Genome-wide association and functional studies identify the *DOT1L* gene to be involved in cartilage thickness and hip osteoarthritis

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Hip osteoarthritis (HOA) is one of the most disabling and common joint disorders with a large genetic component that is, however, still ill-defined. To date, genome-wide association studies (GWAS) in osteoarthritis (OA) and specifically in HOA have yielded only few loci, which is partly explained by heterogeneity in the OA definition. Therefore, we here focused on radiographically measured joint-space width (JSW), a proxy for cartilage thickness and an important underlying intermediate trait for HOA. In a GWAS of 6,523 individuals on hip-JSW, we identified the G allele of rs12982744 on chromosome 19p13.3 to be associated with a 5% larger JSW ($P = 4.8 \times 10^{-10}$). The association was replicated in 4,442 individuals from three United Kingdom cohorts with an overall meta-analysis *P* value of 1.1×10^{-11}. The SNP was also strongly associated with a 12% reduced risk for HOA ($P = 1 \times 10^{-4}$). The SNP is located in the *DOT1L* gene, which is an evolutionarily conserved histone methyltransferase, recently identified as a potentially dedicated enzyme for Wnt target-gene activation in leukemia. Immunohistochemical staining of the DOT1L protein in mouse limbs supports a role for DOT1L in chondrogenic differentiation and adult articular cartilage. *DOT1L* is also expressed in OA articular chondrocytes. Silencing of *Dot1l* inhibited chondrogenesis in vitro. *Dot1l* knockdown reduces proteoglycan and collagen content, and mineralization during chondrogenesis. In the ATDC5 chondrogenesis model system, DOT1L interacts with TCF and Wnt signaling. These data are a further step to better understand the role of Wnt-signaling during chondrogenesis and cartilage homeostasis. DOT1L may represent a therapeutic target for OA.

complex disease | joint development | synovial joint | common variant | pleiotropism

Osteoarthritis (OA), the most common, age-related disease of the synovial joints, results in a substantial reduced quality of life because of pain and disability. Current clinical management of OA focuses on pain control. In severe cases, joint prosthesis surgery may be the unique solution. There are currently no targeted therapies that maintain homeostasis of the joint or stimulate cartilage repair. OA is characterized by progressive destruction of articular cartilage, subchondral bone sclerosis, and osteophyte formation, and has a large genetic component, which varies between the joint studied (1).

Several genome-wide association studies (GWAS) on OA have been published, but up to now few signals have been identified with reproducible association (2–6). In Caucasians, only three loci reach the genome-wide significance (GWS) threshold. These loci include a variant influencing expression of *GDF5* (2, 3), a locus on chromosome 7q22 near the orphan receptor *GPR22* (4, 5), and a variant in *MCF2L* (6). The low number of identified loci can be explained by relatively low power caused by insufficient sample sizes and by phenotype heterogeneity, which is a well-known problem in epidemiology of OA (7). The diagnosis of OA is based on a combination of parameters, including both clinical features (pain and stiffness) and a structural damage score (the most widely used is the Kellgren and Lawrence score), which includes formation of new bone spurs (osteophyte formation) and reduction of the joint-space width (JSW), indicating cartilage degradation. JSW is considered to be the surrogate for cartilage thickness in the joint and change in minimal JSW (mJSW) is the primary structural endpoint used in clinical trials and epidemiological studies of knee and hip OA (8–10).

In this study we combined GWAS and functional studies to identify genes involved in cartilage thickness and osteoarthritis. We first performed a discovery GWAS on mJSW of the hip in 6,523 participants from the Rotterdam cohorts I and II (RS-I and RS-II) and replication included populations from three independent United Kingdom studies ($n = 4,442$) in which mJSW was measured (see Table S1 for cohort specifics). Additionally, we analyzed association of the genetic variants with hip OA (HOA) in 3,717 cases and 10,013 controls. Furthermore, we carried out functional genetic studies using cell-culture experiments in human and mouse tissues.

Results

A GWAS on mJSW of the hip was performed in 6,523 participants from the RS-I and RS-II (Table S1).

We applied extensive quality-control measures (see Table S2 for details on quality control and exclusion criteria), leaving a total of 2,455,290 SNPs for association analysis. Genomic control inflation factors for the *P* values of the RS-I and RS-II GWAS were low ($\lambda = 1.02$ and 1.01, respectively), and the interquantile-quantile plot (Fig. S1) also indicated no substantial population stratification

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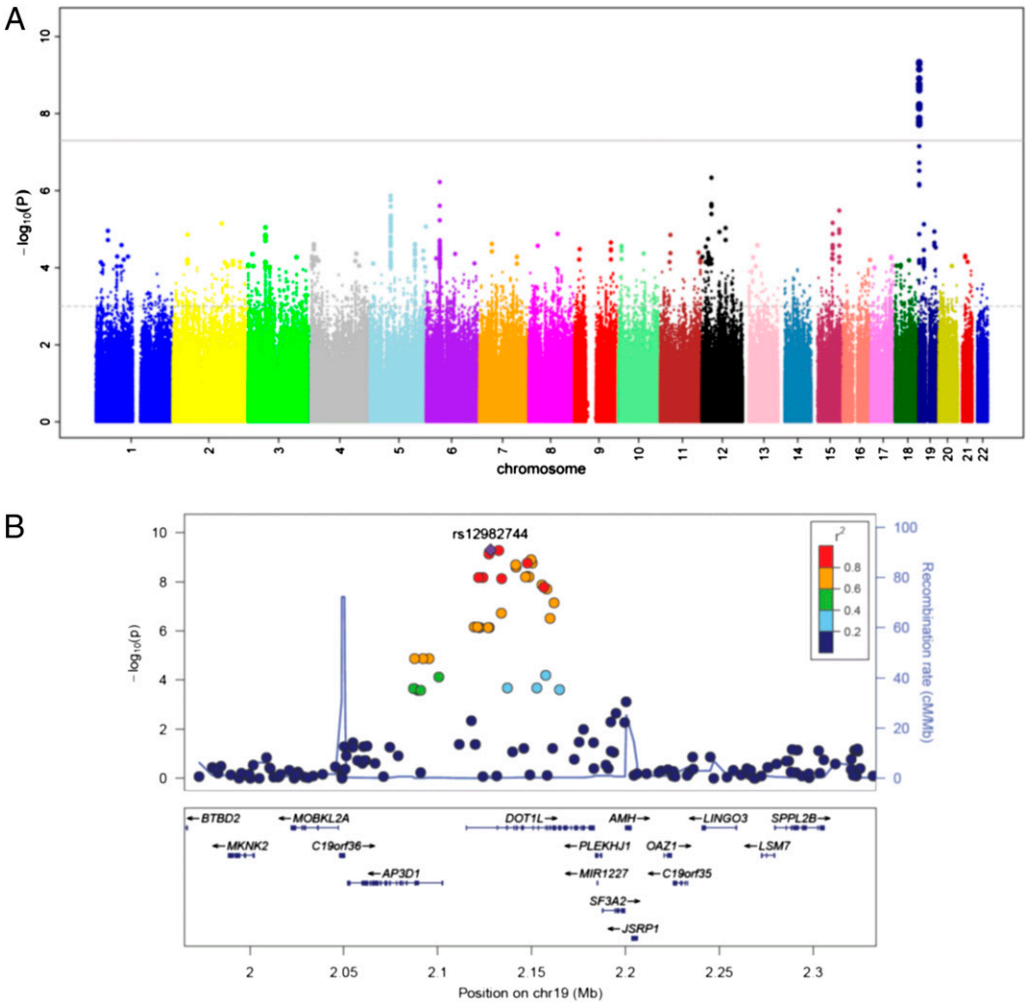

Fig. 1. (*A*) Association results by chromosome. The −log *P* values for each of the 2.5 million tests performed as part of the genome-wide association of minimal joint space (MJS) of the hip. The black solid horizontal line corresponds to *P* value threshold of 5×10^{-8} (GWS). (*B*) Regional association plot for the locus of JSW (19 p31.3). SNPs are plotted by position in a 400-kb window against association with mJSW (−log10 *P*). The purple diamond highlights the most significant SNP in discovery analysis. Blue peaks indicate recombination rates. The SNPs surrounding the most significant SNP are color coded to reflect their LD with this SNP (from pairwise r^2 values from the HapMap CEU). Genes, exons and the direction of transcription from the University of California at Santa Cruz genome browser are depicted underneath the plot.

because of cryptic relatedness, population substructure, or other biases. After meta-analyzing the association results of RS-I and RS-II, we identified a significant association on chromosome 19 that satisfied our GWS threshold of $P < 5 \times 10^{-8}$ (Fig. 1*A*). A total of 18 SNPs were GWS and clustered around one locus on chromosome 19p13.3. The top SNP rs12982744 ($P = 4.5 \times 10^{-10}$) is localized in the first intron of the gene *DOT1*-like, histone H3 methyltransferase (*DOT1L*). This SNP is in high linkage disequilibrium (LD) with the other 17 GWS SNPs representing the same signal (Fig. 1*B*). We additionally found eight loci with suggestive evidence for association ($5 \times 10^{-8} < P < 1 \times 10^{-5}$) (Table S3).

To validate the association with *DOT1L*, we performed a replication study using three independent United Kingdom studies: TwinsUK, Chingford, and the Genetics of Osteoarthritis and Lifestyle (GOAL) (n = 4,442 in total) (Table S1). Association between rs12982744 and mJSW in the replication cohorts was analyzed by linear regression including age and sex as covariates. The association of rs12982744 with mJSW was replicated (β: 0.07 mm/allele; $P = 9 \times 10^{-3}$) (Fig. 2).

Results from the Rotterdam Studies and the replication cohorts were combined in a joined meta-analysis. The combined analysis including discovery and replication studies showed strong evidence for association of the *DOT1L* locus with minimal JSW in the general population (β: 0.09 mm/allele; $P = 1.1 \times 10^{-11}$, $I^2 = 0\%$). These associations were corrected by age and sex. The minor G allele of rs12982744 [minimum allele frequency (MAF) = 0.39] is associated with an increased JSW of 0.09 mm per copy of the G allele. This finding implicates that homozygote carriers of the rs12982744 G allele have ~5% thicker cartilage than the reference group.

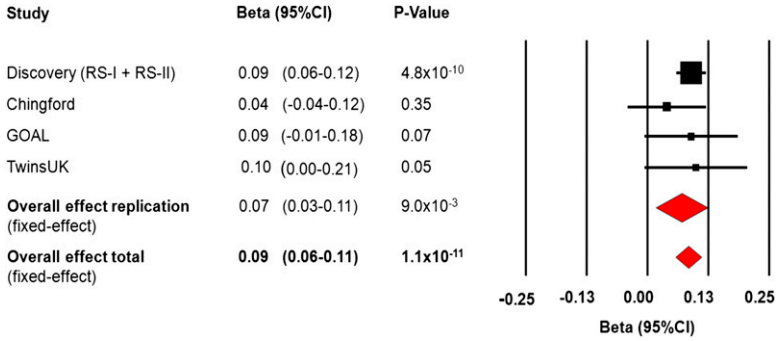

Fig. 2. Forest plots for rs12982744. Black squares represent effect estimate and 95% CI for each study, and the red diamond is a summary effect estimates. mJSW measurements units are in millimeters.

We further investigated whether rs12982744 was influencing the risk for HOA. This influence was examined in all of the five studies described previously and one additional large case-control study (Nottingham); the total sample size was 3,717 cases and 10,013 controls for this analysis (Table S1). Risk for HOA was calculated using logistic regression analysis and was adjusted for age and sex. As shown in Fig. 3, the minor allele of rs12982744 was significantly associated with a 12% reduced risk for HOA [odds ratio (OR) = 0.88, confidence interval (CI) 0.82–0.94; $P = 1.5 \times 10^{-4}$, $I^2 = 0\%$; analysis adjusted for age and sex], with consistent effects in all cohorts studied. Additional adjustment for height did not affect the association (OR = 0.88, CI 0.82–0.94; $P = 1.1 \times 10^{-4}$). We also observed that in people without radiographic HOA the association with mJSW was present (Table S4) (β: 0.06 mm, SE: 0.011; $P = 7.3 \times 10^{-9}$). This finding suggests that the association with cartilage thickness is present already before onset of OA, and possibly implicates involvement of this DNA-variant on the articular cartilage during development and growth.

The G allele of the identified SNP (rs12982744) was previously found to be associated with increased height (11). This finding is in line with the thicker cartilage that was found in the present study. We therefore tested whether our findings with mJSW were affected by differences in stature by including height as a covariate in the analysis. This inclusion did not substantially change the results, which suggests that this locus has independent pleiotropic effects on height as well as mJSW of the hip.

The associated polymorphisms are annotated in the *DOT1L* gene (Fig. 1*B*). DOT1L is an evolutionarily conserved histone methyltransferase, identified as an essential and dedicated enzyme for Wnt target-gene activation in the intestine and needed for the expression of genes that require high levels of Wnt signaling in *Drosophila* (12, 13). We hypothesized that *DOT1L* is the culprit gene underlying the association with mJSW and height by influencing chondrogenic differentiation, which is important in growth and joint formation.

We examined the function of *Dot1l* during chondrogenesis in ATDC5 cells, which exhibit a multistep process of chondrogenic differentiation analogous to that observed during endochondral bone formation (14, 15). As depicted in Fig. 4*A* and Fig. S2, ATDC5 cells stably transfected with plasmid overexpressing shmiRNA directed against *Dot1l* (Dot1l^{-}) synthetized less sulfated proteoglycans than control cells, demonstrated by the weaker Alcian blue and Safranin O staining, respectively decreased by 1.35- and 2.5-fold. Moreover, mineralization in the micromasses was less efficient, as shown by the 1.4-fold decrease in Alizarin red staining, which was restricted to the core of the micromasses in Dot1l^{-} cells. Collagen content, revealed by Sirius red staining, was also 1.8-fold reduced in these cells. These data indicate that chondrogenesis is severely affected by *Dot1l* knockdown. These observations were supported by mRNA analyses. Indeed, type II collagen expression was not increased in cells with *Dot1l* knockdown, and type X collagen and aggrecan induction was 3.3-fold and 4-fold reduced compared with normal ATDC5 cells (Fig. 4*B*). Interestingly, type I collagen levels were 1.7-fold higher in *Dot1l*$^{-}$ cells at day 21. Moreover, evaluation of matrix metalloproteinases (MMPs) mRNA level allowed to show a different pattern in *Dot1l*$^{-}$ cells compared with controls, as seen in Fig. S3. Indeed, *Mmp9* expression was dramatically increased in *Dot1l*$^{-}$ cells (35-fold increase at day 21), but *Mmp13* was decreased by 1.7-fold at the same time. *Mmp2* expression did not differ between *Dot1l*$^{-}$ and controls.

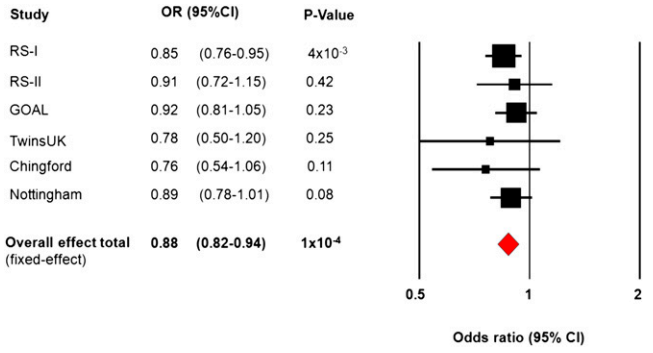

Fig. 3. Risk for HOA. Values represent OR and 95% CI.

Because DOT1L was previously linked to β-catenin signaling, we investigated whether mRNA expression of Wnt target genes was affected in *Dot1l*$^{-}$ cells. As seen in Fig. 4*C*, *Tcf1* levels (positively regulated by Wnt/β-catenin signaling) were increased in control ATDC5s at day 7 (2.5-fold), but no induction was detected in *Dot1l*$^{-}$ cells. Other Wnt target genes, *Axin2* and *c-Myc*, followed the same pattern (Fig. S4). Moreover, osteocalcin level (negatively regulated by Wnt/β-catenin signaling) was increased by 2.8-fold at day 21 in control cells, but the up-regulation was of 6.2-fold in *Dot1l*$^{-}$ ATDC5s (2.2-fold more than in control cells). Taken together, these elements suggest a role for DOT1L in the Wnt/β-catenin signaling cascade in developing chondrocytes.

Coimmunoprecipitation experiments strengthened these observations, because DOT1L was found to directly interact with transcription factor 4 (TCF4), a transcription factor interacting with β-catenin (Fig. 4*D*). These functional analyses seemed relevant in vivo, because DOT1L is very strongly present during chondrogenesis in mouse developing limbs and still found in articular cartilage as seen in Fig. 4*E*. Interestingly, *DOT1L* mRNA was clearly detected in adult human chondrocytes freshly extracted (without any passage) from articular cartilage obtained from patients with osteoarthritis (Fig. S5).

Discussion

This study identified a genetic variant in the *DOT1L* gene robustly associated with JSW and HOA. We used an in vitro chondrogenesis model and ex vivo expression studies in mice to functionally characterize the role of *Dot1l* in chondrogenesis. We found that DOT1L is involved in chondrogenic differentiation, presumably through its role in canonical Wnt-signaling.

DOT1 is an evolutionarily conserved histone methyltransferase, which was initially identified as a disruptor of telomeric silencing in *Saccharomyces cerevisiae* (16). The mammalian homolog, DOT1L, has been shown to be required for embryogenesis, hematopoiesis, and cardiac function (17–20). DOT1L was recently identified as an essential and dedicated enzyme for Wnt target-gene activation in the intestine and needed for the expression of genes that require high levels of Wnt signaling in *Drosophila* (12, 13). We are unique in providing evidence that demonstrates a role for DOT1L in chondrogenesis. Knockdown of *Dot1l* resulted in a reduced chondrogenic differentiation in the ADTC5 cells. We additionally observed a pronounced reduction in expression of Wnt-targeted genes. Together with the proven physical interaction of DOT1L and TCF4 proteins, this finding suggests that Dot1l influenced chondrogenic differentiation by regulating transcription of Wnt target genes. The differential effect of *Dot1l* silencing on different MMPs further highlights its complex role in cartilage biology.

Wnt signaling is critical in the formation of cartilage and bone and in the development of the synovial joint (21) Mutants in the Wnt have been shown to cause developmental abnormalities early in life [see for example WNT3 (22)]. Variants with a less dramatic effect on function, such as the one identified in this study, result in a mild phenotype with late onset. The same has been observed in the BMP-signaling pathway (another key developmental pathway). Mutations in the *GDF5* gene result in severe chondrodysplasia and skeletal malformations (23), and a milder variant that influences *GDF5* expression levels, results in a slightly elevated risk for knee OA later in life (2, 24).

The exact same variant that we found associated with cartilage thickness has previously been found associated with height in both young and old individuals (11, 25), which suggests a role in skeletal formation. Although the specific differentiation process in the growth plate and articular cartilage are different, common signaling pathways, such as the Wnt cascades, are involved (26). Interestingly the association between the *DOT1L* genetic variant and cartilage thickness was present also in people without OA. This finding indicates that the association with cartilage thickness is present already before onset of OA, and possibly implicates involvement of this DNA-variant on normal formation of the articular cartilage during development, in agreement with a role for this variant in skeletal development.

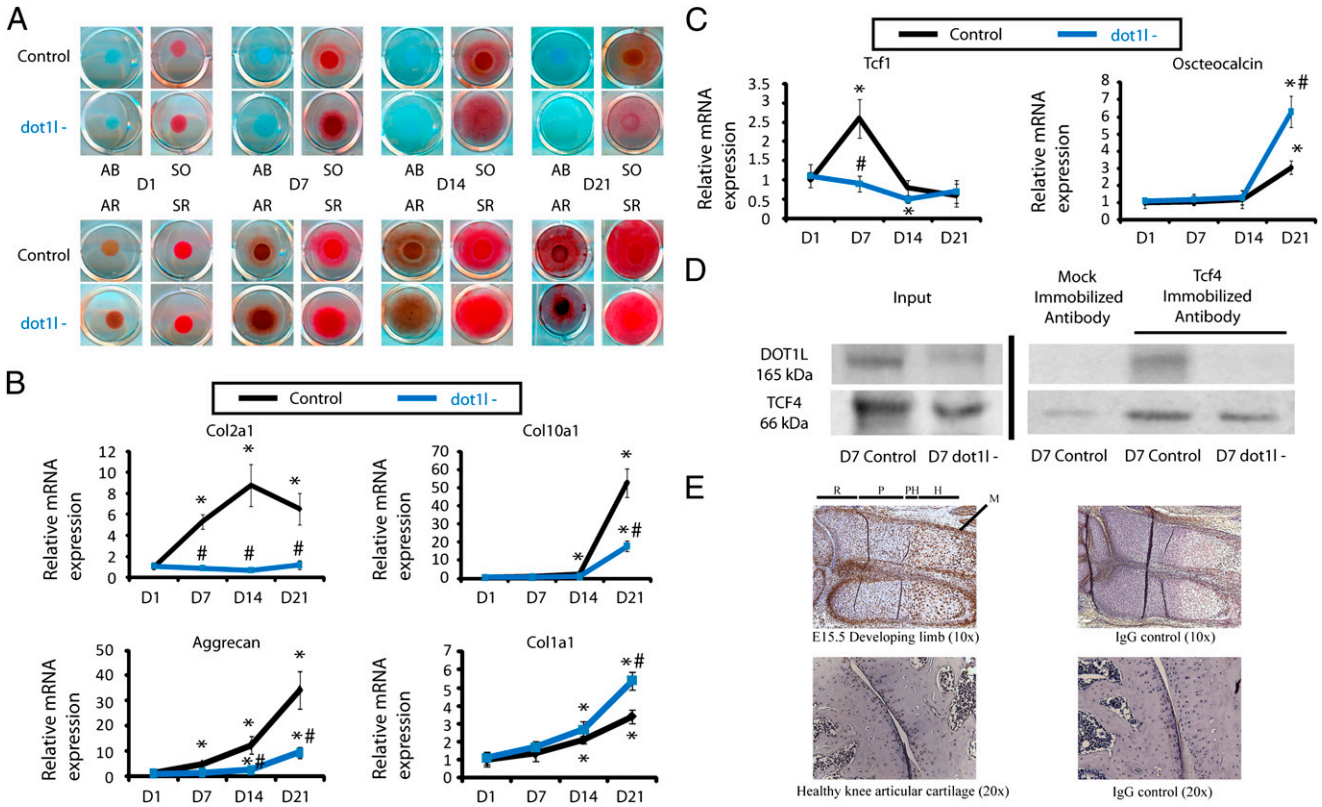

Fig. 4. Functional analysis of *Dot1l* during chondrogenesis. Stable ATDC5 clones were established using either the control noninterfering pGIPZ or the pGIPZ-shmiRNA directed against mouse *Dot1l*. Three different antibiotic-resistant clones were selected. Knockdown efficiency was assessed by quantitative RT-PCR. Stably-transfected ATDC5 clones were cultured as micromasses as described previously (14, 15). Each condition was performed in triplicate. Total RNA from was isolated after 1, 7, 14, or 21 d. Data presented are representative of the three independent clonal colonies. Results are expressed as the mean ± SD of three independent replicates. Comparisons were made by ANOVA, followed by Fisher's *t* post hoc test. Statistically significant differences vs. day 1 are indicated as $^{*}P < 0.05$, and vs. control-transfected cells as $^{\#}P < 0.05$. (*A*) *Dot1l* knock-down reduces proteoglycan and collagen content, and mineralization during chondrogenesis. Stainings were performed on ATDC5 micromass cultures stably transfected with either control or *Dot1l* shmiRNA producing vector, over 21 d. AB, Alcian blue; AR, Alizarin red; SO, Safranin O; SR, Sirius red. (*B*) *Dot1l* knock-down reduces mRNA expression of markers of chondrogenesis. mRNA levels were normalized to *S29* (reference gene) ($n = 3$). Quantitative real-time PCR conditions and primers are available upon request. (*C*) *Dot1l* knock-down affects Wnt signaling during chondrogenesis. mRNA levels of Wnt target genes *Tcf1* and osteocalcin were normalized to *S29* (reference gene) ($n = 3$). (*D*) DOT1L interacts with the Wnt signaling pathway TCF4. Coimmunoprecipitation of DOT1L and TCF4 using 100 μg of total proteins (input) from micromass cultures (at day 7) of either control or *Dot1l* knocked-down cells. Proteins were isolated from ATDC5 micromasses. Coimmunoprecipitations were performed and 20 μL of elution fraction was probed after protein binding on either mock (donkey anti-goat IgG) or TCF4 column-immobilized antibody. (*E*) DOT1L is expressed during joint development and in mature articular cartilage of mice. Immunohistochemistry on paraffin-embedded EDTA decalcified adult knee sections and nondecalcified embryonal sections, was performed with rabbit anti-Dot1L antibody (5 μg/mL). After overnight incubation of the sections at 4 °C, 1:100 peroxidase goat anti-rabbit IgG was applied and peroxidase activity was determined using DAB. In the developing limb (embryonic day 15.5), expression was detected in resting (R), proliferating (P), prehypertrophic (PH), and hypertrophic (H) chondrocytes, as well as in the mesenchyme surrounding the bones (M). Immunohistochemistry also detected expression in articular cartilage chondrocytes in healthy mice knee (age 9 wk). IgG as a negative control is also shown.

OA is a complex disease with a large genetic component. Twins studies have shown that the influence of genetic factors for HOA is about 60% (1). Nevertheless, it has been difficult to find genes involved in OA and especially in HOA. From the few genetic signals found, only one has shown a modest association with HOA (6). *GDF5* polymorphisms (3) and a locus on chromosome 7q22 near the *GPR22* gene (4) have been consistent associated with knee OA only across different European populations. Recently, a locus on chromosome 13 localized in the *MCF2L* gene that regulates a nerve growth factor points to pronounced association with OA affecting the knee and less significantly for HOA (6). These few signals have been found using the traditional composite definitions of OA, which have features of structural damage to the joint (Kellgren and Lawrence score of 2 or more, including joint replacement) as well as clinical parameters, such as pain, which may lead to considerable heterogeneity and consequently low power.

In the case of HOA, where degeneration of articular cartilage is the most important feature, the approach to identify genetic variants of OA studying only one of the components of the physiopathology (cartilage thickness) can result in less heterogeneity in the phenotype definition, and therefore in more power to pick up true signals. Both, intrarater and interrater reliability has been significantly higher for joint space measurement than for Kellgren and Lawrence scoring (10, 27), and the findings that decline of JSW in OA proceeds in a linear manner (28) and that JSW is predictive of long-term progression of joint-space narrowing (29) make measurement of JSW suitable for clinical trials and prioritize the identification of genes responsible for cartilage formation and homeostasis.

The effect we report of the DOT1L variant on cartilage thickness is modest, similar in magnitude to most of the identified variants involved in risk of complex diseases. Consequently, one might prematurely anticipate that the clinical relevance for this variant is by default small. However, the effect size of an identified variant does not necessarily reflect importance of the gene for a disease. Variants that strongly disrupt pivotal genes are unlikely to result in a late-onset disease that affects 40% of the population over the age of 70. As DOT1L function is linked to Wnt signaling, genetic variation may not only contribute to cartilage thickness with reduced cartilage volume, a likely risk factor for OA development, but also to the deleterious processes

that are activated when osteoarthritis is progressing. Accordingly, DOT1L might be a target for the design of new anti-OA drugs that could be used in the prevention and treatment of OA. Additionally, carriers of the DOT1L variant might have a different response to possible treatments targeting cartilage repair.

Another potential application of OA genetics is improved measurement of the disease process in combination with other variants of modest but consistent effect forming a "genetic risk score." A previous study has suggested that when several genetic markers are added up, the aggregated genetic risk is substantial and similar in magnitude to classic risk markers, such as obesity or knee injury (30), and which may help to identify individuals at risk for OA years before disease onset.

Our results are not directly generalizable to other ethnicities, such as the Asian population. Asian populations have a high prevalence of large-joint OA despite a much lower prevalence of obesity, suggesting etiological differences with regards to European-descent patients. Specifically, strong evidence of heterogeneity in the genetic contribution to OA between Asian and European populations has been widely reported (3, 31–34). In particular, no loci influencing HOA have been consistently reported in both ethnic groups. Although the lack of inclusion of Asian patients may reduce the generalizability of our results, it has the advantage, (by concentrating on a clearly defined phenotype in homogenous Caucasian samples of Dutch and United Kingdom origin) of reducing heterogeneity and thus achieving sufficient statistical power.

Considering the known important function of the Wnt signaling pathway in cartilage and bone formation and the role of DOT1L in chondrogenesis here presented, DOT1L may represent a therapeutic target for modulation, and thus therapeutic intervention in OA. It is apparent that DOT1L and its associated methylation activity are regulated in an extremely complex way. As such, the regulation of DOT1L activity and the functional consequences of manipulation of DOT1L need to be further elucidated before efficient treatments can be developed. Future studies are therefore warranted to determine how to target DOT1L in a selective and tissue-specific manner. There are already initiatives for targeting DOT1L in other pathologies, having in mind that DOT1L has a key role in other normal cellular processes (35). This might represent an exciting opportunity for the development of disease modifying drugs for OA.

Materials and Methods

GWAS Meta-Analysis. Genotyping of the samples in the discovery cohorts (RS-I and RS-II) was carried out with the Illumina HumanHap 550v3 Genotyping BeadChip. The Beadstudio GenCall algorithm was used for genotype calling and quality-control procedures, as described previously (36). The following quality-control inclusion filters were applied: call rate ≥97.5%, MAF ≥1%, *P* for Hardy-Weinberg equilibrium $<1 \times 10^{-6}$ (see Table S2 for details on quality control and exclusion criteria). The total number of genotyped SNPs that passed these filters was 512,349 for RS-I and 466,389 for RS-II. Imputation was done with reference to HapMap release 22 CEU using the maximum-likelihood method implemented in MACH (http://www.sph.umich.edu/csg/abecasis/MACH/index.html). Analysis of imputed genotype data accounted for uncertainty in each genotype prediction by using the dosage information from MACH. For this analysis, MACH2QTL was used via GRIMP (37), which uses genotype dosage value (0–2, continuous) as a predictor in a linear-regression framework. Genomic control correction was applied to the SEs and *P* values before meta-analysis. We included only imputed SNPs that had a good imputation quality leaving a total of 2,455,290. The summary statistics of RS-I and RS-II were meta-analyzed using METAL applying inverse-variance methodology assuming fixed effects, with Cochran's Q and I2 metrics used to quantify between-study heterogeneity (www.sph.umich.edu/csg/abecasis/metal).

The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant.

Replication Analysis. All samples from the TwinsUK cohort for this study were genotyped with the HumanHap610Q (Illumina). The following quality-control filters were applied: call rate ≥98%, MAF ≥ 1%, *P* for Hardy-Weinberg equilibrium $\geq 1 \times 10^{-6}$ (Table S2). The total number of genotyped SNPs that passed these filters was 598,207. Imputation was done with reference to HapMap release 22 CEU using the IMPUTE software package (v2) (38). For the GOAL, Nottingham, and Chingford study participants, genomic DNA was extracted from peripheral blood leukocytes of affected individuals and controls using standard protocols. Genotyping was carried out by Kbioscience. SNPs were genotyped using the KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos.

Association between rs12982744 and mJSW in the replication cohorts was analyzed by linear regression, including age and sex as covariates. In addition, separate analyses were carried out including age, sex, and height as covariates. The R version 2.10.1 (The R Foundation for Statistical Computing http://www.r-project.org/) was used for analysis.

Results from the Rotterdam studies and the replication cohorts were combined in a joined meta-analysis using inverse variance weighting with METAL, as described above. We declared results GWS at $\alpha = 5 \times 10^{-8}$ after adjusting for all common variant tests in the human genome.

The replication studies [TwinsUK, Genetics of Osteoarthritis and Lifestyle (GOAL) study, Chingford study and Nottingham case-control study] were approved by their respective institutional review boards and informed consent was obtained from all participants involved.

Cell Culture Experiments. ATDC5 cells were cultured in growth medium (1:1 mixture of DMEM and Ham's F-12 medium) (Gibco) containing 1% (vol/vol) antibiotic-antimycotic (Gibco), 5% FBS (Gibco), 10 μg/mL human transferrin (Sigma) and 3×10^{-8} M sodium selenite (Sigma). Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C.

Stable ATDC5 clones were established using either the control non-interfering pGIPZ (Thermo Fisher) or the pGIPZ-shmiRNA directed against mouse *Dot1l* construct (Thermo Fisher). Arrest-In transfection reagent (Thermo Fisher) was used for transfection. After 24 h, selection with 1 μg/mL puromycin (Invitrogen) was initiated and continued for 10 d. In the end, three different antibiotic resistant clonal colonies were isolated and grown independently. Knockdown efficiency was assessed by quantitative RT-PCR.

Stably transfected ATDC5 clones were cultured as micromasses: trypsinized cells were resuspended in medium at a concentration of 2×10^7 cells/mL. Three drops of 10 μL of this cell suspension were placed in a well of a standard 24-well culture plate. The cells were allowed to adhere for 3 h at 37 °C, and then 0.5 mL medium was added to each well. For induction of chondrogenesis, the cells were cultured in growth medium containing 1% (vol/vol) antibiotic-antimycotic, 5% FBS, supplemented with an ITS premix containing 10 μg/mL insulin, 5 μg/mL human transferrin, and 3×10^{-8} M sodium selenite for 2 wk (Gibco). Next, 5 μg/mL human transferrin (Sigma) was additionally added to reach a final concentration of 10 μg/mL Alpha-MEM medium (Gibco) containing 5% (vol/vol) FBS (Gibco), and the same mix of insulin, human transferrin and sodium selenite was added supplemented with 7 mM β-glycerolphosphate (Sigma) from day 14 until day 21. The medium was replaced daily. Each condition was performed in triplicate. Total RNA from ATDC5 cell micromasses was isolated after 1, 7, 14, or 21 d in culture using the Nucleospin RNA II kit (Macherey-Nagel). Some ATDC5 micromasses were fixed in 95% ice-cold methanol for 30 min at 4 °C. After washing with water, the micromasses were stained for 1 h in either Alcian blue [0.1% Alcian Blue 8GX, (Sigma) in 0.1 M HCl pH 0.2], Safranin O (Klinipath), Alizarin red [1% Alizarin Red S (Sigma) in water pH 4.2] or Sirius red [0.1% Direct Red 80 (Sigma) in a saturated aqueous solution of picric acid]. To remove unbound staining, cells were washed with water until the washing solution remained colorless.

Coimmunoprecipitation Analyses. Proteins were isolated from ATDC5 micromasses using the IP Lysis/Wash buffer (Thermo Fisher) supplemented with 5% (vol/vol) Protease Mixture Inhibitor (Sigma) and 1 mM phenylmethanesulfonyl (Sigma). After two homogenization cycles (7 s) with an ultrasonic cell disruptor (Microson; Misonix), total cell lysates were centrifuged 10 min at 13,000 × *g*, and supernatant containing proteins was collected. Coimmunoprecipitation were performed using the ProFound Co-Immunoprecipitation Kit (Thermo Scientific). Columns were conditioned following the manufacturer's recommendations, to activate a gel slurry retained in a spin-column system, ensuring the proper binding of antibodies. Antibody binding to the column was performed using 100 μg of either a mock antibody (donkey anti-goat IgG) as a control or an anti-TCF4 antibody (Millipore) in the gel slurry, followed by an overnight incubation at 4 °C under constant mixing. The day after, the columns were washed, and 100 μg of the lysate's proteins were incubated for 2 h at room temperature. After four washings, retained proteins were eluted using 50 μL of Elution Buffer (Thermo Fisher) pH 3, and stored at −80 °C.

Western Blot Analyses. Twenty microliters of the elution fraction supplemented with Laemmli Buffer (Sigma) was heated for 5 min at 95 °C, chilled at room temperature, and separated on a 4–12% Bis-Tris gel (Invitrogen). Proteins were then transferred onto a polyvinylidene fluoride membrane (Millipore). After 2 h in blocking buffer [TBS-0.1% Tween (TBST) supplemented with 5% nonfat dry milk], membranes were washed three times with TBST and incubated overnight at 4 °C with primary antibodies. The antibody against DOT1L (Abcam) was used at a 1/1,000 dilution, and antibody against TCF4 (Millipore) was used at a 1/500

dilution. After three washings with TBST, each blot was incubated for 1 h at room temperature with either anti-rabbit IgG (for DOT1L) or anti-mouse IgG (for TCF4) conjugated with HRP (both from Jackson ImmunoResearch Laboratories) at 1/10,000 dilution in blocking buffer. After four washings in TBST, protein bands were detected by chemiluminescence with the SuperSignal West Femto Maximum Sensitivity Substrate system (Thermo Scientific) according to manufacturer's recommendations. Images were acquired with the LAS-3000 mini CCD camera (Fujifilm).

cDNA Synthesis and Quantitative Real-Time PCR. CDNA (cDNA) was synthesized of 500 ng RNA isolated from ATDC5 micromasses using the RevertAid H minus First Strand cDNA synthesis kit (Fermentas). The MaximaSYBRgreen qPCR master mix system (Fermentas) was used to analyze differential mRNA expression of *Col2a1*, *Col10a1 Col1a1*, Aggrecan, *Tcf1* and Osteocalcin, *Mmp2*, *Mmp9*, and *Mmp13* (primers available upon request) in the ATDC5 micromasses. To assess *Dot1l* knockdown efficiency, primers were: forward 5′-CGAGGAAATCCCAGATCTCA-3′, reverse 5′-ATGGCCCGGTTGTATTTGT-3′. The following PCR conditions were used: incubation for 10 min at 95 °C followed by 40 amplification cycles of 15 s of denaturation at 95 °C followed by 45 s of annealing-elongation at 60 °C. Melting curve analysis and 1% agarose gel migration of amplicons were performed to determine the specificity the PCR. Results are expressed using the comparative threshold method (39) and were normalized to housekeeping gene *S29* mRNA level (forward 5′-CCAGCAGCTCTACTGGAGTCA-3′, reverse 5′-GCCTATGTCCTT-CGCGTACT-3′). Expression of *DOT1L* was also analyzed in articular chondrocytes (freshly isolated) from OA patients undergoing knee prosthesis surgery.

Statistical Analysis and Cell Culture Experiments. Data presented are representative of the three independent clonal colonies. Results are expressed as the mean ± SD of three independent replicates. Comparisons were made by ANOVA, followed by Fisher's *t* post hoc test, using the Statview 5.0 software (SAS Institute). A value of $P < 0.05$ was considered significant.

Immunohistochemistry on Mouse Tissues. Immunohistochemistry on paraffin-embedded EDTA decalcified adult knee sections and nondecalcified embryonal sections was performed with rabbit anti-DOT1L antibody (Ab64077; Abcam) (5 μg/mL). After overnight incubation of the sections at 4 °C, 1:100 peroxidase goat anti-rabbit IgG (Jackson Immunoresearch) was applied for 30 min and peroxidase activity was determined using DAB. Rabbit IgG (Santa Cruz Biotechnologies) was used as negative controls.

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