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The Cleidocranial Dysplasia-Related R131G Mutation in the Runt-Related Transcription Factor RUNX2 Disrupts Binding to DNA But Not CBF-β

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ABSTRACT

Cleidocranial dysplasia (CCD) is caused by haploinsufficiency in *RUNX2* function. We have previously identified a series of RUNX2 mutations in Korean CCD patients, including a novel R131G missense mutation in the Runt-homology domain. Here, we examine the functional consequences of the RUNX2^{R131G} mutation, which could potentially affect DNA binding, nuclear localization signal, and/or heterodimerization with core-binding factor-β (CBF-β). Immunofluorescence microscopy and western blot analysis with subcellular fractions show that RUNX2^{R131G} is localized in the nucleus. Immunoprecipitation analysis reveals that heterodimerization with CBF-β is retained. However, precipitation assays with biotinylated oligonucleotides and reporter gene assays with RUNX2 responsive promoters together reveal that DNA-binding activity and consequently the transactivation of potential of RUNX2^{R131G} is abrogated. We conclude that loss of DNA binding, but not nuclear localization or CBF-β heterodimerization, causes RUNX2 haploinsufficiency in patients with the RUNX2^{R131G} mutation. Retention of specific functions including nuclear localization and binding to CBF-β of the RUNX2^{R131G} mutation may render the mutant protein an effective competitor that interferes with wild-type function. J. Cell. Biochem. 110: 97–103, 2010.

KEY WORDS: RUNX2; RUNX2^{R131G}; CORE-BINDING FACTOR-β (CBF-β); CLEIDOCRANIAL DYSPLASIA (CCD); SUBCELLULAR LOCALIZATION; HETERODIMERIZATION; DNA-BINDING ACTIVITY; TRANSACTIVATION

Runt-related transcription factor RUNX2 [van Wijnen et al., 2004] controls normal bone formation by regulating growth and maturation of osteoblasts [Komori et al., 1997; Otto et al., 1997; Ducy et al., 1997; Banerjee et al., 1997; Choi et al., 2001; Pratap et al., 2003; Galindo et al., 2005; Young et al., 2007; Lou et al., 2009]. RUNX2 has several functional domains, including the N-terminal runt-domain which is responsible for both DNA binding and heterodimerization with CBF-β [Bae et al., 1994; Crute et al., 1996] and an immediately adjacent nuclear localization signal (NLS) [Kagoshima et al., 1993; Thirunavukkarasu et al., 1998]. The C-terminus of RUNX2 contains a transactivation domain [Thirunavukkarasu et al., 1998; Yagi et al., 1999], a nuclear matrix targeting signal [Zaidi et al., 2001], and a VWRPY peptide that supports transcriptional repression [Javed et al., 2000], as well as

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interacting domains for a number of co-regulatory proteins that transduce cell signaling pathways and support activation of RUNX2 target genes [Lian et al., 2006]. The C-terminal domain is essential for Runx2 function as evidenced by studies showing that loss of the Runx2 C-terminus causes complete loss of bone [Choi et al., 2001], a phenotype that is virtually indistinguishable from that of Runx2 null mice [Komori et al., 1997; Otto et al., 1997]. Mutations in both the N-terminal DNA binding domain and C-terminal transcriptional regulatory domains have been linked to Cleidocranial dysplasia (CCD; MIM 119600) indicating that RUNX2 is essential for early patterning and post-natal formation of the human skeleton [Mundlos et al., 1997; Zhou et al., 1999; Zhang et al., 2000; Otto et al., 2002; Kim et al., 2006].

CCD is an autosomal dominant inherited human bone dysplasia that is characterized by the absence of hypoplastic clavicles, persistently open or delayed closure of sutures, supernumerary teeth, and short stature [Lee et al., 1997; Mundlos et al., 1997; Kim et al., 2006]. Although many RUNX2 mutations from CCD patients have been identified [Mundlos et al., 1997; Zhang et al., 2000; Nagata and Werner, 2001; Tahirov et al., 2001; Otto et al., 2002; Yoshida et al., 2002, 2003; Puppin et al., 2005; Cunningham et al., 2006; Kim et al., 2006; Matheny et al., 2007; Li et al., 2009; Ryoo et al., 2009], genotype–phenotype relationships and the molecular pathology of RUNX2 mutations that cause CCD have remained unclear. Rigorous functional studies are required with distinct CCD-related RUNX2 mutations to validate postulated pathological effects on the osteogenic activity of RUNX2. Furthermore, natural amino acid variations that yield skeletal phenotypes characteristic of human CCD represent clinically validated RUNX2 mutants that will enable structure–function analysis of its multiple molecular activities.

Previously, we characterized 11 CCD patients with characteristic dental and skeletal abnormalities that were linked to mutations in the coding sequence of the RUNX2 [Kim et al., 2006]. Four novel mutations were identified including a splice donor site mutation (IVS1 + 1G > A) that produced an internal deletion of RUNX2 (RUNX2Δe1) resulting in partial loss of nuclear localization, as well as dysfunctions in DNA binding and transactivation [Kim et al., 2006]. In this study, we analyzed the functional consequences of a previously identified missense mutation of RUNX2 (RUNX2^{R131G}) in the Runt domain. We show that this mutation abolishes sequence-specific DNA binding and transcriptional enhancement. However, RUNX2^{R131G} retains nuclear localization and heterodimerization with CBF-β, suggesting that the CCD phenotype may result from competitive inhibition with wild-type RUNX2 for CBF-β and other transcriptional co-regulators in chromatin-related subnuclear microenvironments.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

LipofectAMINE™, α-minimum essential medium (α-MEM) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Nuclear and cytoplasmic extraction reagent was acquired from PIERCE Biotechnology (Rockford, IL). Nitrocellulose membrane and ECL™ Western blotting detection reagents were obtained from GE Healthcare (Bucks, UK). Mouse IgG monoclonal antibodies for hemagglutinin (HA; BAbCO, Richmond, CA), Myc (Invitrogen), Lamin B1 (Santa Cruz, CA), β-actin, as well as anti-mouse IgG (H + L) conjugated with Alexa Fluor® 555 (Invitrogen), goat anti-mouse IgG (Santa Cruz), Protein G sepharose (Santa Cruz) and Streptavidin-conjugated agarose CL4B (Sigma, Springfield, MO) were acquired from the indicated suppliers. A chemiluminescence assay kit was obtained from Tropix (Bedford, MA).

CONSTRUCTION OF HA-TAGGED RUNX2^{R131G}

Amino acid substitution mutation of RUNX2^{R131G} was generated by a two-step PCR approach [Kim et al., 2006] using the forward primer 5′-GCC CTC GCA CTG GGG CTG CAA CAA GAC CCT GCC-3′ and the reverse primer 5′-CCG TCC CAG AAC AAC GTC GGG GTC ACG CTC CCG-3′. PCR products and the RUNX2^{R131G} mutation were verified by sequencing using T7 promoter primer 5′-TAA TAC GAC TCA CTA TAG GG-3′ and BGH reverse primer 5′-TAG AAG GCA CAG TCG AGG-3′.

Recombinant proteins were expressed using pcDNA3.1-HA-RUNX2, pcDNA3.1-HA-RUNX2^{R131G}, and pCMV-Myc-CBF-β vectors. For transactivation studies, pOC1050-Luciferase and pGL3-6XRUNX2-Luc reporter gene constructs were used as previously described [Kim et al., 2006].

CELL CULTURE AND TRANSIENT TRANSFECTION

Chinese hamster ovary (CHO) cells were cultured in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. HeLa cells and HEK293T cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For transient transfections, we seeded CHO, HeLa or HEK293T cells at a density of 4×10^4 cells per well of a 8-well-chamber slide or 4×10^5 cells a well of 6-well-culture plate. Plasmids were transfected using LipofectAMINE™ according to the manufacturer's instructions. At 3 h post-transfection, complete growth medium was added to each transfected well and the cells were incubated at 37°C for 18 h.

WESTERN BLOT ANALYSIS

HeLa or HEK293T cells were seeded and transiently transfected with HA-RUNX2 or HA-RUNX2^{R131G} expression vectors with LipofectAMINE™ as described above. Nuclear and cytoplasmic extracts were prepared using NE-PER® nuclear and cytoplasmic extraction reagents (PIERCE Biotechnology) according to the manufacturer's protocol. Nuclear and cytoplasmic extracts (20 μg each) were separated by 10% SDS–PAGE and transferred onto nitrocellulose membrane. Blocking of transferred nitrocellulose membrane was performed in 5% non-fat dried milk in TBS-T (1× TBS containing 0.1% Tween-20) for 1 h at room temperature (RT). Membrane was incubated for 1 h at RT or overnight at 4°C with mouse monoclonal anti-HA IgG (diluted 1:2,000 in TBS-T), and then incubated for 1 h at RT with peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:4,000 in TBS-T). The blot was developed with Amersham ECL™ reagent. Goat anti-Lamin B1 or mouse anti-β-actin

antibodies were used as internal controls on the same membrane after deprobing of the membrane.

IN SITU IMMUNOFLUORESCENCE MICROSCOPY

HA-RUNX2 and HA-RUNX2^{R131G} expression vectors were transfected into HeLa cells. After 24 h, cells were washed with PBS and treated with 0.1% Triton X-100 for 5 min. After fixation of cells with 4% paraformaldehyde for 5 min, cells were incubated with mouse monoclonal anti-HA antibody to final concentration of 1:100 for 1 h at RT. Cells were then washed with PBS thrice and incubated with goat anti-mouse IgG conjugated with Alexa Fluor 555 for 1 h at RT. Cover slides were mounted with Prolong® Gold antifade reagent (Invitrogen) after three rinses with PBS. Immunofluorescent signals were captured using a Nikon light microscope (Nikon, Japan).

CO-IMMUNOPRECIPITATION ANALYSIS

HeLa or HEK293T cells were seeded and transiently transfected with HA-RUNX2 and Myc-CBF-β or HA-RUNX2^{R131G} and Myc-CBF-β expression vectors. Protein G bead slurry was added to 200 μg of nuclear extracts and incubated at 4°C for 1 h with rotation to eliminate non-specific-binding protein. After spin down, supernatant was incubated with mouse monoclonal anti-HA IgG or mouse monoclonal anti-Myc IgG at 4°C for 1 h with rotation. Newly prepared protein G bead slurry was added to the reacted supernatant and incubated at 4°C overnight with rotation. After centrifugation, Protein G beads were rinsed four times using wash buffer (50 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 10% glycerol, and 1% NP-40). 6× SDS-sample loading buffer (30 μl) was added to washed protein G beads and samples were heated to 100°C for 5 min. Boiled samples were separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. Blocking of transferred nitrocellulose membrane was performed in 5% non-fat dried milk in TBS-T for 1 h at RT, incubated for 1 h at RT. As above, blots were incubated for 1 h at RT or overnight at 4°C with mouse monoclonal anti-HA IgG or anti-Myc IgG (diluted 1:2,000 in TBS-T). Membranes were then incubated for 1 h at RT with the peroxidase-conjugated goat anti-mouse IgG antibodies (diluted 1:3,000 in TBS-T). The blot was developed with Amersham ECL™ Reagents. Antibodies for Lamin B1 or β-actin antibodies were used as internal controls on the same membrane after deprobing of the membrane.

DNA AFFINITY PROTEIN-BINDING ASSAY (DAPA)

HeLa or HEK293T cells were transiently transfected with pcDNA3.1-HA-RUNX2 or pcDNA3.1-HA-RUNX2^{R131G} expression vectors. Biotinylated wild type- or mutant RUNX2-binding site oligonucleotides (20 μg) corresponding to the −156/−112 segment of the mouse osteocalcin promoter [Kim et al., 2003] were added to 100 μg of nuclear extracts and incubated at RT for 1 h with rotation. Streptavidin immobilized on agarose CL-4B slurry (50%, 30 μl) was added to binding reactions with nuclear extracts and oligonucleotides and the mixtures were incubated at RT for 1 h with rotation and the beads were recovered by microcentrifugation. After three rinses with cold PBS, 30 μl of 6× SDS-sample loading buffer was added to protein/DNA complexes attached to the sepharose CL-4B. Samples were boiled for 5 min, separated by 10% SDS–PAGE and transferred onto nitrocellulose membranes. Blocking of transferred nitrocellulose membrane was performed in 5% non-fat dried milk in TBS-T for 1 h at RT, incubated for 1 h at RT or overnight at 4°C with mouse monoclonal anti-HA IgG or mouse monoclonal anti-Runx2 serum [Pratap et al., 2003] (diluted 1:2,000 in TBS-T), and then the membrane was incubated for 1 h at RT with the peroxidase-conjugated goat anti-mouse IgG antibodies (diluted 1:3,000 in TBS-T).

The RUNX2-binding site nucleotides sequences used in this study were as follows: wild-type RUNX2, Forward 5′-GAT CCG CTG CAA TCA CCA ACC ACA GCA-3′, Reverse 5′-GCG ACG TTA GTG GTT GGT GTC GTC TAG-3′; Mutant RUNX2, Forward 5′-GAT CCG CTG CAA TCA CCA AGA AC AGC A-3′, Reverse 5′-GCG ACG TTA GTG GTT CTT GT CGT CTA G-3′.

PROMOTER ACTIVITY ASSAY

HeLa Cells were transiently transfected with the pcDNA3.1-HA-RUNX2 or pcDNA3.1-HA-RUNX2^{R131G} expression plasmids (0.5 μg) plus either the osteocalcin gene pOC1050-Luciferase or pGL3-6XRUNX2-Luciferase reporters using LipofectAMINE PLUS reagent as previously described [Kim et al., 2006]. The pSV-β-gal plasmid expressing β-galactosidase was also co-transfected as an internal control for transfection efficiency. Luciferase activity was measured using D-luciferin as substrate in luciferase reaction buffer (15 mM potassium phosphate buffer, pH 7.5, 15 mM $MgSO_4$, 25 mM glycine, 4 mM EGTA, 1 mM ATP and 1 mM dithiothreitol) and normalized relative to β-galactosidase activity measured using a chemiluminescent assay kit (Tropix). Luminescence was measured by an AutoLumat LB953 instrument (EG&G Berthold, Walloc, Finland).

RESULTS

NUCLEAR LOCALIZATION OF RUNX2^{R131G}

The RUNX2^{R131G} mutation, which converts arginine 131 to glycine due to a dC to dG basepair substitution at nucleotide 391, was previously identified in a 13-year-old female Korean CCD patient [Kim et al., 2006] with supernumerary teeth and open fontanelle (data not shown). The location of this mutation within the conserved Runt homology domain predicts that the RUNX2^{R131G} mutation may affect one or more essential functions of RUNX2.

RUNX2 as well as RUNX1 and RUNX3 contain a NLS that is located immediately carboxyl-terminal to the Runt domain (amino acids 218–234 in RUNX2) [Thirunavukkarasu et al., 1998]. RUNX2 is localized in the nucleus and organized in subnuclear foci throughout the nucleus, as well as the peri-nucleolar region [Javed et al., 2000; Zaidi et al., 2001, 2004; Young et al., 2007]. Some RUNX2 mutations cause CCD by perturbing nuclear localization [Quack et al., 1999; Zhou et al., 1999; Otto et al., 2002; Sakai et al., 2002; Yoshida et al., 2003]. Interestingly, the mutation in RUNX2^{R131G} is localized in a putative NLS (-HWRCNKTLP-) and a RUNX2 N-terminal deletion mutant RUNX2Δe1 is in the cytoplasm [Kim et al., 2006]. To determine the localization of RUNX2^{R131G} at the single cell level, we expressed an epitope-tagged HA-RUNX2 wild-type protein and the R131G mutant protein in HeLa, CHO or HEK293 cells (Fig. 1 and data not shown). The subcellular location of the two proteins was examined by in situ

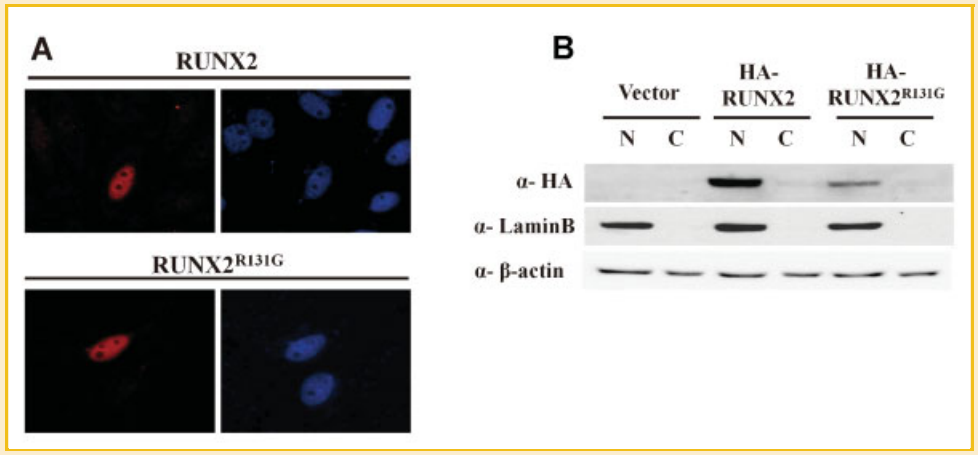

Fig. 1. Subcellular localization of RUNX2 and RUNX2^{R131G}. HA-tagged RUNX2 and RUNX2^{R131G} plasmids were transiently transfected in HeLa cells and the expression of the encoded proteins was observed by immunofluorescence microscopy. In situ immunofluorescence staining was performed with Alexa Fluor 555-labeled primary HA antibody. The DAPI panel shows staining of nucleus in whole-cell preparations (A). Nuclear (N) and cytoplasmic (C) extracts from CHO cells transfected with HA-tagged RUNX2 or RUNX2^{R131G} plasmid were used for western blot analysis with an HA antibody. Antibodies for Lamin B and β-actin were used as markers, for respectively, nuclear and cytoplasmic compartments, and internal loading controls (B).

immunofluorescence microscopy and Western blotting of subcellular fractions with HA- or RUNX2-antibody. The results show that both RUNX2^{R131G} and wild-type RUNX2 are localized in nuclei of HeLa cells (Fig. 1A), as well as CHO or HEK293 cells (data not shown). Biochemically, both RUNX2^{R131G} and the wild-type protein are predominantly present in the nuclear fraction (Fig. 2B). Thus, the subcellular compartmentalization of RUNX2 in the nucleus is not perturbed by the CCD-related mutation.

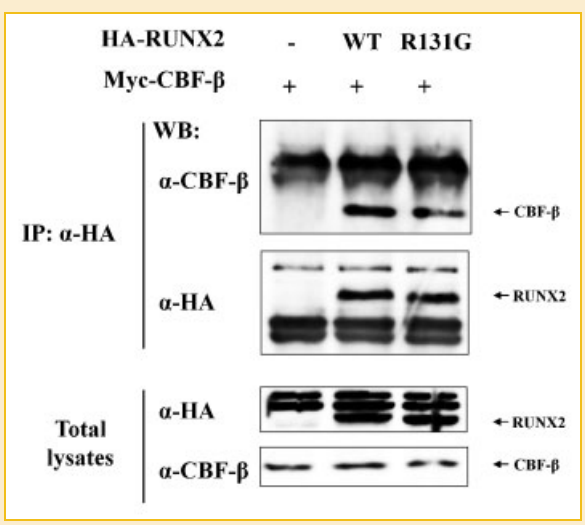

Fig. 2. Heterodimerization of RUNX2^{R131G} with CBF-β. HA-tagged RUNX2 or RUNX2^{R131G} and Myc-tagged CBF-β plasmid were co-transfected in HEK293 cells and proteins were immunoprecipitated with antibodies to HA. Immunoprecipitated proteins or total lysates were separated in 10% SDS-PAGE transferred, and immunoblotted with antibodies to either CBF-β or HA.: Mock Plasmid; WT: HA-tagged wild-type RUNX2 plasmid; R131G: HA-tagged RUNX2^{R131G} plasmid.

THE RUNX2^{R131G} MUTANT DOES NOT BIND TO THE RUNX RECOGNITION MOTIF BUT HETERODIMERIZES WITH CBF-β

According to the three-dimensional structure of the Runt domain, the R131G site is located in β3 strand which contacts the DNA at the consensus sequence 5′-TGTGGTT-3′ [Tahirov et al., 2001]. This location is structurally analogous to the R80C mutation in RUNX1 that has been observed in one acute myelogenous leukemia patient [Osato et al., 1999]. The RUNX1^{R80C} mutant shows abrogation of DNA-binding activity without affecting heterodimerization with CBF-β. Therefore, we performed immunoprecipitation analysis and DNA-binding assays (DAPA) to assess whether the RUNX2^{R131G} mutation affects either heterodimerization with CBF-β or binding to a RUNX-binding consensus site in a target gene promoter. Immunoprecipitates with either wild-type RUNX2 or RUNX2^{R131G} but not samples obtained from untransfected control cells, clearly contain CBF-β (Fig. 2). CBF-β was not detected in RUNX2 untransfected control cells. With respect to DNA-binding activity, the wild-type RUNX2 but not the RUNX2^{R131G} mutant interact with the RUNX2-binding consensus site oligonucleotide in DAPA assays (Fig. 3A), yet both proteins were each robustly expressed in the nucleus (Fig. 3B). These results indicate that RUNX2^{R131G} has the ability to heterodimerize with CBF-β, but is incapable of DNA binding to the RUNX consensus motif.

TRANSACTIVATION OF RUNX2^{R131G} FOR TARGET GENES

The loss of DNA-binding activity but retention of heterodimerization potential with CBF-β observed for the RUNX2^{R131G} mutant predicts that the transactivation function of RUNX2^{R131G} on RUNX target gene promoters is abrogated. We tested this prediction using a natural promoter of the osteocalcin gene or a chimeric reporter with six tandemly linked RUNX2 sites (6XRUNX2 promoter; also referred to as 6XOSE reporter). In transient transfection assays, wild-type RUNX2 increases promoter activity of the osteocalcin gene and 6XRUNX2 promoter (Fig. 4). However, the RUNX2^{R131G} mutant does

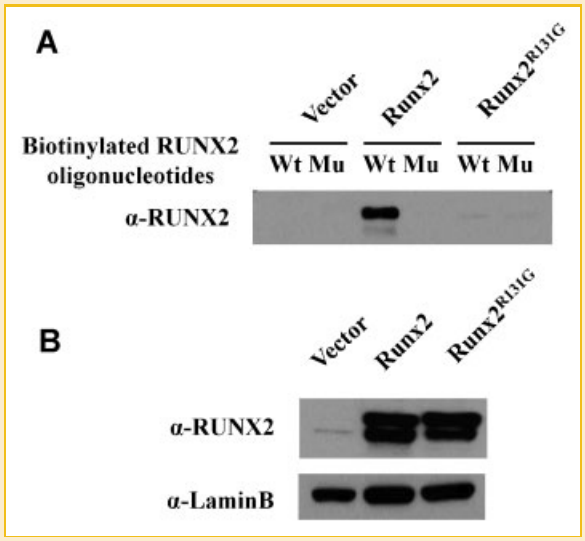

Fig. 3. DNA-binding activity of RUNX2 or RUNX2^{R131G}. HA-tagged RUNX2 or RUNX2^{R131G} proteins were expressed in HeLa cells. Nuclear extracts from transfected HeLa cells were incubated with biotinylated wild type (Wt) or mutant (Mu) RUNX2-binding oligonucleotides for 1 h at RT. Streptavidin-coated Sepharose CL4B beads were added to nuclear protein/DNA complexes and incubated at RT for 1 h with rotation. Reacted (A) or non-reacted (B) nuclear extracts were separated in 10% SDS–PAGE, transferred, and immunoblotted with antibodies to RUNX2. Lamin B antibody was used as internal loading control.

not have transactivation potential on either the osteocalcin or 6XRUNX2 promoters (Fig. 4). These results demonstrate that the transactivation function of RUNX2^{R131G} is abrogated consistent with the defect in DNA-binding activity.

DISCUSSION

This study shows that loss of DNA-binding activity is the primary molecular mechanism by which a novel RUNX2^{R131G} mutation causes CCD, based on examination of the molecular and cellular properties of the mutant protein. Mutation of arginine 131 in RUNX2^{R131G} is expected to perturb putative NLS within the Runt domain of RUNX2 [Kim et al., 2006]. However, our results show that the RUNX2^{R131G} is not aberrantly localized in the cytoplasm, but rather shows normal nuclear localization of RUNX2^{R131G}. Functional analysis of RUNX2^{R131G} revealed that heterodimerization with CBF-β is not perturbed, but that DNA binding and transactivation are abrogated in RUNX2^{R131G} (Fig. 5).

The Runt domain encodes the evolutionarily conserved protein structure that defines the RUNX family [Bae et al., 1994; Tahirov et al., 2001]. CCD mutations have clearly shown that natural amino acid substitutions in the Runt domain can affect sequence-specific DNA binding and/or heterodimerization with CBF-β and target DNA [Otto et al., 2002]. Our observation that the RUNX2^{R131G} mutant is capable of heterodimerizing with CBF-β in co-immunoprecipitation assays but does not bind the RUNX2 recognition motif is consistent with structural models for the Runt domain that predict that R131 is in proximity to DNA and not directly at the protein–protein interaction surface with CBF-β. CCD mutations have been correlated with three main DNA-binding peptides in the RUNX2 Runt domain: the $\beta_{A'-B}$ loop (V125–V138) that includes R131, the $\beta_{E'-F}$ loop (R190–S196), and three arginine residues that are part of the NLS (R225, R228, and R229) [Otto et al., 2002]. Specific mutations (R176W, F183S, K204N, T206I, R211W, and R211Q) in RUNX2 are postulated to perturb DNA binding without affecting heterodimerization with CBF-β [Yoshida et al., 2003]. Our present results establish that the RUNX2^{R131G} mutation also distinguishes between DNA-binding activity and CBF-β heterodimerization.

Most hematopoietic missense mutations in RUNX1 involve DNA-contacting residues in the Runt domain. For instance, Osato et al. [1999] reported a missense mutation resulting in conversion of arginine 80 to cysteine in RUNX1 (RUNX1^{R80C}), which corresponds to arginine 131 of RUNX2, in a blastic phase patient with chronic myeloid leukemia. Similar to our results, this RUNX1^{R80C} is active in heterodimerization with CBF-β, but shows neither DNA binding nor trans-activation. According to genotype–phenotype correlation

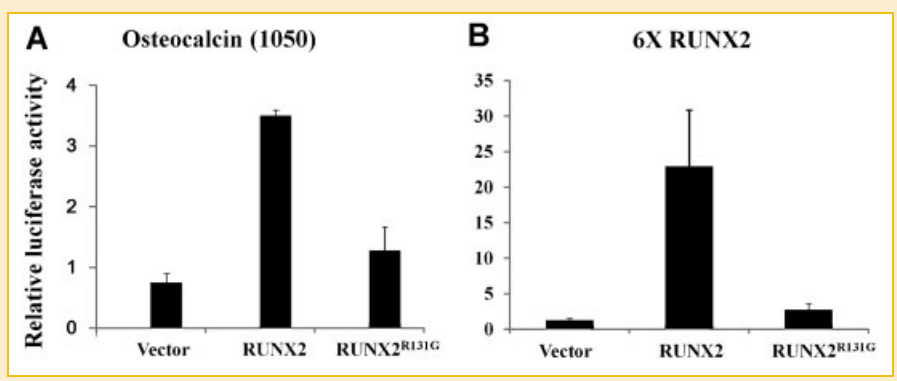

Fig. 4. Trans-activation of RUNX2 or RUNX2^{R131G}. HeLa cells were co-transfected with HA-RUNX2 or HA-RUNX2^{R131G} and the osteocalcin gene promoter derived pOC1050-luciferase (A) or 6XRUNX2-luciferase (B) reporters. The pSV-βgal plasmid expressing β-galactosidase was also co-transfected as an internal control for transfection efficiency. Total lysates were extracted from transfected HeLa cells and luciferase and β-galactosidase activities were assayed. Bars represent the average ratios of luciferase to β-galactosidase activity. The standard deviations obtained from three independent transfections of one representative experiment are represented by error bars. The values of relative luciferase activity represent the mean ± SD.

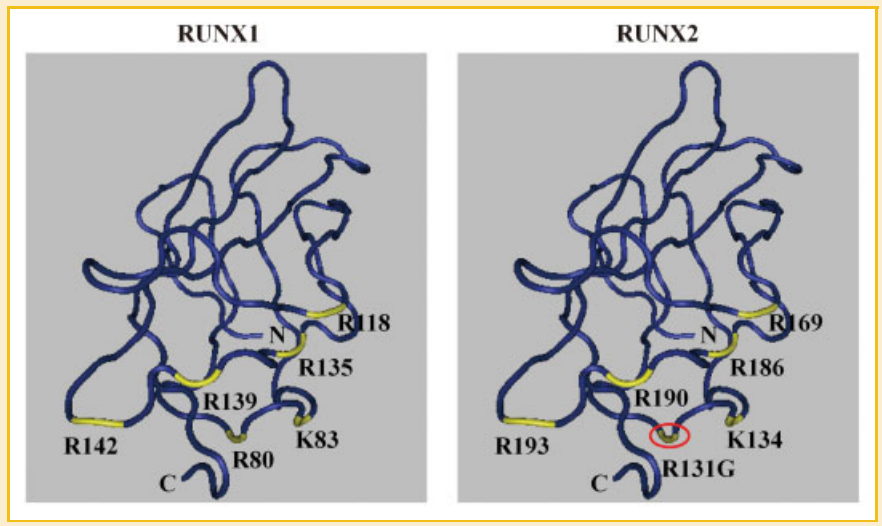

Fig. 5. Three-dimensional structure of RUNX1 and RUNX2 Runt domain. The three-dimensional structures of Runt domains from RUNX1 and RUNX2 were annotated based on amino acid numbering for each protein. Red circle indicates the novel missense mutation site (R131G) and yellow color points to DNA-binding residues. The three-dimensional structures were obtained from NCBI (CDD pfam00853).

studies of RUNX1 mutations in AML patients, RUNX1^{R80C} has been classified as a strong dominant negative mutation [Yoshida et al., 2003]. In contrast, CCD mutations exhibit haploinsufficiency effects instead of dominant negative effects. For this reason, it has been hypothesized that the CCD and hematopoietic disease mutations in RUNX2 and RUNX1, respectively, are functionally distinct [Matheny et al., 2007]. Our data suggest that the RUNX2^{R131G} mutant may act as a functionally defective competitive inhibitor consistent with dosage insufficiency rather than dominant negative interference with wild-type function. Because RUNX1^{R80C} and RUNX2^{R131G} have similar structural and biochemical properties (i.e., loss of DNA binding and retention of heterodimerization), the molecular basis for the clinical outcomes in leukemia (for RUNX1 mutations) or CCD (for RUNX2) may perhaps be fundamentally similar and be due to dosage insufficiency. However, the different clinical phenotypes observed for distinct RUNX2 mutants in CCD could be directly due to differences in functional properties of the mutants. We propose that differences in the competency to bind DNA or co-regulators, as well as sequestration in subnuclear or cytoplasmic compartments, may each contribute to the effectiveness of distinct mutants as competitive inhibitors and the phenotypic penetrance of RUNX2 mutations in the clinical manifestation of CCD.

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