Myeloid Zinc Finger 1 and GA Binding Protein Co-Operate with Sox2 in Regulating the Expression of Yes-Associated Protein 1 in Cancer Cells

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ABSTRACT

The transcription factor (TF) yes-associated protein 1 (YAP1) is a major effector of the tumor suppressive Hippo signaling pathway and is also necessary to maintain pluripotency in embryonic stem cells. Elevated levels of YAP1 expression antagonize the tumor suppressive effects of the Hippo pathway that normally represses YAP1 function. High YAP1 expression is observed in several types of human cancers and is particularly prominent in cancer stem cells (CSCs). The stem cell TF Sox2, which marks and maintains CSCs in osteosarcomas (OSs), promotes YAP1 expression by binding to an intronic enhancer element and YAP1 expression is also crucial for the maintenance of OS stem cells. To further understand the regulation of YAP1 expression in OSs, we subjected the YAP1 intronic enhancer to scanning mutagenesis to identify all DNA cis-elements critical for enhancer function. Through this approach, we identified two novel TFs, GA binding protein (GABP) and myeloid zinc finger 1 (MZF1), which are essential for basal YAP1 transcription. These factors are highly expressed in OSs and bind to distinct sites in the YAP1 enhancer. Depletion of either factor leads to drastically reduced YAP1 expression and thus a reversal of stem cell properties. We also found that YAP1 can regulate the expression of Sox2 by binding to two distinct DNA binding sites upstream and downstream of the Sox2 gene. Thus, Sox2 and YAP1 reinforce each others expression to maintain stemness and tumorigenicity in OSs, but the activity of MZF1 and GABP is essential for YAP1 transcription.

INTRODUCTION

The transcriptional coactivator yes-associated protein 1 (YAP1), is one of the major effectors of the tumor suppressive Hippo pathway, which is conserved crucial regulator of organ size and cell proliferation, whose ultimate upstream initiating signals are still unknown [1–4]. The Hippo protein kinase cascade ultimately leads to phosphorylation of its transcriptional effectors, YAP1 and TAZ, which as a result are inactivated and excluded from the cell nucleus [2, 5]. Thus, Hippo pathway activity causes repression of gene expression and the pathway is generally considered a negative regulator of growth [6]. YAP1, which is not a DNA binding protein and has to interact with other DNA binding cofactors to activate transcription is often highly expressed in cancer cells where its high expression antagonizes Hippo signaling by swamping the ability of the LATS kinases to phosphorylate it and cause its transcriptional inactivation [5, 7, 8]. YAP1 is necessary to maintain growth in rapidly proliferating tissues during embryogenesis.
and is required to maintain pluripotency in embryonic stem cells (ESCs) [9]. It is also highly expressed in a variety of solid tumors where it appears to mark cancer stem cells (CSC) to promote proliferation and inhibit differentiation [10–12]. Thus, high YAP1 expression exerts pro-proliferative effects escaping the restraint of Hippo pathway signaling, generally by complexing with its TEA Domain family (TEAD) TEAD coactivator and stimulating expression of TEAD target genes [13, 14].

We have studied the role and regulation of YAP1 and the Hippo pathway in osteosarcomas (OSs) and found that the transcription factor (TF) Sox2, which marks and maintains tumor initiating CSC, antagonizes the Hippo pathway by regulating the expression of three main players in the pathway; Kibra WWC2 domain containing protein (WWC1) and Neurofibromin 2 (NF2) and YAP1, whose expression is induced by Sox2 [11]. YAP1 is a major downstream effector of Sox2 [15]. Its activation or inactivation mimic similar changes in Sox2 expression on tumorigenicity, stemness, or differentiation of OS cells and YAP1 constitutive expression can compensate for the loss of Sox2 expression [11, 16].

While the positive regulation of YAP1 expression by Sox2 is significant, YAP1 transcription proceeds also in the absence of Sox2. Given the importance of YAP in the maintenance of CSC and in OSs, we wished to determine which other pathways or TFs regulate YAP1 expression in OS, and whether such factors operated together or independently of Sox2. We focused on the intronic enhancer in the YAP1 gene which is a target of Sox2. Extensive mutagenesis of this enhancer identified two distinct novel DNA elements that are essential for enhancer activity and YAP1 gene expression. They are the target of two TFs, GA binding protein (GABP) and myeloid zinc finger 1 (MZF1) that are highly expressed in OS. Depletion of either factor leads not only to strongly reduced YAP1 expression but also to a reversal of many transformed properties of OS cells. Additionally, we have found that YAP1 can also regulate Sox2 transcription by interacting with TEAD on two TEAD binding DNA elements near the Sox2 gene. Thus, many TFs contribute to high YAP1 expression in cancer cells and Sox2 and YAP1 reinforce each others expression to produce malignancy and stemness in OS cells.

**Materials and Methods**

**Plasmids and Reagents**

Flag-MZF1 cDNA plasmid was a kind gift of Dr. Doppler (Munich, Germany). mGABPα and mGABPβ-cDNA were purchased from Origene. GABP small interfering RNA (siRNA) and MZF1 short hairpin RNA (shRNA) were purchased from Dharmacon Lafayette, CO, USA and Sigma-Aldrich St. Louis, MO 63178 USA respectively. Primary antibodies against MZF1, GABP, Sox2, and YAP1 purchased from Biornbyt San Francisco, CA, USA (orb-324165). Proteintech Rosemont, IL 60018, USA (21542-1-AP), Millipore Billerica ME 01821 United States (ab-5603), and CST (4912). Sca-1 and IgG antibodies were purchased from e-biosciences San Diego, CA 92121 United States (12–59–8181) and Santa Cruz Dallas, TX, USA (sc-2025), respectively. YAP lentiviruses were generated as described previously [15].

**Generation of Reporter Constructs and Assay of Luciferase Activity**

pGL3 plasmids expressing the firefly luciferase gene driven by the mutated or wild type (WT) YAP1 intronic enhancer were generated by site directed mutagenesis and used to transfect cells in 24-well plates. Cells were cotransfected using Lipofectamine 2000 (Life Technologies) with 200 ng or 500 ng of the YAP1 enhancer plasmid in 293 and OB1 cells, respectively, together with 10 ng of PGL4.74 vector containing a Renilla reporter gene as an internal control and then cultured for 24 hours. To measure luciferase reporter gene activity using SpectraMax M5 plate reader, cells were washed two times with phosphate-buffered saline (PBS), lysed, and the luciferase activities were measured using the Dual Luciferase assay kit (Promega, E1910). Luciferase activity was normalized to Renilla activity to account for variations in transfection efficiency. TF binding to DNA sequences were predicted by PROMO and Transfac softwares.

**Site-Directed Mutagenesis**

Mutations were created by using the QS Site-Directed Mutagenesis Kit (NEB) as per manufacturer instructions. NEB primer design tool was used to design the primers to generate mutant reporter constructs as listed in the Supporting Information Table S1.

**Cell Culture**

The osteoprogenitor cell line OB1, has been described previously [15, 17]. The Human Osteosarcoma cell line LM7 was obtained from Dr. E. Kleinerman, Anderson Cancer Center, Houston, TX. The murine OS (mOS) cells used in this study (mOS-482 and mOS-202) were derived from spontaneous OSs as described previously [18]. All cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. To obtain MZF1 depleted cells, lentivirus vectors containing scrambled or MZF1-specific shRNA (Sigma Aldrich) were used to infect mOS-482 cells in the presence of 8 μg/ml polybrene for 24 hours. Following infection, cells were selected with 4 μg/ml puromycin for 96 hours.

**Sphere Assay**

Cells expressing scrambled or MZF1 shRNA or MZF1 shRNA + YAP1 lentiviruses were used as described previously [17].

**BrdU Proliferation Assay**

Cells were plated in eight-well multichamber slides and grown overnight. Next day, 5′-bromo-2′-deoxyuridine (BrdU) was added to final concentration of 10 μM for 4 hours. Cells were then fixed with 4% Paraformaldehyde (PFA) for 30 minutes and washed with PBS. Cells were then incubated in permeabilization buffer for 10 minutes on ice and washed with wash buffer. Cells were then stained using anti BrdU antibody (Amersham Cat# RPN 202) in the presence of DNase I for 30 minutes at 37°C. Finally, cells were stained with anti-mouse alexafluor 568 (cat # A 11004) from invitrogen. Stained slides were analyzed using a Leica CTR 5000 microscope.

**Analysis of Sox2-Sca-1 Double-Positive Cells**

Flow cytometry of intracellular Sox2 and membrane Sca-1 double-labeled control and MZF1 depleted cells was carried out as described in ref. 17.

**Chromatin Immunoprecipitation Assays**

Chromatin immunoprecipitation (ChiP) assays were performed using a ChiP assay kit (Active Motif, MA), according to
guide RNA (gRNA) and puromycin resistance, while a separate plasmid was cloned into the single gRNA scaffold. Lentiguide-Puro and LentiCas9-Blast plasmids PLP1, PLP2, and VSVG (Invitrogen cat # 497500) as transfer plasmids Lentiguide-Puro and LentiCas9-Blast that delivers hSpCas9 and blasticidin resistance was used to first integrate Cas9 into the mOS-202 cell line. Briefly, the Lentiguide-Puro vector was used for RT-PCR are listed in Supporting Information Table S2.

Electromobility Shift Assay

For electrophoretic mobility shift assay (EMSA), nuclear protein using NE-PER® Nuclear and Cytoplasmic Extraction kit (Thermo Scientific Pierce) was extracted from mOS-482 cells and incubated with biotin-labeled WT and mutated DNA probes corresponding to mZF1 and GABP binding elements, respectively. A competition assay was performed using 100-fold excess unlabeled WT probe. Supershift was obtained by addition of 1 μg of GABP antibody (Proteintech, AJ1374a). After incubation, binding reactions were separated on a 4% non-denaturing polyacrylamide gel, followed by transfer to a positively charged nylon membrane (Hybond). Protein/DNA complex was crosslinked to membrane using UV crosslinking. Biotin detection was performed following the manufacturer’s protocol (Pierce # 89880). Primers used for EMSAs are listed in Supporting Information Table S2.

Differentiation Assay

In vitro osteogenic differentiation of cells expressing scrambled or mZF1 shRNA or MZF1 shRNA + YAP1 lentiviruses was carried out as described previously [17].

Construction of CRISPR/Cas9 gRNA Plasmids

Lentiguidu-Puro/LentiCas9-Blast (two vector system) was purchased from Addgene and used following manufacturer instruction. In particular, Lentiguidu-Puro plasmid express guide RNA (gRNA) and puromycin resistance, while a separate lentiviral construct LentiCas9-Blast that delivers hSpCas9 and blasticidin resistance was used to first integrate Cas9 into mOS-202 cell line. Briefly, the Lentiguidu-Puro vector was digested using BsmBI enzyme, and a pair of annealed oligos was cloned into the single gRNA scaffold. Lentiguidu-Puro without gRNA was used as control (WT). To make lentivirus, the transfer plasmids Lentiguidu-Puro and LentiCas9-Blast were cotransfected into HEK293FT cells with the packaging plasmids PLP1, PLP2, and VSVG (Invitrogen cat # 497500) as per manufacturer instruction's. gRNAs sequences are as follows: YAP1-gRNA 5'-TGATGACCAGAGGAGGAGG-3'; gRNA targeting MZF1 binding site within YAP1 enhancer region 5'-GGGGAGAGGGAGGGAGG-3'.

Following infection, DNA from selected colonies was harvested and amplified by PCR with the following primers to confirm deletion: YAP1-exon1 FW: 5'-AAGGATGCAAGGGG-ATG-3', YAP1-exon1 RV: 5'-TTGAAGAGGCTCCACTGAGT-3'; YAP1-exon2 FW: 5'-CTCCTGAGACTCAGCAGCCAG-3', YAP1-exon2 RV: 5'-TCCCCACTGCCTTCCTCCTC-3'. The PCR products were purified and sequenced by Macrogen (http://www.macrogen.com) and alignment data were analyzed by ClustalW software.

Gene-Expression Analysis by RT-PCR and Western Blotting

Total RNA was extracted from cultured cells using RLT lysis buffer (Qiagen cat# 74104) and 500 ng total RNA was reverse transcribed to cDNA with First Strand cDNA Synthesis Kit (BioRad Cat# 170–8891), as per manufacturers’ instructions. Quantitative real-time PCR (RT-PCR) was performed with the BioRad sequence detection system using SYBR green (BioRad). Experiments were repeated three times and gene expression levels were determined by the delta delta Ct method, after normalization to expression. Primers used for RT-PCR are listed in the Supporting Information Table. Western blot analyses were performed as described previously [15]. Primers used for RT-PCR are listed in Supporting Information Table S3.

Results

GABP and MZF1 Transcriptionally Regulate YAP1 Expression

Sox2-mediated induction of YAP1 gene expression depends on its binding to an enhancer element located in the first gene intron of YAP1, a 243-bp region which contains several Sox2 binding sites [15]. Sox proteins generally require the cooperation of partner factors that bind DNA in the vicinity of the Sox binding site to enhance transcription [19]. To identify other TFs that regulate enhancer activity either independently or in cooperation with Sox2 in OS, we used scanning mutagenesis to introduce 21 mutations in this enhancer. Figure 1A shows a schematic representation of the enhancer nucleotide sequence and its location in the YAP1 gene. Transient transfection studies using a firefly luciferase reporter plasmid whose activity is controlled by the fibroblast growth factor-4 minimal promoter show that the presence of the WT YAP1 enhancer leads to sustained luciferase expression both in 293 cells, which do not express Sox2 (Fig. 1B) as well as in OB1 cells overexpressing Sox2 (Fig. 1C). The luciferase activities of mutants m1, m4, and m19, which correspond to Sox2 binding sites, were reduced to 50%-60% of WT, and stimulation by Sox2 on each of these mutants was attenuated, confirming the individual contribution of each conserved Sox2 binding motifs in the activation of YAP1. Interestingly, mutation of the Sox2 binding site at mt 11, whose sequence is not conserved, had no effect. Mutations at positions m8, m14, and m15 strongly affected the luciferase activity of the reporter plasmid in both cell types. Overexpression of Sox2 did not significantly stimulate the m8, m14, and m15 mutant plasmid activity in 293 cells, indicating that these motifs are critical for enhancer activity. Our binding motif analysis using promo and transfac software revealed that among the DNA sequences whose mutations cause low activity, m8 appears to contain MZF1...
binding motifs, while m14 and m15 resemble GA binding protein (GABP) binding motifs.

**MZF1 and GABP Bind to the YAP1 Enhancer and Stimulate YAP1 Expression**

To verify MZF1 and GABP binding to the 243 bp YAP1 enhancer, we performed nonradioactive EMSA as shown in Figure 2A. Nuclear extracts from 482 mOS cells were incubated with biotin-labeled DNA probes corresponding to the putative GABP or MZF1 binding sequences, respectively. In both cases, a specific shifted band was detected only in the presence of WT probe indicating that a binding activity that recognizes this sequence is present in the OS nuclear extract. An anti-GABP antibody produces a supershifted band identifying the activity as GABP. DNA sequences with a common G-rich core are recognized by the MZF1 factor and indeed a shifted complex was detected after incubation of WT probes corresponding to the putative MZF1 binding region with nuclear lysates, but not with probes containing mutated MZF1 binding sites (Fig. 2B). To further confirm the binding of MZF1 to the YAP1 enhancer region, ChIP-PCR was performed. Formaldehyde crosslinked chromatin complexes from 293 cells overexpressing FLAG tagged MZF1 were immunoprecipitated with FLAG antibody and the captured genomic DNA was analyzed by PCR using primers spanning the MZF1 motifs of the enhancer. The results showed binding of MZF1 to specific DNA sequences within the YAP1 enhancer region (Fig. 2C). Together with the mutagenesis data, these experiments suggest that GABP and MZF1 are important regulators of YAP1 expression. To determine that MZF1 and GABP can activate YAP1 expression, we co-transfected plasmids expressing MZF1 or GABP with the YAP1 enhancer reporter plasmid in 293 cells and observed a significant increase in reporter expression (Fig. 2D), indicating that GABP and MZF1 promote YAP1 enhancer activity and they are more potent than Sox2 in this assay.

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MZF1 and GABP Have High Expression in Osteosarcoma

Functional properties of GABP and MZF1 are dependent on the cellular type and environment, and a meta-analysis of MZF1 data [35] shows that this gene is often amplified in cancer, including sarcomas. We determined the expression level of these two factors by Western blot in OS cells. As shown in Figure 3A, both MZF1 and GABP are highly expressed in several murine and human OS cell lines as compared to OB1 cells. We further assessed in vivo expression of MZF1 and GABP in a spontaneous osteosarcoma by immunohistochemistry. Both MZF1 and GABP show strong nuclear expression in OS cells compared with the adjacent normal tissues (Fig. 3B). Oncomine data (Supporting Information Fig. S1) show that both MZF1 and GABP mRNAs are highly expressed in human sarcomas.

Depletion of GABP and MZF1 Strongly Reduces YAP Expression and Stemness Properties

To determine whether depletion of these TFs could disrupt YAP1 expression, we depleted MZF1 using shRNA in both murine OS cell line mOS482 and human OS cell line LM7 and found decreased YAP1 expression both at the mRNA (Fig. 4A) and protein levels (Fig. 4B). Similar results were obtained when we knocked down heterodimer functional complex GABPα/β using siRNA in OS cells (Fig. 4C, 4D), revealing that the depletion of MZF1 and GABP had a potent effect on YAP1 expression.

We have shown that the mOS cell lines used in this work contain a population of tumor initiating CSCs, which are capable of forming spheres in suspension culture in serum-free medium [17]. These OS sphere forming cells (also referred to as sarcospheres or osteospheres) have increased tumorigenic properties [21]. Since YAP1 silencing abrogates self renewal ability of CSCs in OS [11] as well as in other CSCs, we investigated whether MZF1 knockdown also affects sphere formation in OS. We found that depletion of MZF1 strongly reduces sphere formation and cell proliferation (Fig. 5A–5C). Cells positive for the stem cell antigen Sca-1 are thought to represent tumor initiating cells [18] and we showed previously that Sox2 marks the Sca1 positive population in OS [17]. As shown in Figure 5D, the majority of the cells coexpressed both Sca-1 and Sox2 and depletion of MZF1 significantly decreases the double positive cell population. YAP1 downregulation also results in a

Figure 2. Electrophoretic mobility shift assay (EMSA) of the binding of GA binding protein (GABP) and myeloid zinc finger 1 (MZF1) transcription factor to the yes-associated protein (YAP) enhancer region. EMSA was performed using 5 μg nuclear extract from 482 murine osteosarcoma cells and biotin-labeled oligonucleotides corresponding to the putative binding sites of GABP (A) and MZF1 (B) in the YAP1 enhancer, respectively. For competition, a 100-fold excess of unlabeled wild type oligo was used. Super shift analysis using GABP antibodies (Lane 5) was performed as described in Materials and Methods. Specific complexes are indicated with arrows; non-specific bands and supershifted bands are indicated by NS and Supershift, respectively. (C): Chromatin immunoprecipitation–polymerase chain reaction assays confirming the binding of Flag-MZF1 to the YAP1 enhancer region in 293 cells. Antibodies used for immunoprecipitation are indicated above the lanes. (D): A reporter plasmid expressing luciferase under the control of the fibroblast growth factor 4 minimal promoter and the YAP enhancer was transfected into 293 cells in combination with different plasmids expressing MZF1, Sox2, and GABP cDNA. *, pc < .05. Abbreviations: GABP, GA binding protein; MT, mutant; NS, non-specific; WT, wild-type.
shift to the osteoblastic lineage in the differentiation ability of OS cells. To determine whether MZF1 expression affects osteogenesis, we determined the ability of the parental and MZF1 knockdown OS cells to differentiate to osteoblastic lineage (Fig. 5E). Parental OS cells, or those expressing scrambled shRNAs, are impaired in their ability to differentiate into osteoblasts. However, the MZF1 shRNA-expressing cell lines rapidly differentiate into mature osteoblasts, as shown by an increase expression of alkaline phosphatase staining and of Osterix (Fig. 5F).

To further demonstrate that the reduction in CSC properties of the MZF1 KD cells was due to reduced YAP expression, we introduced a YAP lentivirus for the constitutive expression of YAP in the MZF1 KD cells and observed a complete return to high self renewal, sphere formation, and reduced osteogenic differentiation (Fig. 5).

Figure 3. GA binding protein (GABP) and myeloid zinc finger 1 (MZF1) are highly expressed in osteosarcomas (OSs). GABP and MZF1 expression level in OS; (A): Western blot analyses of GABPs and MZF1 in human and murine OS cell lines as well as in OB1 osteoblasts. (B): Immunohistochemistry (IHC) staining was used to identify the expression of GABP and MZF1. Representative images of GABP and MZF1 staining are shown in a spontaneous OS developing in a bone-specific Rb/p53-knockout mouse. IHC with GABP (bottom panels) and MZF1 (top panels) antibodies demonstrate that GABP and MZF1 have strong nuclear expression in OS. Magnification, ×40; scale bar = 100 μm. The results are representative of three independent tumors. Abbreviations: GABP, GA binding protein; MZF1, myeloid zinc finger 1; OS, osteosarcomas.

MZF1 Binding to the YAP1 Enhancer Is Essential for the Endogenous Expression of YAP1

To verify the importance of MZF1 binding to the YAP1 enhancer, we used CRISPR/Cas9 technology to create deletions in the GC rich MZF1 binding region within the 243 bp YAP1 enhancer. Lentivirus vectors containing gRNAs targeting the conserved MZF1 binding motifs and conferring puromycin resistance were introduced into OS cells expressing Cas9 (Fig. 6A). Puromycin selection allowed us to isolate clones in which sequencing revealed deletions only in the YAP1 enhancer region next to the gRNA target sequences (Fig. 6B). mRNA expression analysis from such clones (clone 1 and clone 2) show significant reduction of YAP1 mRNA expression whereas clone 3 where the region is intact retains expression of YAP1.
Fig. 6C). Consequently to the reduction of YAP1 expression (Fig. 6D), osteosphere formation was greatly impaired, but was restored by the introduction of a constitutively expressed YAP (Fig. 6E). To determine whether depletion of MZF1 binding site affects osteogenesis, we determined the ability of the OS cells with deletions of the MZF1 binding region to differentiate. MZF1 binding site depleted clones rapidly differentiate into mature osteoblasts, as shown by an increase expression of alkaline phosphatase staining (Fig. 6F) and introduction of a constitutively expressed YAP inhibits such differentiation. Together, these results demonstrate that MZF1 binding to the 243 bp YAP1 enhancer is crucial for endogenous YAP1 expression and for the CSC phenotype (Fig. 6).

YAP1 Regulates Sox2 Expression in a Feed Forward Loop

Knockdown of MZF1 expression resulted not only in the down-regulation of YAP expression but also of Sox2. Furthermore, in unrelated experiments, we had observed that a transient KO of the YAP1 gene in mOS cells produced a strong reduction in Sox2 expression (Fig. 7A). Therefore, we tested the hypothesis that YAP1 could regulate the expression of Sox2. Toward this purpose, YAP1 was depleted in OS cells by stable transfection of YAP1 shRNA, and indeed, we observed that depletion of YAP1 reduces the expression of Sox2 (Fig. 7B). The literature contains two reports of YAP1 regulation of Sox2 expression. In cardiomyocytes YAP1 was reported to bind to regulatory elements downstream of the Sox2 ORF [22], while in lung cancer cells binding was identified at an upstream regulatory enhancer [16]. To investigate the interaction of YAP1 with either of these two putative YAP1-TEAD binding sites in OS cells, we performed ChIP-PCR using anti-YAP1 antibodies. We could demonstrate YAP1 binding to either of the two reported regions at conserved TEAD binding motifs in mOS cells (Fig. 7C, 7D). Thus, YAP1 may regulate Sox2 expression through two distinct regulatory elements, and Sox2 and YAP1 function in a feed forward regulatory loop to maintain CSCs in OS.

**DISCUSSION**

YAP1 is necessary to maintain pluripotency in ESC and is over-expressed in a variety of solid tumors where it appears to mark CSCs to promote self renewal and inhibit differentiation [8, 10–12, 23]. In recent years, our understanding of the complexity of YAP1 regulation has expanded, with the identification of more regulatory components and with evidence that YAP1 can perform functions independent of the Hippo pathway. Our previous studies on the regulation of the Hippo pathway in OSs had shown that two upstream regulators of the Hippo pathway (Nf2 and WWC1) as well as the downstream effector YAP1, are directly regulated by Sox2 [11, 15], resulting in a strong antagonism of Hippo signaling in these tumors. The results presented here show that mutagenesis of the YAP1 intronic enhancer that is targeted by Sox2 identified two additional DNA elements that are essential for enhancer function. They interact with two distinct TFs, GABP, a heteromeric member of the ets family of TFs, that binds to a GA-rich binding site (GGAAG) in DNA and consist of two unrelated subunits: GABPa and GABPβ [24], and MZF1 which binds to a GC rich site...
in DNA [25]. In this report, we also identify a new mechanism through which YAP1 can regulate Sox2 transcription by interacting with TEAD on two binding elements on the Sox2 gene.

MZF1 has been found to be amplified in several cancers and its overexpression inhibits apoptosis and promotes oncogenesis [20, 26, 27] while silencing of MZF1 leads to reduced tumor growth [28]. The mechanisms by which MZF1 exerts an oncogenic effect are still unclear. It can regulate protein kinase C α [28] and stimulate Axl expression to induce metastasis in solid cancer [29]. Our present results strongly suggest that MZF1 could promote tumor formation through stimulation of YAP1 expression. GABP has been linked to the regulation of diverse

**Figure 5.** High myeloid zinc finger 1 (MZF1) expression is required in osteosarcoma (OS) stem cells. (A): Western blot analyses of MZF1, yes-associated protein 1 (YAP1), and Sox2 in murine OS (mOS) 482 cells (Control or MZF1 short hairpin RNA (shRNA) or MZF1 shRNA + YAP1 lentiviruses) as indicated in the lane above. (B): MZF1 knockdown decreases osteosphere-forming ability and infection with YAP1 lentivirus in MZF1 shRNA expressing cells rescues osteosphere-forming defect. (C): Sh-MZF1 mOS482 cells have reduced cell proliferation and YAP1 rescues proliferation defect caused by MZF1 depletion as determined by 5′-bromo-2′-deoxyuridine (BrdU) incorporation. A representative image of 4′,6-diamidino-2-phenylindole and BrdU-positive cells; magnification, ×40; scale bar = 100 μm; *, p < .05. (D): Flow cytometry analysis of Sh-MZF1 mOS cells stained with antibodies against Sox2 and Sca-1. (E): Osteogenic differentiation assay in cells expressing different levels of YAP1: mOS-482 cells expressing (scramble, MZF1 shRNA, and MZF1 shRNA + YAP1 lentivirus) were plated in osteogenic medium. Osteogenic differentiation was detected using alkaline phosphatase staining and (F) by increased mRNA expression of Osf2 and ALP. *, p < .05. Abbreviations: BrdU, 5′-bromo-2′-deoxyuridine; MZF1, myeloid zinc finger 1; WT, wild-type; YAP1, yes-associated protein 1.
functional classes of genes, including many genes that encode key cell-cycle control proteins [30]. GABP has been shown to activate the expression of YAP1 in liver cells [31] through its interaction with a DNA element different from the one described here. Other mechanisms appear to be involved in the GABP-mediated regulation of YAP1 activity. Previous studies have shown that Lats1, the inhibitory YAP1 kinase of the Hippo pathway, can phosphorylate GABPb in vitro, thereby interfering with GABP nuclear translocation [31]. Here, we show that both GABP and MZF1 are highly expressed in OS and they exert their...

Figure 6. Myeloid zinc finger 1 (MZF1) binding to the yes-associated protein (YAP) enhancer is essential for the endogenous expression of YAP. (A): Schematic representation showing the guide RNA used to target the conserved MZF1 binding site in the YAP enhancer. (B): DNA sequencing of the YAP enhancer region in three independent clones isolated after puromycin selection of murine osteosarcoma (mOS) 202 cells expressing Cas9 and transduced with lentivirus vectors carrying the guide RNA shown above and puromycin resistance factors. (C): YAP1 mRNA expression level in the three clones compared with control. (D): Western blot analyses showing YAP1 expression in mOS 202 cells with a deletion in the MZF1 binding site (clone 2) and infected with YAP1 lentivirus. (E): Depletion of MZF1 binding site decreases osteosphere forming ability and infection with YAP1 lentivirus in MZF1 short hairpin RNA expressing cells rescues this defect. (F): Osteogenic differentiation assay: mOS-202 cells expressing scrambled or MZF1 binding site depleted cells or MZF1 depleted cells infected with YAP1 lentivirus were plated in osteogenic medium. Osteogenic differentiation was detected using alkaline phosphatase staining. Abbreviations: MZF1, myeloid zinc finger 1; WT, wild-type; YAP1, yes-associated protein 1.
oncogenic potential via YAP1. The depletion of either one results in reduction of YAP1 expression and decreased cell proliferation. This is particularly interesting because multiple signaling networks such as Wnt, bone morphogenic protein, Notch, and transforming growth factor-β [32] are engaged in regulatory crosstalk with the Hippo pathway and determine the phenotype of CSC in solid cancers. We have demonstrated previously that high YAP1 activity maintains CSCs in OS [11]. Downregulation of MZF1 produces an opposing effect by downregulating YAP1 expression, which decreases the stem cell fraction and restores osteogenic differentiation. Given the low viability of the MZF1 and GABP knockdown cells, we did not test their tumorigenicity in mouse xenografts, but we believe that our results indicate strongly that the major effect of MZF1 or GABP depletion is the reduction in the tumor stem cell fraction through inhibition of YAP expression.

Loss of nuclear expression of MZF1 has been associated with more advanced clinical stages in oral squamous cell carcinoma indicating that MZF1 can act as a negative regulator of tumorigenesis [33], while we observed both GABP and MZF1 have nuclear expression, and induce tumor growth through the activation of YAP1 in OS. Previous studies indicated that YAP1 may associate with promoters of genes which are important for the pluripotency of ESCs [9, 34], through the mediation of the TEAD TF [9, 14]. In line with this notion, two reports in the literature indicated that YAP1 can regulate Sox2 expression in cardiomyocytes and lung cancer cells, respectively. We show here that YAP1 also regulates Sox2 expression in OS cells, creating a feed forward loop that is important in the maintenance of the CSC population (Fig. 7E). Interestingly, while the two studies mentioned above identified different YAP1 binding sites as responsible for the YAP1 regulation of Sox2 transcription, our experiments showed that in OS cells YAP1 binds both these elements. Additionally, the lung cancer cells study reported a cooperative action of Sox2 with Oct4 in the activation of Sox2 expression but, as reported previously [11], Oct4 is not expressed in OSs and, thus, cannot play a role in our system.

The upregulation of Sox2 by YAP1 together with our previous findings where we showed that YAP1 is a direct transcriptional target of Sox2 in osteoprogenitors and MSCs suggests that these factors have a reciprocal positive regulation that is important to determine the CSC phenotype in OS cells.

**CONCLUSION**

High levels of expression of the transcriptional coactivator YAP1 antagonize the tumor suppressive effects of the Hippo pathway.
pathway and are important in the maintenance of cancer stem cells (CSC). We show here that in osteosarcomas YAP1 expression is regulated by the transcription factors Sox2, GABP, and MZF1 that together determine the basal and inducible levels of transcription of the YAP1 gene. Inactivation of MZF1 or GABP leads to reversal of the CSC phenotype through downregulation of YAP1 expression. Furthermore YAP1 can also directly regulate the expression of Sox2 creating a feed forward loop that fuels the CSC phenotype.

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AUTHORS CONTRIBUTIONS

N.K.V.: designed and performed experiments, wrote the manuscript, provided intellectual input, read, and approved the manuscript; A.G. and G.M.: performed experiments, provided intellectual input, read, and approved the manuscript; U.B.R.: designed experiments, provided intellectual input, read, and approved the manuscript; A.M. and C.B.: designed experiments and wrote the manuscript, provided intellectual input, read, and approved the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.