-293 cells of promoter deletion constructs and vector expressing zebrafish *ets1* (Fig. 4C). A 2 fold increase of luciferase activity was observed in C3 upon expression of *ets1*, indicating that it is a positive albeit weakly regulator of *bmp2* transcription. Deletion of the region containing 3 of the responsive elements in C5 and C7 constructs (-1502/-1496; -984/-980; -847/-843) did not affect Ets1 transactivation of *bmp2* promoter, suggesting that those sites are most likely not functional. On the contrary, deletion of the region containing the responsive element located at (-64/-60) in the C8 construct decreased luciferase activity to basal levels. Although this should be confirmed (e.g. through site-directed mutagenesis of the specific DNA binding elements), we propose that the -64/-60 binding site is functional and accounts for the totality of Ets1 activity. Members of ETS family of transcription factors are expressed at the onset of bone formation (Raouf and Seth, 2000; Uhlén et al., 2015) and are co-expressed with *BMP2* in several cell lines including the HEK-293 (see the Human Protein Atlas database at www.proteinatlas.org). ETS family members have been associated with mechanisms regulating osteogenic and chondrogenic processes in vertebrates (Gao et al., 2005; Rosa et al., 2014). In mice, ETS1 has been shown to cooperate with other regulatory proteins to modulate transcription of bone and cartilage related genes – e.g. with RUNX2 to regulate osteopontin gene transcription (Miyake et al., 1998), and with retinoic acid receptor to regulate RA-induced expression of PTHrP (Karperien et al., 1997). The possibility of Ets1 cooperating with Runx3 in the regulation of *bmp2* transcription was evaluated through the co-transfections of *ets1* and *runx3* expression vectors with

promoter constructs, but no cooperative effect was observed in our experimental system (data not shown). Our data suggest, for the first time that Ets1 is able to activate seabream *bmp2* transcription.

Conclusions

We have collected within the scope of this work valuable data towards a better understanding of the transcriptional regulation of BMP2 gene. The high conservation of BMP2 gene structure among vertebrates – in particular the presence of a 5' non-coding exon – and the prediction of similar binding sites for RUNX/MEF2/SOX9/ETS1 transcription factors in gilthead seabream and human BMP2 genes suggest that these results, collected in a fish system, are probably valid in other vertebrate system, in particular in human. Functional analysis of promoter-luciferase constructs suggests that 5' flanking region of gilthead seabream gene contains several responsive elements for selected transcription factors and therefore corresponds to a functional *bmp2* promoter, while intron I might contribute to silence promoter activity. The functionality of several binding sites for bone and cartilage related factors predicted in silico was confirmed *in vitro*, highlighting the relevance of performing in silico analysis prior to functional assays. Runx3 is a negative regulator of *bmp2* gene transcription and its activity is enhanced by the co-factor Cbfβ. Similarly, Sox9b and Mef2c, in a Sox9-dependent manner, are also negative regulators of *bmp2* transcription, while Ets1 is a positive regulator, although a weak one. Current knowledge on the transcriptional regulation of seabream *bmp2* promoter has been summarized in Fig. 5. Although the data reported here will require further studies, it provides new evidences on the regulation of BMP2 transcriptional activity by bone- and cartilage- related transcription factors.

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References

Abrams, K.L., Xu, J., Nativelle-Serpentini, C., Dabirshahsahebi, S., Rogers, M.B., 2004. An evolutionary and molecular analysis of Bmp2 expression. J. Biol. Chem. 279, 15916–15928. doi:10.1074/jbc.M313531200

Agarwal, P., Verzi, M.P., Nguyen, T., Hu, J., Ehlers, M.L., McCulley, D.J., Xu, S.-M., Dodou, E., Anderson, J.P., Wei, M.L., Black, B.L., 2011. The MADS box transcription factor MEF2C regulates melanocyte development and is a direct transcriptional target and partner of SOX10. Development 138, 2555–2565. doi:10.1242/dev.056804

Asahina, I., 2014. Review Bone Morphogenetic Proteins : Their History and Characteristics 283–286.

Bacolla, A., Larson, J.E., Collins, J.R., Li, J., Milosavljevic, A., Stenson, P.D., Cooper, D.N., Wells, R.D., 2008. Abundance and length of simple repeats in vertebrate genomes are determined by their structural properties. Genome Res. 18, 1545–1553. doi:10.1101/gr.078303.108

Baldi, P., Baisnee, P.-F., 2000. Sequence analysis by additive scales: DNA structure for sequences and repeats of all lengths. Bioinformatics 16, 865–889. doi:10.1093/bioinformatics/16.10.865

Banday, A.R., Azim, S., Tabish, M., 2012. Differentially expressed three non-coding alternate exons at 5' UTR of regulatory type I beta subunit gene of mouse. Mol. Biol. Rep. 39, 3375–3383. doi:10.1007/s11033-011-1108-4

Barrett, L.W., Fletcher, S., Wilton, S.D., 2013. Untranslated gene regions and other non-coding elements. SpringerBriefs Biochem. Mol. Biol., SpringerBriefs in Biochemistry and Molecular Biology 1, 1–57. doi:10.1007/978-3-0348-0679-4

Blache, P., van de Wetering, M., Duluc, I., Domon, C., Berta, P., Freund, J.-N., Clevers, H., Jay, P., 2004. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. J. Cell Biol. 166, 37–47. doi:10.1083/jcb.200311021

Blake, T., Adya, N., Kim, C.-H., Oates, A.C., Zon, L., Chitnis, A., Weinstein, B.M., Liu, P.P., 2000. Zebrafish homolog of the leukemia gene CBFβ: its expression during embryogenesis and its relationship to scl and gata-1 in hematopoiesis. Blood 96, 4178–4184.

Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J., Nohe, A., 2011. Bone morphogenetic proteins: a critical review. Cell. Signal. 23, 609–620. doi:10.1016/j.cellsig.2010.10.003

Carreira, A.C., Lojudice, F.H., Halcsik, E., Navarro, R.D., Sogayar, M.C., Granjeiro, J.M., 2014. Bone morphogenetic proteins: facts, challenges, and future perspectives. J. Dent. Res. 93, 335–345. doi:10.1177/0022034513518561

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., Werner, T., 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21, 2933–2942. doi:10.1093/bioinformatics/bti473

Chandler, R.L., Chandler, K.J., McFarland, K.A., Mortlock, D.P., 2007. Bmp2 transcription in osteoblast progenitors is regulated by a distant 3' enhancer located 156.3 kilobases from the promoter. Mol. Cell. Biol. 27, 2934–2951. doi:10.1128/MCB.01609-06

Chekmenev, D.S., Haid, C., Kel, A.E., 2005. P-Match: transcription factor binding site search by combining patterns and weight matrices. Nucleic Acids Res. 33, W432–W437. doi:10.1093/nar/gki441

Choi, K.-Y., Kim, H.-J., Lee, M.-H., Kwon, T.-G., Nah, H.-D., Furuichi, T., Komori, T., Nam, S.-H., Kim, Y.-J., Ryoo, H.-M., 2005. Runx2 regulates FGF2-induced Bmp2 expression during cranial bone development. Dev. Dyn. 233, 115–121. doi:10.1002/dvdy.20323

Conceição, N., Laizé, V., Simões, B., Pombinho, A.R., Cancela, M.L., 2008. Retinoic acid is a negative regulator of matrix Gla protein gene expression in teleost fish Sparus aurata. Biochim. Biophys. Acta 1779, 28–39. doi:10.1016/j.bbagrm.2007.11.003

Conceição, N., Simões, B., Cancela, M.L., 2013. Functional analysis of the two Runx3 promoters in osseous and non-osseous cells: implications for tissue/differentiation specific transcription of distinct isoforms, in: Bone Abstracts. p. 273. doi:10.1530/boneabs.1.PP273

Dalcq, J., Pasque, V., Ghaye, A., Larbuisson, A., Motte, P., Martial, J.A., Muller, M., 2012. RUNX3, EGR1 and SOX9B form a regulatory cascade required to modulate BMP-signaling during cranial cartilage development in zebrafish. PLoS One 7, e50140. doi:10.1371/journal.pone.0050140

Dathe, K., Kjaer, K.W., Brehm, A., Meinecke, P., Nürnberg, P., Neto, J.C., Brunoni, D., Tommerup, N., Ott, C.E., Klopocki, E., Seemann, P., Mundlos, S., 2009. Duplications involving a conserved regulatory element downstream of BMP2 are associated with brachydactyly type A2. Am. J. Hum. Genet. 84, 483–492. doi:10.1016/j.ajhg.2009.03.001.

Doolittle, W.F., Sapienza, C., 1980. Selfish genes, the phenotype paradigm and genome evolution. Nature 284, 601–603. doi:10.1038/284601a0

Drissi, H., Luc, Q., Shakoori, R., Lopes, S.C.S., Choi, J.Y., Terry, A., Hu, M., Jones, S., Neil, J.C., Lian, J.B., Stein, J.L., Van Wijnen, A.J., Stein, G.S., 2000. Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. J. Cell. Physiol. 184, 341–350. doi:10.1002/1097-4652(200009)184:3<341::AID-JCP8>3.0.CO;2-Z

Dy, P., Wang, W., Bhattaram, P., Wang, Q., Wang, L., Ballock, R.T., Lefebvre, V., 2012. Sox9 directs hypertrophic maturation and blocks osteoblast differentiation of growth plate chondrocytes. Dev. Cell 22, 597–609. doi:10.1016/j.devcel.2011.12.024

Feng, J.Q., Chen, D., Ghosh-Choudhury, N., Esparza, J., Mundy, G.R., Harris, S.E., 1997. Bone morphogenetic protein 2 transcripts in rapidly developing deer antler tissue contain an extended 5′ non-coding region arising from a distal promoter. Biochim. Biophys. Acta - Gene Struct. Expr. 1350, 47–52. doi:10.1016/S0167-4781(96)00178-9

Ferraresso, S., Vitulo, N., Mininni, A.N., Romualdi, C., Cardazzo, B., Negrisolo, E., Reinhardt, R., Canario, A.V.M., Patarnello, T., Bargelloni, L., 2008. Development and validation of a gene expression oligo microarray for the gilthead sea bream (Sparus aurata). BMC Genomics 9, 580. doi:10.1186/1471-2164-9-580

Fonseca, V.G., Rosa, J., Laizé, V., Gavaia, P.J., Cancela, M.L., 2011. Identification of a new cartilage-specific S100-like protein up-regulated during endo/perichondral mineralization in gilthead seabream. Gene Expr. Patterns 11, 448–455. doi:10.1016/j.gep.2011.07.003

Gagniuc, P., Ionescu-Tirgoviste, C., 2012. Eukaryotic genomes may exhibit up to 10 generic classes of gene promoters. BMC Genomics 13, 512. doi:10.1186/1471-2164-13-512

Gao, Y., Ganss, B.W., Wang, H., Kitching, R.E., Seth, A., 2005. The RING finger protein RNF11 is expressed in bone cells during osteogenesis and is regulated by Ets1. Exp. Cell Res. 304, 127–135. doi:10.1016/j.yexcr.2004.10.031

Gemayel, R., Vinces, M.D., Legendre, M., Verstrepen, K.J., 2010. Variable tandem repeats accelerate evolution of coding and regulatory sequences. Annu. Rev. Genet. 44, 445–477. doi:10.1146/annurev-genet-072610-155046

Ghosh-Choudhury, N., Choudhury, G.G., Harris, M.A., Wozney, J., Mundy, G.R., Abboud, S.L., Harris, S.E., 2001. Autoregulation of mouse BMP-2 gene transcription is directed by the proximal promoter element. Biochem. Biophys. Res. Commun. 286, 101–108. doi:10.1006/bbrc.2001.5351

González-Mariscal, J. a, Gallardo-Gálvez, J.B., Méndez, T., Álvarez, M.C., Béjar, J., 2014. Cloning and characterization of the Mx1, Mx2 and Mx3 promoters from gilthead seabream (Sparus aurata). Fish Shellfish Immunol. 38, 311–317. doi:10.1016/j.fsi.2014.03.031

Gu, T., Goetz, T.L., Graves, B.J., Speck, N.A., 2000. Auto-Inhibition and partner proteins, core-binding factor beta (CBFbeta) and Ets-1, modulate DNA binding by CBFalpha 2 (AML1). Mol. Cell. Biol. 20, 91–103. doi:10.1128/MCB.20.1.91-103.2000

Haque, R., Chong, N.W., Chaurasia, S.S., Klein, D.C., Iuvone, P.M., 2004. Retinal melatonin biosynthesis: Interactions of A/T–rich regions and CRE–like sequences

contribute to cAMP–dependent regulation of the chicken AANAT promoter. Investig. Ophtalmol. Vis. Sci. 45, 659.

Heller, L.C., Li, Y., Abrams, K.L., Rogers, M.B., 1999. Transcriptional regulation of the Bmp2 gene. J. Biol. Chem. 274, 1394–1400. doi:10.1074/jbc.274.3.1394

Helvering, L.M., Sharp, R.L., Ou, X., Geiser, A.G., 2000. Regulation of the promoters for the human bone morphogenetic protein 2 and 4 genes. Gene 256, 123–138. doi:10.1016/S0378-1119(00)00364-4

Hino, J., Takao, M., Takeshita, N., Konno, Y., Nishizawa, T., Matsuo, H., Kangawa, K., 1996. cDNA cloning and genomic structure of human bone morphogenetic protein-3B (BMP-3b). Biochem. Biophys. Res. Commun. 223, 304–310. doi:10.1006/bbrc.1996.0889

Hogan, B.L., 1996. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. 10, 1580–1594. doi:10.1101/gad.10.13.1580

Javed, A., Bae, J.-S., Afzal, F., Gutierrez, S., Pratap, J., Zaidi, S.K., Lou, Y., van Wijnen, A.J., Stein, J.L., Stein, G.S., Lian, J.B., 2008. Structural coupling of Smad and Runx2 for execution of the BMP2 osteogenic signal. J. Biol. Chem. 283, 8412–22. doi:10.1074/jbc.M705578200

Karperien, M., Farih-Sips, H., Löwik, C.W., de Laat, S.W., Boonstra, J., Defize, L.H., 1997. Expression of the parathyroid hormone-related peptide gene in retinoic acid-induced differentiation: involvement of ETS and Sp1. Mol. Endocrinol. 11, 1435–1448. doi:10.1210/mend.11.10.9997

Katti, M. V., Ranjekar, P.K., Gupta, V.S., 2001. Differential distribution of simple sequence repeats in eukaryotic genome sequences. Mol. Biol. Evol. 18, 1161–1167. doi:10.1093/oxfordjournals.molbev.a003903

Kawai, S., Sugiura, T., 2001. Characterization of human bone morphogenetic protein (BMP)-4 and -7 gene promoters: activation of BMP promoters by Gli, a sonic hedgehog mediator. Bone 29, 54–61. doi:10.1016/S8756-3282(01)00470-7

Kawakami, Y., Rodriguez-León, J., Izpisúa Belmonte, J.C., 2006. The role of TGFβs and Sox9 during limb chondrogenesis. Curr. Opin. Cell Biol. 18, 723–729. doi:10.1016/j.ceb.2006.10.007

Kingsley, D.M., 1994. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. 8, 133–146. doi:10.1101/gad.8.2.133

Kube, D., Laser, H., von Knethen, A., Tesch, H., 1999. The AT-rich region between -54 to -66 is important for the promoter activity of interleukin-10 in Epstein-Barr virus positive Burkitt's lymphoma cells. Genes Immun. 1, 105–114. doi:10.1038/sj.gene.6363641

Kundu, M., Javed, A., Jeon, J.-P., Horner, A., Shum, L., Eckhaus, M., Muenke, M., Lian, J.B., Yang, Y., Nuckolls, G.H., Stein, G.S., Liu, P.P., 2002. Cbfβ interacts with Runx2 and has a critical role in bone development. Nat. Genet. 32, 639–644. doi:10.1038/ng1050

Laizé, V., Gavaia, P., Cancela, M., 2014. Fish: a suitable system to model human bone disorders and discover drugs with osteogenic or osteotoxic activities. Drug Discov. Today Dis. Model. 10.1016/j.ddmod.2014.08.001. doi:10.1016/j.ddmo0.1016/j.ddmod.2014.08.001

Liao, J., Hu, N., Zhou, N., Lin, L., Zhao, C., Yi, S., Fan, T., Bao, W., Liang, X., Chen, H., Xu, W., Chen, C., Cheng, Q., Zeng, Y., Si, W., Yang, Z., Huang, W., 2014. Sox9 potentiates BMP2-induced chondrogenic differentiation and inhibits BMP2-induced osteogenic differentiation. PLoS One 9, e89025. doi:10.1371/journal.pone.0089025

Mackie, E.J., Ahmed, Y.A., Tatarczuch, L., Chen, K.-S., Mirams, M., 2008. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int. J. Biochem. Cell Biol. 40, 46–62. doi:10.1016/j.biocel.2007.06.009

Marques, C.L., Fernández, I., Viegas, M.N., Cox, C.J., Martel, P., Rosa, J., Cancela, M.L., Laizé, V., 2015. Comparative analysis of zebrafish bone morphogenetic proteins 2, 4 and 16: molecular and evolutionary perspectives. Cell. Mol. Life Sci. in press. doi:10.1007/s00018-015-2024-x

Marques, C.L., Rafael, M.S., Cancela, M.L., Laizé, V., 2007. Establishment of primary cell cultures from fish calcified tissues. Cytotechnology 55, 9–13. doi:10.1007/s10616-007-9098-8

Miyake, S., Morii, E., Komori, T., Kawahata, H., Sugimoto, M., Terai, K., Shimizu, H., Yasui, T., Ogihara, H., Yasui, N., Ochi, T., Kitamura, Y., Ito, Y., Nomura, S., 1998. Transcriptional regulation of osteopontin gene in vivo by PEBP2alphaA/CBFA1 and ETS1 in the skeletal tissues. Oncogene 17, 1517–1525. doi:10.1038/sj.onc.1202064

Nishi, T., Itoh, S., 1986. Enhancement of transcriptional activity of the Escherichia coli trp promoter by upstream A + T-rich regions. Gene 44, 29–36. doi:10.1016/0378-1119(86)90039-9

Orgel, L.E., Crick, F.H.C., 1980. Selfish DNA: the ultimate parasite. Nature 284, 604–607. doi:10.1038/284604a0

Pombinho, A.R., Laizé, V., Molha, D.M., Marques, S.M.P., Cancela, M.L., 2004. Development of two bone-derived cell lines from the marine teleost Sparus aurata; evidence for extracellular matrix mineralization and cell-type-specific expression of matrix Gla protein and osteocalcin. Cell Tissue Res. 315, 393–406. doi:10.1007/s00441-003-0830-1

Pugh, B.F., Tjian, R., 1991. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. Genes Dev. 5, 1935–1945. doi:10.1101/gad.5.11.1935

Rafael, M.S., Laizé, V., Cancela, M.L., 2006. Identification of Sparus aurata bone morphogenetic protein 2: molecular cloning, gene expression and in silico analysis of protein conserved features in vertebrates. Bone 39, 1373–1381. doi:10.1016/j.bone.2006.06.021

Raouf, A., Seth, A., 2000. Ets transcription factors and targets in osteogenesis. Oncogene 19, 6455–6463. doi:10.1038/sj.onc.1204037

Rosa, J.T., Cancela, M.L., Laizé, V., 2014. Ets1 regulates the transcription of a cartilage-specific S100 protein in gilthead seabream. J. Appl. Ichthyol. 30, 707–712. doi:10.1111/jai.12534

Rosen, V., 2009. BMP2 signaling in bone development and repair. Cytokine Growth Factor Rev. 20, 475–480. doi:10.1016/j.cytogfr.2009.10.018

Shapiro, J.A., von Sternberg, R., 2005. Why repetitive DNA is essential to genome function. Biol. Rev. 80, 227–250. doi:10.1017/S1464793104006657

Shore, E.M., Xu, M.Q., Shah, P.B., Janoff, H.B., Hahn, G. V, Deardorff, M.A., Sovinsky, L., Spinner, N.B., Zasloff, M.A., Wozney, J.M., Kaplan, F.S., 1998. The human Bone Morphogenetic Protein 4 (BMP-4) gene: molecular structure and transcriptional regulation. Calcif. Tissue Int. 63, 221–229. doi:10.1007/s002239900518

Simon, M., Feliers, D., Arar, M., Bhandari, B., Abboud, H.E., 2002. Cloning of the 5’-flanking region of the murine bone morphogenetic protein-7 gene. Mol. Cell. Biochem. 233, 31–37. doi:A:1015546615027

Smale, S.T., Kadonaga, J.T., 2003. The RNA polymerase II core promoter. Annu. Rev. Biochem. 72, 449–479. doi:10.1146/annurev.biochem.72.121801.161520

Sohaskey, C.D., Zuckert, W.R., Barbour, A.G., 1999. The extended promoters for two outer membrane lipoprotein genes of Borrelia spp. uniquely include a T-rich region. Mol. Microbiol. 33, 41–51. doi:10.1046/j.1365-2958.1999.01443.x

Soung, D.Y., Dong, Y., Wang, Y., Zuscik, M.J., Schwarz, E.M., O’Keefe, R.J., Drissi, H., 2007. Runx3/AML2/Cbfa3 regulates early and late chondrocyte differentiation. J. Bone Miner. Res. 22, 1260–1270. doi:10.1359/jbmr.070502

Spender, L.C., Whiteman, H.J., Karstegl, C.E., Farrell, P.J., 2005. Transcriptional cross-regulation of RUNX1 by RUNX3 in human B cells. Oncogene 24, 1873–1881. doi:10.1038/sj.onc.1208404

Sugiura, T., 1999. Cloning and functional characterization of the 5′-flanking region of the human bone morphogenetic protein-2 gene. Biochem. J. 338, 433–440. doi:10.1042/0264-6021:3380433

Tamada, H., Kitazawa, R., Gohji, K., Kamidono, S., Maeda, S., Kitazawa, S., 1998. Molecular cloning and analysis of the 5′-flanking region of the human bone morphogenetic protein-6 (BMP-6). Biochim. Biophys. Acta 1395, 247–251. doi:10.1016/S0167-4781(97)00191-7

Tiago, D.M., Marques, C.L., Roberto, V.P., Cancela, M.L., Laizé, V., 2014. Mir-20a regulates in vitro mineralization and BMP signaling pathway by targeting BMP-2 transcript in fish. Arch. Biochem. Biophys. 543, 23–30. doi:10.1016/j.abb.2013.12.009

Tomilin, N. V., 2008. Regulation of mammalian gene expression by retroelements and non-coding tandem repeats. Bioessays 30, 338–348. doi:10.1002/bies.20741

Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C.A., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., Feilitzen, K. Von, Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., Heijne, G. Von, Nielsen, J., Pontén, F., 2015. Tissue-based map of the human proteome. Science (80-.). 347, 1260419–1260419. doi:10.1126/science.1260419

Walker, F.O., 2007. Huntington's Disease. Semin. Neurol. 27, 143–150. doi:10.1055/s-2007-971176

Wang, S., Furmanek, T., Kryvi, H., Krossøy, C., Totland, G.K., Grotmol, S., Wargelius, A., 2014. Transcriptome sequencing of Atlantic salmon (Salmo salar L.) notochord prior to development of the vertebrae provides clues to regulation of positional fate, chordoblast lineage and mineralisation. BMC Genomics 15, 141–158. doi:10.1186/1471-2164-15-141

Wang, S., Wang, Q., Crute, B.E., Melnikova, I.N., Keller, S.R., Speck, N.A., 1993. Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol. Cell. Biol. 13, 3324–3339. doi:10.1128/MCB.13.6.3324

Warren, A.J., Bravo, J., Williams, R.L., Rabbitts, T.H., 2000. Structural basis for the heterodimeric interaction between the acute leukaemia-associated transcription factors AML1 and CBFbeta. EMBO J. 19, 3004–3015. doi:10.1093/emboj/19.12.3004

Wigner, N.A., Soung, D.O.Y., Einhorn, T.A., Drissi, H., Gerstenfeld, L.C., 2013. Functional Role of Runx3 in the Regulation of Aggrecan Expression During Cartilage Development. doi:10.1002/jcp.24396

Wu, J., Kubota, J., Hirayama, J., Nagai, Y., Nishina, S., Yokoi, T., Asaoka, Y., Seo, J., Shimizu, N., Kajiho, H., Watanabe, T., Azuma, N., Katada, T., Nishina, H., 2010. p38 Mitogen-activated protein kinase controls a switch between cardiomyocyte and neuronal commitment of murine embryonic stem cells by activating myocyte

enhancer factor 2C-dependent bone morphogenetic protein 2 transcription. Stem Cells Dev. 19, 1723–1734. doi:10.1089/scd.2010.0066

Yang, S., Wei, D., Wang, D., Phimphilai, M., Krebsbach, P.H., Franceschi, R.T., 2003. In vitro and in vivo synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. J. Bone Miner. Res. 18, 705–15. doi:10.1359/jbmr.2003.18.4.705

Yoshida, C.A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K., Yamana, K., Zanma, A., Takada, K., Ito, Y., Komori, T., 2004. Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes Dev. 18, 952–963. doi:10.1101/gad.1174704

Zheng, L., Iohara, K., Ishikawa, M., Into, T., Takano-Yamamoto, T., Matsushita, K., Nakashima, M., 2007. Runx3 negatively regulates Osterix expression in dental pulp cells. Biochem. J. 405, 69–75. doi:10.1042/BJ20070104

Zheng, M., Zhu, J., Lu, T., Liu, L., Sun, H., Liu, Z., Tian, J., 2013. p300-mediated histone acetylation is essential for the regulation of GATA4 and MEF2C by BMP2 in H9c2 cells. Cardiovasc. Toxicol. 13, 316–322. doi:10.1007/s12012-013-9212-4

Figure captions

Figure 1. Schematic representation of BMP2 gene structure. Exons are displayed as *gray boxes* (non-coding exons) and *black boxes* (coding exons) and indicated with arabic numbers in gilthead seabream scheme, and as *thick solid lines* in other species. Introns are displayed as *thin solid lines* and indicated with roman numbers. Phase of intron II insertion is indicated in a *white triangle*. *Dashed lines* indicate translation initiation and termination sites (aligned according to gilthead seabream sites ATG and TGA, respectively). Accession numbers of BMP2 gene sequences in Ensembl database: Human (*Homo sapiens*, ENSG00000125845); Mouse (*Mus musculus*, ENSMUSG00000027358); Green anole (*Anolis carolinensis,* ENSACAG00000003113); Zebra finch (*Taeniopygia guttata*, ENSTGUG00000006434); Spotted gar (*Lepisosteus oculatus*, ENSLOCG00000016442); Zebrafish (*Danio rerio*, ENSDARG00000041430; *bmp2b*) and fruit fly (*Drosophila melanogaster dpp*, FBgn0000490).

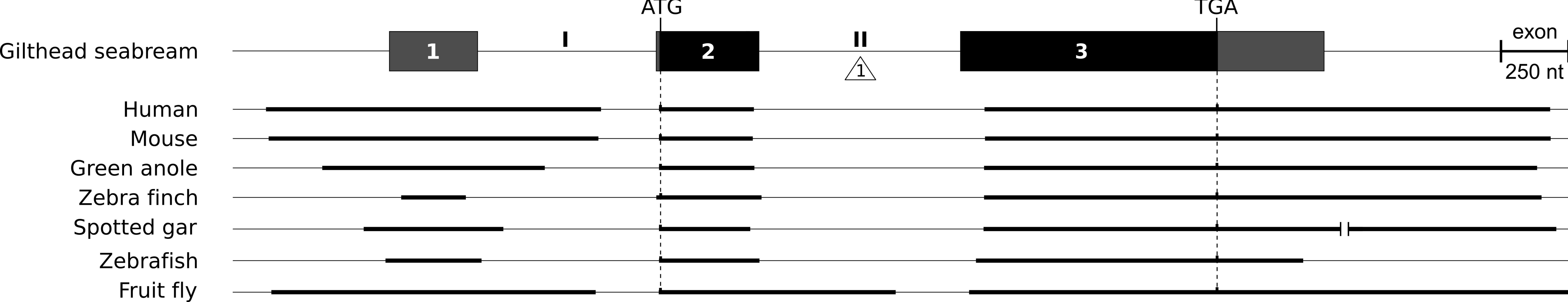
Figure 2. Basal activity of gilthead seabream *bmp2* promoter constructs in HEK-293 cells. Promoter deletion constructs (C1 to C8) are presented on the left side as *light gray boxes*. Nucleotide positions are given according to currently known transcription start site (*TSS*). *White boxes* indicate repetitive sequences (motif and number of repetitions are indicated on the top of each box). Non-coding and coding exons are displayed as *dark gray* and *black boxes*, respectively. *ATG* indicates translation initiation. *Black circles* indicate *in silico* predicted Sp1 sites. Luciferase activity (Fluc/Rluc; n = 4) is presented as fold change over the activity of promoter less pGL3 basic vector. *N.A.*, not active. *Letters* indicate values significantly different (one-way ANOVA followed by Tukey's post-test; $P<0.05$).

Figure 3. Effect of Runx3 and Cbfβ on the transcriptional activity of gilthead seabream *bmp2* promoter, determined from luciferase activity of promoter constructs. **A**, Schematic representation of *bmp2* promoter constructs with putative Runx binding sites indicated as *gray circles*. *E1*, exon 1. **B**, Luciferase activity (Fluc/Rluc) of promoter constructs co-transfected with expression vectors carrying zebrafish *runx3* or *cbfβ* transcripts in HEK-293 cells. Values are presented as fold induction over the basal

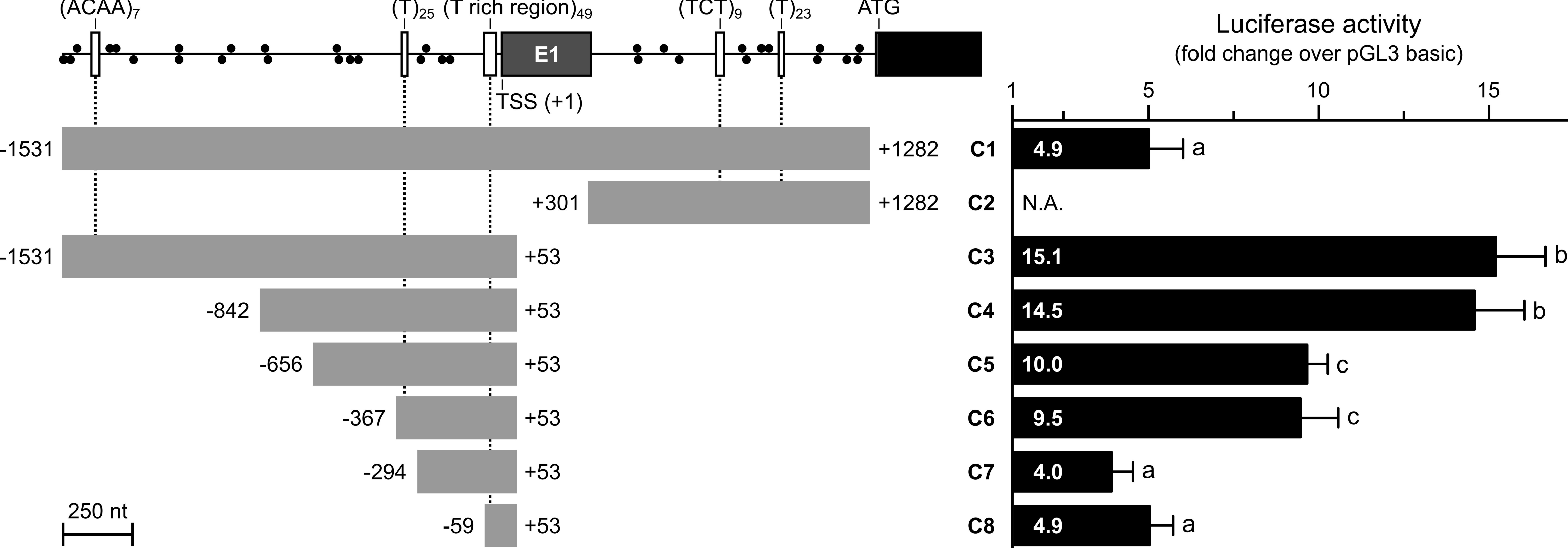
activity of each construct. *Gray bars* represent values that are not significantly different from the basal activity. *Asterisks* indicate values significantly different for the same construct. Different *letters* indicate values significantly different between constructs for the same transcription factor (one-way ANOVA followed by Tukey's post-test; *P*<0.05).

Figure 4. Effect of Mef2c, Sox9b and Ets1 on the transcriptional activity of gilthead seabream *bmp2* promoter determined from luciferase activity of promoter constructs. **A**, Schematic representation of *bmp2* promoter constructs with putative Mef2, Sox9 and Ets1 binding sites indicated as *circles*. *E1*, exon 1. **B**, Luciferase activity (Fluc/Rluc) of promoter constructs co-transfected with expression vectors carrying zebrafish *mef2c* (1:1 mixture of *mef2ca* and *mef2cb*) and/or *sox9b* transcripts in HEK-293. **C**, Luciferase activity (Fluc/Rluc) of promoter constructs co-transfected with expression vector carrying zebrafish *ets1* in HEK-293. Values are presented as fold induction over the basal activity of each construct. *Gray bars* represent values that are not significantly different from the basal activity. *Asterisks* indicate values significantly different for the same construct. Different *letters* indicate values significantly different between constructs for the same transcription factor (one-way ANOVA followed by Tukey's post-test; *P*<0.05).

Figure 5. Schematic representation of proposed transactivation of gilthead seabream *bmp2* transcription by Runx3, Cbfβ, Mef2c, Sox9b and Ets1. *E1*, exon 1. *Arrows* and *intersected lines* indicate activation and repression, respectively. Fold changes in luciferase activity are indicated above the line respective to each transcription factor or pairs of transcription factors.



ATG
TGA
Gilthead seabream
1
I
2
II
1
3
exon
250 nt
Human
Mouse
Green anole
Zebra finch
Spotted gar
Zebrafish
Fruit fly



(ACAA)7
(T)25
(T rich region)49
(TCT)9
(T)23
ATG
E1
TSS (+1)
-1531
+1282
+301
+1282
-1531
+53
-842
+53
-656
+53
-367
+53
-294
+53
-59
+53
250 nt
Luciferase activity
(fold change over pGL3 basic)
1
5
10
15
C1
4.9
a
C2
N.A.
C3
15.1
b
C4
14.5
b
C5
10.0
c
C6
9.5
c
C7
4.0
a
C8
4.9
a

A

− + − + + + +

E1

C3 Luc

C5 Luc

C7 Luc

C8 Luc

+ sense

− anti-sense

B

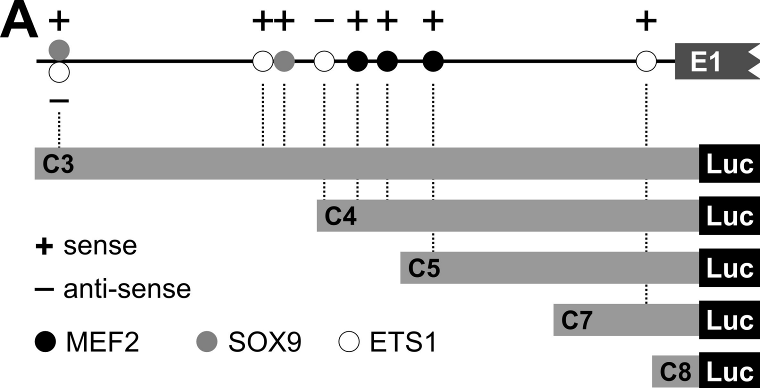
Luciferase activity
(fold change over basal activity)

0.125 0.25 0.5 1 2

		RUNX3	CBFβ
C3	a	−	+
	b	+	−
	c	+	+
C5	a	−	+
	b	+	−
	c'	+	+

* * *

* * *



B

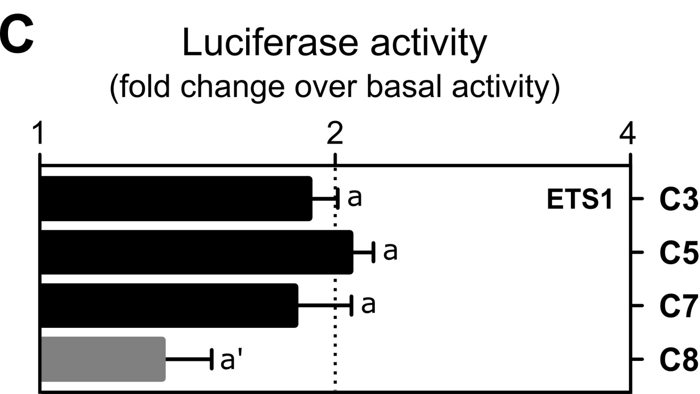
Luciferase activity
(fold change over basal activity)

0.25 0.5 1 2

MEF2C SOX9b

		MEF2C	SOX9b
C3	a	+	−
	b	−	+
	c	+	+
C4	a'	+	−
	b'	−	+
	c'	+	+
C5	a'	+	−
	b'	−	+
	c'	+	+

*



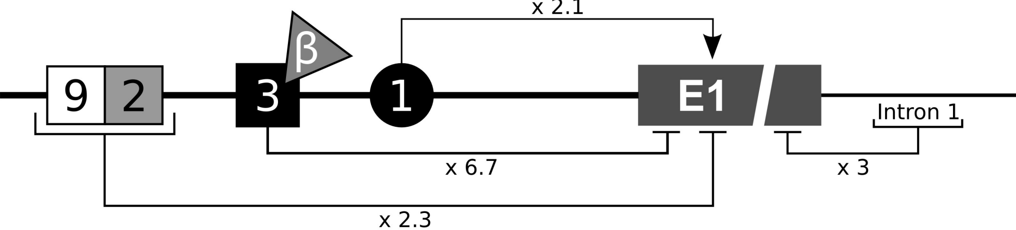

x 2.1
9
2
3
β
1
E1
Intron 1
x 6.7
x 3
x 2.3
9 SOX9b
3 RUNX3
1 ETS1
2 MEF2C
β CBFβ

Table 1. PCR primers used in this study

Name	Sequence (5’-3’)*
SauBMP2_1Rv	AAGTCTTGGTCAGCGCGGAAACGAA
SauBMP2_2Rv	GGCCTGCGCCTCAGTCCAAACATATT
SauBMP2_3Rv	GGCTCGCGTAGGCAGGACCGATCTA
SauBMP2_4Rv	GGATAAGTCCCGTGGCACCTTCCAGC
SauBMP2_*Hin*dIII_5Rv	CACGCAAGCTTCACTGGTGCTGAGGTATT
SauBMP2_*Hin*dIII_6Rv	CACGCAAGCTTGGCTCGCGTAGGCAGGACCGATCTA
SauBMP2_*Xho*I_1Fw	CCGGAGCTCGAGCCGCCTGCCCCACCATCAT
SauBMP2_*Kpn*I_2Fw	CCGGAGGGTACCGCCTCCTCGTCAGGTAAA
SauBMP2_*Xho*I_3Fw	CCGGAGCTCGAGTCACGTTCACGGGGAAGCATTGC
SauBMP2_*Xho*I_4Fw	CCGGAGCTCGAGGTGTGAGTTTCCAGGATGTGTA
SauBMP2_*Xho*I_5Fw	CCGGAGCTCGAGTTTGTTTGACATGAGAAGGGG
SauBMP2_*Xho*I_6Fw	CCGGAGCTCGAGGAGGTGCTTTATCGCGGACA
SauBMP2_*Xho*I_7Fw	CCGGAGCTCGAGACTGCTCTCTCTCGTGTTC

*Underlined sequences indicate recognition site for endonucleases cited in primer name

Supplementary figure 1. Basal activity of gilthead seabream *bmp2* promoter constructs in mouse chondrocyte ATDC5 and seabream branchial arch-derived ABSa15 cells. Luciferase activity (Fluc/Rluc; n=4) is presented as fold change over the activity of promoter less pGL3 basic vector. *N.A.*, not active. Letters indicate values significantly different (one-way ANOVA followed by Tukey's post-test; *P*<0.05).