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Lauriane Bonnet, Esma Karkeni, Charlène Couturier, Julien Astier, Julie Dalifard, et al.. Gene Expression Pattern in Response to Cholecalciferol Supplementation Highlights Cubilin as a Major Protein of 25(OH)D Uptake in Adipocytes and Male Mice White Adipose Tissue. Endocrinology, 2018, 159 (2), pp.957-966. 10.1210/en.2017-00650 . hal-01998528

HAL Id: hal-01998528
https://amu.hal.science/hal-01998528

Submitted on 29 Jan 2019

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Gene expression pattern in response to cholecalciferol supplementation highlights cubilin as a major protein of 25(OH)D uptake in adipocytes and male mice white adipose tissue.

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Abbreviated title: Cholecalciferol impacts 25(OH) uptake in adipocytes.

Key terms: vitamin D, metabolism, adipose tissue, adipocyte, nutrition, cubilin, VDR

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Disclosure statement: The authors have nothing to disclose.

Abstract

It is well established that the active form of vitamin D, i.e. 1,25$(OH)_2$D, regulates the expression of genes involved in its own metabolism and transport in the kidney, and possibly in the liver. However, little is known about the transcriptional impact of cholecalciferol supplementation on white adipose tissue (WAT) and adipocytes, which are a major site of vitamin D and 25(OH)D storage in the organism. To fill this gap, we investigated the impact of cholecalciferol supplementation in WAT via a panel of genes coding for enzymes and proteins involved in vitamin D metabolism and uptake.

Mice supplemented with cholecalciferol (15 000 IU/kg of body weight/day) for 4 days showed decreased mRNA levels of proteins involved in cholecalciferol metabolism (Cyp24a1, Cyp27a1) and decreased cubilin mRNA levels in WAT. These data were partly confirmed in 3T3-L1 adipocytes incubated with 1,25$(OH)_2$D. The down-regulation of cubilin mRNA observed in WAT and in 3T3-L1 was confirmed at protein level in WAT and at mRNA level in human primary adipocytes. VDR agonist (EB1089) and RNA interference approaches demonstrated that VDR was involved in this regulation. Furthermore, chemical inhibitor and by RNA inference analysis demonstrated that cubilin was involved in 25(OH)D uptake by adipocytes.

This study established an overall snapshot of the genes regulated by cholecalciferol in mouse WAT and cell-autonomously in adipocytes. We highlighted that the regulation of cubilin expression is mediated by a VDR-dependent mechanism, and we demonstrated that cubilin is involved in 25(OH)D uptake by adipocytes.

Introduction

Vitamin D, or calciferol, is a hormone that is synthesized in the epidermis after exposure to UVB radiation or can be obtained from eating fatty fish (1,2). After intestinal uptake (3), dietary vitamin D reaches the bloodstream. Both dietary and endogenous cholecalciferol undergo their first hydroxylation in the liver by 25-hydroxylases including Cyp2r1—although Cyp27a1, Cyp3a11 and Cyp2j6 are also involved (4)—to produce 25-hydroxyvitamin D (25(OH)D), the major circulating form of vitamin D (5). 25-hydroxylation is generally regarded as poorly regulated (6), even if 25-hydroxylase activity was decreased by $1,25(OH)_2D$ in rat liver (7). In plasma, calciferol and 25(OH)D are principally bound to the vitamin D-binding protein (DBP, encoded by the Gc gene) [87% of 25(OH)D], their transport protein, and albumin [13% of 25(OH)D], although a small quantity remains unbound [<1% of 25(OH)D] (8,9). 25(OH)D is taken up by the kidney, where a fraction is 1α-hydroxylated by Cyp27b1 to produce $1,25(OH)_2D$, the active form of vitamin D. This uptake is mediated by the megalin/cubilin complex (10), which involves other proteins such as disabled 2 (Dab2) (11) and amnionless (Amn) (12). CYP27B1 activity in the kidney is positively regulated by parathyroid hormone and low calcium levels and deactivated by fibroblast growth factor 23 and $1,25(OH)_2D$ itself through a negative feedback mechanism (13,14).

In target tissues, 25(OH)D and $1,25(OH)_2D$ can be catabolized by 24-hydroxylases (Cyp24a1) to generate inactive metabolites (15). In the kidney, this step is auto-regulated, and cholecalciferol supplementation induced Cyp24a1 expression (16,17). The molecular mechanisms have been unraveled and shown to involve transcriptional regulation mediated by the vitamin D receptor (VDR), which binds $1,25(OH)_2D$ with high affinity. After heterodimerization with the retinoic acid receptor, the resulting complex can bind to vitamin D

response elements (VDRE) in the promoter region of regulated genes, and induce their transcriptional activation or repression (18).

Vitamin D and 25(OH)D are stored mainly in white adipose tissue (WAT), plasma and skeletal muscle (19). The uptake of vitamin D and its metabolites in preadipocytes and skeletal muscle cells has recently been described (20), and involved megalin (21). In adipocytes, vitamin D and 25(OH)D are not only stored in lipid droplets (22) but could also be converted to active metabolites (23,24) that are able to modulate adipocyte biology (25-27). Indeed, adipocytes have been shown to express most of the genes involved in vitamin D metabolism, such as 25-hydroxylases (23,24), 1α-hydroxylase (24, 28), megalin (20), Cyp24a1 and Vdr (23,29). Given that in liver and in kidney, vitamin D regulates its own metabolism at a transcriptional level via its active metabolite $1,25(OH)_2D$, it is highly likely that similar regulations occur in WAT. Nevertheless, $1,25(OH)_2D$-mediated transcriptional regulation of genes involved in vitamin D metabolism in adipocytes and in WAT has only been only partly established so far.

The main goal of this work was to study the overall impact of cholecalciferol supplementation on the regulation of genes involved in its own metabolism and uptake *in vivo* in WAT and *in vitro* in adipocytes. This approach brought key insight into the gene expression pattern of cubilin, and we went on to investigate the underlying molecular mechanism involved. We also demonstrated that cubilin is involved in 25(OH)D uptake by adipocytes.

Materials and methods

Reagents – DMEM was obtained from Life Technologies, and fetal bovine serum (FBS) was obtained from PAA Laboratories. Isobutylmethylxanthine, dexamethasone, and insulin were bought from Sigma-Aldrich. TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Life Technologies. SYBR Green reaction buffer was purchased from Eurogentec (Liege, Belgium). [3H]-25(OH)D (161 Ci/mmol for specific activity) was sourced from PerkinElmer (Waltham, MA).

Animal experiments – The protocol received approval from the local ethics committee. Six-week-old male C57BL/6J mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France), fed ad libitum with control food (chow diet A04 from Safe-diets) and had full access to drinking water. Male mice were used to avoid the cyclic hormonal changes associated with the estrus cycle in female mice. Animals were maintained at 22°C under a 12h/12h light/dark cycle with a 20% humidity level. Mice were supplemented with cholecalciferol (15 000 IU/kg of body weight/day; Sigma-Aldrich, Saint-Quentin-Fallavier, France) for the cholecalciferol group (Cholecalciferol, n=6 mice) or with vehicle alone (olive oil) for control group (Control, n=8 mice) for 4 days, by gavage (total volume of 200 µL), as previously described (30,31). Weight gain was measured daily. After 4 days of treatment, the mice were fasted overnight and blood was collected by cardiac puncture under anesthesia. After euthanasia, tissues (kidney, liver and epididymal WAT) were collected, weighed, and stored at -80°C.

Cell culture and treatment – 3T3-L1 preadipocytes (American Type Culture Collection, VA) were seeded in 3.5 cm-diameter dishes at a density of 15×10^4 cells/well and grown in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2-humidified atmosphere, as previously

described (32,33). After two-day confluence, 3T3-L1 (day 0) were stimulated for 48 h with 0.5 mM isobutylmethylxanthine, 0.25 µmol/L dexamethasone, and 1 µg/mL insulin in DMEM supplemented with 10% FBS to induce differentiation. The cultures were successively treated with DMEM supplemented with 10% FBS and 1 µg/mL insulin.

Human preadipocytes (isolated from female subcutaneous adipose tissue biopsies) supplied by Promocell (Heidelberg, Germany) were cultured and differentiated into adipocytes according to the company's instructions. Briefly, cells were seeded at a density of 5000 cells/cm² in Preadipocyte Growth Medium and grown until confluence, then allowed to differentiate for 3 days in Preadipocyte Differentiation Medium. Mature adipocytes were cultivated in Adipocyte Nutrition Medium for another 11 days, as previously reported (34).

To examine the regulation of genes coding for proteins involved in cholecalciferol metabolism, both human and murine adipocytes were incubated with $1,25(OH)_2D$ (1, 10 and 100 nM) dissolved in absolute ethanol for 24 hours or with EB1089 (10, or 100 nM), a VDR agonist, for 24 hours.

RNA interference – 3T3-L1 differentiated cells seeded in 24 or 12-well plates were transfected with either targeted siRNA (against VDR or cubilin, respectively) or a non-targeting siRNA according to the manufacturer's instructions (Dharmacon, Lafayette, CO) using INTERFERin (Polyplus-transfection) for 24 h, as previously described (30).

Uptake of 25(OH)D by adipocytes –3T3-L1 adipocytes were incubated with [3H]-25(OH)D at a concentration of 11.25 nCi/mL and with 50 nM of non-radiolabeled 25(OH)D (Sigma-Aldrich, St Louis, MO) in DMEM supplemented with 1 µg/mL insulin in the presence of either 1.9 µM DBP, 0.125% bovine serum albumin (BSA) or ethanol (control condition). These 3T3-L1 adipocytes were treated with 10 or 100 nM of $1,25(OH)_2D$ or with 100 or 500 nM of

receptor-associated protein (RAP), an inhibitor of the megalin-cubilin complex. After 16 h of incubation, cells were lysed and radioactivity was measured by liquid scintigraphy. The results were expressed as counts per minute (CPM) per well.

RNA extraction and real-time qPCR – Total RNA was extracted from the liver, kidney and epididymal WAT or from cells using TRIzol reagent (Life Technologies, Courtaboeuf, France). One µg of total RNA was used to synthesize cDNAs using random primers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Courtaboeuf, France). Real-time quantitative PCR analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA), as previously described (35). For each condition, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (CT) method (36). Sequences of the primers used in this study are reported in supplemental data (Supplemental Table 1).

Mouse cubilin protein quantification – Quantity of cubilin protein in WAT was determined using a specific ELISA kit (Mouse cubilin, Mybiosource, San Diego, CA) according to the manufacturer's protocol.

***In-silico* promoter analysis** – The MatInspector software implemented in the Genomatix suite (www.genomatix.de) was used to perform *in-silico* identification of VDR response elements within human and mouse cubilin promoter regions. Briefly, human and murine cubilin promoters were extracted directly from the Genomatix ElDorado Database (www.genomatix.de). These promoter regions correspond to loci identified by their ElDorado reference ID. Response elements were identified on these promoter regions using the MatInspector software workflow (37).

Cholecalciferol, 25(OH)D and 1,25(OH)2D quantification in plasma and WAT – All quantifications were performed using LC-MS/MS as per the protocol below.

Preparation of analytical and deuterated standards – A working solution of deuterated analytes was prepared at 0.02 ng/mL of each internal standard (IS), i.e. d3-cholecalciferol, d3-25(OH)D and d3-1,25(OH)2D). Primary stock solutions of cholecalciferol, 25(OH)D and 1,25$(OH)_2$D standards were prepared at concentrations of 100, 50 and 10 ng/mL, respectively, in ethanol, and stored at -80°C in the dark. Calibration curves were prepared by serial dilution of the three analyte stock solutions to obtain calibration standards from 0 to 75 ng/mL, then adding 1.5 µL of the working solution of deuterated analytes to each dilution. After complete evaporation of solvent, derivatization was performed. A one-step derivatization was employed to improve the ionization efficiency of the metabolites using Amplifex diene as reagent (38). Amplifex (30 µL) was added to the dried sample above, vortexed for 15 s, and incubated for 30 min at ambient temperature. Next, 30 µL of deionized water was added, vortexed for 15 s, and transferred for LC injection. Calibration curves were plotted as peak area ratio of the vitamin D metabolite to the respective IS versus a range of analyte concentrations.

Preparation of plasma – Sample preparation was adapted from Wang et al. (39). As cholecalciferol and its metabolites are light-sensitive, the extraction procedure was conducted under low light. After thawing on ice, mice plasmas were centrifuged at 11,000 rpm for 15 min at 4°C, then 100 µL of each sample was transferred to a glass test tube containing 10 µL of deuterated standard working solution. Proteins were precipitated by adding acetonitrile (ACN), vortex-mixed, and centrifuged for at 3,000 *g* for 10 min. The supernatant was moved to another glass tube, and the volume was reduced to half under a nitrogen stream. Then, 5 mL of ethyl acetate was added to the solution for liquid-liquid extraction. After shaking vigorously, samples were centrifuged at 590 *g* for 20 min, and the upper organic layer was transferred to a fresh

glass tube and reduced under nitrogen stream. The samples were then derivatized as described above.

Epididymal WAT preparation – Sample preparation was adapted from Lipkie et al. (40). First, 25 µL of deuterated standard working solution was added to tissue homogenates (50 mg of tissue ground within 1 mL of PBS) in glass test tube. Acetonitrile (ACN) was added, vortex-mixed for 5 min, and centrifuged at 6,000 *g* for 5 min. Then, methyl tert-butyl ether (MTBE) was added, vortexed for 5 min, centrifuged, and the upper organic layer was collected into a fresh glass tube. The extraction was repeated twice, and the combined supernatants were dried under nitrogen. Oasis HLB SPE cartridges (Waters, Guyancourt, France) were conditioned with ethyl acetate, methanol (MetOH) and H_2O. The sample was reconstituted with 1 mL of MetOH and 1 mL of K_2HPO_4 (0.4 M), and added onto the cartridge. The cartridge was washed with H_2O and 70% MetOH, and then dried for 2 min under vacuum. Tips were washed with ACN, and analytes were eluted with ACN and dried under nitrogen. After complete evaporation of solvent, the samples were derivatized as described above.

LC-MS/MS analysis – Accurate mass measurements were performed on the Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Heated Electrospray Ionization (H-ESI II) probe. Thermo Xcalibur 3.0.63 software was used for instrument setup, control of the LC-MS system during acquisition, and data treatment. Tune Q Exactive Plus 2.5 software was used for direct control of the mass spectrometer.

Samples were injected onto a Hypersil GOLD C18 column (2.1 × 100 mm; Thermo Scientific, Les Ulis, France). Flowrate was 0.4 mL/min, and injection volume was 5 µL. The mobile phase was composed of A = ultrapure water with 0.1% formic acid (FA) (v/v), and B = ACN with 0.1% FA (v/v). Starting conditions were A = 70% and B = 30%, held for 4 min. A linear gradient was applied until 10.0 min where A = 35% and B = 65% which was held until 12.0 min, at 14 min A=0% and B = 100% until 16 min. Starting conditions were re-implemented at 18 min.

The SRM transitions used for quantification for each analyte were: 716.5→657.5 (Cholecalciferol), 719.5→660.5 (d3-Cholecalciferol), 732.5→673.4 (25(OH)D), 735.5→676.4 (d3-25(OH)D), 751.5→692.4 (d3-1,25$(OH)_2$D) and 748.5→689.4 (1,25$(OH)_2$D).

Data was validated for linearity and repeatability data (Supplemental Table 2).

Statistical analysis – Data are presented as mean ± SEM. Significant differences between control group and treatment group were determined using a Student's *t*-test or ANOVA followed by the Tukey-Kramer post hoc test, all using Statview software (SAS Institute, Cary, NC). $p<0.05$ was considered statistically significant.

Results

Cholecalciferol supplementation modifies expression of genes involved in cholecalciferol metabolism and uptake in WAT and adipocytes.

To examine the impact of cholecalciferol supplementation on the regulation of genes coding proteins involved in its own metabolism in WAT, wild-type C56BL/6J male mice were supplemented with cholecalciferol (15 000 IU/kg of body weight/day) for 4 days. Body weight and absolute and relative organ weights were not modified by the treatment (Table 1). As expected, supplemented mice showed higher plasma concentrations of cholecalciferol, 25(OH)D and 1,25$(OH)_2$D (26.22, 6.32 and 9.94-fold, respectively, Table 1) and increased cholecalciferol and 25(OH)D in WAT (4.87-fold and 3.41-fold, respectively; Table 1). WAT from supplemented mice also had a higher quantity of 1,25$(OH)_2$D, but the difference compared to control mice did not reach statistical significance (Table 1).

The expression of genes coding for vitamin D metabolism proteins (Supplemental Table 3) was measured by real-time PCR in liver, kidney and epididymal WAT (eWAT). In liver, no major difference in gene expression was observed between the two groups except a decrease of mRNA expression of cubilin (Cubn) in cholecalciferol-supplemented mice (0.44-fold, Figure 1A). In kidney, Cyp24a1 and Vdr mRNA levels were increased whereas Cyp27b1, Gc and Cubn mRNA were decreased in cholecalciferol-supplemented mice (20.68, 1.78, 0.96, 0.33 and 0.18-fold respectively, Figure 1B). Interestingly, in eWAT, a decrease of Cyp24a1, Cyp27a1 and Cubn gene expression was observed in cholecalciferol-supplemented mice compared to controls (0.59, 0.34 and 0.62-fold, respectively, Figure 1C). Note that genes not mentioned as regulated in the different tissues were not modified by cholecalciferol supplementation.

To study the effect of 1,25$(OH)_2$D-mediated cell-autonomous regulation on adipocyte gene expression, 3T3-L1 adipocytes were treated with different doses of 1,25$(OH)_2$D (1, 10 and 100

nM) for 24 hours (Figure 2 and Supplemental Table 4). Cyp27a1 and Cubn mRNA levels were decreased in cells treated with 10 and 100 nM of 1,25$(OH)_2$D compared to control cells (0.36 and 0.34-fold for Cyp27a1 and 0.34 and 0.56-fold for cubilin, respectively). Conversely, Cyp24a1 mRNA expression increased strongly with the higher dose (489.88-fold for 100 nM), similarly to Vdr mRNA (2.87 and 10.35-fold for 10 and 100 nM, respectively).

The regulation of cubilin expression in adipocytes is VDR-dependent.

To gain further insight into the mechanism of cholecalciferol uptake by WAT/adipocytes, we focused analysis on the regulation of cubilin. The down-regulation (0.28-fold) of Cubn mRNA was confirmed in human primary white adipocytes incubated with 1,25$(OH)_2$D (100 nM for 24 h; Figure 3A). The impact of the cholecalciferol supplementation on cubilin protein was confirmed by ELISA in mouse WAT, which showed 0.23-fold-lower cubilin protein in cholecalciferol-supplemented mice (Figure 3B). 3T3-L1 adipocytes were incubated with EB1089, a specific VDR agonist, for 24 h (Figure A4). mRNA level of Cubn was decreased in adipocytes treated with 10 and 100 nM of EB1089 (0.73 and 0.78-fold, respectively), thus supporting the putative role of VDR in this regulation. In addition, *in silico* analysis with MatInspector software unveiled the location of several VDRE in both human and mouse cubilin promoters (Table 2). To confirm the involvement of VDR in this regulation, 3T3-L1 adipocytes were transfected with either a small interfering RNA (siRNA) oligonucleotide directed against VDR or a non-silencing control for 24 h. qPCR confirmed that the RNA interference was efficient, and a significant decrease of VDR expression was observed (0.83-fold; Figure 4B). In addition, the transfection with siRNA-targeting VDR completely blunted the 1,25$(OH)_2$D-mediated inhibition of Cubn mRNA level (100 nM of 1,25$(OH)_2$D for 24 h; Figure 4C).

25(OH)D endocytosis is mediated by cubilin and regulated by 1,25(OH)$_2$D in 3T3-L1 adipocytes.

To highlight the functional role of cubilin for 25(OH)D uptake, we undertook a preliminary experiment to measure 25(OH)D uptake in 3T3-L1 adipocytes. Cells were incubated for 16 h without (control) or with [3H]-25(OH)D and non-labelled 25(OH)D (50 nM) under its free form (dissolved in ethanol) or complexed with different proteins: DBP or BSA. CPM served reflected the uptake of 25(OH)D. The highest uptake of 25(OH)D was obtained with BSA as vehicle (44-fold compared to control). A significant but lower uptake was obtained with free form and DBP conditions (22.8-fold and 4.4-fold respectively, Figure 5A).

To confirm the involvement of cubilin receptor in [3H]-25(OH)D endocytosis, cells were treated with RAP, a specific inhibitor of the megalin/cubilin complex. When [3H]-25(OH)D was solubilized in ethanol, there was no difference between RAP-treated conditions and controls (Figure 5B). However, when [3H]-25(OH)D was bound to BSA or DBP, there was a strong decrease of [3H]-25(OH)D uptake (0.23-fold and 0-49 fold, respectively; Figure 5C and 5F). In a second set of experiments, 3T3-L1 adipocytes were transfected with either a siRNA directed against Cubilin (siCubilin) or a non-targeted siRNA (siNT) used as control for 24 h. [3H]-25(OH)D uptake in complexes with ethanol, BSA or DBP was measured for 16 h. [3H]-25(OH)D uptake was decreased in the presence of siCubilin compared to siNT when the BSA was used as vehicle (0.63-fold, Figure 5E).

To study the effect of modulating cubilin expression on [3H]-25(OH)D uptake, we incubated the cells with 1,25(OH)$_2$D (10 or 100 nM) and measured the uptake of [3H]-25(OH)D solubilized in ethanol (Figure 5F) or bound to BSA (Figure 5G) or DBP (Figure 5H). In all conditions, incubation with 100 nM of 1,25(OH)$_2$D decreased uptake of [3H]-25(OH)D in adipocytes (0.04, 0.19 and 0.45-fold, respectively). Note that even if statistically significant,

293 the 1,25$(OH)_2$D-induced modification of ethanol-solubilized [3H]-25(OH)D uptake was
294 quantitatively negligible (Figure 5F).
295 .

Discussion

Here we used targeted gene profiling to show that cholecalciferol regulates the expression of several genes involved in cholecalciferol metabolism and uptake in WAT in response to a short-term cholecalciferol supplementation. Among regulated genes, we report, for the first time, the negative regulation of Cubn gene. Additional experiments in 3T3-L1 adipocytes demonstrated that Cubn regulation was VDR-dependent. Finally, we demonstrated that cubilin is involved in 25(OH)D uptake in adipocytes.

To analyze the regulatory effect of cholecalciferol supplementation on gene expression in WAT, mice received cholecalciferol for 4 days by oral gavage. Cholecalciferol was diluted in olive oil to assure better absorption of this lipophilic molecule (41). In this study, no difference of total body mass or organ mass (liver and AT) was detected between groups, but as expected, plasma concentrations of cholecalciferol, 25(OH)D and $1,25(OH)_2D$ in WAT concentrations of cholecalciferol and 25(OH)D were strongly increased in cholecalciferol-supplemented mice. Despite a clear tendency to increase, the quantity of $1,25(OH)_2D$ in WAT of supplemented mice was not statistically different from control mice. In agreement with previously published data (42), cholecalciferol supplementation strongly induced kidney mRNA expression of Cyp24a1 and Vdr and decreased mRNA levels of Cyp27b1, thus validating our experimental conditions. Interestingly, we also observed a decrease of Gc and Cubn gene expression in the kidney. This regulation, if confirmed at protein level, could result in a decrease in renal recycling of cholecalciferol and its metabolites, thus constituting a way to eliminate excess cholecalciferol from the plasma. In the liver, we only observed a decrease of Cubn expression decreased, but there was no change in the expression of mRNA coding for other enzymes of hepatic cholecalciferol metabolism. This decrease of Cubn expression suggests that the uptake of these

molecules could be regulated in the liver at a transcriptional level, through a negative feedback mechanism, thus limiting their hepatic uptake. We also gained an overview of the transcriptional effect of cholecalciferol supplementation in epididymal WAT. Our results showed a decrease of Cyp27a1 and Cyp24a1 mRNA levels, suggesting a putative decrease of 25-hydroxylation and inactivation of metabolites. Cyp24a1 induction has already been reported (28), but this is the first report of Cyp27a1 repression. This novel finding result will require further investigations, especially to confirm the real contribution of Cyp27a1 in the adipose metabolism of cholecalciferol. Interestingly, we also found a specific down-regulation of Cubn mRNA levels while other partners in the endocytosis complex (megalin, Dab2 and Amn) were not transcriptionally affected.

To demonstrate the direct effect of $1,25(OH)_2D$ on these regulations, we used murine 3T3-L1 adipocytes. In these cells, we observed an up-regulation of Vdr and Cyp24a1, both of which are well-known VDR target genes (43), thus validating our experimental model. The fact that Cyp24a1 was decreased *in vivo* but strongly increased *in vitro* is surprising, but could be due to indirect regulations that simultaneously occurred *in vivo* whereas induction *in vitro* only resulted from direct VDR-mediated induction m (43) Interestingly, the patterns of Cyp27a1 and Cubn regulation were reproduced *in vitro*, confirming the direct and cell-autonomous nature of the regulation. While it is well documented that most of the enzymes of cholecalciferol metabolism are expressed in adipocytes (25), including Vdr (29), 25-hydroxylation enzymes (23,24), 1α-hydroxylation enzyme (24,44) and megalin (20), here we report that certain putative actors of hepatic 25-hydroxylation (4) are not expressed in adipocytes. This is notably the case of Cyp2r1 and Cyp3a11, which were not detected in our conditions, in agreement with Zoico et al. who did not detect Cyp2r1 in 3T3-L1 cells (24). The ability of adipocytes to produce 25(OH)D has been demonstrated (23,24), but the enzyme involved has not yet been identified.

Based on our results, i.e. down-regulation of Cyp27a1, which could be considered as a negative feed-back, we could posit that Cyp27a1 is a major contributor to 25(OH)D production in adipocytes, but further investigations are needed.

The down-regulation of Cubn mRNA levels in response to cholecalciferol was confirmed not only in 3T3-L1 adipocytes but also in human primary adipocytes and in mouse WAT. The cubilin protein is known to play a crucial role in 25(OH)D uptake, since mutations causing cubilin dysfunction lead to urinary excretion of 25(OH)D (10). Indeed, cubilin participates together with megalin (45), Dab-2, an intracellular adaptor protein, and AMN, a transmembrane protein (11,12), in the endocytosis of 25(OH)D, notably in proximal tubules of the glomerulus. We identified mRNA coding for megalin, Dab-2 and AMN in adipocytes and WAT, but we did not observe any modification in expression levels. The detection of megalin mRNA does not fit with Abboud et al.'s report that megalin is expressed in preadipocytes but not in adipocytes (20). This discrepancy could be due to cell culture model specifics, but it nevertheless clearly demonstrates the existence of the megalin/cubilin complex in adipocytes.

To investigate the molecular mechanism involved in Cubn regulation, several approaches were combined. First, the use of a specific VDR agonist (EB1089) led to similar down-regulation of Cubn expression compared to 1,25$(OH)_2$D, suggesting that the regulation described *in vitro* is mediated by VDR. This involvement was demonstrated by the RNA interference experiments implemented here using siRNA targeted against VDR. Furthermore, an *in silico* analysis (MatInspector in the Genomatix suite) confirmed the presence of putative VDREs within the murine and human promoters of cubilin.

To study the involvement of cubilin in 25(OH)D uptake by adipocytes, experiments were undertaken using radiolabeled 25(OH)D. In the physiological context, plasma 25(OH)D is either bound to DBP (87% of total 25(OH)D) or albumin (13% of total 25(OH)D) or else

considered unbound (“free form”) (>1% of total 25(OH)D) (7). In preliminary experiments, the ability of different vehicles to deliver 25(OH)D to adipocytes was tested. We observed that the best vehicle for 25(OH)D was BSA, followed by ‘free form’ (mimicked here by an ethanolic solution) then DBP. These data suggest that BSA-complexed 25(OH)D is easily absorbed by cells. Note too that the free form can also be internalized in adipocytes. Finally, it appears that the DBP is probably not the best way to deliver 25(OH)D to adipocytes but corresponds to a 25(OH)D storage site in plasma, as previously suggested (46).

To confirm the involvement of cubilin in 25(OH)D uptake by adipocytes, two strategies were implemented. First, we used RAP (inhibitor of megalin/cubilin complex (47)), and second, we used an RNA interference approach. Interestingly, 25(OH)D uptake in complex with BSA or DBP was decreased by RAP and, to a lesser extent, siRNA directed against Cubn (especially for 25(OH)D-DBP complexes that were not impacted by siRNA). Note that 25(OH)D uptake of the free from (in ethanol) was not impacted by RAP or siRNA, suggesting that the uptake of unbound 25(OH)D occurs independently of the megalin/cubilin pathway. Finally, to confirm that cubilin regulation is involved in 25(OH)D uptake, adipocytes were incubated with $1,25(OH)_2D$. Interestingly, this incubation led to a decrease of cubilin expression and was associated with a decrease of 25(OH)D uptake. Taken together, these data provide strong evidence that cubilin is involved in 25(OH)D uptake by adipocytes. From a physiological point of view, these data suggest that a negative feedback regulation occurs in WAT to control the uptake of cholecalciferol and its metabolites via a modulation of cubilin expression. This kind of limitation of cholecalciferol and metabolite storage, which is generally assumed to be a passive mechanism due to lipophilicity, suggests that cholecalciferol and 25(OH)D storage in WAT is actually tightly controlled and regulated.

Here we demonstrate for the first time that there is a coordinated overall regulation of genes coding for enzymes involved in the cholecalciferol metabolism in WAT and in adipocytes. Our

data also demonstrate that cubilin is involved in 25(OH)D uptake in adipocytes, and suggest that this mechanism is transcriptionally regulated, thus extending our knowledge of adipocyte cholecalciferol metabolism.

Figure legends

Figure 1: Effect of cholecalciferol supplementation on the expression of cholecalciferol metabolism genes in liver, kidney and adipose tissue.

Expression of genes coding for proteins involved in cholecalciferol metabolism relative to 18S ribosomal RNA in the liver (A), kidney (B) and epididymal white adipose tissue (WAT; C) of control mice or cholecalciferol-supplemented mice (control n=9, cholecalciferol n=6). Values are reported as means ± SEM. * $p < 0.05$ for an unpaired Student's *t*-test.

Figure 2: Effect of $1,25(OH)_2D$ incubation on expression of cholecalciferol metabolism genes in 3T3-L1 adipocytes.

3T3-L1 adipocytes were incubated with $1,25(OH)_2D$ (1, 10 and 100 nM) for 24 h (A to E). Expression of genes coding for proteins involved in cholecalciferol metabolism relative to 18S ribosomal RNA. Values are reported as means ± SEM. Bars not sharing the same letter were significantly different in a Tukey-Kramer post hoc test at $p < 0.05$.

Figure 3: Cubilin mRNA level is down-regulated in human adipocytes and at protein level in mice adipose tissue.

(A) Human primary white adipocytes were incubated with 100 nM of $1,25(OH)_2D$ for 24 h. Expression of cubilin relative to 18S ribosomal RNA. (B) Cubilin protein quantification performed by ELISA tests in epididymal white adipose tissue (WAT) of mice (control n=9, cholecalciferol n=6). Values are reported as means ± SEM. * $p < 0.05$ for an unpaired Student's *t*-test.

Figure 4: The regulation of cubilin expression is VDR-dependent.

(A) 3T3-L1 adipocytes were incubated with EB1089, a vitamin D receptor agonist (VDR) (10 and 100 nM) for 24 h. (B) The 3T3-L1 adipocytes were transfected with either a small interfering RNA (siRNA) oligonucleotide for VDR or a non-silencing control for 24 h. The efficiency of RNA interference against VDR was determined by qPCR. (C) These cells were transfected with siRNA and incubated with 100 nM of $1,25(OH)_2D$ for 24h. Values are reported as means ± SEM. Bars not sharing the same letter were significantly different in a Tukey-Kramer post hoc test at $p < 0.05$. * $p < 0.05$ for an unpaired Student's *t*-test.

Figure 5: 25(OH)D uptake in 3T3-L1 adipocytes is mediated by cubilin.

3T3-L1 adipocytes were incubated with control (Ctrl) or with 25(OH)D solubilized in ethanol (free form) or complexed with bovine serum albumin (BSA) or vitamin D-binding protein (DBP) (A). Cells were incubated with receptor-associated protein (RAP) (100 or 500 nM) and 25(OH)D was solubilized in ethanol (B) or complexed to BSA (C) or DBP (D) for 16 h. 3T3-L1 cells were transfected with siRNA (non-targeted (siNT) or directed against VDR (siVDR)) and incubated with 25(OH)D solubilized in ethanol (free form) or complexed with BSA or DBP (E). Cells were incubated with $1,25(OH)_2D$ (10 and 100 nM) and 25(OH)D was solubilized into ethanol (F) or complexed to BSA (G) or DBP (H). In each experiment, 25(OH)D uptake was quantified by measuring the number of counts per minute per well by liquid scintillation. Values are reported as means ± SEM. Bars not sharing the same letter were significantly different in a Tukey-Kramer post hoc test at $p < 0.05$. * $p < 0.05$ for an unpaired Student's *t*-test.

Table 1: Morphological and biological parameters of mice.

	Control mice	Cholecalciferol-supplemented mice
Body weight (g)	21.9 ± 0.37	21.1 ± 0.90
Liver weight (mg)	976.9 ± 29.09	955.5 ± 57.74
Liver weight/body weight ratio	0.0446 ± 0.0007	0.0451 ± 0.0009
Adipose tissue weight (mg)	242.9 ±10.12	281.0 ± 32.63
Adipose tissue weight/body weight ratio	0.0111 ± 0.0005	0.0136 ± 0.002
Serum cholecalciferol (ng/mL)	2.58 ± 0.60	67.65 ± 9.66 *
Serum 25(OH)D (ng/mL)	16.79 ± 0.98	106.16 ± 18.60 *
Serum 1,25(OH)$_2$D (pg/mL)	68.6 ± 16.95	682.2 ± 122.11 *
AT cholecalciferol quantity (ng)	158 ± 22.01	770.24 ± 175.28 *
AT 25(OH)D quantity (ng)	27.3 ± 1.05	93.13 ± 30.91 *
AT 1,25(OH)$_2$D quantity (pg)	1.58 ± 0.31	2.77 ± 0.68

Values are reported as means ± SEM. Student's *t*-test. p values: *, $p < 0.05$.

Table 2: VDRE sequences and location identified with MatInspector software within human and mouse cubilin promoters.

	Sequence	ElDorado reference ID	Position of the VDRE
Human promoter	gtttcaaaGGTCaaatagataatga	GXP_271874 (-)	17171654_17172330
Mouse promoter	tcaagagGATTcaaaggcaacttca	GXP_425459 (-)	13491712_13492424

In silico analysis with MatInspector software of human and mouse cubilin promoters. The position of the VDRE (referred to by a start _ end number) corresponds to its location within the input sequence (ElDorado reference ID).

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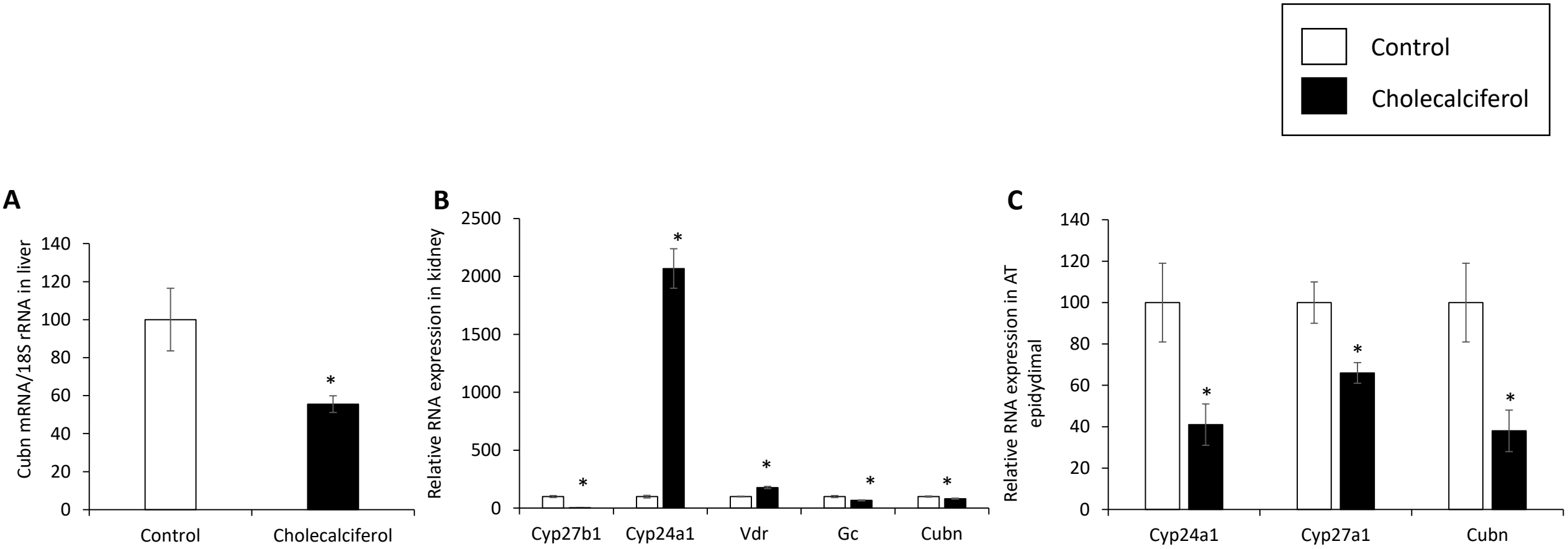
Figure 1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120
100
80
60
40
20
0
*
Control
Cholecalciferol
B
Relative RNA expression in kidney
2500
2000
1500
1000
500
0
*
*
*
*
*
Cyp27b1
Cyp24a1
Vdr
Gc
Cubn
C
Relative RNA expression in AT epididymal
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100
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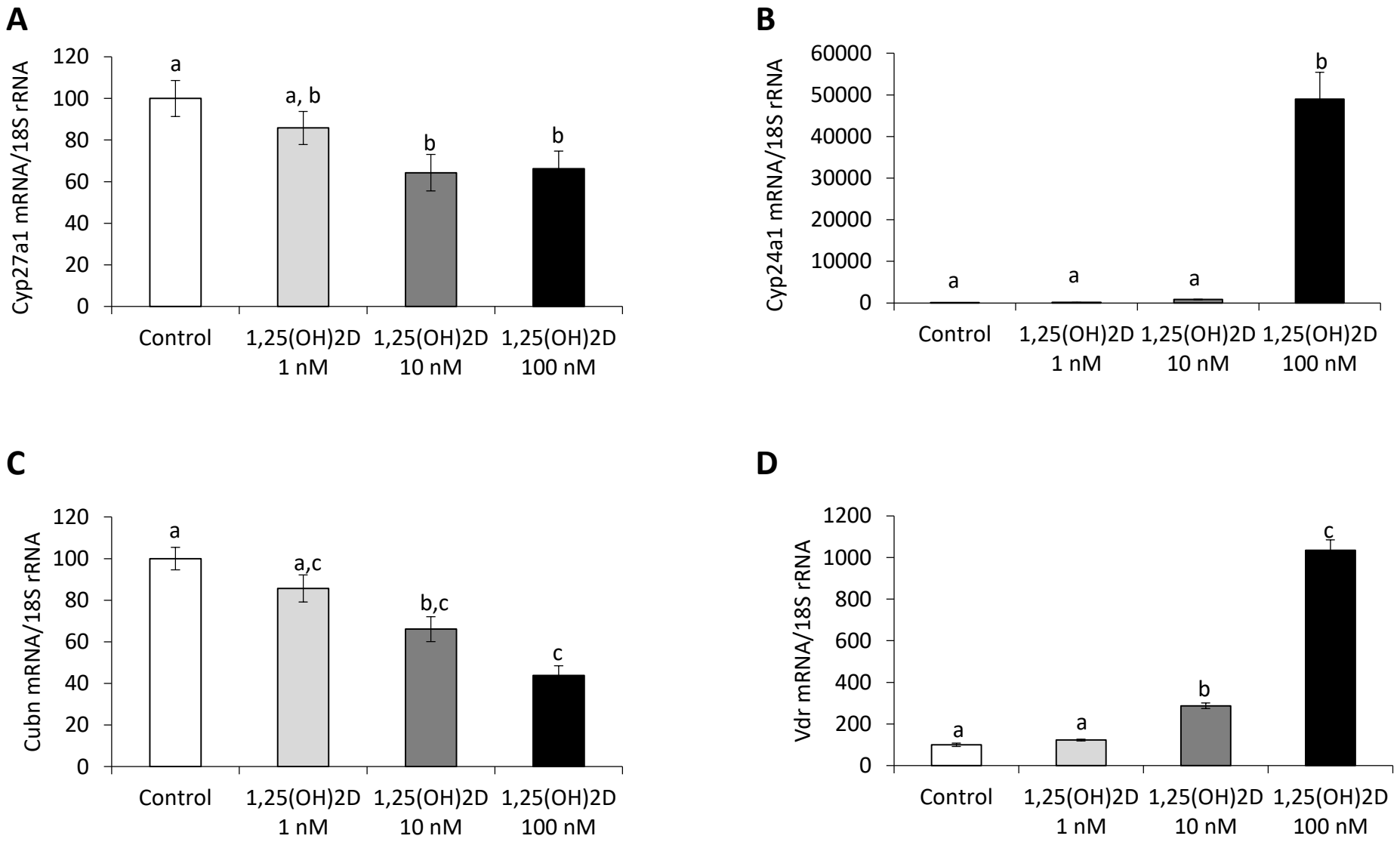
60
40
20
0
*
*
*
Cyp24a1
Cyp27a1
Cubn

Figure 2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100
80
60
40
20
0
a
a, b
b
b
Control
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1,25(OH)2D 10 nM
1,25(OH)2D 100 nM
B
Cyp24a1 mRNA/18S rRNA
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50000
40000
30000
20000
10000
0
a
a
a
b
Control
1,25(OH)2D 1 nM
1,25(OH)2D 10 nM
1,25(OH)2D 100 nM
C
Cubn mRNA/18S rRNA
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100
80
60
40
20
0
a
a,c
b,c
c
Control
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1,25(OH)2D 10 nM
1,25(OH)2D 100 nM
D
Vdr mRNA/18S rRNA
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1000
800
600
400
200
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b
c
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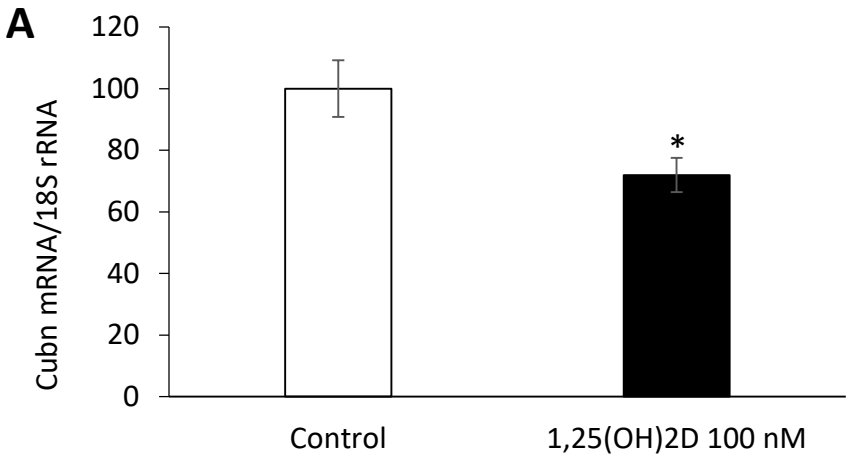
Figure 3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Cubn mRNA/18S rRNA
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Control
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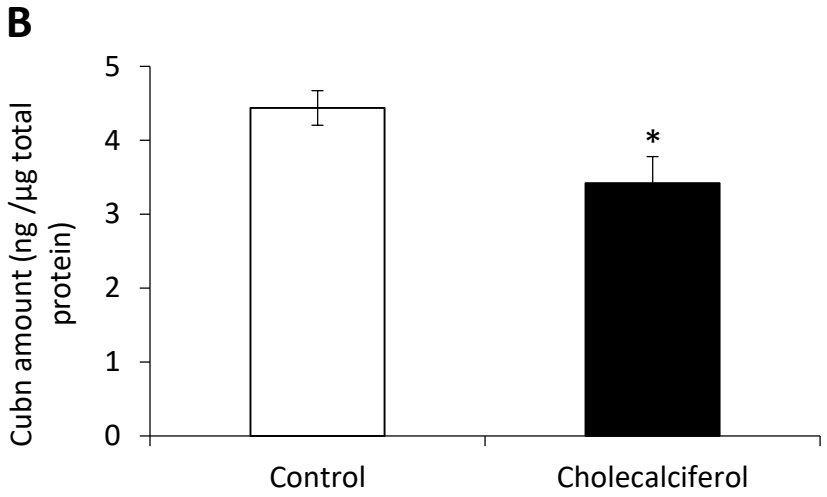
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Cubn amount (ng /µg total protein)
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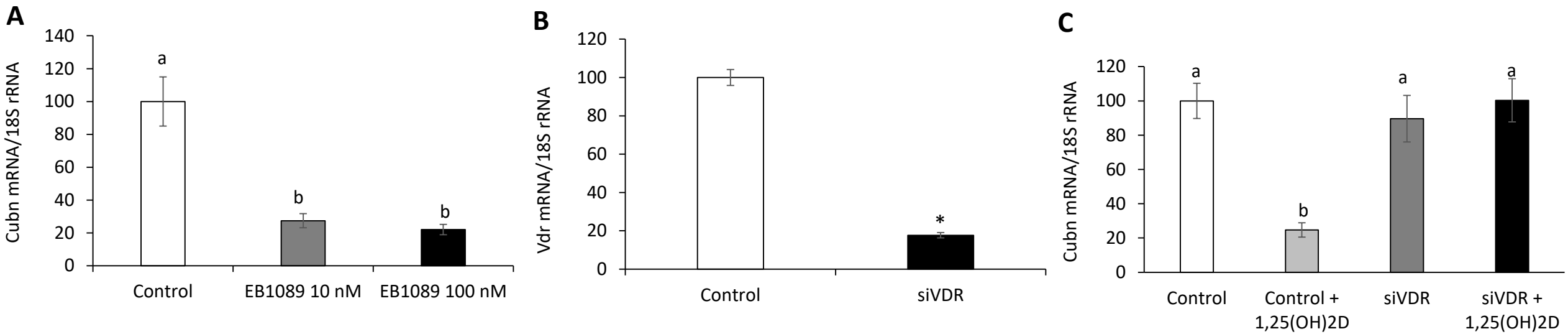
Control
Cholecalciferol

Figure 4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Cubn mRNA/18S rRNA
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80
60
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20
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a
b
b
Control
EB1089 10 nM
EB1089 100 nM
B
Vdr mRNA/18S rRNA
120
100
80
60
40
20
0
*
Control
siVDR
C
Cubn mRNA/18S rRNA
120
100
80
60
40
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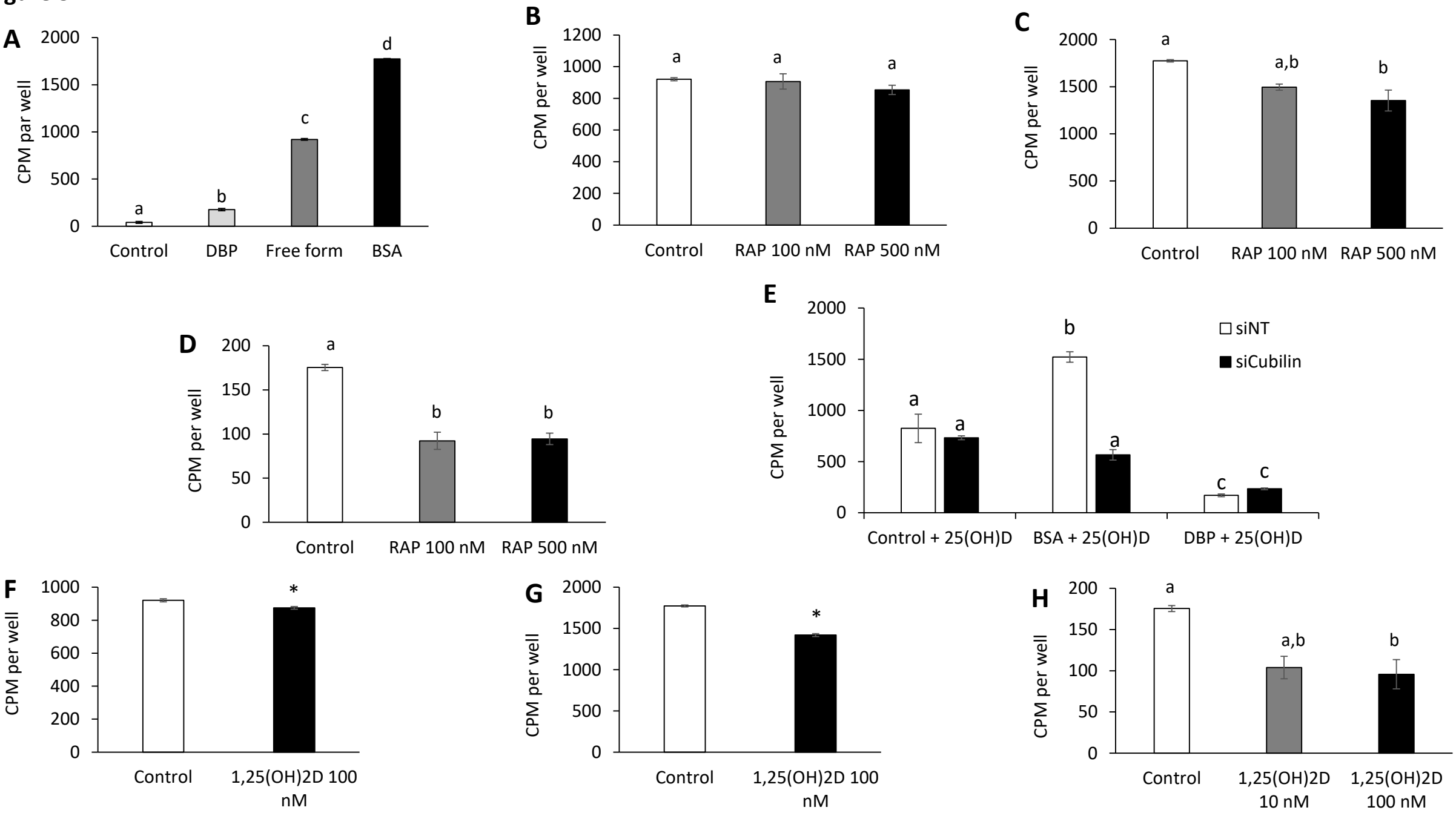
b
a
a
Control
Control + 1,25(OH)2D
siVDR
siVDR + 1,25(OH)2D

Figure 5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1500
1000
500
0
a
b
c
d
Control
DBP
Free form
BSA
B
CPM per well
1200
1000
800
600
400
200
0
a
a
a
Control
RAP 100 nM
RAP 500 nM
C
CPM per well
2000
1500
1000
500
0
a
a,b
b
Control
RAP 100 nM
RAP 500 nM
D
CPM per well
200
150
100
50
0
a
b
b
Control
RAP 100 nM
RAP 500 nM
E
CPM per well
2000
1500
1000
500
0
a
a
b
a
c
c
siNT
siCubilin
Control + 25(OH)D
BSA + 25(OH)D
DBP + 25(OH)D
F
CPM per well
1000
800
600
400
200
0
*
Control
1,25(OH)2D 100 nM
G
CPM per well
2000
1500
1000
500
0
*
Control
1,25(OH)2D 100 nM
H
CPM per well
200
150
100
50
0
a
a,b
b
Control
1,25(OH)2D 10 nM
1,25(OH)2D 100 nM

Supplemental tables

Supplemental table 1: Primers sequences

Gene	Forward sequence	Reverse sequence
Cyp2r1	TTTGTCGGCAACATCTGCT	TGCCTCCAAGATCTAAACTGAAA
Cyp3a11	TGAATATGAAACTTGCTCTCACTAAAA	CCTTGTCTGCTTAATTTCAGAGCT
Cyp27a1	CCTCACCTATGGGATCTTCATC	TTTAAGGCATCCGTGTAGAGC
Cyp2j6	CCCTCTACCCAGAAGTCCAA	TTCTGGCCAATCACCCTATC
Cyp24a1	AAGCCTACGCGCTGATGAT	CACGGGCTTCATGAGTTTC
Cyp27b1	AGTGGGGAATGTGACAGAGC	GGAGAGCGTATTGGATACCG
Megalin	GATGGATTAGCCGTGGACTG	TCCGTTGACTCTTAGCATCTGA
Cubn	GCCATCCAGATGCAACCT	GGTGCAGACAGGCAACAAG
Vdr	AACCCCTCATAAAGTTCCAGGT	CTGTACCCCAGGTCGGTCT
Gc	CTACCTCAGAGGATTGCATGG	CTTTTTGGATAAGTTTTGACAGATTTT
Dab2	GCAGTCGAACTTTCTGCATCTC	GGTGTTACTGGGACCGTACCT
Amn	AGACAGTCACGCCATCTCG	GAGGCCAGGACCAACTCC.
18S rRNA	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG

Supplemental table 2: Method validation for LC-MS/MS analysis of cholecalciferol metabolites after Amplifex derivatization.

Analytes	Linear range ng/ml	LOD ng/ml	LOQ ng/ml	Inter-assay CV%	Intra-assay CV%
Cholecalciferol	0.78-50	0.049	0.78	14.4	6.0
25OH D	0.1-12.5	0.025	0.1	12.5	11.0
$1,25(OH)_2D$	0.02-2.5	0.005	0.02	5.5	11.6

Supplemental table 3: Effect of cholecalciferol supplementation on gene expression of cholecalciferol metabolism in liver, kidney and epididymal white adipose tissue.

	Liver		**Kidney**		**Epididymal WAT**	
	Control	Cholecalciferol	Control	Cholecalciferol	Control	Cholecalciferol
Cyp27a1	100 ± 6	109 ± 6			100 ± 10	66 ± 5 *
Cyp2r1	100 ± 8	92 ± 5			100 ± 8	83 ± 16
Cyp2j6	100 ± 6	109 ± 8			100 ± 8	96 ± 8
Cyp3a11	100 ± 9	76 ± 9			100 ± 33	103 ± 38
Cyp24a1			100 ± 11	2068 ± 171 *	100 ± 19	41 ± 10 *
Cyp27b1			100 ± 9	4 ± 0 *	100 ± 25	78 ± 22
Vdr	100 ± 10	79 ± 6	100 ± 4	178 ± 10 *	100 ± 16	77 ± 14
Gc	100 ± 6	115 ± 6	100 ± 9	67 ± 4 *	100 ± 44	36 ± 11
Megalin	100 ± 7	93 ± 7	100 ± 5	98 ± 3	100 ± 25	50 ± 9
Cubilin	100 ± 16	56 ± 4 *	100 ± 5	82 ± 4 *	100 ± 19	38 ± 10 *
Dab2	100 ± 8	106 ± 5	100 ± 4	82 ± 5	100 ± 5	73 ± 9
Amn	100 ± 10	93 ± 8	100 ± 8	75 ± 5	100 ± 21	119 ± 32

Expression of genes coding for proteins involved in cholecalciferol metabolism relative to 18S ribosomal RNA in liver, kidney and epidydimal white adipose tissue (WAT) of control mice or cholecalciferol supplemented mice (control n=9, cholecalciferol n=6). Values are presented as means ± SEM. For unpaired Student's t-test, p values: *, $p < 0.05$.

Supplemental table 4: Dose effect of $1,25(OH)_2D$ incubation on gene expression of cholecalciferol metabolism in 3T3-L1 adipocytes.

	3T3-L1 adipocytes			
	Control	$1,25(OH)_2D$ 1 nM	$1,25(OH)_2D$ 10 nM	$1,25(OH)_2D$ 100 nM

Cyp27a1	100 ± 9 [a]	100 ± 9 [a]	64 ± 9 [b]	66 ± 9 [b]
Cyp2j6	100 ± 9 [a]	103 ± 9 [a]	105 ± 4 [a]	91 ± 4 [a]
Cyp3a11	100 ± 37 [a]	80 ± 13 [a]	86 ± 22 [a]	97 ± 34 [a]
Cyp24a1	100 ± 10 [a]	138 ± 22 [a]	856 ± 46 [a]	48 988 ± 6453 [b]
Cyp27b1	100 ± 5 [a]	95 ± 13 [a]	80 ± 16 [a]	85 ± 9 [a]
Vdr	100 ± 8 [a]	123 ± 4 [a]	287 ± 13 [b]	1035 ± 50 [c]
Gc	100 ± 17 [a]	60 ± 5 [b]	70 ± 10 [a,b]	101 ± 6 [a]
Megalin	100 ± 10 [a]	86 ± 10 [a]	93 ± 10 [a]	97 ± 9 [a]
Cubn	100 ± 5 [a]	86 ± 7 [a,c]	66 ± 6 [b,c]	44 ± 5 [b]

Expression of genes coding for proteins involved in cholecalciferol metabolism relative to 18S ribosomal RNA in 3T3-L1 adipocytes. Values are presented as means ± SEM. Values not sharing the same letter were significantly different in ANOVA and Tukey-Kramer post hoc test $p < 0.05$.