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Dkk2 has a role in terminal osteoblast differentiation and mineralized matrix formation

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Human and mouse genetic and *in vitro* evidence has shown that canonical Wnt signaling promotes bone formation, but we found that mice lacking the canonical Wnt antagonist Dickkopf2 (Dkk2) were osteopenic. We reaffirmed the finding that canonical Wnt signaling stimulates osteogenesis, including the differentiation from preosteoblasts to osteoblasts, in cultured osteoblast differentiation models, but we also found that canonical Wnts upregulated the expression of Dkk2 in osteoblasts. Although exogenous overexpression of Dkk before the expression of endogenous canonical Wnt (Wnt7b) suppressed osteogenesis in cultures, its expression after peak Wnt7b expression induced a phenotype resembling terminal osteoblast differentiation leading to mineralization. In addition, osteoblasts from *Dkk2*-null mice were poorly mineralized upon osteogenic induction in cultures, and Dkk2 deficiency led to attenuation of the expression of osteogenic markers, which could be partially reversed by exogenous expression of Dkk2. Taken together with the finding that *Dkk2*-null mice have increased numbers of osteoids, these data indicate that Dkk2 has a role in late stages of osteoblast differentiation into mineralized matrices. Because expression of another Wnt antagonist, FRP3, differs from Dkk2 expression in rescuing Dkk2 deficiency and regulating osteoblast differentiation, the effects of Dkk2 on terminal osteoblast differentiation may not be entirely mediated by its Wnt signaling antagonistic activity.

Osteoporosis is a public health problem[1–3]. Most fractures that occur in people more than 65 years of age are due to osteoporosis[2,4]. Peak bone mass is a key determining factor for the risk of osteoporotic fracture[5], and genetic factors contribute substantially to the variance in peak bone mass. The gene *LRP5* was recently determined to regulate bone mass. Loss-of-function mutations in *LRP5* are associated with osteoporosis-pseudoglioma syndrome, an autosomal recessive disorder[6], and hypermorphic alleles of this gene are associated with high bone mass (HBM) phenotypes[7–9]. Moreover, mice in which *Lrp5* was inactivated by gene targeting had phenotypes similar to those of individuals with osteoporosis-pseudoglioma syndrome[10], and the transgenic overexpression of G171V mutant LRP5 in mice resulted in HBM[11]. Furthermore, mouse primary osteoblasts showed reduced responsiveness to Wnt and low proliferation indices in the absence of LRP5 (ref. 10). These results, together with the finding that inactivation of the Wnt antagonist sFRP1 enhances trabecular bone accrual[12], support the conclusion that canonical Wnt signaling has an important role in osteogenesis.

The Wnt family of secretory glycoproteins comprises many developmentally important signaling molecules[13–15]. Wnt pathways are also closely linked to tumorigenesis[14,16,17]. Studies using fruit flies, African clawed frogs and mammalian cells have established a canonical signaling pathway: Wnt proteins bind to cell-surface receptors Frizzled (Fz) and LRP5/6 (refs. 15,18–20). Through mechanisms that are not well characterized but may involve interactions between LRP5/6 and axin[21,22] and between Fz and Dvl[23], glycogen synthase kinase 3–dependent phosphorylation of β-catenin is suppressed, leading to the stabilization of β-catenin and activation of gene transcription[15,24,25]. Genome projects have identified 19 Wnt genes in mammals. Not all the Wnts have been tested for the ability to stabilize β-catenin and activate LEF-1/TCF-mediated gene transcription, and among those tested, not all can activate the canonical pathway. Representative mammalian canonical Wnts are Wnt1, Wnt3, Wnt3a, Wnt7a and Wnt7b.

Wnt signaling is also tightly regulated by a large number of antagonists, including the dickkopf (Dkk), frizzled-related protein (FRP), Wnt inhibitory factor, cerberus and WISE/sclerostin families[26]. The first Dkk protein, *Xenopus laevis* Dkk1, was initially identified as a Wnt antagonist with a role in head formation[27]. To date, four Dkk proteins have been identified in mammals[28,29], but only the first two members (Dkk1 and Dkk2) have been well documented to function as antagonists to canonical Wnt signaling. Both Dkk1 and Dkk2

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Table 1 DXA analysis of 2-month-old mice

Sex	*Dkk2* genotype (*n*)	Femur (g cm^{-2})	%*	*P**	Body (g cm^{-2})	%*	*P**
Male	+/+ (17)	0.0734 ± 0.0065	7.3	0.021	0.0503 ± 0.0029	7.0	0.001
	+/– (25)	0.0745 ± 0.0055	8.7	0.001	0.0501 ± 0.0023	6.7	0.000
	–/– (17)	0.0680 ± 0.0062			0.0467 ± 0.0026		
Female	+/+ (17)	0.0694 ± 0.0050	7.6	0.021	0.0494 ± 0.0025	5.5	0.027
	+/– (25)	0.0688 ± 0.0068	6.7	0.074	0.0488 ± 0.0033	4.3	0.135
	–/– (17)	0.0640 ± 0.0062			0.0468 ± 0.0036		

*Relative to *Dkk2*$^{-/-}$ mice.

antagonize canonical Wnt signaling by simultaneously binding to LRP5/6 and a single-transmembrane protein called kremen[30–33]. The second, but not the first, cysteine-rich domains of Dkk1 and Dkk2 are responsible for canonical Wnt signaling antagonism[34,35].

Although genetic evidence from both humans and mice has established a role for canonical Wnt signaling in bone development[6–9], the mechanism by which the canonical Wnt regulates osteogenesis is not known. Our initial observation that mice lacking the Wnt antagonist Dkk2 are osteopenic led us to determine that canonical Wnts upregulate Dkk2 expression during osteoblast differentiation and that Dkk2 regulates some of the processes required for terminal osteogenic differentiation. These findings provide not only a mechanism for the regulation of osteogenesis by canonical Wnt signaling but also an explanation for the phenotype of *Dkk2*-null mice.

RESULTS

Dkk2 deficiency leads to osteopenia

To investigate the biological relevance of Dkk2, we generated a mouse line that lacks Dkk2 using the conventional gene-targeting technique. We inactivated the gene *Dkk2* by replacing the four coding exons with the neomycin-resistance gene. We confirmed *Dkk2* deficiency by PCR analysis of *Dkk2* and by the failure to detect *Dkk2* mRNA using RT-PCR or Dkk2 protein by western-blot analysis in differentiated bone marrow stromal osteoblasts (BMSOs) from *Dkk2*-null mice (data not shown). *Dkk2*-null mice were viable and had no grossly abnormal phenotype. Histological examination of various tissues showed no apparent anomalies except hyperplasia of eyelid epithelial cells, which may be explained by the high level of expression of Dkk2 in the eyes of mouse embryos[28,29].

Because *Lrp5*-null mice are osteoporotic and mice lacking the Wnt antagonist FRP-1 have HBM[10,12], we anticipated that mice lacking Dkk2 should have HBM. We examined whole-body and femoral bone mineral density (BMD) using dual-energy X-ray absorptiometry (DXA) in wild-type, *Dkk2*$^{+/-}$ and *Dkk2*$^{-/-}$ mice. Total femoral BMD was substantially lower in *Dkk2*-null mice than in wild-type or *Dkk2*$^{+/-}$ mice (**Table 1**) of both sexes. Whole-body BMD was also substantially lower in *Dkk2*-null mice than in wild-type littermates (**Table 1**). To confirm these observations, we carried out peripheral qualitative computerized tomography (pQCT) analysis of distal femur metaphysis and found that total, trabecular and cortical bone volumetric mineral contents were 17%, 14% and 16% lower, respectively, in *Dkk2*-null mice compared with *Dkk2*$^{+/-}$ mice (**Fig. 1a–c**). We found no statistically significant differences in pQCT measurements between wild-type and *Dkk2*$^{+/-}$ mice (data not shown). Consistent with the pQCT data, histomorphometric analyses of proximal tibial metaphyseal trabecular bones showed that trabecular bone volume and trabecular bone number were 33% and 31% lower, respectively, and trabecular separation was 52% greater in *Dkk2*-null mice than in *Dkk2*$^{+/-}$ mice (**Fig. 1d–f**). These results indicate that Dkk2 deficiency results in osteopenia rather than HBM.

Dkk2 deficiency causes defects in mineralization

Bone histomorphometric analyses of proximal tibial metaphyseal trabecular bones of the same mice used for pQCT and DXA analyses also showed that osteoid surface was greater (+96%; **Fig. 1g,j–m**) in *Dkk2*$^{-/-}$ mice than in *Dkk2*$^{+/-}$ mice, without increases in osteoblast surface (**Fig. 1h**) or osteoblast number (1.55 ± 0.155 osteoblast number mm^{-1} for wild-type, 0.85 ± 0.21 for *Dkk2*$^{-/-}$; $P = 0.21$).

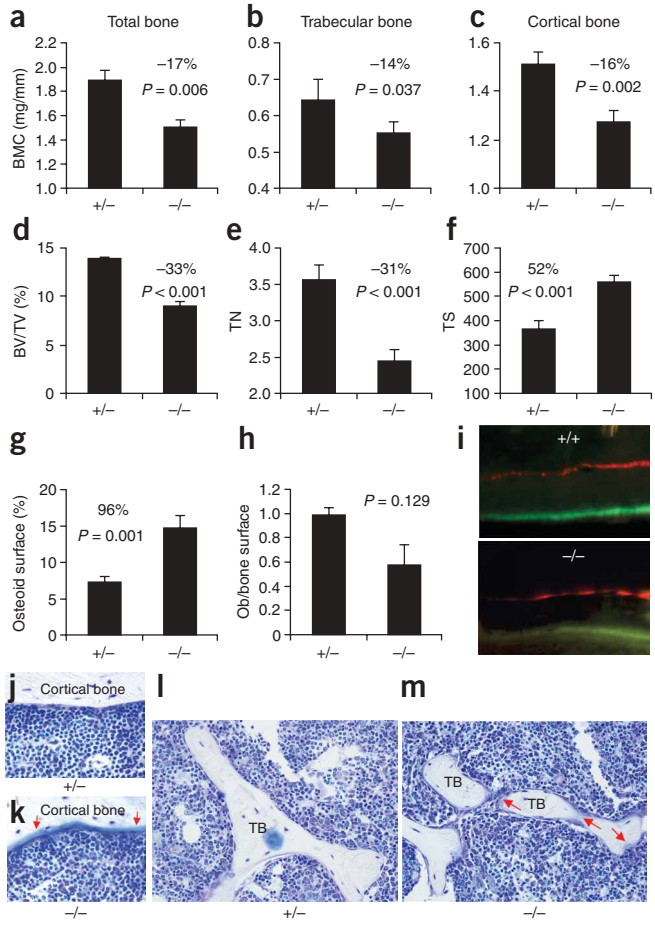

Figure 1 *Dkk2*-null mice are osteopenic. Distal femoral metaphyseal volumetric BMCs of total (**a**), trabecular (**b**) and cortical (**c**) bones were determined by pQCT analysis. Trabecular bone volume (BV/TV; **d**), trabecular bone number (TN; **e**) and separation (TS; **f**), percent osteoid surface (**g**) and osteoblast (Ob) surface/bone surface (**h**) were determined by bone histomorphometric analysis of proximal tibial trabecular bones ($n > 12$). (**i**) Calcein and xylenol orange double staining of 4-month-old male wild-type and *Dkk2*$^{-/-}$ mice. The distance between green and red staining represents mineral apposition rate. (**j–m**) Histological examination of proximal tibial metaphysis. Sections were stained with toludine blue. Osteoid surfaces are indicated with arrows. TB, trabecular bone.

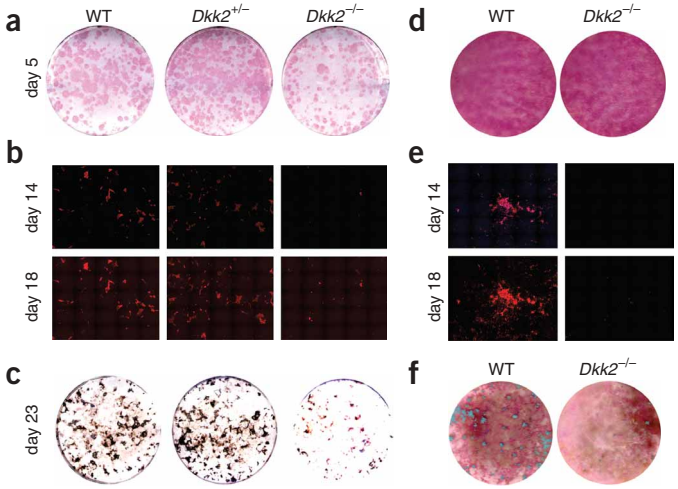

Figure 2 Osteoblasts derived from *Dkk2*-null mice show poor mineralization in cultures. (**a–f**) Parallel cultures of BMSOs (**a–c**) and calvarial osteoblasts (**d–f**) from wild-type and *Dkk2*-null mice were stained with an alkaline phosphatase substrate at day 5 (**a**,**d**), with xylenol orange at days 14 and 18 (**b**,**e**) or silver nitrate at day 23 (**c**,**f**). Cultures in **f** were also stained for alkaline phosphatase activity followed by silver staining. Differentiation was induced at day 5. (**g**) Calvarial osteoblasts from 5-d-old wild-type and *Dkk2*-null pups were transfected with the LEF-1 reporter gene and induced to osteogenic differentiation. Luc activity was determined 2 d after transfection in the presence or absence of Wnt3a protein (6-h treatment).

Mineral apposition rate (20%; **Fig. 1i**) and bone formation rate (33%) in the proximal tibial metaphyseal trabecular bone were lower in *Dkk2*-null mice compared with *Dkk2*$^{+/-}$ mice. These data suggest that Dkk2 deficiency may affect terminal osteoblast differentiation and mineralized matrix formation. This conclusion is corroborated by the observations that *Dkk2*-null osteoblasts derived from both bone marrow (**Fig. 2a–c**) and calvaria (**Fig. 2d–f**) showed delayed mineralization upon osteogenic induction in cultures compared with wild-type or *Dkk2*$^{+/-}$ osteoblasts. Given that *Dkk2*-null mice consistently yielded less (~20–30%) alkaline phosphatase–positive colonies (**Fig. 2a**) and had similar osteoblast numbers as did wild-type and *Dkk2*$^{+/-}$ mice, the increases in osteoid surfaces are probably not due to increases in osteoblast cells. In addition, there were no substantial changes in the levels of serum calcium, phosphate and bone-derived alkaline phosphatase or in the expression levels of calvarial, long bone or vertebrate alkaline phosphatase between wild-type and *Dkk2*-null mice (**Supplementary Fig. 1** online and data not shown). Therefore, we conclude that *Dkk2* has an important role in terminal osteoblast differentiation leading to the formation of mineralized matrices.

Canonical Wnts stimulate osteogenesis in cultures

This conclusion seems to contradict the idea that canonical Wnt signaling has a positive role in bone development. We sought to resolve this paradox by determining whether Dkk2 deficiency led to an increase in Wnt signaling. We transfected calvarial osteoblasts with a LEF reporter gene that has been widely used to gauge canonical Wnt activity[36–38]. Osteoblast cells from *Dkk2*-null mice had a higher basal activity and were more permissive to Wnt stimulation than cells from wild-type mice (**Fig. 2g**), indicating that Dkk2 deficiency results in an increase in Wnt signaling. We then attempted to confirm the positive role of canonical Wnt signaling in osteogenesis by examining the effect of canonical Wnts on differentiation of BMSOs and calvarial osteoblasts. Consistent with genetic evidence, addition of Wnt3a (a canonical Wnt) conditioned medium or adenoviruses expressing Wnt1 (a different canonical Wnt) to BMSO (**Fig. 3a**,**b**) and calvarial osteoblast (**Supplementary Fig. 2** online) cultures led to more mineralization. In addition, Wnt1 stimulated the expression of two widely used markers for osteoblast differentiation: bone sialoprotein and osteocalcin (**Fig. 3c**). Therefore, canonical Wnt proteins can stimulate osteogenesis in the primary osteoblast culture.

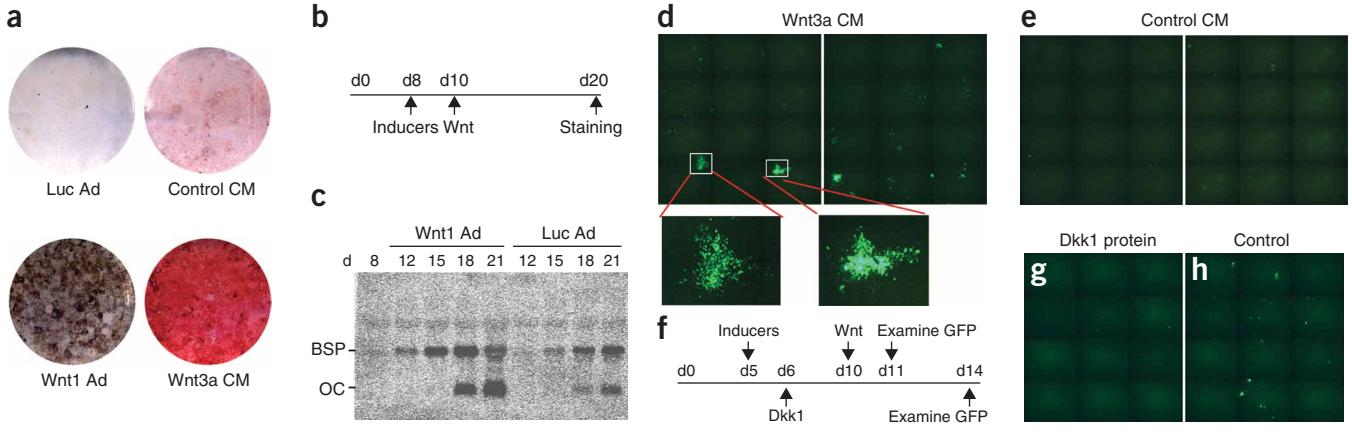

Figure 3 Canonical Wnts stimulate osteogenesis and osteoblast differentiation. (**a**) Canonical Wnts stimulate mineralization. BMSOs isolated from 3-month-old wild-type mice were infected with control (Luc) or Wnt1 adenovirus (Ad) or were treated with Wnt3a or control conditioned medium (CM) and were stained for mineralization at the indicated times (**b**). (**c**) Northern-blot analysis of expression of osteocalcin (OC) and bone sialoprotein (BSP) at different stages of differentiation. Luc, control adenovirus. (**d–h**) BSMO cultures established from 2.3GFP mice were treated with Wnt3a (**d**) or control (**e**) conditioned medium (CM) at the indicated times (**f**). The expression of GFP was examined 24 h later. In a separate experiment, recombinant Dkk1 protein (0.1 μM; **g**) or control buffer (**h**) was added at the indicated times (**f**).

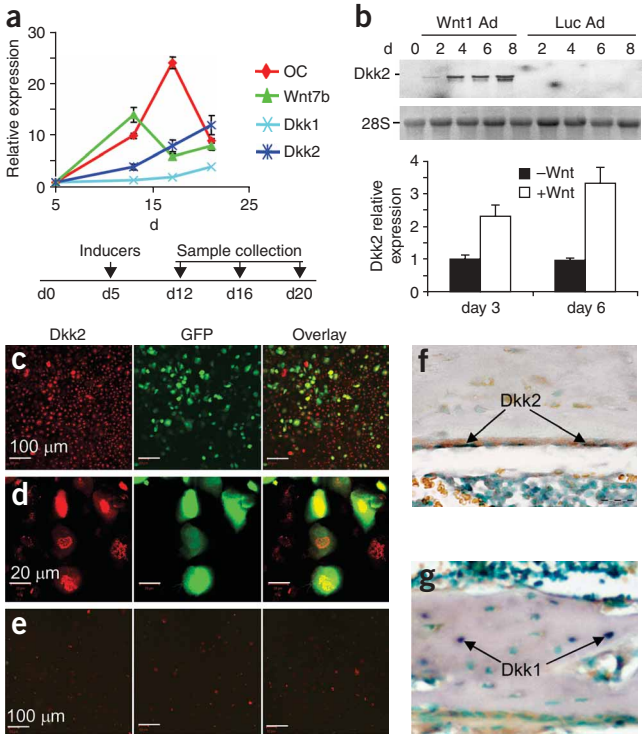

Figure 4 Dkk expression during osteoblast differentiation. (**a**) The expression levels of osteocalcin (OC), Wnt7b, Dkk1 and Dkk2 in differentiating BMSO cultures relative to those at day 5 were determined by QPCR. (**b**) Wnt1 stimulates Dkk2 expression in osteoblastic MC3T3 cells and calvarial osteoblasts. MC3T3 cells were induced to osteogenic differentiation and infected with adenovirus (Ad) that expressed Wnt1 or a control (Luc). Total RNA was prepared and analyzed by Northern blotting with *Dkk2* cDNA as the probe (upper panels). Calvarial osteoblasts were cultured to confluence and induced to osteogenic differentiation. They were then stimulated with Wnt3a. RNA was collected at days 3 and 6 after induction. Relative levels of Dkk2 expression were determined by QPCR. (**c–e**) Detection of Dkk2 protein in differentiating osteoblast by immunostaining BMSO cultures derived from 2.3GFP mice with a Dkk2-specific monoclonal antibody. The cultures were induced to osteogenic differentiation at day 5 and stained at day 15. (**c**) An area containing a GFP nodule. (**d**) High magnification view of **c**. (**e**) An area without GFP nodules. (**f**,**g**) Detection of Dkk1 and Dkk2 expression in mouse bones by *in situ* hybridization. The sections were made from the fibula area of a 3-week-old mouse. *In situ* hybridization was carried out using cRNA probes prepared from the full-length *Dkk1* and *Dkk2* cDNAs.

Canonical Wnt proteins stimulate osteoblast proliferation[10], but we found that they also stimulate osteoblast differentiation. When BMSO cultures derived from mice carrying a GFP transgene controlled by a 2.3-kb *Col1a1* promoter (called 2.3GFP mice), which is activated only in mature osteoblasts[39], were stimulated by Wnt3a conditioned medium, nodules of GFP-positive cells appeared within 24 h (**Fig. 3d**). This did not occur in cell cultures treated with the control conditioned medium (**Fig. 3e**,**f**), although GFP-positive nodules did appear 3–5 d later (**Fig. 3g**,**h**). To determine whether Wnt could directly regulate the 2.3-kb *Col1a1* promoter, we tested a 2.3-kb *Col1a1* promoter–luciferase (Luc) reporter gene construct. Wnt3a did not activate this reporter construct in NIH3T3 cells (data not shown), suggesting that this promoter is not a direct target for the canonical signaling pathway. In addition, after treatment with Wnt3a conditioned medium, only a few patches of cells became GFP-positive in the BMSO culture, even though all the cells contained the same GFP transgene (**Fig. 3d**). This observation suggests that only those osteoblasts that developed to a certain stage were permissive for stimulation by the canonical Wnts to undergo further differentiation. Some of the GFP-positive nodules contained hundreds of cells; this rapid increase in the number of GFP-positive cells could not have resulted solely from proliferation. Therefore, it is reasonable to conclude that canonical Wnts promote differentiation into more mature osteoblast cells.

We previously showed that differentiating BMSOs produce a canonical Wnt, Wnt7b[40]. The expression levels of Wnt7b changed substantially during BMSO differentiation; Wnt7b expression peaked 8 d after induction of differentiation and then fell (**Fig. 4a**). This canonical Wnt, possibly together with other unidentified canonical Wnts, may be responsible for endogenous stimulation of differentiation to 2.3GFP-positive mature osteoblasts. This idea is supported by the observation that addition of purified recombinant Dkk1 (**Fig. 3g**) or infection with Dkk2-expressing adenoviruses (data not shown) before the onset of Wnt7b expression greatly reduced the number of GFP-positive nodules.

Dkk expression during osteoblast differentiation and in bone

To understand better the roles of Dkk1 and Dkk2 in the regulation of osteogenesis, we first examined their expression during BMSO differentiation using quantitative PCR (QPCR). Dkk2 expression levels were elevated throughout differentiation, and Dkk1 expression was also increased but primarily at a later stage of differentiation (**Fig. 4a**). These results indicate that expression of Wnt7b precedes that of Dkk2 and Dkk1, suggesting that canonical Wnts might regulate the expression of Dkk1 and Dkk2. When we infected cells of the osteoblast-like cell line MC3T3 with adenovirus expressing Wnt1, we observed marked increases in the expression of Dkk2 (~20-fold; **Fig. 4b**) but not that of Dkk1 (data not shown). We also observed increases in Dkk2 expression in primary cultured calvarial osteoblasts (**Fig. 4b**).

To confirm that osteoblasts express Dkk2, we immunostained the BMSO cultures from 2.3GFP mice using an antibody to Dkk2. GFP expression was used as a marker for osteoblasts and their differentiation stages. At the time when many GFP-positive nodules were observed, Dkk2 staining was often colocalized with these GFP-positive nodules (**Fig. 4c**,**d**). In areas where there were no clusters of GFP-positive cells, Dkk2 staining was low (**Fig. 4e**). Therefore, the elevation of Dkk2 expression coincides with the expression of 2.3GFP, which depends on endogenous canonical Wnts (**Fig. 3g**,**h**).

The patterns of expression of Dkk1 and Dkk2 observed in the BMSO cultures are consistent with their expression patterns *in vivo*. We examined the expression of Dkk1 and Dkk2 in mouse long bones by *in situ* hybridization. Dkk1 expression was primarily detected in osteocytes (**Fig. 4g**), whereas Dkk2 expression was readily detected in osteoblast cells but not in osteocytes (**Fig. 4f**).

Dkk2 deficiency affects osteoblast gene expression

The regulation of Dkk2 expression during osteoblast differentiation and the requirement of Dkk2 for efficient mineralization of cultured osteoblasts might explain the apparent paradox between the positive role of canonical Wnt signaling and the osteopenic phenotype caused by Dkk2 deficiency. Canonical Wnt could upregulate the expression of Dkk2 during osteoblast differentiation, which could in turn regulate osteoblast terminal differentiation into mineralized matrix. To investigate further the roles of Dkk2 in this terminal differentiation process, we examined the effect of Dkk2 deficiency on the expression of a number of osteogenic markers in calvarial osteoblast cultures that are more homogeneous than BMSO cultures and better represent the late

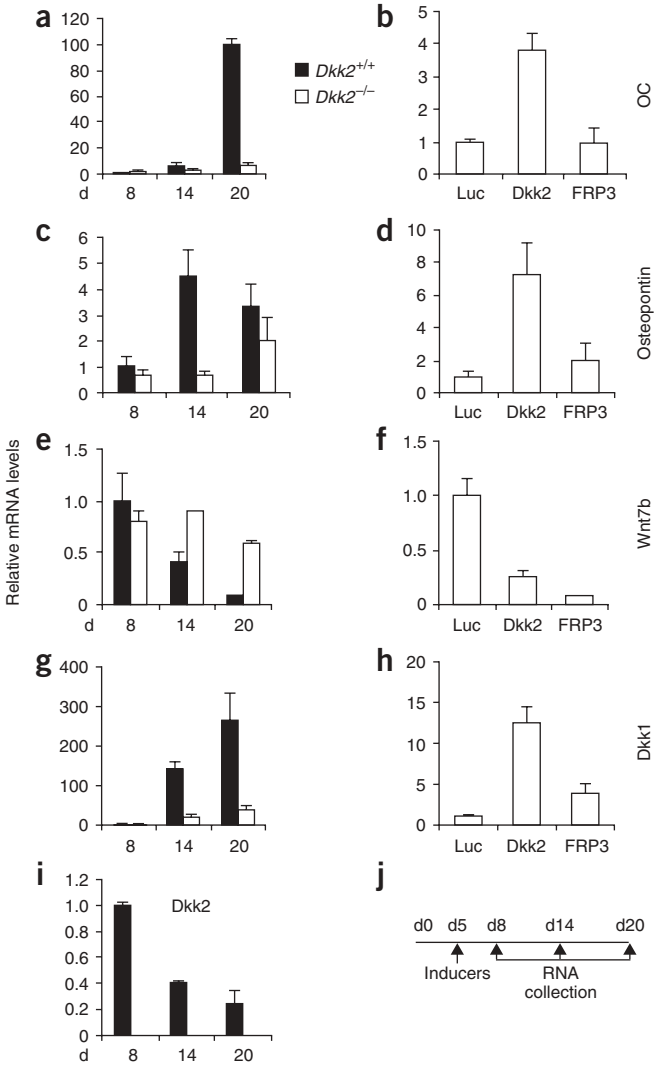

Figure 5 Expression of osteogenic markers Wnt7b, Dkk1 and Dkk2 in differentiating osteoblasts. Calvarial osteoblast cultures were established from wild-type and *Dkk2*-null mice and induced to undergo differentiation. RNA samples were prepared at days 8, 14 and 20 (**a**,**c**,**e**,**g**,**i**), and expression of osteocalcin (OC; **a**), osteopontin (**c**), Wnt7b (**e**), Dkk1 (**g**) and Dkk2 (**i**) relative to that at day 5 was determined by QPCR. (**j**) Relevant time points of the experiment. Calvarial osteoblast cultures from *Dkk2*-null mice were also infected with adenoviruses expressing Luc, Dkk2 or FRP3 at day 5 (**b**,**d**,**f**,**h**). Cells were collected for RNA preparation at day 14 for analysis of gene expression by QPCR.

stages of osteoblast development. Expression of osteocalcin and osteopontin in calvarial osteoblast cultures was markedly reduced in *Dkk2*$^{-/-}$ mice compared with wild-type mice (**Fig. 5a**,**c**). Adenovirus-mediated expression of Dkk2 could reverse the reduction in the expression of osteocalcin and osteopontin (**Fig. 5b**,**d**), suggesting that Dkk2 has a role in the regulation of expression of osteogenic marker genes. Notably, adenoviral expression of FRP3, a different Wnt antagonist, had little effect in the reversal of the gene expression caused by Dkk2 deficiency (**Fig. 5b**,**d**). This suggests that Dkk2 may have functions other than antagonizing canonical Wnts. We also examined the expression of Wnt7b during the differentiation of calvarial osteoblasts. Expression of Wnt7b (**Fig. 5e**) and Dkk2 (**Fig. 5i**) declined throughout differentiation in wild-type osteoblasts, but expression of Wnt7b persisted in *Dkk2*-null osteoblasts (**Fig. 5e**). Virus-mediated expression of both Dkk2 and FRP3 was able to suppress the expression of Wnt7b in *Dkk2*-null osteoblasts (**Fig. 5f**), indicating that the reduction in expression of Wnt7b could result from the inhibition of canonical Wnt signaling. Like BMSO differentiation, calvarial osteoblast differentiation was associated with an increase in Dkk1 expression (**Fig. 5g**), which was blunted in cells lacking Dkk2; expression of Dkk2, and, to a much lesser extent, FRP3, restored Dkk1 expression (**Fig. 5h**,**j**). The ability of Dkk2 expression to restore the effect of Dkk2 deficiency on gene expression indicates that these effects are the direct results of the lack of Dkk2. The fact that FRP3 expression mimics only some of the effects produced by Dkk2 expression suggests that Dkk2 may not function merely as a canonical Wnt antagonist. Nevertheless, all these data are consistent with the idea that Dkk2 deficiency hampers the progress of osteoblast differentiation.

Stimulation of mineralization by Dkk2

Knowing that Dkk2 expression could lead to increases in the expression of some osteogenic markers, we speculated that overexpression of Dkk2 at an appropriate stage of osteoblast differentiation could stimulate terminal differentiation of osteoblasts. To test this idea, we infected calvarial osteoblasts and BMSOs with adenoviruses expressing Dkk2 or Luc as a control (**Fig. 6e**). Infection with Dkk2-expressing adenoviruses induced a phenotype similar to mineralization (*i.e.*, increased the staining for mineral deposits; **Fig. 6b**,**d**), whereas infection with control viruses did not (**Fig. 6a**,**c**). In addition, Fourier-transformed infrared spectroscopic analysis showed that the mineral to matrix ratio in Dkk2-treated cultures was the same as in control cultures (**Supplementary Fig. 2** online); both were bone-like hydroxyapatite. Infection with a Dkk1-expressing adenovirus also resulted in an increase in calcium staining as assayed by xylenol orange, but this staining was considerably weaker than that resulting from infection with Dkk2-expressing adenovirus (data not shown). We obtained similar results with expression of Dkk2 in MC3T3 cells (data not shown). Infection with FRP3-expressing adenoviruses, like infection with Luc-expressing adenoviruses, did not induce any mineralized matrix (data not shown).

Roles of Dkk2 in osteoclast development

Bone histomorphometric analysis also showed that Dkk2 deficiency led to a substantial increase in the number of osteoclasts (**Fig. 7a**). To determine whether this effect of Dkk2 deficiency is autonomous to osteoclasts, we examined bone marrow osteoclast cultures established from Dkk2 and wild-type mice. We observed no obvious differences in osteoclast differentiation in cultures from wild-type versus *Dkk2*-null mice in the presence of exogenous RANKL and M-CSF (**Fig. 7b**), suggesting the effect of Dkk2 deficiency on the number of osteoclasts *in vivo* may be nonautonomous. Osteoblasts produce factors, including RANKL, OPG and M-CSF, that regulate osteoclast differentiation and activity. Therefore, a possible explanation for the increase in osteoclasts in *Dkk2*-null mice may be that attenuation in osteoblast terminal differentiation caused by Dkk2 deficiency results in changes in the production of M-CSF, OPG and RANKL. To test this possibility, we examined the expression of M-CSF, OPG and RANKL during the differentiation of calvarial osteoblasts. RANKL expression in *Dkk2*-null osteoblasts was highest 8 d after induction of differentiation (**Fig. 7c**), the same time at which normal Dkk2 expression is highest during the differentiation of control cultures (**Fig. 5i**). Expression of M-CSF started to decrease 8 d after induction of differentiation in

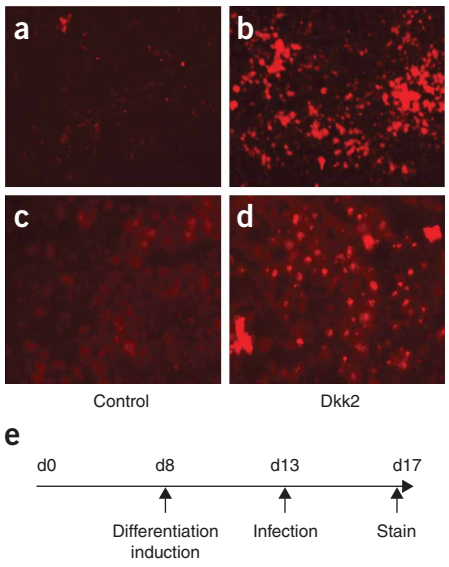

Figure 6 Dkk2 stimulates mineralization. Primary calvarial osteoblast (**a**,**b**) and BMSO (**c**,**d**) cultures were infected with control (**a**,**c**) or Dkk2-expressing (**b**,**d**) adenovirus and stained with xylenol orange at the indicated time points (**e**).

wild-type osteoblasts but did not decline until 11 d after induction of differentiation in *Dkk2*-null osteoblasts (**Fig. 7d**). We observed no substantial differences in expression of OPG between wild-type and *Dkk2*-null osteoblasts (**Fig. 7e**). These results suggest that delayed mineralization of osteoblasts may lead to increases in the number of osteoclasts, at least in part as the result of increased production of osteoclast stimuli from less mature osteoblasts.

DISCUSSION

Here we described mechanisms by which the canonical Wnt and its antagonist Dkk2 regulate late stages of osteoblast differentiation (**Fig. 7f**). Our study of *Dkk2*-null mice identified unexpected bone-related phenotypes. Contrary to the conventional prediction, mice lacking Dkk2 were osteopenic and did not have increased bone mass. By investigating the expression of Dkk2 and the effects of Dkk2 deficiency, partial suppression and overexpression on osteogenic processes, we conclude that Dkk2, whose expression is upregulated by canonical Wnt signaling, is important in late stages of osteogenic differentiation, particularly the formation of mineralized matrix.

We found ample evidence that Dkk2 has a role in osteoblast terminal differentiation. Although we do not know precisely the mechanisms by which Dkk2 is involved in terminal osteoblast differentiation, our results suggest that Dkk2 may be involved through both canonical Wnt signaling antagonism–dependent and –independent mechanisms. Dkk2 is a potent antagonist for canonical Wnt signaling[30,33,34], and Dkk2 seemed to act as a canonical Wnt antagonist during osteogenesis, because increases in canonical Wnt signaling were observed in osteoblasts isolated from *Dkk2*-null mice (**Fig. 2g**). The effects of Dkk2 deficiency on expression of Wnt7b and Dkk1 could be partially reversed by virus-mediated expression of Dkk2 and FRP3, a different Wnt antagonist. This finding supports the idea that the involvement of Dkk2 in osteoblast terminal differentiation occurs, at least in part, through its antagonism of canonical Wnt signaling that may be involved in removing cells from the cell cycle.

Although the effects of Dkk2 on osteoblast differentiation seem to be mediated in part by its Wnt-antagonistic effect, we believe that the role of Dkk2 in osteoblast differentiation is not limited to its Wnt-antagonistic effect for two reasons. First, overexpression of Dkk2, but not FRP3, could lead to mineralization phenotypes (**Fig. 6b**,**d**). Second, expression of Dkk2, but not FRP3, resulted in rescuing the expression of two osteogenic markers, osteocalcin and osteopontin (**Fig. 5a–d**). The possibility that Dkk molecules may not function only as Wnt antagonists, but also in other roles, was previously described. Dkk1 may be involved in the regulation of cardiac myocyte differentiation in a mechanism independent of its Wnt-antagonistic effect[41], and Dkk2 has agonistic effects on the Wnt–β-catenin pathway[34,35,42]. Results showing that inactivation or activation of β-catenin in osteoblasts through expression of Cre driven by the 2.3-kb *Col1a1* promoter had little effect on osteoblast functions[43] further support the idea that Dkk2 regulates osteoblast terminal differentiation in canonical Wnt-independent mechanisms. This study[43], together with our observation that canonical Wnt stimulates osteoblast transition to the 2.3-kb *Col1a1* promoter active state, also suggests that the effect of canonical Wnt signaling on osteoblasts may be exerted mainly before the 2.3-kb *Col1a1* promoter is active. Although Dkk2 had a very weak agonistic effect on the Wnt–β-catenin pathway in the overexpression system, we do not think that Dkk2 regulates osteoblast terminal differentiation through this agonistic effect on canonical Wnt signaling. This agonistic effect of Dkk2 depends on overexpression of LRP6, and overexpression of LRP5 had no effect[34,35]. In addition, in osteogenic differentiation, the expression levels of LRP6 decrease,

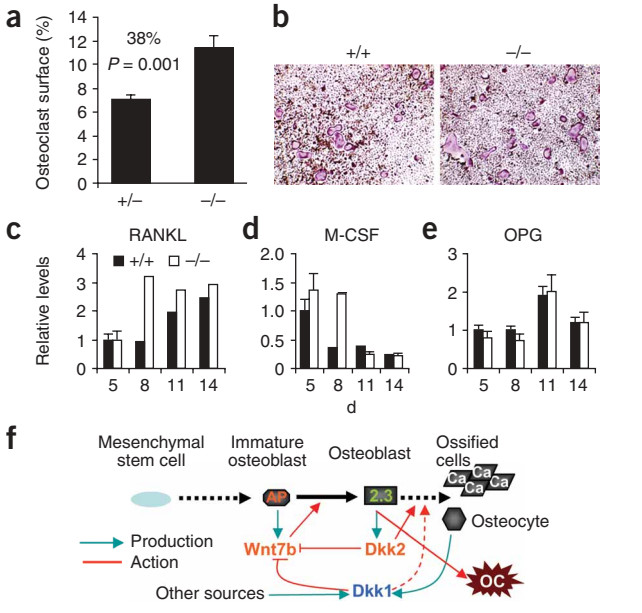

Figure 7 Effects of Dkk2 deficiency on osteoclast activity, and a model for the involvement of Wnt and Dkk in the regulation of osteogenesis. (**a**) Bone histomorphometric analysis showed an increase in osteoclast surfaces in proximal tibial trabecular bones of *Dkk2*-null mice. (**b**) Dkk2 deficiency does not affect osteoclast differentiation in bone marrow cultures containing RANKL and M-CSF. (**c–e**) *Dkk2*-null calvarial osteoblasts produced more M-CSF and RANKL than wild-type cells during their differentiation, whereas OPG was expressed at similar levels. (**f**) A model describing the involvement of Wnt and Dkk in osteogenic differentiation. Differentiating osteoblasts produce canonical Wnts, which stimulate proliferation and differentiation of immature osteoblasts into mature osteoblasts. Meanwhile, Wnt stimulates the production of Dkk2, which has a role in terminal osteoblast differentiation into mineralized matrix.

and those of LRP5 increase (**Supplementary Fig. 3** online). Therefore, it is very unlikely that this effect of Dkk2 has a considerable role in osteoblast terminal differentiation. Moreover, Dkk1, which has never been shown to have any agonistic effect on the Wnt–β-catenin pathway, could also stimulate the mineralization of cultured calvarial osteoblasts and BMSOs (data not shown). Dkk1 expression in osteoblasts is not tightly coupled with Wnt7b expression; therefore, it may be secondary to Dkk2 in terms of regulating osteoblast mineralization. But we believe that it may partially compensate for the lack of Dkk2 *in vivo*, because even though *Dkk2*-null osteoblasts fail to differentiate to the mineralized matrix in cultures, mice lacking Dkk2 are still able to develop ossified bones, though with lower density. The compensation may also explain the differences in the effects of Dkk2 deficiency on osteogenic marker expression *in vivo* and *in vitro*. Although Dkk2 deficiency had substantial effects on expression of osteocalcin and osteopontin expression *in vitro*, it did not substantially affect expression *in vivo* (data not shown). It would be of great interest to determine the role of Dkk1 in osteoblast differentiation and bone remodeling. Unfortunately, this study depends on bone-specific gene targeting, as mice lacking Dkk1 die as embryos[44].

In summary, this study showed that Dkk2, whose expression is tightly regulated during the osteogenic differentiation, probably by canonical Wnts, has an important role in terminal osteoblast differentiation into mineralized matrices. This study also reaffirms the positive role of canonical Wnts in promoting osteogenesis. Notably, we identified Wnt7b, the only known canonical Wnt whose expression is regulated during osteoblast differentiation. On the basis of the expression timing and regulation of Wnt7b, Dkk2, Dkk1 and osteogenic markers; the effects of Dkk2 deficiency on the expression of Wnt7b, Dkk1 and osteogenic markers; and the effects of Dkk2 and canonical Wnts on osteogenesis, we believe that canonical Wnts, including Wnt7b, and Dkk2 form a signaling circuitry required for osteogenic differentiation. In other words, canonical Wnts function as stimulators of proliferation and mid-stage differentiation, whereas Dkk2 acts as the terminal differentiation agent whose expression is directly regulated by canonical Wnts (**Fig. 7e**). In addition to osteoblast terminal differentiation, Dkk2 seems to affect osteogenic progenitor cells and osteoclasts also. The effects on osteoclasts may result from the increases in the expression of osteoclast-active cytokines, including M-CSF and RANKL, due to the delay in the terminal differentiation of osteoblasts in *Dkk2*-null mice. The reduction in the number of alkaline phosphatase–positive clones in *Dkk2*-null mice may result from a decrease in osteoblastic progenitor cell proliferation rather than an increase in apoptosis (**Supplementary Fig. 4** online). This conclusion is consistent with the previous finding that Dkk1 is involved in the re-entry of mesenchymal stem cells into the cell cycle[45]. The precise role of Dkk in the regulation of mesenchymal stem cell biology needs further investigation.

METHODS

Generation of *Dkk2*-null mice. We generated *Dkk2*-null mice using the standard gene targeting approach. We used a neomycin-resistance gene to replace four coding exons of *Dkk2* by homologous recombination in mouse embryonic stem cells. We aggregated the embryonic stem cells into mouse embryos and implanted them into foster female mice. After serial breeding schemes, we obtained *Dkk2*-null mice. We used littermate wild-type, $Dkk2^{+/-}$ and $Dkk2^{-/-}$ mice in all experiments. All experiments involving mice were approved by the institutional Animal Care Committee of the University of Connecticut Health Center.

Measurement of static and dynamic bone formation in mice. To measure BMD and bone mineral content, we anesthetized mice (4-month-old males; $n > 17$), measured total femoral and whole-body bone mineral content (g) and bone area (cm^2) using the PIXImus small animal DXA system (GE-Lunar) and calculated BMD. We excluded heads from whole-body measurements. We carried out pQCT analyses using a Stratec XCT Research system (Norland Medical Systems). We examined the distal femoral metaphysis of right femur from each mouse to obtain volumetric bone mineral area, content and density of the trabecular, cortical and total bone. We imaged a 1-mm-thick cross-section of the distal femoral metaphysis at 2.5 mm proximal from the distal end[46].

We carried out static and dynamic histomorphometry after injecting calcein and alizarin 7 and 2 d before necropsy. We dissected tibiae from 4-month-old male mice ($n > 17$) and fixed them in 70% ethanol. For studies of trabecular bone, we prepared undecalcified, methyl methacrylate–embedded longitudinal sections of the proximal tibial metaphysis at thicknesses of 4 μm and 8 μm for trabecular bone histomorphometry as described previously[47]. We stained the 4-μm sections with toludine blue stain and used them for measurements of bone mass, structure, osteoblast number and surface, and bone resorption–related indices. We did not stain the 10-μm sections and used them for the measurement of mineralizing surface, mineral apposition rate and bone formation rates. We determined bone morphometric parameters as described previously[47].

Primary calvarial and BMSO and osteoclast cultures. We generated mouse calvarial osteoblast cultures from 5-d-old mice and BMSO cultures as previously described[39] and induced them to undergo osteogenic differentiation in the presence of 8 mM β-glycerophosphate and 50 μg ml^{-1} ascorbic acid. For BMSO cultures, we also added dexamethasone (10 pM) as a differentiation inducer. We changed the medium every 2 d.

Quantitative PCR analysis. We isolated total RNA using the TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. For QPCR analysis, we reverse-transcribed RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). We carried out QPCR using QuantiTect SYBR Green PCR kit (Qiagen) on a DNA Engine OPTICON (MJ Research Inc.) instrument. We used β-actin as an internal reference for each sample. Using a formula previously described[48], we normalized the relative change in mRNA levels against β-actin mRNA levels.

***In situ* hybridization.** We used the full-length coding region of Wnt7b to synthesize antisense and sense probes and labeled the probes with digoxigenin using an RNA Labeling Kit (Roche). Sections of the tibia from a 3-week-old mouse were dewaxed, rehydrated, fixed with 4% paraformaldehyde, treated with 2% glycine and proteinase-K, acetylated using an acetic anhydride–TEA solution and then hybridized with a digoxigenin-labeled probe. After washing the sections twice with 50% formamide, 5× saline sodium citrate and 5% SDS for 30 min at 70 °C and once with 50% formamide and 2× saline sodium citrate for 30 min at 65 °C, we incubated them with antibody to digoxigenin conjugated with alkaline phosphatase and then with Nitro Blue etrazolium/4-bromo-5-chloro indolylphosphate, which yields a purple-blue color. We also counterstained the sections with methyl green (nuclei) and orange G (cytoplasm).

5-bromodeoxyuridine (BrdU) incorporation and apoptosis of osteogenic progenitor cells. We measured BrdU incorporation and apoptosis of osteogenic progenitor cells using Roche Biochemical's Cell Proliferation and Homogeneous Caspases Assay kits. We seeded 10^8 wild-type and *Dkk2*-null marrow cells per 10-cm dish. Three days later, we washed dishes, collected adherent cells after trypsinization and reseeded them into 96-well plates for the BrdU incorporation and apoptosis assays. For BrdU incorporation, we labeled cells with BrdU for 4 h the next day and determined the levels of incorporated BrdU in accordance with the instructions of the Cell Proliferation Assay kit (Roche). For the apoptosis assay, we determined caspase activity the next day using the Homogeneous Caspases Assay kit (Roche). The results for both assays were normalized against protein contents, which were determined by crystal violet staining of parallel plates. We did the assays in quadruplicate.

Measurement of serum alkaline phosphatase, calcium and phosphorus. We collected blood from five 3-month-old wild-type and *Dkk2*-null mice. We determined serum bone-derived alkaline phosphatase levels as described[49] with

some modifications. We measured alkaline phosphatase activity using chemiluminescence with the CSPD substrate from PerkinElmer. We determined levels of serum calcium and phosphorus using a VETACE Clinical Chemistry Analyzer (Alfa Wasserman Inc.).

Fourier-transformed infrared spectroscopic analysis of culture samples. We allowed mineralized matrix in the culture dishes to air-dry in the dish, added KBr (spectral grade, Fisher Chemical) to the dried cultures and used KBr pellets for spectroscopic analysis. We collected infrared spectra using a Thermo-4700 Fourier-transform infrared Spectrometer (Thermo-Nicolet) under nitrogen purge. For illustrative purposes, we normalized spectra so that the area under the amide I peak was constant.

Note: Supplementary information is available on the Nature Genetics website.

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The authors declare competing financial interests (see the *Nature Genetics* website for details).

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