The microtubule-associated protein DCAMKL1 regulates osteoblast function via repression of Runx2

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The microtubule-associated protein DCAMKL1 regulates osteoblast function via repression of Runx2

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Osteoblasts are specialized cells of mesenchymal origin that synthesize the extracellular matrix proteins required for mineralization of the embryonic skeleton (Erlebacher et al., 1995; Karsenty and Wagner, 2002). The high synthetic capacity of the osteoblast is also essential for maintaining bone mass during postnatal remodeling (Harada and Rodan, 2003). As bone accrues damage as the result of physiological exposure to mechanical stress, multinucleated giant cells termed osteoclasts localize at these sites and resorb the existing bone through an acidic and enzymatic process (Boyle et al., 2003). Osteoblasts are then recruited to sites of resorption where they secrete both collagenous and noncollagenous proteins that form the extracellular matrix that will give rise to newly mineralized bone (Martin et al., 2009). Functional dysregulation of osteoblasts during this remodeling process can affect bone density and contributes to the pathogenesis of skeletal disorders, such as osteoporosis, the most common skeletal disease worldwide (Zaidi, 2007). Thus, numerous regulatory pathways that control osteoblast differentiation and function exist to ensure that skeletal formation and remodeling proceed unperturbed.

Several regulatory mechanisms influencing osteoblast differentiation center on controlling the activity of the transcription factor Runx2 (Lian et al., 2006). The expression of Runx2 in mesenchymal precursors is essential for their commitment to the osteoblast lineage as demonstrated by the lack of both mature osteoblasts and a mineralized skeleton in Runx2−/− mice (Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997). Although the presence of a single copy of Runx2 is sufficient for mineralization to occur

Abbreviations used: 3-D, three-dimensional; Alp, alkaline phosphatase; API, Alp index; MSC, mesenchymal stem cell; TRAP, tartrate-resistant acid phosphatase.

Osteoblasts are responsible for the formation and mineralization of the skeleton. To identify novel regulators of osteoblast differentiation, we conducted an unbiased forward genetic screen using a lentiviral–based shRNA library. This functional genomics analysis led to the identification of the microtubule–associated protein DCAMKL1 (Doublecortin–like and CAM kinase–like 1) as a novel regulator of osteogenesis. Mice with a targeted disruption of Dcamkl1 displayed elevated bone mass secondary to increased bone formation by osteoblasts. Molecular experiments demonstrated that DCAMKL1 represses osteoblast activation by antagonizing Runx2, the master transcription factor in osteoblasts. Key elements of the cleidocranial dysplasia phenotype observed in Runx2+/− mice are reversed by the introduction of a Dcamkl1-null allele. Our results establish a genetic linkage between these two proteins in vivo and demonstrate that DCAMKL1 is a physiologically relevant regulator of anabolic bone formation.

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in vivo, Runx2+/- mice still exhibit delayed closure of the fontanelles and clavicular hypoplasia (Otto et al., 1997). The skeletal phenotype of Runx2+/- mice resembles the cleido-cranial dysplasia phenotype arising from Runx2 haploinsufficiency in humans (Mundlos et al., 1997). The presence of these skeletal pathologies in both humans and mice underscores the importance of precise regulation of Runx2 levels and activity during osteogenesis.

Although a significant number of molecules, like Runx2, have been described to regulate osteoblast biology, the complex temporal signaling networks that govern this process suggest that many regulators of osteogenesis remain to be identified. Historically, molecules critical for osteoblast differentiation and function have been identified through single gene mutations in humans and mice. Although this approach results in the identification of genes that are biologically relevant to the skeletal system, it is limited by the rate at which new genes are identified. Using a high-throughput forward genetic approach could accelerate the identification of additional molecules and signaling pathways that regulate osteoblast biology. Unbiased functional genomics approaches using RNAi-based loss-of-function screens have been successful in identifying key molecules that regulate several physiological and pathological processes (Moffat et al., 2006; Vasudevan et al., 2009; Kim et al., 2010). We therefore used arrayed libraries of lentiviral-based shRNAs to assess individual gene function during osteoblast differentiation in vitro.

DCAMKL1 (Doublecortin-like and CAM kinase–like 1) is a serine-threonine kinase of the CAMK family, showing homology to Doublecortin (DCX), which is mutated in X-linked lissencephaly (Omori et al., 1998; Burgess et al., 1999; Matsumoto et al., 1999; Sossey-Alaoui and Srivastava, 1999). An evolutionarily conserved Doublecortin (DC) domain that is able to bind to tubulin and enhance microtubule polymerization exists in the N terminus of DCAMKL1 and DCX. Both DCAMKL1 and DCX are highly expressed in the developing brain and may function together to regulate microtubules involved in neuronal migration. Dcamkl1-deficient mice are viable and fertile and display modest neural abnormalities (Deuel et al., 2006; Koizumi et al., 2006), suggesting that Doublecortin may provide partial functional compensation. Subsequent studies have demonstrated that DCAMKL1 exhibits variable expression patterns in embryonic and adult tissues outside of the central nervous system, suggesting that this protein may control additional physiological processes (May et al., 2008; Gerbe et al., 2009; Itzkovitz et al., 2012).

In this study, we describe a previously unknown role for DCAMKL1 within the skeletal system to regulate osteoblast functions and bone formation. We identified DCAMKL1 through a functional genomics approach that used RNAi-based loss-of-function screens to assess individual gene function during osteoblast differentiation in vitro. Mice lacking Dcamkl1 exhibit an increase in osteoblast numbers, elevated bone mass, and increased rates of bone formation, which confirm that DCAMKL1 is a negative regulator of anabolic bone formation. This occurs via the ability of DCAMKL1 to control osteoblast differentiation through antagonism of Runx2 transcriptional activity. Analysis of Dcamkl1/Runx2 compound mutant mice revealed partial rescue of the clavicular hypoplasia, runting, and calvarial hypomineralization seen in Runx2 haploinsufficiency. These results provide genetic evidence that DCAMKL1 is an important novel regulator of Runx2, the master transcription factor for the osteoblast lineage.

### RESULTS

#### A functional genomics approach to identify novel regulators of osteoblast differentiation

To identify unique genes that dictate the differentiation of mesenchymal stem cells (MSCs) into the osteoblast lineage, we initiated an unbiased screen that used a lentiviral-based shRNA library that targeted ~1,500 different human kinases, phosphatases, and receptors with each gene in the library being covered by four to five separate shRNAs (http://www.broadinstitute.org/rnai/trc/lib; Moffat et al., 2006). This screen was conducted in primary human MSCs that under the appropriate culture conditions can be reproducibly differentiated along the osteoblast lineage. As the human MSCs differentiate into osteoblasts, they up-regulate expression of the characteristic osteoblast marker alkaline phosphatase (Alp), which can be quantified via a simple colorimetric assay (Fig. 1A). We used this characteristic of hMSCs to analyze the capability of the various shRNAs to alter differentiation into the osteoblast lineage. As proof of principle that hMSC lineage commitment can be altered, we demonstrated that transduction of these cells with Runx2-specific shRNAs resulted in reduced osteogenesis, as shown by staining with Fast blue (Fig. 1B). For the large-scale screen, Alp levels of lentivirus-infected MSCs were quantified after a 7-d culture period and were normalized to cell number with Alamar blue (Fig. 1C). An Alp index (API) is assigned to each shRNA sample, and a list of candidates were identified in the screen based on the API of known genes that regulate MSC differentiation (see Materials and methods). Our screen and subsequent analysis demonstrated robust induction of Alp levels by shRNA directed against Dcamkl1, a member of the doublecortin family of microtubule-associated proteins (Omori et al., 1998; Burgess et al., 1999; Matsumoto et al., 1999; Sossey-Alaoui and Srivastava, 1999). We next asked whether reduction of DCAMKL1 protein levels by individual shRNAs correlated with osteoblast phenotype. As shown in Fig. 1D, those hairpins that augmented Alp levels also reduced Dcamkl1 protein levels, as measured by Western blot analysis. More importantly, Dcamkl1-targeted shRNAs also induced elevated levels of mineral deposition as determined by von Kossa staining (Fig. 1E). Collectively, these data demonstrate that knockdown of Dcamkl1 by shRNA hairpins can augment the differentiation of osteoblasts from undifferentiated human mesenchymal precursors in vitro.

#### DCAMKL1 regulates mouse osteoblast differentiation in vitro

To further explore the function of DCAMKL1 in osteoblastogenesis, we sought to establish whether this protein functions in the mouse skeletal system. In situ hybridization of
cell types that control bone formation and that differentiation and function of osteoblasts in vitro are sensitive to the endogenous levels of DCAMKL1.

**Dcamkl1**−/− mice exhibit elevations in bone mass and bone anabolism

Mice deficient in Dcamkl1 have previously been generated and described to be viable and fertile (Deuel et al., 2006; Koizumi et al., 2006). To examine the effect of Dcamkl1 on skeletal development, we first analyzed the growth plate of the Dcamkl1−/− mice by hematoxylin and eosin (H&E) staining, revealing that Dcamkl1−/− mice have a well-organized growth plate and that the growth plate architecture of these mice is indistinguishable from that of WT controls (Fig. 3A). To further determine the in vivo effects of Dcamkl1 expression within the skeletal system, we used quantitative computed tomography (µ-QCT) to determine whether differences can be observed in skeletal elements isolated from Dcamkl1−/− mice when compared with skeletal elements from WT control mice. As shown in Fig. 3 (B–F), the distal femurs of Dcamkl1−/− mice exhibited increases in trabecular bone volume (BV/TV), trabecular number
To assess whether the increase in osteoblasts is related to expansion of a pool of osteoprogenitor cells, flow cytometric analysis of this population was performed. Recently, a population of osteoprogenitor cells with surface markers CD45$^{-}$Tie2$^{-}$αv$^{+}$CD105$^{+}$Thy1.2$^{-}$ (CD105$^{+}$Thy1$^{-}$) was isolated from bone marrow cells (Chan et al., 2009). This population of cells can produce bone through endochondral ossification. To assess whether there are more osteoprogenitors in the bone marrow of Dcamkl1$^{-/-}$ mice, bone cells were isolated from WT and Dcamkl1$^{-/-}$ mice and analyzed using flow cytometry. Dcamkl1 knockout embryos showed similar numbers of CD105$^{+}$Thy1$^{-}$ osteoprogenitor cells when compared with littermate controls.
Figure 3. Elevated bone mass present in mice lacking Dcamkl1. (A) H&E staining of distal femur of 4-wk-old WT and Dcamkl1−/− mice showing the growth plate architecture of these mice. The data are representative of two independent experiments. (B) 3-D µ-QCT image of distal femurs isolated from 9-wk-old male Dcamkl1−/− and WT control mice. (C–F) Analysis of µ-QCT femur images from 9-wk-old male WT and Dcamkl1−/− mice for bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and cortical thickness (C.Th). Results are presented as the mean ± SD of six mice per group. (G) von Kossa staining and histomorphometry analysis of proximal tibia from 9-wk-old male Dcamkl1−/− and control mice. Results are representative of five mice per group. (H) Representative FACS profiles on pregated, CD45−Ter119− lineage cells harvested from long bone WT and Dcamkl1 knockout embryos. Percentages represent the percentage of cells within that quadrant. Values are mean ± SD of biological replicates. (I) Dual calcein labeling of tibial bone from WT and Dcamkl1−/− mice was visualized by fluorescent microscopy. Results are representative of five mice per group. (J) von Kossa staining of WT and Dcamkl1−/− osteoblast cultures at day 16. (K–O) Analysis of Tnalp (K), Bsp (L), Ocn (M), and Sost (O) via qPCR in WT and Dcamkl1−/− osteoblast cultures. Results are presented as the mean ± SD of triplicates of cells pooled from three mice per group and are representative of two independent experiments. Statistical analysis was performed using an unpaired Student's t test: *P < 0.05. Bars: (A) 100 μm; (B and G) 300 μm; (I) 10 μm; (J) 20 μm.

(Fig. 3 H and Fig. S1). Thus, by comparing the number of osteoprogenitors with the results of histomorphometry analysis, we conclude that DCAMKL1 likely functions during osteoblast differentiation, after commitment to the osteoblast lineage. Thus, increased numbers of osteoblasts and enhanced bone formation in Dcamkl1−/− mice reflect accelerated osteoblast differentiation, which is consistent with the results of our in vitro knockdown experiments (Fig. 1, D and E; and Fig. 2, D–F).
Additionally, bone formation rates were elevated in Dcamkl1<sup>−/−</sup> mice compared with control mice. This was caused by an increase in both mineral apposition rate and mineralizing surface as determined by dual-calcine labeling (Fig. 3 I and Table 1). To further verify that the increased bone mass observed in Dcamkl1<sup>−/−</sup> mice is the effect of augmented osteoblast activity, WT and Dcamkl1<sup>−/−</sup> bone marrow stromal cells were differentiated into osteoblasts. The primary osteoblast cultures from Dcamkl1<sup>−/−</sup> mice demonstrated an increased level of mineralized matrix production (Fig. 3 J). Consistent with the enhanced mineralized matrix formation, expression of characteristic osteoblast markers including tissue-nonspecific Alp (Tnap1), bone sialoprotein (Bsp), and osteocalcin (Ocn) were increased in Dcamkl1<sup>−/−</sup> osteoblasts when compared with WT osteoblasts (Fig. 3, K–M). The increased bone mass observed in the Dcamkl1<sup>−/−</sup> mice may be caused by alteration of Wnt–LRP5 pathways. We also examined the expression of the secreted WNT antagonist SOST and did not find obvious difference (Fig. 3 O). These data confirm our previous in vitro observation that Dcamkl1-specific shRNAs can augment osteoblast differentiation and establish that the increased bone mass observed in the Dcamkl1<sup>−/−</sup> mice arises from elevated osteoblast activity.

The increased bone mass present in the long bones of Dcamkl1<sup>−/−</sup> mice could also arise through impaired osteoclast differentiation and/or function. To address this, we analyzed tibia from 4-wk-old WT and Dcamkl1<sup>−/−</sup> mice for the presence of tartrate-resistant acid phosphatase (TRAP)–positive osteoclasts. In comparison with age-matched WT controls, Dcamkl1<sup>−/−</sup> mice showed similar numbers of TRAP-positive cells (Fig. 4, A and B). Further confirming these in vivo findings, bone marrow harvested from Dcamkl1<sup>−/−</sup> mice and cultured in the presence of osteoclast inducers RANKL (receptor activator of NF-κB ligand) and M-CSF resulted in the formation of a similar number of multinucleated TRAP-positive osteoclasts when compared with bone marrow from WT mice (Fig. 4 C). Further analysis of these cultures via quantitative PCR (qPCR) revealed that the osteoclast–specific markers cathepsin K, β3-integrin, and calcitonin receptor were induced to similar levels in WT and Dcamkl1<sup>−/−</sup> cultures when these cells were cultured under conditions that promote osteoclastogenesis (Fig. 4, D–F). Collectively, these results suggest that the elevated bone mass observed in Dcamkl1<sup>−/−</sup> mice is attributed to increased bone formation by osteoblasts as opposed to impaired bone resorption by osteoclasts.

**Table 1.** Histomorphometry analysis of Dcamkl1<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (n = 6)</th>
<th>Dcamkl1&lt;sup&gt;−/−&lt;/sup&gt; (n ≥ 5)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>BV/TV (%)</td>
<td>6.13 ± 0.74</td>
<td>10.39 ± 1.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.018</td>
</tr>
<tr>
<td>Tb.Th (µm)</td>
<td>28.22 ± 1.01</td>
<td>34.23 ± 1.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.055</td>
</tr>
<tr>
<td>Tb.N (l/mm)</td>
<td>2.18 ± 0.25</td>
<td>3.01 ± 0.29</td>
<td>0.055</td>
</tr>
<tr>
<td>Tb.Sp (µm)</td>
<td>464 ± 56</td>
<td>316 ± 39</td>
<td>0.055</td>
</tr>
<tr>
<td>MS/BS (%)</td>
<td>36.68 ± 1.80</td>
<td>42.16 ± 1.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.045</td>
</tr>
<tr>
<td>MAR (µm/d)</td>
<td>1.81 ± 0.05</td>
<td>2.16 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.033</td>
</tr>
<tr>
<td>BFR/BS (µm/µm&lt;sup&gt;2&lt;/sup&gt;/yr)</td>
<td>242 ± 13</td>
<td>331 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.004</td>
</tr>
<tr>
<td>BFR/BV (%)/yr</td>
<td>1.71 ± 36</td>
<td>2.016 ± 142&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.048</td>
</tr>
<tr>
<td>BFR/BV (%)/yr</td>
<td>105 ± 12</td>
<td>184 ± 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>10.19 ± 2.14</td>
<td>15.55 ± 1.27</td>
<td>0.057</td>
</tr>
<tr>
<td>N.Ob/T.Ar (l/mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>33.96 ± 5.19</td>
<td>81.52 ± 14.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.010</td>
</tr>
<tr>
<td>N.Ob/BS (l/mm)</td>
<td>8.68 ± 1.62</td>
<td>13.26 ± 1.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.042</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>5.56 ± 1.01</td>
<td>5.88 ± 0.94</td>
<td>0.824</td>
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<tr>
<td>O.Th (µm)</td>
<td>2.49 ± 0.33</td>
<td>2.40 ± 0.29</td>
<td>0.854</td>
</tr>
<tr>
<td>Oc.S/BS (µm)</td>
<td>0.84 ± 0.21</td>
<td>0.57 ± 0.14</td>
<td>0.303</td>
</tr>
<tr>
<td>N.Oc/T.Ar (l/mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>1.74 ± 0.59</td>
<td>1.54 ± 0.38</td>
<td>0.783</td>
</tr>
<tr>
<td>N.Oc/BS (l/mm)</td>
<td>0.37 ± 0.08</td>
<td>0.24 ± 0.06</td>
<td>0.205</td>
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<sup>a</sup>P < 0.05 compared with WT, unpaired Student’s t test.
<sup>b</sup>P < 0.01 compared with WT, unpaired Student’s t test.

DCAMKL1 regulates the activity of Runx2 in osteoblasts

We next sought to elucidate the mechanism underlying control of osteoblast differentiation by DCAMKL1. Runx2 is a transcription factor essential for osteoblast differentiation and functions. We therefore reasoned that DCAMKL1 might exert its inhibitory influence on osteoblast biology through antagonism of Runx2. To address this question, we asked whether overexpression of DCAMKL1 could alter the ability of Runx2 to activate transcription from the well-characterized OSE2 luciferase reporter construct that contains multimerized Runx2-binding sites (Ducy et al., 1997). As shown in Fig. 5 A, ectopic expression of DCAMKL1 did indeed inhibit Runx2
the kinase domain but lacking the doublecortin domains was unable to block transactivation by Runx2 (Fig. 5 E). A full-length DCAMKL1 mutant harboring a point mutation that renders the kinase region nonfunctional but retains its microtubule-polymerizing activity was also capable of inhibiting Runx2 activity in vitro (Fig. 5 F), indicating that DCAMKL1 represses Runx2 through its ability to promote microtubule polymerization, not its kinase activity.

We next questioned whether DCAMKL1 can promote microtubule polymerization in osteoblasts. To address this, osteoblasts were transfected with a construct expressing EGFP or EGFP-tagged DCAMKL1 constructs. Osteoblasts transfected with EGFP-DCAMKL1 formed numerous dendrite-like processes that were rich in DCAMKL1 expression (Fig. 5 H). Staining the cultured cells with anti-tubulin antibody revealed that these dendrite-like processes containing DCAMKL1 were also rich in tubulin (Fig. 5 H). Similar to what was observed with full-length DCAMKL1, transfection of the EGFP-DCL mutant protein that only expresses the doublecortin domain also resulted in the formation of tubulin-rich, dendrite-like processes (Fig. 5 I). As a control, transfection of EGFP or EGFP-CPG16 did not facilitate the formation of tubulin-rich, dendrite-like processes (Fig. 5, G and J). These data demonstrate that the doublecortin domains of DCAMKL1 are required for both microtubule polymerization and for inhibition of Runx2. Given that a previous study has demonstrated that the stabilization of microtubules results in the cytoplasmic transactivation of this reporter gene. In contrast, DCAMKL1 had no effect on the transcriptional activation of ATF4, another key transcription factor in osteoblast biology (Fig. 5 B). Interestingly, we were unable to detect an association between DCAMKL1 and Runx2 in communoprecipitation experiments (Fig. 5 C), suggesting that the repression of Runx2 function by DCAMKL1 occurs in the absence of a direct interaction between these two proteins.

To gain further insight into how DCAMKL1 controls Runx2 activity, we sought to identify those structural domains of DCAMKL1 that are required to repress Runx2 activity. DCAMKL1 contains a pair of unique doublecortin domains in the N terminus that facilitate its association with microtubules and promote their polymerization (Fig. 5 D; Burgess and Reiner, 2000; Lin et al., 2000). DCAMKL1 also contains a functional Ca\(^{2+}\)/calmodulin-dependent (CAM)–like kinase domain in its C terminus, although physiologically relevant substrates for this kinase have yet to be described (Fig. 5 D; Lin et al., 2000; Burgess and Reiner, 2002; Shang et al., 2003). Using a series of truncated mutant proteins of DCAMKL1 in the same luciferase reporter system described above, we found that the inhibitory function of DCAMKL1 requires the presence of the microtubule-associating doublecortin domains (Fig. 5, E and F). As shown in Fig. 5 E, expression of a C-terminal truncated DCAMKL1 mutant containing the doublecortin domains (DCL) retained its ability to repress Runx2 transactivation. In contrast, a truncated DCAMKL1 mutant containing the kinase domain but lacking the doublecortin domains was unable to block transactivation by Runx2 (Fig. 5 E). A full-length DCAMKL1 mutant harboring a point mutation that renders the kinase region nonfunctional but retains its microtubule-polymerizing activity was also capable of inhibiting Runx2 activity in vitro (Fig. 5 F), indicating that DCAMKL1 represses Runx2 through its ability to promote microtubule polymerization, not its kinase activity.

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sequestration of Runx2 (Pockwinse et al., 2006), we questioned whether DCAMKL1 may repress Runx2 transcriptional activity through sequestering Runx2 in the cytoplasm. To assess this, GFP-tagged DCAMKL1, GFP-tagged DCL mutant, or a control GFP expression vector were transfected into cells expressing myc epitope–tagged Runx2. Analysis of cells transfected with the control GFP expression vector revealed that Runx2 localization was predominantly nuclear.
and the subcellular distribution of Runx2 was not perturbed by GFP (Fig. 5 K). Similar to the observation described above, ectopic expression of the full-length DCAMKL1 or the DCL mutant promoted the formation of dendrite-like processes (Fig. 5, L and M). However, Runx2 was still retained in the nucleus of those cells expressing full-length DCAMKL1 or the DCL mutant (Fig. 5, L and M).

Multiple studies have documented that the regulation of Runx2 transcriptional activity is influenced through posttranslational modifications of specific residues within this protein (Bae and Lee, 2006; Jonason et al., 2009). We therefore asked whether DCAMKL1 can alter the phosphorylation pattern of Runx2. For this, we purified Runx2 from 293T cells that either coexpressed DCAMKL1, the DCL mutant of DCAMKL1, or cells treated with the well-characterized microtubule-stabilizing agent taxol (Fig. 6 A). Mass spectroscopy analysis of the purified Runx2 protein revealed that the presence of DCAMKL1 resulted in the phosphorylation of serine residue 125 (Ser125; Fig. 6, B and C). Interestingly, Ser125 phosphorylation is also detectable on Runx2 when it is purified from taxol-treated cells and from cells cotransfected with the DCL mutant of Dcamkl1 but is not detectable in control cells (Fig. 6 B). To further test whether the inhibition of Runx2 by DCAMKL1 is through the phosphorylation of Runx2 at Ser125, we constructed a mutant Runx2 in which Ser125 was mutated to glutamic acid to mimic phosphorylation of Ser125. Strikingly, Runx2 S125E mutation substantially decreased Runx2 activity. More importantly, DCAMKL1 could not further decrease the activity of the Runx2 S125E mutant (Fig. 6 D). A previous study has established that phosphorylation of Ser125 in Runx2 renders Runx2 transcriptionally inert (Phillips et al., 2006). Considering that the microtubule stabilizing agent Taxol could also induce Ser125 phosphorylation, our data support the conclusion that Runx2 inhibition by DCAMKL1 occurs through the induction of microtubule stabilization and the phosphorylation of Runx2 at this negative-regulatory serine residue.

**Generation and analysis of Dcamkl1/Runx2 compound mutant mice reveal a genetic interaction in vivo**

Based on the in vitro findings described above, we asked whether DCAMKL1 is a bona fide regulator of Runx2 in vivo. Runx2 haploinsufficiency in humans and mice results in the syndrome cleidocranial dysplasia, which is characterized by the delayed closure of the fontanelles and clavicular hypoplasia (Otto et al., 1999). Osteoblast differentiation and function may therefore depend on a precise regulation of multiple proteins that control bone mineralization (Zhao et al., 2009). In addition, mice with deletions of Dcamkl1-null allele into Runx2 heterozygous mice reversed aspects of the cleidocranial dysplasia phenotype of these mutant animals. Our results establish a genetic interaction between Dcamkl1 and Runx2 in vivo and demonstrate that DCAMKL1 is a physiologically relevant regulator of Runx2-mediated anabolic bone formation. Our study validates the usefulness of a functional genomics approach to discover novel regulators of osteogenesis.

Our findings demonstrate that DCAMKL1 regulates biological processes outside of the central nervous system. Similar to the function of DCAMKL1 in the central nervous system, this protein can also augment microtubule polymerization in osteoblasts. The inhibitory effects of DCAMKL1 on osteoblast differentiation may be explained by its ability to promote microtubule polymerization, a suggestion supported by previous reports demonstrating that treatment of osteoblast cultures with known inhibitors of microtubule assembly can enhance osteoblast differentiation in vitro (Zhao et al., 2009). Furthermore, it has been demonstrated that treatment of mice with these microtubule assembly inhibitors increased bone mass in vivo through augmentation of bone formation rates, mirroring what is observed in Dcamkl1−/− mice (Zhao et al., 2009). In addition to DCAMKL1, other proteins that associate with microtubules are also expressed in osteoblasts (Chiellini et al., 2008). The transcription of Stathmin-like 2 (Stmn2), a member of the stathmin family of cytosolic phosphoproteins, is up-regulated in hMSCs during osteoblast differentiation. However, in contrast to DCAMKL1, stathmins are a class of microtubule accessory proteins that inhibit microtubule polymerization (Curni et al., 1999). Osteoblast differentiation and function may therefore depend on a precise regulation of multiple proteins that...
Figure 6. DCAMKL1 regulates Runx2 activity, accompanied by Runx2 Ser125 phosphorylation. (A) Coomassie blue staining of purified Flag-Runx2 protein, which is used for the determination of Runx2 phosphorylation sites by mass spectroscopy. 293T cells were cotransfected with Flag-Runx2 and Dcamkl1 or DCL expression plasmids; after 48 h, Flag-Runx2 protein was purified from 293T cells using M2 beads from Sigma-Aldrich. For the Taxol treatment, the cells were treated with 0.1 µM Taxol for 16 h before harvesting the protein. (B) Schematic indicating the Runx2 phosphorylation sites identified by MS spec analysis. (C) Representative MS/MS spectra for Ser125 phosphorylated TDSPNLCVLPHPLWR peptide. The mass difference between the peaks corresponds directly to the amino acid sequence. The predicted fragmentation patterns, which are indicated by b ions (blue) and y ions (red), are included to establish a sequence both forward (b ions) and backward (y ions). MS/MS spectra were searched using the SEQUEST algorithm allowing for phosphorylation of serine, threonine, and tyrosine residues. Each spectrum was matched with a q-value of <1%. (D) Effects of Dcamkl1 on WT Runx2 and the Runx2-S125E mutant were assessed by p6xOSE2-Luc reporter in C3H10T1/2 cells. Results are presented as the mean ± SD of triplicates of cells and are representative of two independent experiments.
control the dynamic nature of microtubule polymerization and depolymerization.

We further demonstrate that DCAMKL1 antagonizes the activity of Runx2 in a transient reporter assay. We found that the ability of DCAMKL1 to repress Runx2 transcriptional activity required the Doublecortin domains of DCAMKL1. The Doublecortin domains are required for the microtubule-polymerizing activity of DCAMKL1, suggesting that Runx2 activity may be sensitive to active polymerization of microtubules. Coexpression of DCAMKL1 with Runx2 resulted in phosphorylation of Runx2 at Ser125. A previous study has demonstrated that phosphorylation of this serine residue inhibits Runx2 activity (Phillips et al., 2006). Mutation of the corresponding serine residue in human Runx2 has also been identified in a patient with cleidocranial dysplasia, suggesting that phosphorylation of this residue can influence Runx2 activity in vitro and in vivo (Wee et al., 2002). Although DCAMKL1 contains a functional kinase domain, phosphorylation of Ser125 on Runx2 was also observed in cells transfected with the DCL mutant, suggesting that the microtubule-stabilizing function of DCAMKL1 is necessary for its ability to induce phosphorylation of Runx2. Furthermore, phosphorylation of Runx2 at Ser125 could be promoted by taxol, a microtubule-stabilizing agent. Understanding how DCAMKL1-mediated microtubule polymerization promotes the phosphorylation of Runx2 is an area of active investigation. It has been established that microtubules can facilitate signaling from the cell surface to the nucleus through retrograde transport of kinases (Moises et al., 2007). Therefore, stabilization of microtubules by DCAMKL1 within osteoblasts may facilitate the localization of certain kinases in the nucleus that promote Runx2 phosphorylation.

A previous study has demonstrated that DCAMKL1 regulates the transition from prometaphase to metaphase through a process that also requires its doublecortin domains (Shu et al., 2006). DCAMKL1 may therefore alter osteoblast differentiation through affecting cell cycle progression. Indeed, commitment of MSCs to the osteoblast lineage requires exiting from the cell cycle, a process which is mediated by Runx2 (Pratap et al., 2003). Altering the DNA-binding and transcriptional activity of Runx2 at various stages of the cell cycle may dictate the stage-specific expression of genes that are essential for driving osteoblast lineage commitment (Qiao et al., 2006). This oscillation of microtubule polymerization and depolymerization, mediated by various mitogen-activated proteins (MAPs) such as DCAMKL1 may coordinate cell cycle with Runx2 transcriptional activity.

Through the generation and analysis of Dcamkl1/Runx2 compound mutant mice, we established that the cleidocranial dysplasia phenotype observed in Runx2+/− mice can be partially reversed by deletion of Dcamkl1 and provided in vivo evidence that Dcamkl1 is a bona fide regulator of Runx2. In addition to regulating osteoblast lineage commitment, Runx2 has also been reported to regulate chondrocyte maturation as well. To examine the effect of Dcamkl1 on chondrocyte development, we analyzed the growth plate of the Dcamkl1−/− mice by Safranin O staining and H&E staining (Fig. 3 A and
not depicted), revealing that Dcamkl1−/− mice have a well-organized growth plate and that the growth plate architecture of these mice is indistinguishable from that of WT controls. The absence of a growth plate phenotype in the Dcamkl1-null mice may result from compensatory mechanisms that arise between DCAMKL1 and other microtubule-associated proteins expressed in these tissues. Interestingly, another member of the doublecortin family of microtubule-associated proteins, DCX, is expressed in chondrocytes (Zhang et al., 2007). Within the central nervous system, significant overlap in the expression of DCAMKL1 and DCX results in a functional redundancy that masks specific phenotypes in the central nervous system that are only observed in Dcamkl1/Dcx compound mutant mice (Deuel et al., 2006; Koizumi et al., 2006; Shu et al., 2006). It is possible that the analysis of mice deficient in both Dcamkl1 and Dcx may reveal additional skeletal phenotypes, further expanding the functions of the microtubule-associated proteins to cell populations outside of the osteoblast.

MATERIALS AND METHODS

Mouse mutants. Compound Dcamkl1/Runx2 mutant mice were generated using the previously described strains of Dcamkl1−/− mice and Runx2−/− mice (Mundlos et al., 1997; Otto et al., 1997; Deuel et al., 2006). Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the institution’s subcommittee on animal care (IACUC).

shRNA screen. For the in vitro osteoblast differentiation screen, hMSCs (Lonza) were maintained and expanded according to the manufacturer’s instructions. For the entire primary and secondary screen, we used hMSCs from the same lot and passage to minimize variability. MSCs were seeded at a specific density in Opti-Lux 96-well plates (BD) in MSC growth media (MSGM) of the same lot and passage to minimize variability. MSCs were seeded at a specific density in Opti-Lux 96-well plates (BD) in MSC growth media (MSGM) and rested overnight. Each well was infected with an individual lentiviral clone that expresses a specific and unique shRNA along with a puromycin resistance gene. In addition to the ~8000 hairpins corresponding to 1500 individual shRNAs directed against GFP, RFP, luciferase, or β-galactosidase, Cells that had been successfully infected with the lentiviral clone were selected after puromycin treatment and cultured for an additional 4 d. Osteoblast differentiation was assessed by measuring levels of Alp. Given the variability in the osteoblast.

Bone morphometry. 9-week-old male WT and Dcamkl1−/− mice were intraperitoneally injected with 20 mg/kg calcine on days 8 and 3 before necropsy. Tibiae were removed, dehydrated in ethanol, infiltrated, and embedded without demineralization in methyl methacrylate. Undecalcified sections were cut at a thickness of 3 µm and mounted unstained for dynamic measurements. Consecutive sections were stained with toluidine blue to quantify bone cell measurements. Histomorphometric analysis was performed using the OsteoMeasure system (Osteometrics, Inc.), and the results were expressed according to standardized nomenclature (Parfitt et al., 1987). A sampling site with an area of ~0.12 mm² was established in the secondary spongiosa of the metaphysis. The dynamic measurements included mineral apposition rate (MAR), mineralizing surface (MS/BS), and bone formation rate expressed per bone surface referent (BFR/BS), bone volume referent (BFR/BV), and tissue volume referent (BFR/TV). Osteoblast surface (Ob.S/BS) and osteoclast surface (Oc.S/BS) were determined as trabecular surface covered by large basophilic cuboidal cells and multinucleated (two or more nuclei) cells, respectively. Osteoblast number (N.Ob) and osteoclast number (N.Oc) were counted and expressed per bone perimeter (B.Pm) and tissue area (T.Ar). Osteoid surface (OS/BS) and osteoid thickness (O.Th) were also measured.
Primary cell culture. Osteoblastic cells were isolated from calvariae of neonatal WT and DsRed2−/− littersmates as previously described (Jones et al., 2006). Calvarial-derived cells were plated in α-MEM + 10% FBS in a 6-well dish. Cells were harvested at a subconfluent stage and replated in a 6-well dish at a concentration of 10⁴ cells/cm² in α-MEM + 10% FBS + 50 µg/ml ascorbic acid + 5 mM β-glycerophosphate. For von Kossa staining, cells were fixed at day 21 of culture with 10% neutral buffered formalin and stained with 5% silver nitrate for 30 min. For Alp, cultures were fixed in 100% ethanol at day 14 of culture and stained using an Alp kit (Sigma-Aldrich) per the manufacturer’s instructions.

In vitro osteoclastogenesis, bone marrow cells were isolated from the femur and tibia of mice in α-MEM (Corning). After red blood cell lysis, cells were washed once and resuspended in α-MEM + 10% FBS and plated in a 48-well plate at a concentration of 2 × 10⁵ cells per 250 µl of α-MEM + 10% FBS. Cells were cultured for 2 d in the presence of 50 ng/ml M-CSF (PeproTech) followed by an additional 5 d in the presence of 50 ng/ml M-CSF and either 25 or 100 ng/ml RANKL (PeproTech). The cells were then fixed and stained for the presence of TRAP using a kit per the manufacturer’s instructions (Sigma-Aldrich).

Transient transfections and reporter gene assays. The mouse MSC line C3H10T1/2 was maintained in DMEM (Corning) + 10% FBS. For transient transfections, cells were seeded overnight in a 12-well dish at a concentration of 8 × 10⁴ cells/well. Cells were then transfected with a luciferase reporter gene plasmid and varied combinations of expression constructs, as indicated, using Effectene transfection reagent (QIAGEN). Total amounts of transfected DNA were kept constant by supplementing with control empty expression vector plasmids as needed. All cells were cotransfected with pRL-TK (Promega) as a normalization control for transfection efficiency. 48 h after transfection, cells were harvested and lysed in 1× Passive Lysis Buffer (Promega). Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). All luciferase experiments were repeated at least three times. C. Walsh (Beth Israel Deaconess Medical Center, Boston, MA) and L.-H. Tsai (Harvard Medical School, Boston, MA) provided the Dcamkl1 expression plasmids. The Runx2 expression plasmids were the gift of G. Karsenty (Columbia University, New York, NY). The multimerized OSE luciferase (fsoOSE2) construct was obtained from B. Olsen (Harvard School of Dental Medicine, Boston, MA).

Mass spectrometry. For phosphorylation site mapping on Runx2, purified Runx2 protein was resolved on SDS-PAGE and visualized by colloidal Coomassie blue. The band containing Runx2 was excised and treated with DTT to reduce disulfide bonds and iodoacetamide to derivatize cysteine residues. The protein was digested in gel by using trypsin and then analyzed by nanoscale-microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Villén and Gygi, 2008). MS/MS spectra were searched using the SEQUEST algorithm (Eng et al., 1994) against a database containing the sequences of all known and common contaminants such as human keratin proteins with static modifications of cysteine carboxymethylation, dynamic modification of methionine oxidation, and serine, threonine, and tyrosine phosphorylation. All peptide matches were filtered based on mass deviation, tryptic state, and XCorr and were confirmed by manual validation.

Online supplemental material. Fig. S1 shows a representative FACS profile of homogenized E17.5 long bone pre-grown for live CD45 Ter119 cells showing CD105 Thy1.2, CD105 Thy1.2. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20111790/DC1.

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