A RUNX2 stabilization pathway mediates physiologic and pathologic bone formation

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The osteoblast differentiation capacity of skeletal stem cells (SSCs) must be tightly regulated, as inadequate bone formation results in low bone mass and skeletal fragility, and over-exuberant osteogenesis results in heterotopic ossification (HO) of soft tissues. RUNX2 is essential for tuning this balance, but the mechanisms of posttranslational control of RUNX2 remain to be fully elucidated. Here, we identify that a CK2/HAUSP pathway is a key regulator of RUNX2 stability, as Casein kinase 2 (CK2) phosphorylates RUNX2, recruiting the deubiquitase herpesvirus-associated ubiquitin-specific protease (HAUSP), which stabilizes RUNX2 by diverting it away from ubiquitin-dependent proteasomal degradation. This pathway is important for both the commitment of SSCs to osteoprogenitors and their subsequent maturation. This CK2/HAUSP/RUNX2 pathway is also necessary for HO, as its inhibition blocked HO in multiple models. Collectively, active deubiquitination of RUNX2 is required for bone formation and this CK2/HAUSP deubiquitination pathway offers therapeutic opportunities for disorders of inappropriate mineralization.
s shown by both disorders of inappropriate bone forma-
tion, such as heterotopic ossification (HO) and disorders of
deficient bone formation, such as osteoporosis, the capa-
city of skeletal stem cells (SSCs) to differentiate into boney-
forming osteoblasts must be tuned in a context and tissue-
dependent manner. One of the best established molecular path-
ways regulating osteogenesis is the transcription factor RUNX2, which is
required for both the commitment of skeletal progeni-
tors to osteoprogenitors and the subsequent differentiation of
osteoprogenitors12,13. As a master transcriptional regulator of
skeletogenesis, RUNX2 is expressed in both mouse and human
skeletal progenitors14,15. Mice with germline deletion of Runx2
(Runx2−/−) show a complete absence of mineralized bone in both
calvaria and long bones, suggesting that RUNX2 is required for
both intramembranous and endochondral bone formation5. In
addition, haploinsufficiency for RUNX2 causes cleidocranial
dysplasia (CCD), as characterized by open fontanels, hypoplastic
clavicles, supernumerary teeth, and short stature, in both humans
and mice6–7. On the other hand, excessive osteoblast differen-
tiation can lead to disorders of ectopic mineralization, and RUNX2
is necessary for the pathogenesis of ectopic mineralization as
shown in human HO patients and mouse HO models8–11. Thus,
fine-tuning of RUNX2 expression and transcriptional activity is
central for both physiologic and pathologic bone formation.
While there are examples of regulating RUNX2 activity and
stability via phosphorylation12,13, acetylation14, or ubiquitina-
tion15–17, how posttranslational mechanisms control RUNX2 in
initial commitment to the osteoblast lineage and subsequently
sustained to drive osteoblast differentiation remain to be fully
elucidated.

Here we identify a key pathway stabilizing RUNX2 via Casein
Kinase 2 (CK2, encoded by Csnk2a2) mediated phosphorylation of
RUNX2 leading to recruitment of the deubiquitinase herpessirus-
associated ubiquitin-specific protease (HAUSP, also known as
USP7). HAUSP was identified as a regulator of the MDM2 and
p53 pathway18,19. HAUSP also controls a wide array of substrates
involved in immune responses, virus replication and infection,
mitosis, DNA replication, and DNA damage repair18–20. CK2 is a
constitutively active serine/threonine kinase that controls a
multitude of signaling proteins linked to cell cycle progression,
cell growth and differentiation21,22. CK2 is typically composed of
a tetrameric complex including two catalytic α (Csnk2a1)- or α′
(Csnk2a2)-subunits and two regulatory β subunits (Csnk2b)23–25.
The two catalytic subunits have high similarity in the catalytic
domain, although there is tissue-specific functional specialization
during embryonic development and as well as functional com-
ensation dependent on their expression levels26. The C-terminal
regions of the β-subunits bind the α-subunits and enhance the
catalytic activity and stability of CK2 complex. Previous in vitro
studies demonstrated a suppressive role of CK2 in osteoblast
differentiation and bone morphogenetic protein (BMP)
signaling27,28. However, its function in the skeleton has not been
studied. Here, we demonstrate that CK2-induced phosphoryla-
tion of RUNX2 recruits the deubiquitinase HAUSP for the sta-
bilization of RUNX2 and that this pathway is required for
physiologic and pathologic bone formation.

Results

CK2 is required for osteoblast differentiation. HO, abnormal
bone formation in soft tissues, occurs sporadically after burns,
traumatic brain injury, fractures and dislocations, and operative
procedures, resulting in restricted joint mobility, severe pain, and
nerve entrapment29–32. HO is postulated to reflect the aberrant
differentiation of soft tissue-resident stem cells to osteoblasts33.
Our data and others demonstrated elevated mRNA levels of
Runx2, a key regulator of osteogenesis, in mouse HO tissues
(Supplementary Fig. 14a), accompanied with a high expression of
RUNX2 protein in human and mouse HO tissues (Fig. 1a and
Supplementary Figs. 1b, 1c, 1f). Since RNAi-mediated knock-
down of RUNX2 prevented HO in mice34,35, we aimed to identify the
pathways controlling RUNX2. Previously, a high-throughput
short hairpin RNA (shRNA) screen was performed in human bone
marrow-derived mesenchymal stromal cells (BMSCs) using
an alkaline phosphatase (ALP) assay, a marker of early osteoblast
derdifferentiation in order to identify kinases and/or phosphatases
required for osteoblast differentiation34,35. In this study, shRNAs
that suppress ALP activity in the primary screen were further
examined in human BMSCs expressing a RUNX2-responsive luciferase
reporter gene (O2G-luc) to identify kinases and/or phosphatases that
specifically control RUNX2 activity/stability. This identified Casein Kinase 2 (CK2, Csnk2a2) as a putative
regulator of BMSC differentiation into osteoblasts and RUNX2
activation. ALP activity was markedly reduced by knockdown of
Csnk2a2 or Csnk2b, but not Csnk2a1 (Fig. 1b). In particular,
endogenous depletion of CK2 showed the strongest effect on ALP activity,
cellular adhesion, mineralization, and osteogenic gene expres-
sion (Fig. 1b–d and Supplementary Fig. 2a). Moreover, these
BMSCs showed a significant reduction in transcriptional activity of
RUNX2 (Fig. 1e, f). We next sought to identify the relevance of
these findings in primary human osteogenic progenitors. While
the human SSCs serving as a source of osteoblasts after growth
plate closure are currently unclear, we noted the presence of cells
bearing the same surface immunophenotype as previously
described murine SSCs (Lin-Thy-CD200 + CD105–) cells3,36
present in bone marrow aspirates (BMA). The osteogenic
potential of this population was substantially decreased when
with the CK2 inhibitor (i-CCK, Casein Kinase II inhibitor
IV) (Supplementary Fig. 3). Thus, CK2 is important for differ-
entiation of SSCs into osteoblasts and RUNX2 activation.

As CK2 is a constitutively active kinase, it is primarily
transcriptionally regulated37,38. Accordingly, both mRNA and
protein levels of CK2 subunits increased during osteoblast
derdifferentiation, whereas their expression was downregulated
in mature chondrocytes and adipocytes (Fig. 1g and Supplementary
Fig. 2b–e). When cultured under chondrogenic conditions,
Csnk2b-deficient BMSCs showed an increase in chondrogenic
potential evidenced by larger chondrocyte pellet size and
upregulated expression of chondrogenic genes (Fig. 1h, i). Likewise,
under adipogenic conditions, formation of lipid
droplets and expression of adipogenic genes were markedly
upregulated by Csnk2b-deficiency (Fig. 1j, k). These results
suggest that CK2 promotes osteoblast differentiation while it
suppresses differentiation into chondrocytes or adipocytes.

CK2 is essential for skeletal development. To explore a role CK2
in skeletal development, expression of Csnk2b was examined at
the stages of early limb and postnatal skeletal development
(Supplementary Fig. 4). Immunohistochemistry (IHC) dem-
strates the expression of Csnk2b in precursor cells residing in
the perichondrium, osteoblasts in the trabecular bone, and
chondrocytes in the growth plate at postnatal day 10 (P10).
Its expression was also detected at the tip of the limb bud of
E11.5 mouse embryos. Thus, Csnk2b is expressed throughout
skeletal development from early embryonic stage to postnatal
stage. Next, we conditionally deleted Csnk2b in the mesenchyme
by crossing Csnk2b (Csnk2bfl/fl)39,40 floxed allele with a Prx1-
Cre41 (Csnk2bPrx1). Csnk2b deletion was validated in the limbs
dissected from P0 Csnk2bPrx1 neonates (Fig. 2a). Severe limb
shortening was observed in E16.5 and P0 Csnk2bPrx1 pups and P0
Csnk2bPrx1 pups died after the birth due to respiratory distress
Alizarin red and alcian blue staining of skeletal preparations revealed that ossification (red) was markedly reduced in the calvaria, scapula, humerus, radius, ulna, femur, tibia, fibula, digit, and sternum of Csnk2bPrxl pups while cartilage (blue) is normally formed in skeleton (Fig. 2b, c and Supplementary Fig. 5b–f). Moreover, the clavicles of Csnk2bPrxl pups were hypoplastic (Fig. 2c, bottom, and Supplementary Fig. 5c, top). Likewise, endochondral ossification of long bones was arrested at the earliest stages of primary ossification center formation (Fig. 2d, e and Supplementary Fig. 5g, h). These skeletal phenotypes are similar to those seen in Runx2Prxl pups, suggesting that CK2 is required for RUNX2 regulation.

Skeletal development occurs through a hierarchy of bone lineage-specific progenitors, and these progenitors can be isolated.
Fig. 1 CK2 is required for BMSC osteoblast commitment. a Immunohistochemistry (IHC) for RUNX2 in human HO tissue. IgG was used for negative staining. Scale bar, 100 μm. b–d Human BMSCs expressing control shRNA (shCtrl) or shRNAs targeting CSNK2A1, -2A1, or -2B (shCSNK2A1, shCSNK2A2, shCSNK2B2) were cultured under osteogenic conditions. ALP activity was examined at day 7 (b), mineralization activity was assessed by alizarin red staining at day 14 (c), and expression of osteogenic genes was assessed by RT-PCR at day 12 (d) after osteogenic culture. b n = 14; c, n = 5; d, n = 4 biologically independent samples. shCtrl or shCSNK2B2-expressing human BMSCs (e) and shCtrl or shCsnk2B-expressing C3H10T1/2 cells (f) were transfected with the RUNX2-responsive reporter gene (OG2-luc) and Renilla in the absence (e) or in the presence (f) of RUNX2 overexpression. Three days after osteogenic culture (e) or 2 days after transfection (f), OG2-luc activity was measured and normalized to a Renilla. (n = 3 biologically independent samples). g Diagram depicting kinetics of CK2 expression over the differentiation of human BMSCs. h, i shCtrl or shCSNK2B2-expressing human BMSCs were cultured under chondrogenic conditions for 21 days. After alcian blue staining, the size of chondrocyte pellets was measured (h), mRNA levels of chondrogenic genes were assessed by RT-PCR (i). Scale bar, 300 μm (h). j, k n = 5 (shCtrl) or 6 (shCSNK2B2); i n = 4 biologically independent samples. j, k shCtrl or shCSNK2B2-expressing human BMSCs were cultured under adipogenic conditions for 12 days. Cells were stained with oil red O (j) and mRNA levels of adipogenic genes were assessed by RT-PCR (k). Scale bar, 100 μm (j), k n = 4 biologically independent samples. Data are representative of two (a) or three (b–f, h, k) independent experiments. One-way ANOVA with Dunnett’s multiple comparisons test (a) and a two-tailed unpaired Student’s t test for comparing two groups (c–f, h, i, k; error bars, SD of biological replicates).

from mouse limbs based on the expression of cell surface markers. Among these progenitors, we isolated homogenous populations of SSCs using recently described FACS strategies from the limbs of E17.5 Csnk2bfl/fl and Csnk2bpri/fl embryos (Supplementary Fig. 6). Csnk2b was efficiently deleted in Csnk2bpri/fl SSCs (Fig. 2f). Absolute numbers of SSCs were comparable between Csnk2bfl/fl and Csnk2bpri/fl embryonic limbs (Fig. 2g). However, while little cell death of these cells was observed, cell proliferation rate in Csnk2bpri/fl SSCs was slightly reduced in the culture (Supplementary Fig. 7a, b), implicating a role of CK2 in SSC survival and expansion within the kidney capsule (Fig. 2h). These phenotypes were also seen in COBs isolated from P5 Csnk2bfl/fl and P0 Csnk2bpri/fl calvaria (Fig. 4e). However, while little cell death of these cells was observed, cell proliferation rate in Csnk2bpri/fl COBs was significantly reduced in these cells and tissues (Supplementary Fig. 7c and Fig. 4f), suggesting that CK2 controls Runx2 expression at a post-translational level. To investigate the role of CK2 in osteoblast differentiation in vitro, primary calvarial osteoblasts (COBs) were isolated from P5 Csnk2bfl/fl pups and infected with lentiviruses expressing vector or cre recombinase. This inducible deletion by cre induction can offer better control of the timing of deletion, and more uniformly synchronous induction of cre activity than relying on the endogenous cre lines. As expected, Cre expression mediated efficient Csnk2b deletion in Csnk2bpri/fl COBs (Fig. 3a). In these COBs, osteoblast differentiation was substantially decreased as determined by ALP activity, mineralization, and expression of osteogenic genes, while levels of Runx2 mRNA were not altered in the absence of Csnk2b (Fig. 3b–d). These phenotypes were also seen in COBs isolated from mice with deletion of Csnk2b in osteoprogenitors (Csnk2b–/–/fl) (Supplementary Fig. 8). Likewise, Csnk2b–/– fl mice displayed osteopenia and specific phenotypes characteristic of Runx2 partial loss-of-function. P10 and 2-month-old Csnk2b–/– fl mice displayed open calvarial fontanelles, hypoplastic clavicles, and reduced body length (Fig. 3e–h and Supplementary Fig. 9a, b), similar to CCD seen in human patients with loss-of-function mutations in RUNX2 or mice with Runx2 haploinsufficiency. In addition, Csnk2b–/– fl mice displayed severe osteopenia in long bones as both the relative amount of trabecular bone and midshaft cortical bone thickness were both substantially reduced along with an increase in bone marrow adipocytes (Fig. 3i–k). Consistent with this, bone formation rate (BFR), mineralization apposition rate (MAR), and osteoblast surface per bone surface (Ob.S/BS) were decreased in Csnk2b–/– fl femurs (Fig. 3l, m), demonstrating reduced differentiation of Csnk2b-deficient osteoblasts. Numbers of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts and serum levels of the bone resorption marker C-terminal telopeptide type I collagen (CTX) were unchanged in 2-month-old Csnk2b–/– fl mice (Supplementary Fig. 9c–e). Taken together, CK2 is critical for osteoblast differentiation in vitro and in vivo, and loss of CK2 produces skeletal phenotypes similar to those seen with loss of Runx2 activity.

CK2 stabilizes RUNX2 in osteoblasts. Next, the ability of Runx2 to induce osteogenesis was assessed in the absence of Csnk2b following overexpression of Runx2. Accompanied with its reduced transcriptional activity (Fig. 1f), Runx2-depleted ALP activity and expression of osteogenic genes were markedly decreased in Csnk2b-deficient COBs, demonstrating that CK2 is required for Runx2-mediated osteoblast differentiation (Fig. 4a, b and Supplementary Fig. 10a). Intriguingly, Csnk2b-deficient COBs showed a significant reduction in protein levels of Runx2 (Fig. 4c) while ubiquitination levels of Runx2 were substantially increased in these cells after treatment with the proteasomal inhibitor MG132 (Fig. 4d). Similarly, a significant reduction in Runx2 protein levels was seen in Csnk2bpri/fl SSCs (Supplementary Fig. 7d, e) and in P0 Csnk2bpri/fl limbs and P0 Csnk2b–/– fl calvaria (Fig. 4e). However, Runx2 mRNA levels were unaltered in these cells and tissues (Supplementary Fig. 7c and Fig. 4f), suggesting that CK2 controls Runx2 expression at a post-translational level.
ALP activity and expression of osteogenic genes were substantially reduced in Tri-A-RUNX2 mutant-expressing COBs (Fig. 4k and Supplementary Fig. 10b), demonstrating that CK2-induced phosphorylation is crucial for RUNX2-mediated osteoblast differentiation. As observed in Csnk2b-deficient SSCs and osteoblasts, protein levels of Tri-A-RUNX2 were markedly reduced relative to WT-RUNX2 despite showing a slight elevation in the corresponding mRNA levels (Fig. 4l, m). This reduction in protein levels was accompanied by an increase in ubiquitination of the RUNX2-Tri-A mutant (Fig. 4n), suggesting that an increased susceptibility of the Tri-A-RUNX2 mutant to proteasomal degradation is
Fig. 2 CK2 is required for bone formation during skeletal development. a Csnk2b mRNA levels in the hindlimbs (femur and tibia) of E17.5 Csnk2bfl/fl and Csnk2bprx1 embryos. (n = 4). b, c Alizarin red/salicin blue staining of skeletal preparations of E17.5 Csnk2bfl/fl and Csnk2bprx1 pups. Scale bars, 250 μm (left) and 50 μm (right, enlarged one). d, e Csnk2b mRNA levels in SCS (CD45−Ter119 Tie2−αV−/−Int1 Thy1−) E13 CD105−CD200+ isolated from E17.5 Csnk2bfl/fl and Csnk2bprx1 embryos (t). Frequency of SCS within the population of total skeletal cells (CD45−Ter119 Tie2−αV−/−Int1 Thy1−) (g). n = 4; f, g Csnk2b mRNA levels in SCS (CD45−Ter119 Tie2−αV−/−Int1 Thy1−) E13 CD105−CD200+ isolated from E17.5 Csnk2bfl/fl and Csnk2bprx1 embryos were stained with H&E (h, j) or Von Kossa (i, j). The arrow highlights the ectopic bone. Scale bars, 200 μm (h); 100 μm (j). i n = 7 or 14. Data are representative of three (a–e, h, j) independent experiments or are pooled from two experiments (f, g, i). A two-tailed unpaired Student’s t test for comparing two groups (a, f, g, i, j) error bars, SD of biological replicates).

HAUSP controls osteogenesis via RUNX2 stabilization. To identify potential regulators of RUNX2 ubiquitination in osteoblasts, affinity purification using Flag-RUNX2 (WT or Tri-A) followed by mass spectrometry was performed after balancing the expression of these two constructs (Supplementary Fig. 11a). Ingenuity pathway analysis (IPA) of RUNX2-binding proteins identified enrichment of several signaling pathways including the protein ubiquitination pathway (red, Fig. 5a). Specifically, this identified SMURF1, a known ubiquitin E3 ligase of RUNX215, and the ubiquitin-specific protease (USP) family including HAUSP, USP9x, USP10, and USP47, as potential RUNX2-binding partners. Expression intensity of these USPs was markedly reduced in the eluate of Flag-RUNX2 (Tri-A) relative to Flag-RUNX2 (WT) (Supplementary Fig. 11b), suggesting that CK2-induced phosphorylation recruits these USPs to RUNX2. Knockdown studies in human BMSCs were used to determine which of these CK2-dependent RUNX2 interaction partners are important for osteoblast differentiation (Fig. 5b). Knockdown of HAUSP or USP24 but not USP9x or USP10 resulted in a significant reduction in ALP activity, mineralization, and expression of osteogenic genes (Fig. 5b–d and Supplementary Fig. 11c). Notably, similar to CK2, knockdown of HAUSP decreased protein but not mRNA levels of RUNX2 (Fig. 5e, f). HAUSP is an evolutionarily conserved deubiquitinating enzyme (DUB) and plays role in multiple cellular processes46. However, the function of HAUSP in osteoblast biology remains largely unknown.

In vitro deubiquitination assays demonstrate that ubiquitination levels of RUNX2 were substantially reduced by either enforced expression of HAUSP or the addition of recombinant HAUSP (rHAUSP) (Fig. 5g, h). Furthermore, co-immunoprecipitation confirmed an interaction between HAUSP and RUNX2 (Fig. 5i). However, unlike HAUSP, USP24 was unable to interact with RUNX2 and reduce ubiquitination levels of RUNX2 (Supplementary Fig. 12), suggesting that HAUSP, not USP24, functions as a bona-fide RUNX2-DUB. This process requires CK2-induced phosphorylation, as the interaction between RUNX2 and HAUSP was markedly reduced in the presence of the RUNX2-Tri-A mutation (Fig. 5i). Likewise, the interaction between rRUNX2 and rHAUSP was enhanced in a cell-free system by the addition of rCK2 and ATP but not rCK2 alone (Fig. 5i). In addition, while enforced expression of HAUSP increased protein levels of RUNX2-WT, the RUNX2-Tri-A mutant was largely refractory to HAUSP-induced stabilization (Fig. 5k). Taken together, CK2-induced phosphorylation of RUNX2 recruits HAUSP, stabilizing RUNX2 through deubiquitination (Fig. 5i).

Hausp haploinsufficiency causes CCD. Similar to knockdown of HAUSP in human BMSCs, Hausp-deficient COBs show a markedly decreased ability to induce osteoblast differentiation as determined by a significant reduction in expression of osteogenic genes, ALP activity, and mineralization (Fig. 6a–c). In addition, RUNX2 protein was reduced in HAUSP-deficient COBs without alteration of the corresponding mRNA levels (Fig. 6d, e), similar to the phenotypes shown in Csnk2b-deficient COBs. Accordingly, Hausp-deficient osteoblasts were refractory to RUNX2-induced transcriptional activation (Fig. 6f) and osteoblast differentiation (Fig. 6g, h and Supplementary Fig. 13a). Thus, HAUSP is permissive for osteoblast differentiation through stabilization of RUNX2.

Since the function of HAUSP in skeletal development was undefined, expression of HAUSP was examined in limb bud from E11.5 embryo and skeletal components (perichondrium, osteoblasts, and chondrocytes) from P10 mouse, and it showed similar expression aspects found in CSNK2B expression (Supplementary Fig. 4). To investigate a role of HAUSP in osteoblasts in vivo, Hauspfl/fl mice were crossed with Osx-Cre mice. Due to the extremely low birth rate of pups with homozygous deletion of Hausp (Hausplox/lox), skeletal phenotypes were studied in mice with a heterozygous deletion of Hausp (Hausp+/lox) (Fig. 6i). As observed in Runx2+/− and Csnk2blox/− mice, Hausplox/−/lox mice developed CCD phenotypes, as characterized by open fontanels, hypoplastic clavicles, and short stature (Fig. 6j, k). In addition, substantial osteopenia was observed in both the trabecular and cortical compartments, which was accompanied by an increase in bone marrow adipocytes (Fig. 6l–n). However, numbers of TRAP-positive osteoclasts and resorption activity in the metaphysis of trabecular bone were not altered in Hausplox/−/lox mice relative to Osx-Cre or Csnk2blox/−/lox mice (Supplementary Fig. 13c, d), demonstrating that osteoclasts are normal in Hausplox/−/lox mice. Thus, similar to CK2, HAUSP controls osteoblast differentiation via RUNX2 stabilization.

Finally, we investigated whether CK2 and HAUSP cooperate to control the RUNX2 pathway in vivo. The genetic interaction between RUNX2, CK2, and HAUSP was examined by comparing skeletal phenotypes of Osx-Cre, Csnk2blox/−/lox, Hausplox/−/lox, Csnk2blox/−/lox, Hausplox/−/lox, Runx2lox/−/lox, Csnk2blox/−/lox, and Runx2lox/−/lox, Hausplox/−/lox mice (Fig. 6o, p and Supplementary Fig. 13b). The CCD phenotypes seen in Hausplox/−/lox mice became more pronounced by additional heterozygous deletion of Csnk2b (Csnk2blox/−/lox, Hausplox/−/lox mice) (Fig. 6o). Of note, chondrocyte development in the growth plate is relatively normal in these mice (Supplementary Fig. 13e). Likewise, addition of heterozygous deletion of Csnk2b (Runx2lox/−/lox,Csnk2blox/−/lox mice) or Hausp (Runx2lox/−/lox, Hausplox/−/lox mice) results in more severe CCD phenotypes in Runx2lox/−/lox mice (Fig. 6p). Thus, these results suggest that CK2 and HAUSP operate in the same pathway to regulate RUNX2 in osteoprogenitors.
Inhibition of the CK2-HAUSP pathway suppresses HO. IHC on human HO tissue identified expression of CSNK2A and HAUSP in osteoblasts on the surface of heterotopic bone (Fig. 7a), which, when taken together with the expression of RUNX2 in the same tissue (Supplementary Fig. 1b), suggests that the CK2-HAUSP-RUNX2 pathway is intact at sites of HO. Expression levels of Csnk2a1 and HauS were all markedly upregulated at the site of injury in a mouse model of acquired HO (Fig. 7b). Thus, we hypothesized that RUNX2 stabilization by the CK2-HAUSP pathway contributes to the pathogenesis of HO and that...
inhibition of this pathway may prevent HO. To test this, mouse models representing the highest incidence forms of HO, muscle injury/BMP-induced HO29,47 and burn injury/Achilles tenotomy-induced HO48,49, were employed in Csnk2bfl/fl, Osx-Cre, and Csnk2bOxOx mice. In Csnk2bfl/fl and Osx-Cre control mice, HO occurred 3 weeks after blunt muscle trauma and administration of recombinant BMP2/7 (BMP2/7) (muscle injury/BMP-induced HO; Fig. 7c). Likewise, Achilles tenotomy following a remote burn injury resulted in HO at the tenotomy site 8 weeks post injury (burn/Achilles tenotomy-induced HO; Fig. 7f). Heterotopic bone was formed via an endochondral pathway including formation of cartilage and adipose tissues and recruitment of hematopoietic elements (Fig. 7e, left and h, top). By contrast, Csnk2bOxOx mice displayed a significant reduction in HO while cartilage or adipose tissue was still formed at the injury site (Fig. 7c–h), demonstrating that CK2 is required for heterotopic bone formation in mouse models of acquired HO. Notably, protein levels of RUNX2 were markedly decreased in the HO areas of Csnk2bOxOx mice relative to Csnk2bfl/fl mice while its mRNA levels were comparable in these HO tissues (Supplementary Fig. 14b–g). Thus, these results suggest that CK2-mediated stabilization of RUNX2 is important for the development of HO.

We next tested whether pharmacological inhibition of the CK2-HAUSP pathway can suppress HO development. Consistent with observations in Csnk2b-deficient COBs, treatment with a small molecule inhibitor of CK2 (i-CK2) reduced BMP2/7-induced ALP activity and mineralization and RUNX2 transcription activity (Supplementary Fig. 15a–c). Similarly, BMP2/7-induced ALP and mineralization activity and RUNX2 protein levels were decreased in osteoblasts by treatment with a small molecule inhibitor of HAUSP (i-OsxF), while levels of RUNX2 mRNA were not altered (Supplementary Fig. 15d–f). Next, we tested whether pharmacological inhibition of the CK2-HAUSP pathway can suppress ectopic bone formation in mouse models of HO. One day after either muscle injury/BMP2/7 injection or burn injury/tenotomy, WT mice were treated daily with a CK2 inhibitor or a HAUSP inhibitor and HO was assessed by microCT (Fig. 7i–l). As expected, vehicle-treated mice developed heterotopic bone at the sites of injured muscle 3 weeks after muscle injury and BMP2/7 injection or 8 weeks after burn injury and Achilles tenotomy. However, when treated with either a CK2 or HAUSP inhibitor, HO was substantially reduced at the sites of injury in both mouse models. These results provide proof-of-principle that inhibition of the CK2-HAUSP-RUNX2 pathway has therapeutic potential to prevent HO. Taken together, the CK2-HAUSP-RUNX2 pathway is a key regulator of osteoblast differentiation and both orthotopic and heterotopic bone formation.

Discussion

This study identifies that a CK2/HAUSP pathway stabilizing RUNX2 is essential for both physiologic and pathologic bone formation. Mechanistically, CK2 phosphorylates RUNX2 at T340, S354, and S387 and phosphorylated RUNX2 in turn recruits the DUB HAUSP, which stabilizes RUNX2 by counteracting ubiquitin-dependent proteasomal degradation (Fig. 5l). Previous studies have shown that ubiquitin E3 ligases, including SMURF1, SMURF2, or WWP1 induce ubiquitin-dependent proteasomal degradation of RUNX2 in osteoblasts and their deficiency in osteoblasts enhances osteoblast differentiation15–17. As CK2-induced phosphorylation of RUNX2 does not alter its interaction with these E3 ligases (Supplementary Fig. 11b), the CK2-HAUSP pathway is likely to operate as an independent, orthogonal mechanism for RUNX2 regulation. Lastly, this study discovers HAUSP as the DUB that stabilizes RUNX2 by suppressing ubiquitin E3 ligase-mediated ubiquitination. We note that, while we identify RUNX2 as a critical substrate for both CK2 and HAUSP in this study, both proteins are likely to have additional substrates that contribute to their regulation of osteogenesis.

HO can result in severely impaired functional recovery after injury as heterotopic bone spans across joints to limit their mobility29–31. Existing therapeutic approaches include bisphosphonates, external beam radiation, or nonsteroidal anti-inflammatory drugs, however each of these approaches can prevent healing of concurrent skeletal injuries and each can be complicated by either suboptimal efficacy or rare but potentially severe toxicities32,50. Given the relatively high incidence of HO, there is an urgent need to develop improved therapeutic agents. The evidence that RUNX2 expression was highly upregulated in both human and mouse HO tissues and that its inhibition prevented HO in mice implies that RUNX2 plays a role in the pathogenesis of HO8–11. Here, we identify a CK2-HAUSP pathway as a druggable pathway that controls RUNX2 for the treatment of HO. Development of acquired HO in two established models, muscle injury/BMP-induced HO29,47 and burn injury/Achilles tenotomy-induced HO48,49 was suppressed by Csnk2b deletion in osteoblasts or by treatment with either a CK2 or HAUSP inhibitor, providing proof-of-principle that inhibition of the CK2-HAUSP-RUNX2 pathway has therapeutic potential to prevent HO. Nonetheless, the long-term therapeutic outcomes and adverse effects of these inhibitors in treating HO require further investigation, as CK2 and HAUSP are involved in diverse cellular processes in addition to osteogenesis.
CK2 is a highly conserved serine/threonine kinase that is involved in various cellular processes. However, in vivo roles for CK2 in the skeleton are largely unknown. In contrast to previous in vitro studies showing that a CK2 inhibitor (CX-4945) or peptides that interfere with the interaction between CK2 and BMPR1a enhanced BMP-induced osteogenesis\textsuperscript{27,28,53,54}, we here find genetic evidence that orthotopic and heterotopic bone formation were substantially reduced in the absence of Csnk2b. Furthermore, in contrast to a previous study showing inhibitory effects of CX-4945 treatment on RANKL-induced osteoclast differentiation in vitro\textsuperscript{28}, osteoclast differentiation and bone mass are normal in mice lacking Csnk2b in osteoclasts (Csnk2b\textsuperscript{fl/fl}; Cathepsin K-cre, Supplementary Fig. 16a), suggesting that CK2 is dispensable for osteoclast activity in vivo. Notably, unlike osteoblasts, CK2 expression was not upregulated during osteoclast differentiation (Supplementary Fig. 16b). Thus, CK2 functions as a positive regulator of osteoblast differentiation with a function specific for osteoblast-lineage cells.
The vascular development in the skeletal system is essential for physiologic bone formation and skeletal fracture repair. Osteoblast-derived angiogenic factors, such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), and SLIT3, are crucial for this process. Given that CK2 plays a positive role in angiogenesis, Csnk2b-sufficient and -deficient COBs were cultured under undifferentiation conditions and subjected for transcriptome analysis, demonstrating down-regulation of a subset of the genes related to angiogenesis and vasculogenesis in the absence of Csnk2b (Supplementary Fig. 17). Of note, expression of key osteoblast-derived angiogenic factors, including Vegfa, Epo, and Slit3 and hypoxia-inducible factor-1 alpha, a key component that mediates reciprocal interaction between angiogenesis and osteogenesis, was not altered in the absence of Csnk2b. Csnk2b-deficient osteoblasts showed reduced expression of antiangiogenic factor chemokine (C-X-C motif) ligand 9 (Cxcl9) and ligand 7 (Cxcl9) (Supplementary Fig. 18). Accordingly, cell migration and tube formation of endothelial cells were relatively comparable in the culture of conditioned medium (CM) obtained from Csnk2b-sufficient or -deficient COBs (Supplementary Fig. 19). These results suggest that osteoblast CK2 activity is dispensable for regulation of angiogenesis and raise the possibility that CK2-mediated regulation of factors traditionally associated with angiogenesis may instead influence other physiological processes.

**Methods**

**Plasmids, antibodies, and cell culture.** Plasmid for Flag/HA-HAUSP was deposited to Addgene by Bert Vogelstein (#16655). HA-HAUSP was generated by sub-cloning from this source. The construct for HA-UBB (Ubiquitin) was deposited to Addgene by Edward Yeh (#18712) and plasmid for His-Ubiquitin was generated by sub-cloning. All constructs encoding shRNAs were purchased from Sigma. CK2-phosphorylation sites (humanNM_004348 pThr340, pSer354, pSer387) were substituted to alanine in every combination of single, double, and triple mutants on human RUNX2 cDNA13 using Quikchange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s instructions.

Antibodies specific to HAUSP (sc-30164), GAPDH (sc-25778), HSPA90 (sc-79477), or ubiquitin (sc-8017) were purchased from Santa Cruz Biotechnology. Antibodies specific to CSNK2A (Cell signaling, 2656), CSNK2B (Abcam, ab133576), RUNX2 (Calbiochem, PC287; Cell signaling, 12556), and USP24 (Proteintech, 13126-1-A) were used according to manufacturers’ instructions. Recombinant human BMP27 (3229-BM) was purchased from R&D systems. Lastly, inhibitors of CK2 (CK2 inhibitor IV and CK2 inhibitor XV) were purchased from Calbiochem (218713) and Selleckchem (S2248), respectively. HAUSP inhibitor (BX41108, 428510) was purchased from Tocris Bioscience.

**ChT1/2 and HEK293T cells** were purchased from ATCC and grown in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 1% penicillin/ streptomycin and 1% nonessential amino acids. Human BMSCs (CD29+, CD44+, CD105+, CD34+, CD45−) were purchased from Cyagen Biosciences and maintained in the growth medium (HUXMX-90011) and cultured in osteogenic medium (GUXMX-90021), chondrogenic medium (GUXMX-90041), or adipogenic medium (GUXMX-90031) according to the manufacturer’s instructions. To confirm chondrocyte differentiation, the chondrocyte pellets were harvested on days 21 and washed with phosphate-buffered saline (PBS), then stained with 1% of alcian blue solution for (Sigma, A3157). To examine adipocytes differentiation and lipid droplet formation, the differentiated cells were fixed with 4% of paraformaldehyde (PFA) for 2 h on ice, then stained with 0.3% of oil red O (Sigma, O0625) for 1 h on ice.

**Mice.** Csnk2bfl/fl mice were previously reported and maintained on C57BL/6 background. Hauspfl/fl mice were generated and maintained on a mixed background of 129Sv and C57BL/6 mice. Runx2fl/fl mice were previously reported. Transgenic mice expressing Cre recombinase under the control of the pro1 promoter (Pro1-cre) or the osterix promoter (Oox-cre) were purchased from Jackson laboratory and were crossed with Csnk2bfl/fl mice or Hauspfl/fl mice. Transgenic mice expressing Cre recombinase under the control of the Cathepsin K promoter (Csk-cre) were a gift from Dy Takashi Nakamura (Tokyo Dental College, Japan). Mouse genotypes were determined by PCR on tail genomic DNA; primer sequences are available upon request. Both male and female mice were used and analyzed in all experiments. Control littermates were analyzed in all experiments. All animals were used in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Well Cornell Medical College subcommittee and the University of Massachusetts Medical School on animal care (IACUC).
MicroCT, radiography, and skeletal preparation. MicroCT was used for qualitative and quantitative assessment of trabecular and cortical bone microarchitecture and performed by an investigator blinded to the genotypes of the animals under analysis. Femurs excised from the indicated mice were scanned using a microCT 35 (Scanco Medical) with a spatial resolution of 7 μm. For trabecular bone analysis of the distal femur, an upper 2.1 mm region beginning 280 μm proximal to the growth plate was contoured. For cortical bone analysis of femur and tibia, a midshaft region of 0.6 mm in length was used. MicroCT scans of skulls, kidneys, and HO in muscle/achilles tendon were performed using isotropic voxel sizes of 12 μm. 3D-reconstruction images were obtained from contoured 2D images by methods based on distance transformation of the binarized images. All images presented are representative of the respective genotypes (n > 5).
**Fig. 5** HAUSP regulates osteoblast differentiation by controlling RUNX2 stability, a. Ingenuity pathway analysis of proteins that interact with RUNX2-WT and RUNX2-Tri-A mutant. **b-d** Human BMSCs expressing control shRNAs or shRNAs targeting the indicated DUBs were cultured under osteogenic conditions for 7 days and ALP activity was assessed (b). Mineralization (c) and mRNA levels of osteogenic genes (d) in human BMSCs expressing control (shCtrl) or HAUSP-targeting shRNAs (shHAUSP) were assessed by alizarin red staining at day 18 and by RT-PCR at day 12, respectively. **b** n = 14, **c** n = 4 biologically independent samples. Protein (e) and mRNA (f) levels of RUNX2 in shCtrl or shHAUSP-expressing human BMSCs. **f** n = 4 biologically independent samples. **g** RUNX2 and His-ubiquitin were transfected into HEK293T cells along with different concentrations of HAUSP. 2 days after transfection, cells were treated with 10 μM MG132 for 6 h, lysed, immunoprecipitated with Ni-NTAagarose, and immunoblotted with anti-RUNX2 antibody. **h** Cell-free deubiquitination assay using recombinant HAUSP (hHAUSP) and ubiquitinated Flag-RUNX2 proteins. Flag-RUNX2 and HA-ubiquitin were co-transfected into HEK293T cells and ubiquitinated Flag-RUNX2 proteins were obtained by immunoprecipitation with anti-Flag-conjugated agarose. **I** HEK293T cells were transfected with RUNX2-WT or RUNX2-Tri-A mutant along with HA-HAUSP and treated with 10 μM MG132 for 6 h prior to lysis. HA-HAUSP immunoprecipitates were immunoblotted with the indicated antibodies. **J** Cell-free interaction analysis using recombinant RUNX2 (rRUNX2), CK2 (rCK2), and HAUSP (rHAUSP). Recombinant proteins were incubated in the absence or presence of ATP for 30 min, immunoprecipitated with anti-RUNX2 antibody and protein G-conjugated agarose, and immunoblotted with the indicated antibodies. **K** RUNX2-WT or RUNX2-Tri-A mutant was transfected into HEK293T cells along with different concentrations of HAUSP and immunoblotted with the indicated antibodies. **L** Schematic diagram depicting a posttranslational regulation of RUNX2 by the CK2/HAUSP pathway in osteoblasts. Data are representative of three (b-k) independent experiments. Ordinary one-way ANOVA with Dunnett’s multiple comparisons test (b) and a two-tailed unpaired Student’s t test for comparing two groups (d, f, error bars, SD of biological replicates).
with distilled water and examined for the presence of calcium deposits. Mineralization was quantified by the acetic acid extraction method.

**Osteoclast differentiation.** For preparation of bone marrow monocytes, femur was dissected from 8-week-old mice, and bone marrow cells collected by flushing were plated overnight in α-MEM with 10% FBS. Non-adherent cells were collected and seeded on a 100 mm dish with M-CSF (20 ng/ml, R&D systems, 416-ML-010). After 48 h, adherent cells were plated and cultured in osteoclast differentiation condition up to 6 days. For osteoclastogenesis, M-CSF (20 ng/ml) and RANKL (10 ng/ml, R&D systems, 462-TEC-010) were added in the growth medium.

**RT-PCR, immunoprecipitation, and immunoblotting.** To prepare RNA samples from the mouse limbs, the hindlimbs were dissected and skin/muscle tissues were removed. The remaining tibia/femurs were chopped and homogenized. For
Fig. 6 HAUSP is required for maturation of osteoprogenitors in vitro and in vivo. a-e COBs isolated from P5 Hausp+/fl/p mutants were pupfed with lentiviruses expressing vector (WT) or Cre recombinase (Hausp KO) and cultured under osteogenic conditions. RT-PCR analysis to measure mRNA levels of Hausp and osteogenic genes (a) and ALP activity (b, left) and staining (b, right) were performed 7 days after the culture. Mineralization was assessed by alizarin red staining 16 days after the culture (c). Protein (d) and mRNA (e) levels of RUNX2 were assessed in these COBs. Scale bar, 100 μm (b, a, e; n = 4; b, c; n = 9). f shCtrl or shHausp-expressing C3H10T1/2 cells were transfected with OG2-luc and Renilla in the presence or absence of RUNX2 overexpression. Two days after transfection, OG2-luc activity was measured and normalized to Renilla. (n = 6 biologically independent samples). Hausp-sufficient or -deficient COBs were infected with lentiviruses expressing vector or RUNX2, cultured under osteogenic conditions for 7 days, and ALP activity (g) and mRNA levels of Bglap2 (h) were assessed. g n = 6; h n = 4 biologically independent samples). I Hausp mRNA levels in P0 Hausp+/fl, Osx-Cre, and Hausp+/fl calvaria. (n = 4); j, l, m MicroCT analysis shows 3D-reconstruction of calvaria (j) and femurs (l) from 2-month-old Hausp+/fl, Osx-Cre and Hausp+/fl male mice. Quantification of femoral bone mass is displayed (m). The arrows indicate hypomineralization areas. Scale bars, 2 mm (j); 500 μm (l). m n = 10 (Hausp+/fl), 5 (Osx-Cre) or 10 (Hausp+/fl). k Alizarin red/alcan blue staining of skeletal preparations of calvaria from 2-month-old Hausp+/fl, Osx-Cre and Hausp+/fl female mice. Scale bar, 2 mm. l H&E-stained longitudinal sections of femurs from 2-month-old Osx-Cre and Hausp+/fl male mice. Scale bar, 50 μm. Alizarin red/alcan blue staining of skeletal preparations of calvaria (top) and clavicles (bottom) from P10 Osx-Cre, Csk2b+/fl, Oxs-Cre, and Csk2b+/fl, Oxs-Cre, Runx2+/fl, Oxs-Cre, Runx2+/fl, and Runx2+/fl, Oxs-Cre, Runx2+/fl mice (o) and Osx-Cre, Runx2+/fl, and Runx2+/fl mice (p). Scale bars, 2 mm (o, p). Data are representative of three (a-i, n-p) independent experiments or are pooled from two experiments (m). A two-tailed unpaired Student’s t test for comparing two groups (a-c, e-i, m, error bars, SD of biological replicates).

Deubiquitination assay. To test the ability of HAUSP to deubiquitinate ubiquitinated RUNX2, plasmids expressing Flag-RUNX2-WT or Flag-RUNX2-Tri-A mutant were transfected into HEK293T cells along with His-ubiquitin-expressing plasmid in the absence or presence of HAUSP-expressing plasmid. After 48 h, cells were treated with 10 μM MG132 (EMD-Millipore, 474970) for 6 h, lysed and sonicated in denaturation buffer (8 M urea, 50 mM tris pH 8.0, 1.0% Triton X-100, 10 mM imidazol, 10 mM β-mercaptoethanol), and immunoprecipitated with Ni-NTA beads. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-RUNX2 antibody.

A cell-free deubiquitination assay was also performed using the purified ubiquitinated RUNX2 and recombinant HAUSP. Ubiquitinated RUNX2 proteins were purified from HEK293T cells expressing Flag-RUNX2 and HA-ubiquitin by immunoprecipitating with Flag-conjugated agarose. These RUNX2 proteins were subsequently incubated with different amounts of recombinant HAUSP (Boston Biochem, E-519) in deubiquitination buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) at 37 °C for 3 h. Immunoblotting was performed to measure ubiquitination levels.

RNA sequencing and analysis. Adapters are removed and reads are aligned to mouse genome GRCm38 by using STAR aligner (ver. 2.3.0e)75 using default parameters and resulting bam files (mapped reads) were sorted and indexed using SAMTools72. Gene counts were obtained by HTSeq-Count73 to sorted bam files, and only unique-mapping reads were used. For dendrogram (heat map) generation, 3850 total genes were clustered into four groups and the gene sets in these groups are described in Supplementary Table 1. Genes without any expression counts in any sample were discarded. The DESeq2 (ver. 1.45) in R package76 was employed to normalize gene count data, and then detect differentially expressed genes (DEG) between each groups with (FDR < 0.05 and absolute log2 fold change >1.5). Functional enrichment analysis was performed on DEG with DAVID (ver. 6.7)77 and biological process GO terms with enrichment p < 0.05 were selected as overrepresented functions. Summary statistics of the fold change analysis in each of the GO categories is described in Supplementary Table 3.

Collection of CM from WT and Csk2b KO COBs. CM from primary WT and Csk2b-deficient COBs was collected at day 0 (undifferentiated COBs) and day 7 (differentiated osteoblasts) after osteogenic induction. CM for each group was collected after three washes with PBS to remove serum and incubated with serum free α-MEM for 24 h. After incubation, each CM sample was collected and filtered with 0.45 μm syringe filter to remove cellular debris.

Endothelial cell culture and functional assays. Mouse bone marrow-derived Endothelial progenitor outgrowth cells (EPOCs) were purchased from BioChain (Z7030031) and maintained in growth medium (BioChain, Z7030035). Cell migration was performed using chemotaxis chamber (Biovision, K906) as manufacturer’s instructions. Briefly, basal medium or CM or FGF–2 (R&D systems, 3139-FG-025) was placed in the bottom wells of the chamber and the upper chamber was loaded with 10,000 cells/well. After incubation for 6 h at 37 °C, the migrated cells were analyzed and quantified.

For tube formation, EPOCs (100,000 cells/well) were plated into 48-well plates coated with growth factor-reduced matrigel (Corning, 354200) and incubated for 24 h at 37 °C in indicated conditions. Total tube length was measured by counting a random field per well with microscopy (5–7 wells per each group).

Statistics and reproducibility. All experiments were carried out at least two or three times, for IHC, immunofluorescence staining, histological staining, skeletal
preparation, flow cytometry, and immunoblotting, representative images are shown. All data are shown as the mean ± Standard Deviation (SD). We first performed the Shapiro–Wilk normality test for checking normal distributions of the groups. If normality tests passed, two-tailed, unpaired Student’s t-test and if normality tests failed, Mann–Whitney tests were used for the comparisons between two groups. For the comparisons of three or four groups, we used one-way ANOVA if normality tests passed, followed by Tukey’s multiple comparison test for all pairs of groups. The GraphPad PRISM software (ver.8.3.0, La Jolla, CA) was used for statistical analysis. \( P < 0.05 \) was considered statistically significant.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request.

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Author contributions

J.M.K. designed, executed, and interpreted the experiments. Y.Y., G.K., R.X., N.L., and J.F.C. performed histology, immunohistochemistry, dynamic histomorphometry, and micro-CT analyses. K.H.P. performed histology on human HO samples. M.S. and B.B. executed the experiments for isolation of skeletal stem cells. H.C. performed transcriptome analyses. O.F.-C. and B.B., and N.K. and W.G. generated and provided Cnkl2 floxed mice and Hapap floxed mice, respectively. T.T. provided Runx2 floxed mice. T.D. and P.B.Y. executed biochemical experiments for BMP signaling. M.B.G. and J.H.S. supervised the research and participated in the manuscript preparation. (ULTR001454), and the Glory Harvest Group.

Competing interests

J.H.S. is a scientific founder of the AAYAA Therapeutics and holds equity in this company. Other authors declare no competing interests.

Additional information

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