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TEThered to Runx: Novel Binding Partners for Runx Factors

Xiaodong Li, **Matthew Decker**, and **Jennifer J. Westendorf***
Departments of Orthopedic Surgery and Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN USA

Abstract

RUNX transcription factors reside in the nuclear matrix where they integrate numerous signaling pathways to regulate gene expression and affect tissue development, regeneration, and tumorigenesis. An affinity purification and proteomic experiment was performed to identify novel Runx2 binding partners. The interactions between Runx2 and two nuclear factors identified in this screen, Ddx5 and CoAA, were previously described. Co-activator activator (CoAA) bound the DNA binding domain of Runx2 and prevented Runx-driven gene expression. The YxxQ motif in CoAA was required for Runx2 interactions. Members of the FET/TET family of proteins, including FUS/TLS and EWSR1, contain a similar motif and were hypothesized to interact with Runx2. Here we provide evidence that FUS/TLS, EWSR1 and the Ewing's sarcoma t(12;21) fusion protein EWS-FLI, bind Runx2 and alter its transcriptional activity. Potential roles of protein complexes containing FET/TET and RUNX family members during tumor formation and mesenchymal progenitor cell differentiation are discussed.

Keywords

CoAA; EWS; EWS-FLI; Ewing's sarcoma; FUS; TLS

Introduction

RUNX family transcription factors play crucial roles in tissue development, regeneration, and tumorigenesis [1;2]. They bind DNA via a highly conserved Runt domain, are tightly associated with the nuclear matrix, and organize the assembly of protein complexes that direct tissue-specific gene expression. *RUNX1* is required for early hematopoiesis and is altered in acute leukemias by point mutations and at least 30 different chromosomal translocations. RUNX2 and RUNX3 are not affected by chromosomal translocations but paradoxically have both oncogenic and tumor suppressor activities. RUNX2 is required for osteoblast development from mesenchymal progenitor cells and is differentially regulated in osteosarcomas [1;3]. Elevated RUNX2 also contributes to murine T cell leukemias and human adult solid tumors [1]. RUNX3 participates in T cell maturation, neuronal and gastrointestinal development, and is a tumor suppressor that is inactivated in several epithelial cancers [1]. Thus, RUNX family members are central players in the development of many tissues and cancers.

Address correspondence to: Jennifer J. Westendorf, Ph.D., Mayo Clinic, 200 First St SW, Rochester, MN 55905, Phone: 507-538-5651; Fax: 507-284-5075, westendorf.jennifer@mayo.edu.

RUNX proteins interact with other transcription factors, transcriptional co-factors, chromatin-modifying enzymes, and nuclear matrix proteins to control gene expression in context-dependent manners [4;5]. To identify novel RUNX binding partners, we recently performed an affinity purification/proteomic screen with tandem-affinity (TAP) tagged Runx2 [6]. Actinin, EPLIN, gelsolin and HSP70 were some of the proteins eluted from TAP-Runx2 immune-complexes and identified with mass spectroscopy. Ddx5 (p68), Ddx17 (p72), and co-activator activator (CoAA) also bound to TAP-Runx2 and were verified as Runx2 binding partners and cofactors in subsequent assays [6;7]. Ddx5 and CoAA each co-localized with Runx2 in subnuclear structures and interacted with Runx2. Functionally, Ddx5 augmented Runx2 transcriptional activity while CoAA repressed Runx factor-driven expression of reporter plasmids.

CoAA has structural similarities to FET/TET family of proteins [8]: Fused in sarcoma/Translocated in liposarcoma (FUS/TLS), Ewing's sarcoma breakpoint region 1 (EWSR1 or EWS), and TATA-binding protein-associated factor (TAF15). These structurally related factors bind to both DNA and RNA to regulate gene expression and splicing [9;10]. Like RUNX1, FET proteins are frequently altered by chromosomal translocations. Approximately 20 chromosomal translations in pediatric sarcomas and leukemias fuse the N-terminus of FET/TET proteins to the DNA binding domain of ETS-related transcription factors [9;10]. The most common translocation, t(11;22), joins EWSR1 to FLI1 and is detected in 85% of Ewing sarcomas [9]. The resulting onco-fusion protein, EWS-FLI, alters gene expression [11], induces myeloid/erythroid leukemias in mice when introduced into hematopoietic cells [12], causes musculoskeletal abnormalities, and accelerates sarcoma formation in vivo when expressed in mesenchymal progenitor cells [13]. Here we show that FUS/TLS, EWSR1 and EWS-FLI interact with RUNX1 and Runx2 and alter RUNX transcriptional activity.

Materials

Plasmids

Runx expression and reporter plasmids were previously described [6;7]. Dr. Lan Ko kindly provided the CMV-FLAG-CoAA expression plasmid [8]. Drs. Ralf Jacknecht, Thomas Kwiatkowski, Christopher Shaw, and Aykut Üren generously shared EWSR1, FUS/TLS and EWS-FLI expression plasmids [14;15;16;17].

GST-Pulldown Assays

Pulldown assays with GST-Runx proteins were performed as previously described [6;7]. ^{35}S-labeled EWSR1, FUS, and EWS-FLI proteins were made in vitro using Promega's TnT system.

Transcription Assays

C2C12 cells were maintained and transiently transfected as previously described [6;7].

Results

FET/TET Family Proteins Bind to RUNX Factors

We previously showed that the YxxQ-rich region of CoAA binds the Runt domain of RUNX proteins [7]. FET/TET family proteins contain similar repeats in their N-termini (Figure 1) [8]. Therefore, we hypothesized that FET proteins would physically interact with RUNX proteins. ^{35}S-radiolabeled FET/TET proteins [FUS, EWSRI, and EWS-FLI (E-F)] were made with in vitro transcription and translation reactions and incubated with bacterially produced GST-RUNX1 or Runx2 fusion proteins. EWSR1, EWS-FLI and FUS/TLS bound both RUNX1 and Runx2 proteins (Figure 2). The Runt domains of RUNX1 (50–179) and Runx2 were necessary for FET/TET protein interactions. Thus, FUS/TLS interacted well with Runx2

constructs containing residues 1–227, 1–327 and 1–383 from the MRIPV isoform. EWSR1 also interacted strongly to Runx2 proteins containing amino acids 1–327 and 1–383, but did not bind well to a shorter protein with only residues 1–227. These data indicate subtle differences between how FUS/TLS and EWSR1 bind RUNX proteins and that sequences C-terminal to the Runt domain augment EWSR1 interactions. The Ewing's sarcoma-associated fusion protein, EWS-FLI, was the strongest RUNX-binding partner in this experiment and associated with the same RUNX proteins as FUS/TLS. FLI1 was recently identified as a modulator of RUNX1 activity in megakaryocytes [18]; thus, the FLI1 portion of fusion protein may increase the affinity of EWS-FLI to RUNX proteins as compared to EWSR1. Together, these data suggest that RUNX and FET family proteins may co-exist in molecular complexes.

FET/TET Family Proteins Modulate the Transcriptional Activity of RUNX Factors

We next asked if the physical interactions between FET/TET and RUNX family proteins had functional consequences on RUNX-driven gene regulation. To determine if FET/TET proteins influenced Runx2 transcriptional activity, we co-transfected osteo/myoblast progenitor C2C12 cells with expression plasmids for Runx2 and CoAA, EWSR1, FUS/TLS or EWS-FLI, as well as with the Runx reporter plasmid, p6OSE2-luc, which contains six Runx binding elements upstream of the luciferase reporter gene. RUNX1 activated this promoter approximately 6-fold and Runx2 stimulated it by 10-fold. (Figure 3A). As we previously reported, CoAA was a very strong inhibitor of RUNX-directed gene expression [7]. EWSR1, FUS/TLS and EWS-FLI repressed RUNX1-dependent activation by approximately 50% and Runx2-dependent activation by 25–50% (Figure 3A). This repression of Runx2 was enhanced when higher concentrations of FUS/TLS expression vectors were added in the transfection mix (Figures 3B). Similar results were obtained with higher amounts of EWSR1 and EWS-FLI (data not shown). Interestingly, FUS/TLS augmented Runx2 activation of the 1.3 kB osteocalcin promoter (Figures 3C). These data demonstrate that FET/TET proteins affect Runx2 transcriptional activity in a context-dependent manner.

Discussion

RUNX factors are context-dependent regulators of transcription that play essential roles in the development of specific tissues. We recently identified several nuclear proteins (e.g. Ddx5 and CoAA) that bind Runx2 and regulate its ability to regulate gene expression with a proteomic approach [6;7]. Interestingly, Ddx5 augmented Runx2 transcriptional activity while CoAA prevented Runx2 from binding DNA. CoAA contains a repetitive YxxQ motif in its C-terminus that is necessary for Runx2 associations. This result inspired the hypothesis that other proteins containing YxxQ repetitive sequences may also interact with Runx proteins. Such motifs are found in the N-termini of FET family members (FUS, EWSR1, and TAF15), as well as in SYT, and SNI/SNF [8] (Figure 1). Here we present data demonstrating that FET family proteins bind RUNX1 and Runx2 and modulate their transcriptional activity. While CoAA blocks RUNX factor activity by interfering with DNA binding [7], the molecular mechanisms whereby FET/TET proteins affect RUNX factor transcription require further investigation; however, repression does not seem to involve histone deacetylases as the Hdac inhibitor, trichostatin A, did not reverse FET/TET repression of RUNX factors (data not shown). The effects of RUNX factors on FET/TET family activities as transcriptional and splicing factors also remain to be determined [9;19]. It is interesting that RUNX proteins bind numerous splicing factors (e.g. Ddx5 [6], CoAA [7], FET/TET proteins and hnRNAs [6]) that co-exist in nuclear complexes [20]. Thus, it is possible that RUNX factors may also regulate RNA splicing or stability.

The physical interactions between RUNX and FET/TET families of transcription factors are of interest because they functionally link two families of proteins that contribute to hematopoietic and mesenchymal tissue development and that are structurally altered by

chromosomal translocations in acute leukemias and undifferentiated sarcomas. *RUNX1 (AML1)*, *FUS/TLS* and *EWSR1* genes were each identified as a result of chromosomal abnormalities in acute myeloid leukemia or sarcomas [21;22;23;24]. Interestingly, no primary tumors have been described with alterations in genes from both families. If RUNX and FET/TET proteins are components of the same molecular pathway, structural alteration of just one factor could disrupt common downstream cellular events. Thus, RUNX and FET/TET protein associations may provide new perspectives into tumorigenesis and therapy.

Ewing's sarcoma family tumors (ESFT) are characterized as small, undifferentiated, round cells within osteolytic lesions. The cellular progenitor of ESFT has been debated since in 1921 when James Ewing described the malignancy as a diffuse endothelioma [25]; however, recent analyses of molecular signatures suggests mesenchymal origin [11;26;27;28;29]. If a premature mesenchymal cell is indeed the cancer stem cell of ESFT, the ability of EWS-FLI and other FET/TET fusion proteins to block RUNX2 activity could be a crucial step in arresting progenitor cell differentiation to the osteoblast lineage. *Runx2* is required for osteoblast development and *Runx2*-deficient chondrocytes have propensity to differentiate into adipocytes [30;31;32]. Runx2 is also necessary for endothelial cell proliferation, VEGF production and vascular invasion of cartilage [33;34;35]. Interestingly, suppression of EWS-FLI expression in an ESFT cell line by RNA interference permitted the expression of osteoblast differentiation genes and matrix mineralization [27]. Moreover, transgenic mice expressing EWS-FLI in primitive mesenchymal cells exhibited impaired differentiation of numerous mesenchymal lineages [13]. Thus, we propose that moderation of RUNX2 activity could be a crucial step in arresting mesenchymal differentiation and the development of EFST.

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References

1. Blyth K, Cameron ER, Neil JC. The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer 2005;5:376–87. [PubMed: 15864279]
2. Zaidi SK, Young DW, Javed A, Pratap J, Montecino M, van Wijnen A, Lian JB, Stein JL, Stein GS. Nuclear microenvironments in biological control and cancer. Nat Rev Cancer 2007;7:454–63. [PubMed: 17522714]
3. Komori T. Regulation of bone development and maintenance by Runx2. Front Biosci 2008;13:898–903. [PubMed: 17981598]
4. Schroeder TM, Jensen ED, Westendorf JJ. Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. Birth Defects Res C Embryo Today 2005;75:213–25. [PubMed: 16187316]
5. Westendorf JJ. Transcriptional co-repressors of Runx2. J Cell Biochem 2006;98:54–64. [PubMed: 16440320]
6. Jensen ED, Niu L, Caretti G, Nicol SM, Teplyuk N, Stein GS, Sartorelli V, van Wijnen AJ, Fuller-Pace FV, Westendorf JJ. p68 (Ddx5) interacts with Runx2 and regulates osteoblast differentiation. J Cell Biochem 2008;103:1438–51. [PubMed: 17960593]
7. Li X, Hoeppner LH, Jensen ED, Gopalakrishnan R, Westendorf JJ. Co-activator activator (CoAA) prevents the transcriptional activity of Runt domain transcription factors. J Cell Biochem 2009;108:378–87. [PubMed: 19585539]
8. Sui Y, Yang Z, Xiong S, Zhang L, Blanchard KL, Peiper SC, Dynan WS, Tuan D, Ko L. Gene amplification and associated loss of 5′ regulatory sequences of CoAA in human cancers. Oncogene 2007;26:822–35. [PubMed: 16878147]
9. Law WJ, Cann KL, Hicks GG. TLS, EWS and TAF15: a model for transcriptional integration of gene expression. Brief Funct Genomic Proteomic 2006;5:8–14. [PubMed: 16769671]

10. Tan AY, Manley JL. The TET Family of Proteins: Functions and Roles in Disease. J Mol Cell Biol. 2009
11. Hancock JD, Lessnick SL. A transcriptional profiling meta-analysis reveals a core EWS-FLI gene expression signature. Cell Cycle 2008;7:250–6. [PubMed: 18256529]
12. Torchia EC, Boyd K, Rehg JE, Qu C, Baker SJ. EWS/FLI-1 induces rapid onset of myeloid/erythroid leukemia in mice. Mol Cell Biol 2007;27:7918–34. [PubMed: 17875932]
13. Lin PP, Pandey MK, Jin F, Xiong S, Deavers M, Parant JM, Lozano G. EWS-FLI1 induces developmental abnormalities and accelerates sarcoma formation in a transgenic mouse model. Cancer Res 2008;68:8968–75. [PubMed: 18974141]
14. Rossow KL, Janknecht R. The Ewing's sarcoma gene product functions as a transcriptional activator. Cancer Res 2001;61:2690–5. [PubMed: 11289149]
15. Erkizan HV, Kong Y, Merchant M, Schlottmann S, Barber-Rotenberg JS, Yuan L, Abaan OD, Chou TH, Dakshanamurthy S, Brown ML, Uren A, Toretsky JA. A small molecule blocking oncogenic protein EWS-FLI1 interaction with RNA helicase A inhibits growth of Ewing's sarcoma. Nat Med 2009;15:750–6. [PubMed: 19584866]
16. Kwiatkowski TJ Jr, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, Davis A, Gilchrist J, Kasarskis EJ, Munsat T, Valdmanis P, Rouleau GA, Hosler BA, Cortelli P, de Jong PJ, Yoshinaga Y, Haines JL, Pericak-Vance MA, Yan J, Ticozzi N, Siddique T, McKenna-Yasek D, Sapp PC, Horvitz HR, Landers JE, Brown RH Jr. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science 2009;323:1205–8. [PubMed: 19251627]
17. Vance C, Rogelj B, Hortobagyi T, De Vos KJ, Nishimura AL, Sreedharan J, Hu X, Smith B, Ruddy D, Wright P, Ganesalingam J, Williams KL, Tripathi V, Al-Saraj S, Al-Chalabi A, Leigh PN, Blair IP, Nicholson G, de Belleroche J, Gallo JM, Miller CC, Shaw CE. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science 2009;323:1208–11. [PubMed: 19251628]
18. Huang H, Yu M, Akie TE, Moran TB, Woo AJ, Tu N, Waldon Z, Lin YY, Steen H, Cantor AB. Differentiation-dependent interactions between RUNX-1 and FLI-1 during megakaryocyte development. Mol Cell Biol 2009;29:4103–15. [PubMed: 19470763]
19. Takahama K, Kino K, Arai S, Kurokawa R, Oyoshi T. Identification of RNA binding specificity for the TET-family proteins. Nucleic Acids Symp Ser (Oxf) 2008:213–4.
20. Pahlich S, Quero L, Roschitzki B, Leemann-Zakaryan RP, Gehring H. Analysis of Ewing sarcoma (EWS)-binding proteins: interaction with hnRNP M, U, and RNA-helicases p68/72 within protein-RNA complexes. J Proteome Res 2009;8:4455–65. [PubMed: 19673543]
21. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci USA 1991;88:10431–10434. [PubMed: 1720541]
22. Crozat A, Aman P, Mandahl N, Ron D. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature 1993;363:640–4. [PubMed: 8510758]
23. Rabbitts TH, Forster A, Larson R, Nathan P. Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. Nat Genet 1993;4:175–80. [PubMed: 7503811]
24. Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, Kovar H, Joubert I, de Jong P, Rouleau G, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 1992;359:162–5. [PubMed: 1522903]
25. Ewing J. Diffuse Endothelioma of Bone. Proc New York Pathol Soc 1921;21:17–24.
26. Riggi N, Cironi L, Provero P, Suva ML, Kaloulis K, Garcia-Echeverria C, Hoffmann F, Trumpp A, Stamenkovic I. Development of Ewing's sarcoma from primary bone marrow-derived mesenchymal progenitor cells. Cancer Res 2005;65:11459–68. [PubMed: 16357154]
27. Tirode F, Laud-Duval K, Prieur A, Delorme B, Charbord P, Delattre O. Mesenchymal stem cell features of Ewing tumors. Cancer Cell 2007;11:421–9. [PubMed: 17482132]
28. Kauer M, Ban J, Kofler R, Walker B, Davis S, Meltzer P, Kovar H. A molecular function map of Ewing's sarcoma. PLoS One 2009;4:e5415. [PubMed: 19404404]

29. Suva ML, Riggi N, Stehle JC, Baumer K, Tercier S, Joseph JM, Suva D, Clement V, Provero P, Cironi L, Osterheld MC, Guillou L, Stamenkovic I. Identification of cancer stem cells in Ewing's sarcoma. Cancer Res 2009;69:1776–81. [PubMed: 19208848]

30. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755–64. [PubMed: 9182763]

31. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 1997;89:765–71. [PubMed: 9182764]

32. Enomoto H, Furuichi T, Zanma A, Yamana K, Yoshida C, Sumitani S, Yamamoto H, Enomoto-Iwamoto M, Iwamoto M, Komori T. Runx2 deficiency in chondrocytes causes adipogenic changes in vitro. J Cell Sci 2004;117:417–25. [PubMed: 14702386]

33. Qiao M, Shapiro P, Fosbrink M, Rus H, Kumar R, Passaniti A. Cell cycle-dependent phosphorylation of the RUNX2 transcription factor by cdc2 regulates endothelial cell proliferation. J Biol Chem 2006;281:7118–28. [PubMed: 16407259]

34. Sun L, Vitolo M, Passaniti A. Runt-related gene 2 in endothelial cells: inducible expression and specific regulation of cell migration and invasion. Cancer Res 2001;61:4994–5001. [PubMed: 11431332]

35. Zelzer E, Glotzer DJ, Hartmann C, Thomas D, Fukai N, Soker S, Olsen BR. Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. Mech Dev 2001;106:97–106. [PubMed: 11472838]

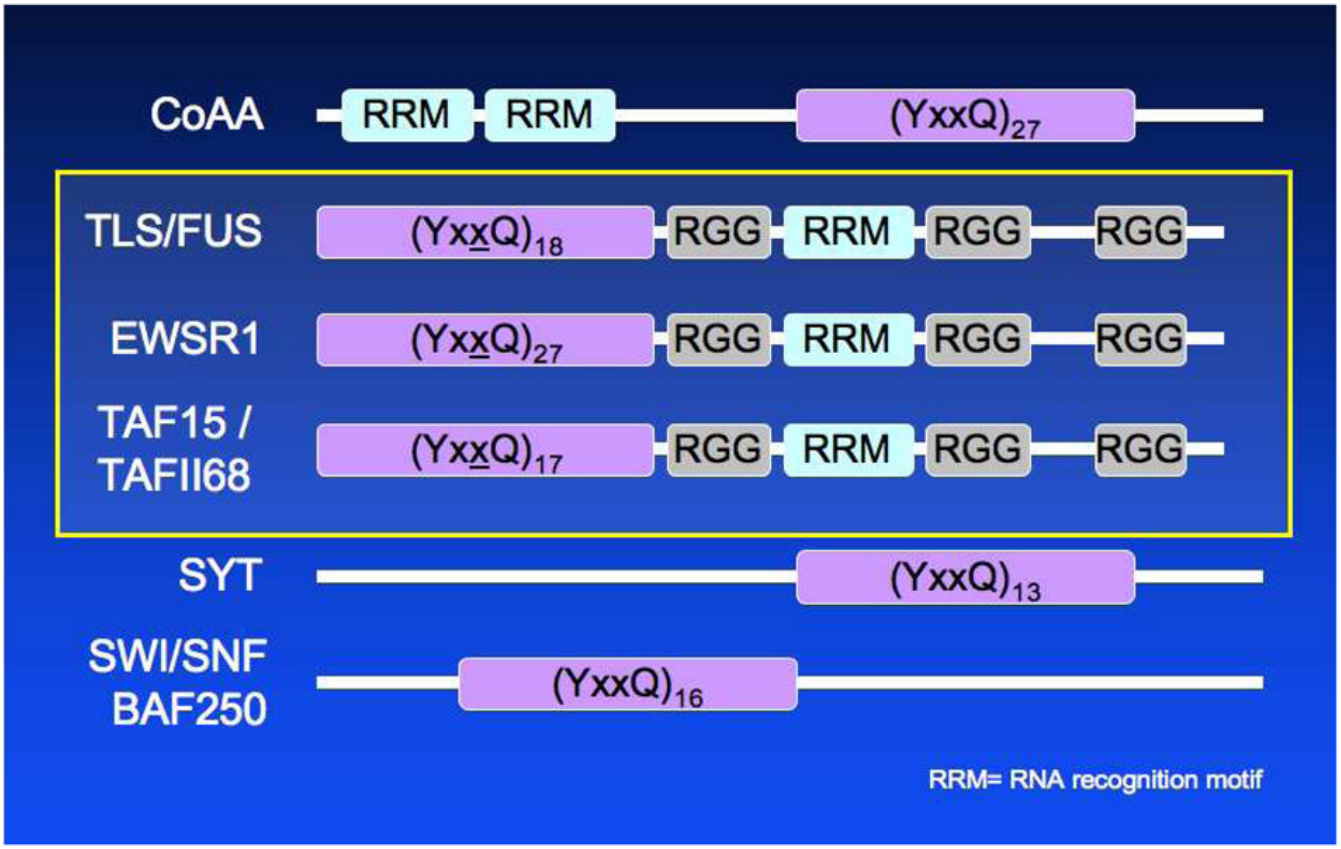

Figure 1. FET/TET proteins share structural similarities to CoAA and other proteins

FET/TET proteins (FUS/TLS, EWSR1 and TAF15) contain multiple YxxQ motifs in their N-termini. Such repeats are also present in CoAA, synovial sarcoma translocation protein (SYT) and SWI/SNF. RRM= RNA recognition motif. All other letters designate amino acids.

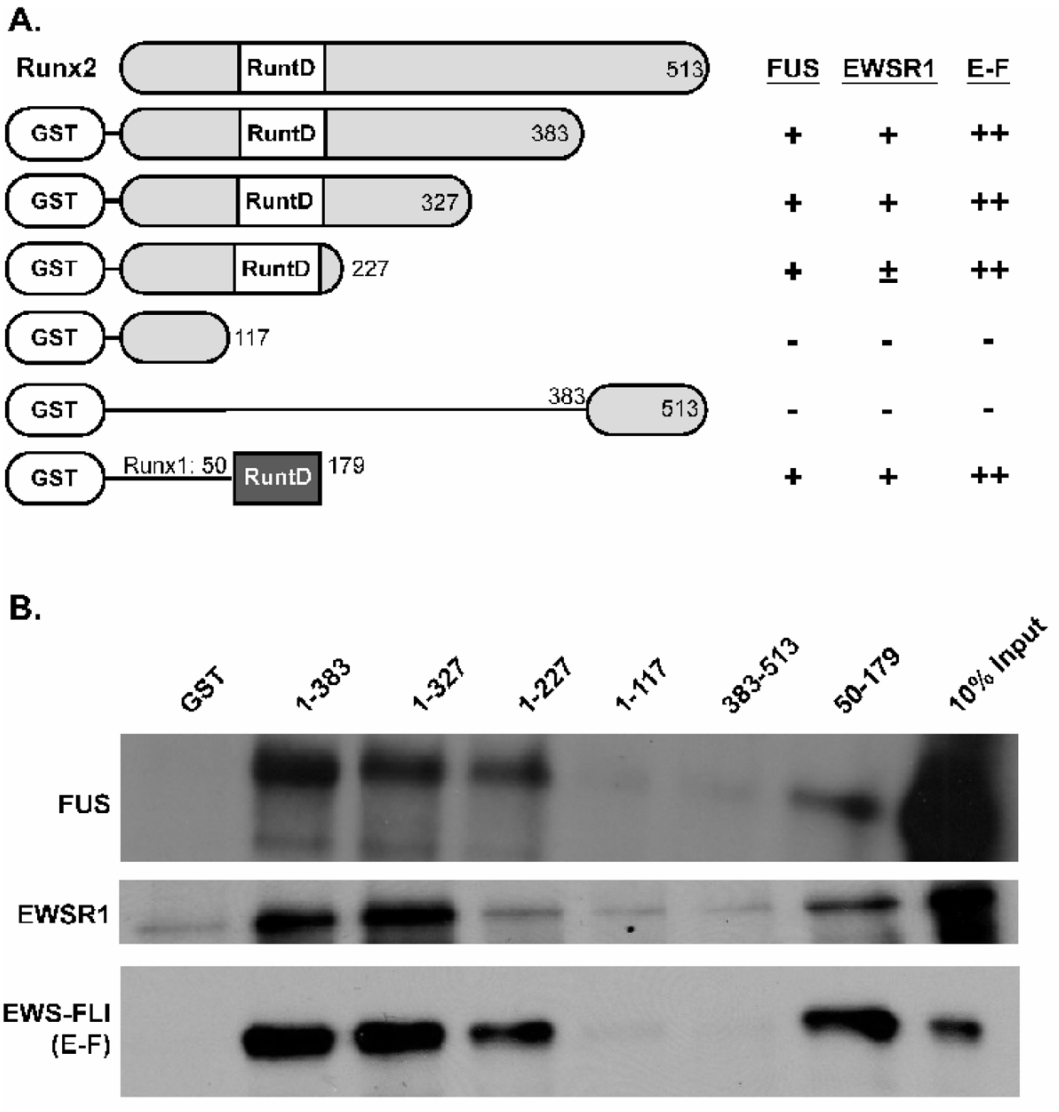

Figure 2. The Runt domains of RUNX1 and Runx2 bind FET/TET proteins

A. This diagram shows the GST-Runx fusion proteins used in this experiment and summarizes their interactions with FET/TET family members. E–F: EWS-FLI; -: no interaction; ±: weak interaction; +: strong interaction; ++ very strong interaction. **B.** FET/TET proteins interact with GST-Runx proteins retaining the Runt domain.

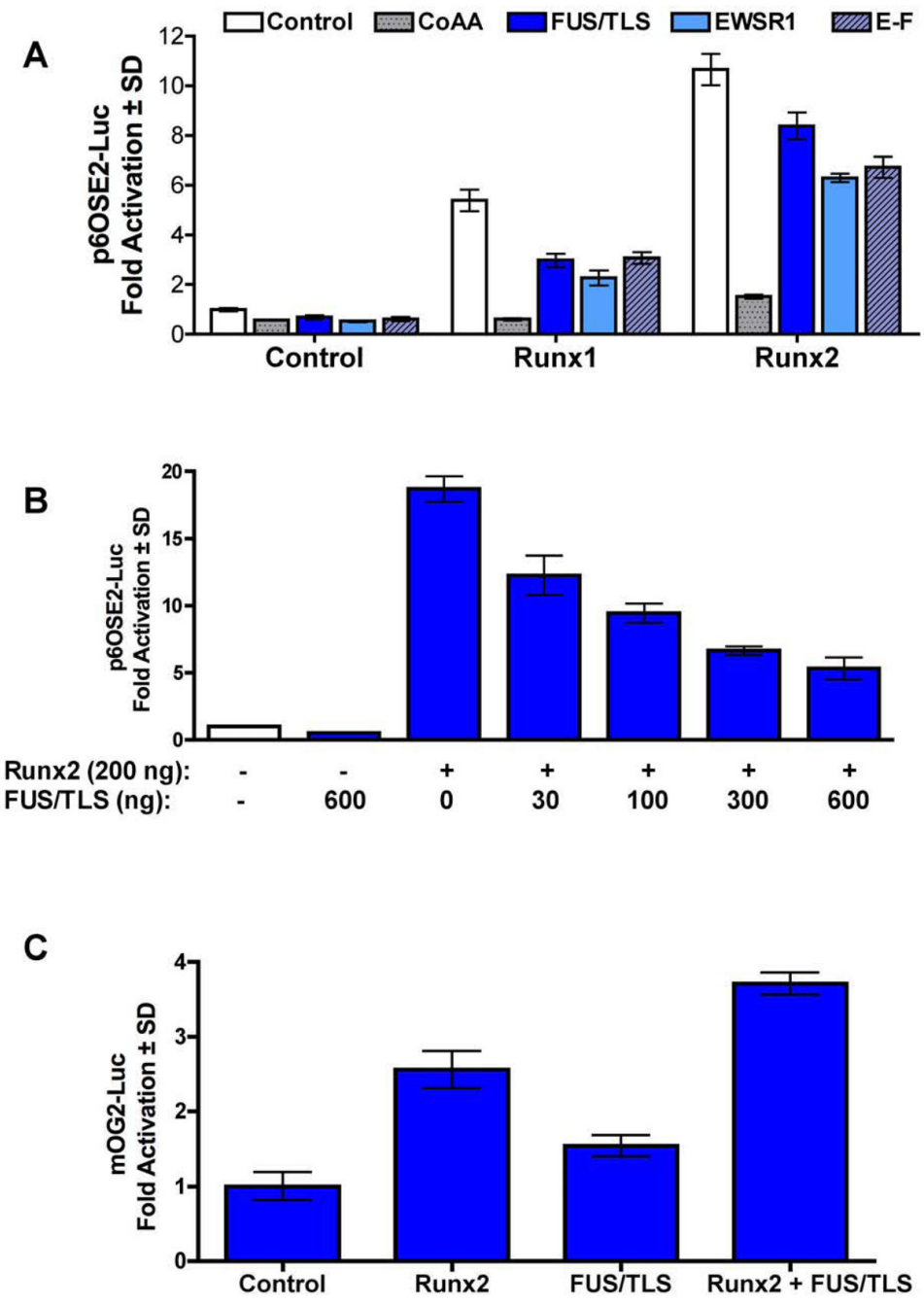

Figure 3. FET/TET proteins alter Runx2 transcriptional activity

A. C2C12 cells were transiently transfected with p6OSE2-Luc (200 ng), pRL-Luc (50 ng), pcDNA3 (Control), pCMV-Runx1 or -Runx2 (200 ng), and either pCMV-CoAA, -FUS/TLS, -EWSR1, or –EWS-FLI (300 ng) using Lipofectamine. Total DNA amounts in the transfections were normalized by the addition of pcDNA3. Firefly and renilla luciferase activities were measured two days later. Firefly luciferase (luc) activity was normalized to renilla luc activity. Data represent the mean of triplicate samples. Results are representative of at least three experiments. **B.** C2C12 cells were transfected as in A, except increasing concentrations of CMV-FUS/TLS was added as indicated. **C.** C2C12 cells were transfected as in A, except

mOG2-Luciferase (200 ng), which contains 1.3 kB of the murine osteocalcin promoter, was used as the primary reporter plasmid.