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

3

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7

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13

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18

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20 **Abstract**

21

22 It is well established that the active form of vitamin D, i.e. 1,25(OH)<sub>2</sub>D, regulates the expression  
23 of genes involved in its own metabolism and transport in the kidney, and possibly in the liver.  
24 However, little is known about the transcriptional impact of cholecalciferol supplementation on  
25 white adipose tissue (WAT) and adipocytes, which are a major site of vitamin D and 25(OH)D  
26 storage in the organism. To fill this gap, we investigated the impact of cholecalciferol  
27 supplementation in WAT via a panel of genes coding for enzymes and proteins involved in  
28 vitamin D metabolism and uptake.

29 Mice supplemented with cholecalciferol (15 000 IU/kg of body weight/day) for 4 days showed  
30 decreased mRNA levels of proteins involved in cholecalciferol metabolism (Cyp24a1,  
31 Cyp27a1) and decreased cubilin mRNA levels in WAT. These data were partly confirmed in  
32 3T3-L1 adipocytes incubated with 1,25(OH)<sub>2</sub>D. The down-regulation of cubilin mRNA  
33 observed in WAT and in 3T3-L1 was confirmed at protein level in WAT and at mRNA level  
34 in human primary adipocytes. VDR agonist (EB1089) and RNA interference approaches  
35 demonstrated that VDR was involved in this regulation. Furthermore, chemical inhibitor and  
36 by RNA inference analysis demonstrated that cubilin was involved in 25(OH)D uptake by  
37 adipocytes.

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

42

43 **Introduction**

44

45 Vitamin D, or calciferol, is a hormone that is synthesized in the epidermis after exposure to  
46 UVB radiation or can be obtained from eating fatty fish (1,2). After intestinal uptake (3), dietary  
47 vitamin D reaches the bloodstream. Both dietary and endogenous cholecalciferol undergo their  
48 first hydroxylation in the liver by 25-hydroxylases including Cyp2r1—although Cyp27a1,  
49 Cyp3a11 and Cyp2j6 are also involved (4)—to produce 25-hydroxyvitamin D (25(OH)D), the  
50 major circulating form of vitamin D (5). 25-hydroxylation is generally regarded as poorly  
51 regulated (6), even if 25-hydroxylase activity was decreased by 1,25(OH)<sub>2</sub>D in rat liver (7). In  
52 plasma, calciferol and 25(OH)D are principally bound to the vitamin D-binding protein (DBP,  
53 encoded by the Gc gene) [87% of 25(OH)D], their transport protein, and albumin [13% of  
54 25(OH)D], although a small quantity remains unbound [ $<1\%$  of 25(OH)D] (8,9). 25(OH)D is  
55 taken up by the kidney, where a fraction is 1 $\alpha$ -hydroxylated by Cyp27b1 to produce  
56 1,25(OH)<sub>2</sub>D, the active form of vitamin D. This uptake is mediated by the megalin/cubilin  
57 complex (10), which involves other proteins such as disabled 2 (Dab2) (11) and amnionless  
58 (Amn) (12). CYP27B1 activity in the kidney is positively regulated by parathyroid hormone  
59 and low calcium levels and deactivated by fibroblast growth factor 23 and 1,25(OH)<sub>2</sub>D itself  
60 through a negative feedback mechanism (13,14).

61 In target tissues, 25(OH)D and 1,25(OH)<sub>2</sub>D can be catabolized by 24-hydroxylases (Cyp24a1)  
62 to generate inactive metabolites (15). In the kidney, this step is auto-regulated, and  
63 cholecalciferol supplementation induced Cyp24a1 expression (16,17). The molecular  
64 mechanisms have been unraveled and shown to involve transcriptional regulation mediated by  
65 the vitamin D receptor (VDR), which binds 1,25(OH)<sub>2</sub>D with high affinity. After  
66 heterodimerization with the retinoic acid receptor, the resulting complex can bind to vitamin D

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

69 Vitamin D and 25(OH)D are stored mainly in white adipose tissue (WAT), plasma and skeletal  
70 muscle (19). The uptake of vitamin D and its metabolites in preadipocytes and skeletal muscle  
71 cells has recently been described (20), and involved megalin (21). In adipocytes, vitamin D and  
72 25(OH)D are not only stored in lipid droplets (22) but could also be converted to active  
73 metabolites (23,24) that are able to modulate adipocyte biology (25-27). Indeed, adipocytes  
74 have been shown to express most of the genes involved in vitamin D metabolism, such as 25-  
75 hydroxylases (23,24), 1 $\alpha$ -hydroxylase (24, 28), megalin (20), Cyp24a1 and Vdr (23,29). Given  
76 that in liver and in kidney, vitamin D regulates its own metabolism at a transcriptional level via  
77 its active metabolite 1,25(OH)<sub>2</sub>D, it is highly likely that similar regulations occur in WAT.  
78 Nevertheless, 1,25(OH)<sub>2</sub>D-mediated transcriptional regulation of genes involved in vitamin D  
79 metabolism in adipocytes and in WAT has only been only partly established so far.

80

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

86 **Materials and methods**

87

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

94

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

107

108 **Cell culture and treatment** – 3T3-L1 preadipocytes (American Type Culture Collection, VA)  
109 were seeded in 3.5 cm-diameter dishes at a density of  $15 \times 10^4$  cells/well and grown in DMEM  
110 supplemented with 10% FBS at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere, as previously

111 described (32,33). After two-day confluence, 3T3-L1 (day 0) were stimulated for 48 h with 0.5  
112 mM isobutylmethylxanthine, 0.25  $\mu\text{mol/L}$  dexamethasone, and 1  $\mu\text{g/mL}$  insulin in DMEM  
113 supplemented with 10% FBS to induce differentiation. The cultures were successively treated  
114 with DMEM supplemented with 10% FBS and 1  $\mu\text{g/mL}$  insulin.

115 Human preadipocytes (isolated from female subcutaneous adipose tissue biopsies) supplied by  
116 Promocell (Heidelberg, Germany) were cultured and differentiated into adipocytes according  
117 to the company's instructions. Briefly, cells were seeded at a density of 5000 cells/cm<sup>2</sup> in  
118 Preadipocyte Growth Medium and grown until confluence, then allowed to differentiate for 3  
119 days in Preadipocyte Differentiation Medium. Mature adipocytes were cultivated in Adipocyte  
120 Nutrition Medium for another 11 days, as previously reported (34).

121 To examine the regulation of genes coding for proteins involved in cholecalciferol metabolism,  
122 both human and murine adipocytes were incubated with 1,25(OH)<sub>2</sub>D (1, 10 and 100 nM)  
123 dissolved in absolute ethanol for 24 hours or with EB1089 (10, or 100 nM), a VDR agonist, for  
124 24 hours.

125

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

130

131 **Uptake of 25(OH)D by adipocytes** – 3T3-L1 adipocytes were incubated with [3H]-25(OH)D  
132 at a concentration of 11.25 nCi/mL and with 50 nM of non-radiolabeled 25(OH)D (Sigma-  
133 Aldrich, St Louis, MO) in DMEM supplemented with 1  $\mu\text{g/mL}$  insulin in the presence of either  
134 1.9  $\mu\text{M}$  DBP, 0.125% bovine serum albumin (BSA) or ethanol (control condition). These 3T3-  
135 L1 adipocytes were treated with 10 or 100 nM of 1,25(OH)<sub>2</sub>D or with 100 or 500 nM of

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

139

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

149

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

153

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



161

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

164 *Preparation of analytical and deuterated standards* – A working solution of deuterated analytes  
165 was prepared at 0.02 ng/mL of each internal standard (IS), i.e. d<sub>3</sub>-cholecalciferol, d<sub>3</sub>-25(OH)D  
166 and d<sub>3</sub>-1,25(OH)<sub>2</sub>D. Primary stock solutions of cholecalciferol, 25(OH)D and 1,25(OH)<sub>2</sub>D  
167 standards were prepared at concentrations of 100, 50 and 10 ng/mL, respectively, in ethanol,  
168 and stored at -80°C in the dark. Calibration curves were prepared by serial dilution of the three  
169 analyte stock solutions to obtain calibration standards from 0 to 75 ng/mL, then adding 1.5 µL  
170 of the working solution of deuterated analytes to each dilution. After complete evaporation of  
171 solvent, derivatization was performed. A one-step derivatization was employed to improve the  
172 ionization efficiency of the metabolites using Amplifex diene as reagent (38). Amplifex (30  
173 µL) was added to the dried sample above, vortexed for 15 s, and incubated for 30 min at ambient  
174 temperature. Next, 30 µL of deionized water was added, vortexed for 15 s, and transferred for  
175 LC injection. Calibration curves were plotted as peak area ratio of the vitamin D metabolite to  
176 the respective IS versus a range of analyte concentrations.

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

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

188 *Epididymal WAT preparation* – Sample preparation was adapted from Lipkie et al. (40). First,  
189 25  $\mu\text{L}$  of deuterated standard working solution was added to tissue homogenates (50 mg of  
190 tissue ground within 1 mL of PBS) in glass test tube. Acetonitrile (ACN) was added, vortex-  
191 mixed for 5 min, and centrifuged at 6,000  $g$  for 5 min. Then, methyl tert-butyl ether (MTBE)  
192 was added, vortexed for 5 min, centrifuged, and the upper organic layer was collected into a  
193 fresh glass tube. The extraction was repeated twice, and the combined supernatants were dried  
194 under nitrogen. Oasis HLB SPE cartridges (Waters, Guyancourt, France) were conditioned with  
195 ethyl acetate, methanol (MetOH) and  $\text{H}_2\text{O}$ . The sample was reconstituted with 1 mL of MetOH  
196 and 1 mL of  $\text{K}_2\text{HPO}_4$  (0.4 M), and added onto the cartridge. The cartridge was washed with  
197  $\text{H}_2\text{O}$  and 70% MetOH, and then dried for 2 min under vacuum. Tips were washed with ACN,  
198 and analytes were eluted with ACN and dried under nitrogen. After complete evaporation of  
199 solvent, the samples were derivatized as described above.

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

205 Samples were injected onto a Hypersil GOLD C18 column ( $2.1 \times 100$  mm; Thermo Scientific,  
206 Les Ulis, France). Flowrate was 0.4 mL/min, and injection volume was 5  $\mu\text{L}$ . The mobile phase  
207 was composed of A = ultrapure water with 0.1% formic acid (FA) (v/v), and B = ACN with  
208 0.1% FA (v/v). Starting conditions were A = 70% and B = 30%, held for 4 min. A linear gradient  
209 was applied until 10.0 min where A = 35% and B = 65% which was held until 12.0 min, at 14  
210 min A=0% and B = 100% until 16 min. Starting conditions were re-implemented at 18 min.

211 The SRM transitions used for quantification for each analyte were: 716.5→657.5  
212 (Cholecalciferol), 719.5→660.5 (d3-Cholecalciferol), 732.5→673.4 (25(OH)D), 735.5→676.4  
213 (d3-25(OH)D), 751.5→692.4 (d3-1,25(OH)<sub>2</sub>D) and 748.5→689.4 (1,25(OH)<sub>2</sub>D).

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

215

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

## 220 **Results**

221

### 222 **Cholecalciferol supplementation modifies expression of genes involved in cholecalciferol** 223 **metabolism and uptake in WAT and adipocytes.**

224 To examine the impact of cholecalciferol supplementation on the regulation of genes coding  
225 proteins involved in its own metabolism in WAT, wild-type C56BL/6J male mice were  
226 supplemented with cholecalciferol (15 000 IU/kg of body weight/day) for 4 days. Body weight  
227 and absolute and relative organ weights were not modified by the treatment (Table 1). As  
228 expected, supplemented mice showed higher plasma concentrations of cholecalciferol,  
229 25(OH)D and 1,25(OH)<sub>2</sub>D (26.22, 6.32 and 9.94-fold, respectively, Table 1) and increased  
230 cholecalciferol and 25(OH)D in WAT (4.87-fold and 3.41-fold, respectively; Table 1). WAT  
231 from supplemented mice also had a higher quantity of 1,25(OH)<sub>2</sub>D, but the difference compared  
232 to control mice did not reach statistical significance (Table 1).

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

243 To study the effect of 1,25(OH)<sub>2</sub>D-mediated cell-autonomous regulation on adipocyte gene  
244 expression, 3T3-L1 adipocytes were treated with different doses of 1,25(OH)<sub>2</sub>D (1, 10 and 100

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

250

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

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

268

269 **25(OH)D endocytosis is mediated by cubilin and regulated by 1,25(OH)<sub>2</sub>D in 3T3-L1**  
270 **adipocytes.**

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

278 To confirm the involvement of cubilin receptor in [3H]-25(OH)D endocytosis, cells were  
279 treated with RAP, a specific inhibitor of the megalin/cubilin complex. When [3H]-25(OH)D  
280 was solubilized in ethanol, there was no difference between RAP-treated conditions and  
281 controls (Figure 5B). However, when [3H]-25(OH)D was bound to BSA or DBP, there was a  
282 strong decrease of [3H]-25(OH)D uptake (0.23-fold and 0-49 fold, respectively; Figure 5C and  
283 5F). In a second set of experiments, 3T3-L1 adipocytes were transfected with either a siRNA  
284 directed against Cubilin (siCubilin) or a non-targeted siRNA (siNT) used as control for 24 h.  
285 [3H]-25(OH)D uptake in complexes with ethanol, BSA or DBP was measured for 16 h. [3H]-  
286 25(OH)D uptake was decreased in the presence of siCubilin compared to siNT when the BSA  
287 was used as vehicle (0.63-fold, Figure 5E).

288 To study the effect of modulating cubilin expression on [3H]-25(OH)D uptake, we incubated  
289 the cells with 1,25(OH)<sub>2</sub>D (10 or 100 nM) and measured the uptake of [3H]-25(OH)D  
290 solubilized in ethanol (Figure 5F) or bound to BSA (Figure 5G) or DBP (Figure 5H). In all  
291 conditions, incubation with 100 nM of 1,25(OH)<sub>2</sub>D decreased uptake of [3H]-25(OH)D in  
292 adipocytes (0.04, 0.19 and 0.45-fold, respectively). Note that even if statistically significant,

293 the 1,25(OH)<sub>2</sub>D-induced modification of ethanol-solubilized [3H]-25(OH)D uptake was  
294 quantitatively negligible (Figure 5F).  
295 .

296 **Discussion**

297

298 Here we used targeted gene profiling to show that cholecalciferol regulates the expression of  
299 several genes involved in cholecalciferol metabolism and uptake in WAT in response to a short-  
300 term cholecalciferol supplementation. Among regulated genes, we report, for the first time, the  
301 negative regulation of *Cubn* gene. Additional experiments in 3T3-L1 adipocytes demonstrated  
302 that *Cubn* regulation was VDR-dependent. Finally, we demonstrated that cubilin is involved in  
303 25(OH)D uptake in adipocytes.

304

305 To analyze the regulatory effect of cholecalciferol supplementation on gene expression in  
306 WAT, mice received cholecalciferol for 4 days by oral gavage. Cholecalciferol was diluted in  
307 olive oil to assure better absorption of this lipophilic molecule (41). In this study, no difference  
308 of total body mass or organ mass (liver and AT) was detected between groups, but as expected,  
309 plasma concentrations of cholecalciferol, 25(OH)D and 1,25(OH)<sub>2</sub>D in WAT concentrations of  
310 cholecalciferol and 25(OH)D were strongly increased in cholecalciferol-supplemented mice.  
311 Despite a clear tendency to increase, the quantity of 1,25(OH)<sub>2</sub>D in WAT of supplemented mice  
312 was not statistically different from control mice. In agreement with previously published data  
313 (42), cholecalciferol supplementation strongly induced kidney mRNA expression of *Cyp24a1*  
314 and *Vdr* and decreased mRNA levels of *Cyp27b1*, thus validating our experimental conditions.  
315 Interestingly, we also observed a decrease of *Gc* and *Cubn* gene expression in the kidney. This  
316 regulation, if confirmed at protein level, could result in a decrease in renal recycling of  
317 cholecalciferol and its metabolites, thus constituting a way to eliminate excess cholecalciferol  
318 from the plasma. In the liver, we only observed a decrease of *Cubn* expression decreased, but  
319 there was no change in the expression of mRNA coding for other enzymes of hepatic  
320 cholecalciferol metabolism. This decrease of *Cubn* expression suggests that the uptake of these



321 molecules could be regulated in the liver at a transcriptional level, through a negative feedback  
322 mechanism, thus limiting their hepatic uptake. We also gained an overview of the  
323 transcriptional effect of cholecalciferol supplementation in epididymal WAT. Our results  
324 showed a decrease of Cyp27a1 and Cyp24a1 mRNA levels, suggesting a putative decrease of  
325 25-hydroxylation and inactivation of metabolites. Cyp24a1 induction has already been reported  
326 (28), but this is the first report of Cyp27a1 repression. This novel finding result will require  
327 further investigations, especially to confirm the real contribution of Cyp27a1 in the adipose  
328 metabolism of cholecalciferol. Interestingly, we also found a specific down-regulation of Cubn  
329 mRNA levels while other partners in the endocytosis complex (megalin, Dab2 and Amn) were  
330 not transcriptionally affected.

331

332 To demonstrate the direct effect of 1,25(OH)<sub>2</sub>D on these regulations, we used murine 3T3-L1  
333 adipocytes. In these cells, we observed an up-regulation of Vdr and Cyp24a1, both of which  
334 are well-known VDR target genes (43), thus validating our experimental model. The fact that  
335 Cyp24a1 was decreased *in vivo* but strongly increased *in vitro* is surprising, but could be due to  
336 indirect regulations that simultaneously occurred *in vivo* whereas induction *in vitro* only  
337 resulted from direct VDR-mediated induction m (43) Interestingly, the patterns of Cyp27a1 and  
338 Cubn regulation were reproduced *in vitro*, confirming the direct and cell-autonomous nature of  
339 the regulation. While it is well documented that most of the enzymes of cholecalciferol  
340 metabolism are expressed in adipocytes (25), including Vdr (29), 25-hydroxylation enzymes  
341 (23,24), 1 $\alpha$ -hydroxylation enzyme (24,44) and megalin (20), here we report that certain putative  
342 actors of hepatic 25-hydroxylation (4) are not expressed in adipocytes. This is notably the case  
343 of Cyp2r1 and Cyp3a11, which were not detected in our conditions, in agreement with Zoico  
344 et al. who did not detect Cyp2r1 in 3T3-L1 cells (24). The ability of adipocytes to produce  
345 25(OH)D has been demonstrated (23,24), but the enzyme involved has not yet been identified.

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

349

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

361 To investigate the molecular mechanism involved in Cubn regulation, several approaches were  
362 combined. First, the use of a specific VDR agonist (EB1089) led to similar down-regulation of  
363 Cubn expression compared to 1,25(OH)<sub>2</sub>D, suggesting that the regulation described *in vitro* is  
364 mediated by VDR. This involvement was demonstrated by the RNA interference experiments  
365 implemented here using siRNA targeted against VDR. Furthermore, an *in silico* analysis  
366 (MatInspector in the Genomatix suite) confirmed the presence of putative VDREs within the  
367 murine and human promoters of cubilin.

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

371 considered unbound (“free form”) (>1% of total 25(OH)D) (7). In preliminary experiments, the  
372 ability of different vehicles to deliver 25(OH)D to adipocytes was tested. We observed that the  
373 best vehicle for 25(OH)D was BSA, followed by ‘free form’ (mimicked here by an ethanolic  
374 solution) then DBP. These data suggest that BSA-complexed 25(OH)D is easily absorbed by  
375 cells. Note too that the free form can also be internalized in adipocytes. Finally, it appears that  
376 the DBP is probably not the best way to deliver 25(OH)D to adipocytes but corresponds to a  
377 25(OH)D storage site in plasma, as previously suggested (46).

378 To confirm the involvement of cubilin in 25(OH)D uptake by adipocytes, two strategies were  
379 implemented. First, we used RAP (inhibitor of megalin/cubilin complex (47)), and second, we  
380 used an RNA interference approach. Interestingly, 25(OH)D uptake in complex with BSA or  
381 DBP was decreased by RAP and, to a lesser extent, siRNA directed against Cubn (especially  
382 for 25(OH)D-DBP complexes that were not impacted by siRNA). Note that 25(OH)D uptake  
383 of the free form (in ethanol) was not impacted by RAP or siRNA, suggesting that the uptake of  
384 unbound 25(OH)D occurs independently of the megalin/cubilin pathway. Finally, to confirm  
385 that cubilin regulation is involved in 25(OH)D uptake, adipocytes were incubated with  
386 1,25(OH)<sub>2</sub>D. Interestingly, this incubation led to a decrease of cubilin expression and was  
387 associated with a decrease of 25(OH)D uptake. Taken together, these data provide strong  
388 evidence that cubilin is involved in 25(OH)D uptake by adipocytes. From a physiological point  
389 of view, these data suggest that a negative feedback regulation occurs in WAT to control the  
390 uptake of cholecalciferol and its metabolites via a modulation of cubilin expression. This kind  
391 of limitation of cholecalciferol and metabolite storage, which is generally assumed to be a  
392 passive mechanism due to lipophilicity, suggests that cholecalciferol and 25(OH)D storage in  
393 WAT is actually tightly controlled and regulated.

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

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

399

400

401 **Figure legends**

402

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

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

409

410 **Figure 2: Effect of 1,25(OH)<sub>2</sub>D incubation on expression of cholecalciferol metabolism**  
411 **genes in 3T3-L1 adipocytes.**

412 3T3-L1 adipocytes were incubated with 1,25(OH)<sub>2</sub>D (1, 10 and 100 nM) for 24 h (A to E).  
413 Expression of genes coding for proteins involved in cholecalciferol metabolism relative to 18S  
414 ribosomal RNA. Values are reported as means  $\pm$  SEM. Bars not sharing the same letter were  
415 significantly different in a Tukey-Kramer post hoc test at  $p < 0.05$ .

416

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

419 (A) Human primary white adipocytes were incubated with 100 nM of 1,25(OH)<sub>2</sub>D for 24 h.  
420 Expression of cubilin relative to 18S ribosomal RNA. (B) Cubilin protein quantification  
421 performed by ELISA tests in epididymal white adipose tissue (WAT) of mice (control n=9,  
422 cholecalciferol n=6). Values are reported as means  $\pm$  SEM. \*  $p < 0.05$  for an unpaired Student's  
423 *t*-test.

424

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

426 (A) 3T3-L1 adipocytes were incubated with EB1089, a vitamin D receptor agonist (VDR) (10  
427 and 100 nM) for 24 h. (B) The 3T3-L1 adipocytes were transfected with either a small  
428 interfering RNA (siRNA) oligonucleotide for VDR or a non-silencing control for 24 h. The  
429 efficiency of RNA interference against VDR was determined by qPCR. (C) These cells were  
430 transfected with siRNA and incubated with 100 nM of 1,25(OH)<sub>2</sub>D for 24h. Values are reported  
431 as means ± SEM. Bars not sharing the same letter were significantly different in a Tukey-  
432 Kramer post hoc test at  $p < 0.05$ . \*  $p < 0.05$  for an unpaired Student's *t*-test.

433

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

435 3T3-L1 adipocytes were incubated with control (Ctrl) or with 25(OH)D solubilized in ethanol  
436 (free form) or complexed with bovine serum albumin (BSA) or vitamin D-binding protein  
437 (DBP) (A). Cells were incubated with receptor-associated protein (RAP) (100 or 500 nM) and  
438 25(OH)D was solubilized in ethanol (B) or complexed to BSA (C) or DBP (D) for 16 h. 3T3-  
439 L1 cells were transfected with siRNA (non-targeted (siNT) or directed against VDR (siVDR))  
440 and incubated with 25(OH)D solubilized in ethanol (free form) or complexed with BSA or DBP  
441 (E). Cells were incubated with 1,25(OH)<sub>2</sub>D (10 and 100 nM) and 25(OH)D was solubilized into  
442 ethanol (F) or complexed to BSA (G) or DBP (H). In each experiment, 25(OH)D uptake was  
443 quantified by measuring the number of counts per minute per well by liquid scintillation. Values  
444 are reported as means ± SEM. Bars not sharing the same letter were significantly different in a  
445 Tukey-Kramer post hoc test at  $p < 0.05$ . \*  $p < 0.05$  for an unpaired Student's *t*-test.

446

447 **Table 1: Morphological and biological parameters of mice.**

448

	Control mice	Cholecalciferol-supplemented mice
<b>Body weight (g)</b>	21.9 ± 0.37	21.1 ± 0.90
<b>Liver weight (mg)</b>	976.9 ± 29.09	955.5 ± 57.74
<b>Liver weight/body weight ratio</b>	0.0446 ± 0.0007	0.0451 ± 0.0009
<b>Adipose tissue weight (mg)</b>	242.9 ± 10.12	281.0 ± 32.63
<b>Adipose tissue weight/body weight ratio</b>	0.0111 ± 0.0005	0.0136 ± 0.002
<b>Serum cholecalciferol (ng/mL)</b>	2.58 ± 0.60	67.65 ± 9.66 *
<b>Serum 25(OH)D (ng/mL)</b>	16.79 ± 0.98	106.16 ± 18.60 *
<b>Serum 1,25(OH)<sub>2</sub>D (pg/mL)</b>	68.6 ± 16.95	682.2 ± 122.11 *
<b>AT cholecalciferol quantity (ng)</b>	158 ± 22.01	770.24 ± 175.28 *
<b>AT 25(OH)D quantity (ng)</b>	27.3 ± 1.05	93.13 ± 30.91 *
<b>AT 1,25(OH)<sub>2</sub>D quantity (pg)</b>	1.58 ± 0.31	2.77 ± 0.68

449

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

451

452

453

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

456

	Sequence	EIDorado reference ID	Position of the VDRE
<b>Human promoter</b>	gtttcaaaGGTCaaatagataatga	GXP_271874 (-)	17171654_17172330
<b>Mouse promoter</b>	tcaagagGATTcaaaggcaactca	GXP_425459 (-)	13491712_13492424

457

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

461

462 **References**

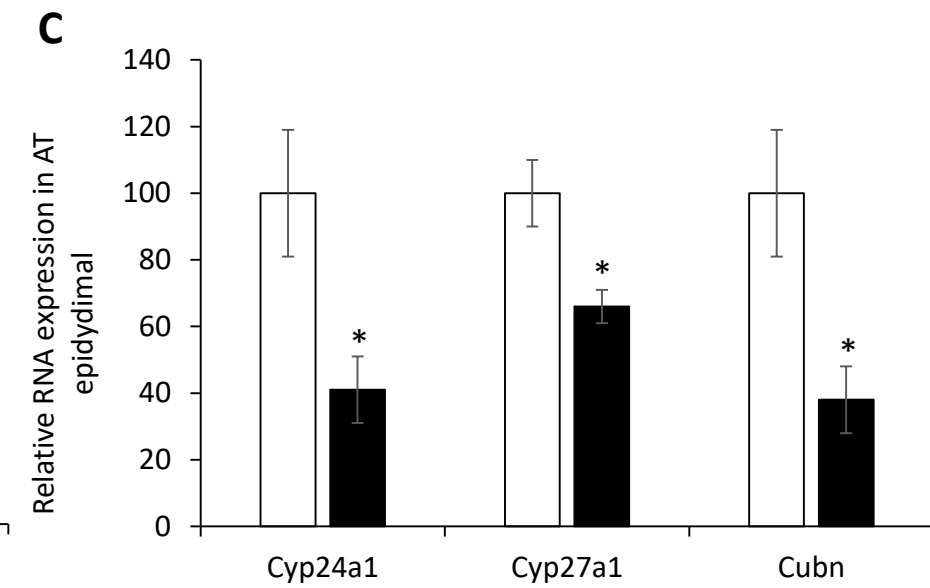
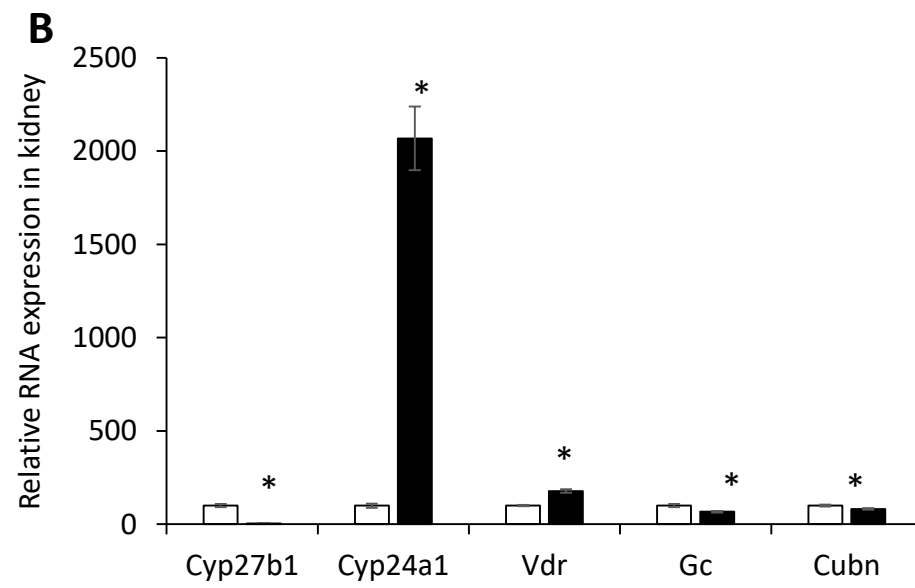
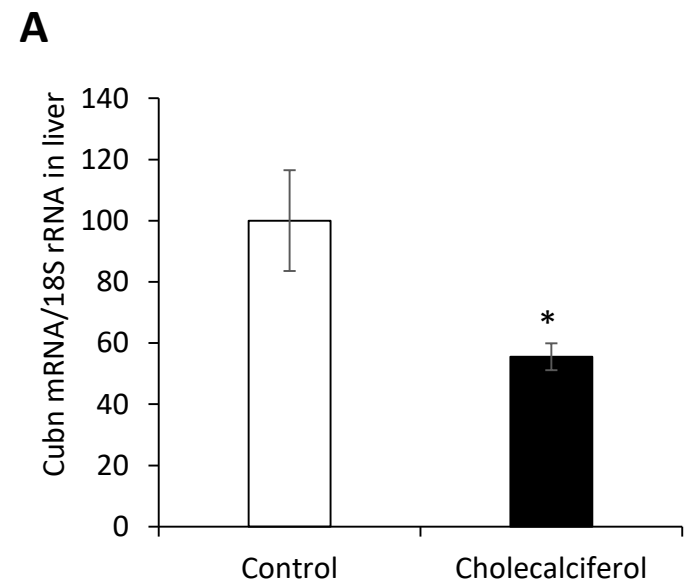
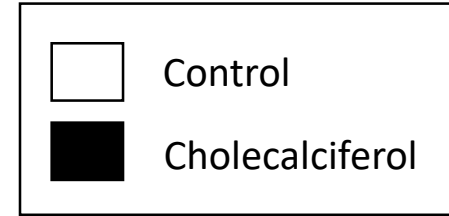
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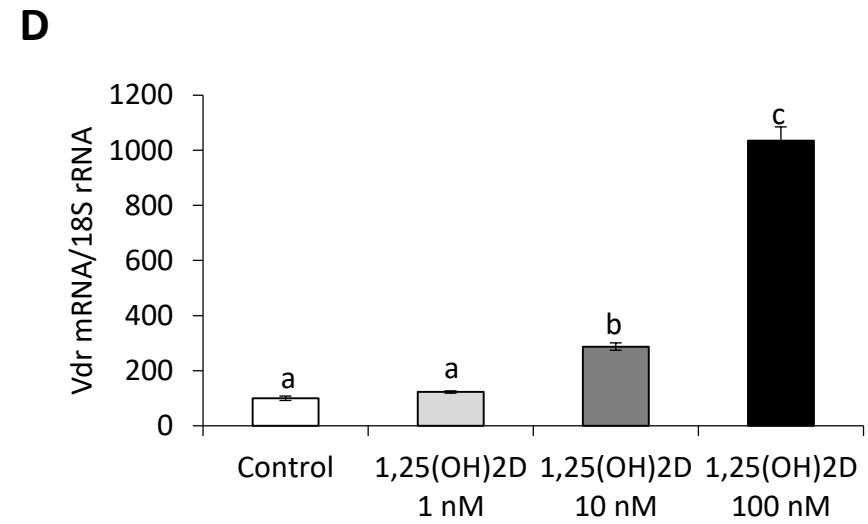
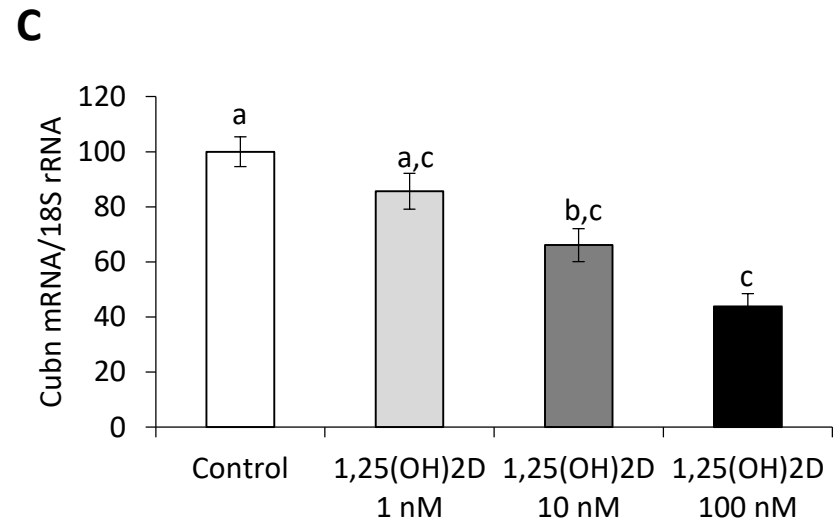
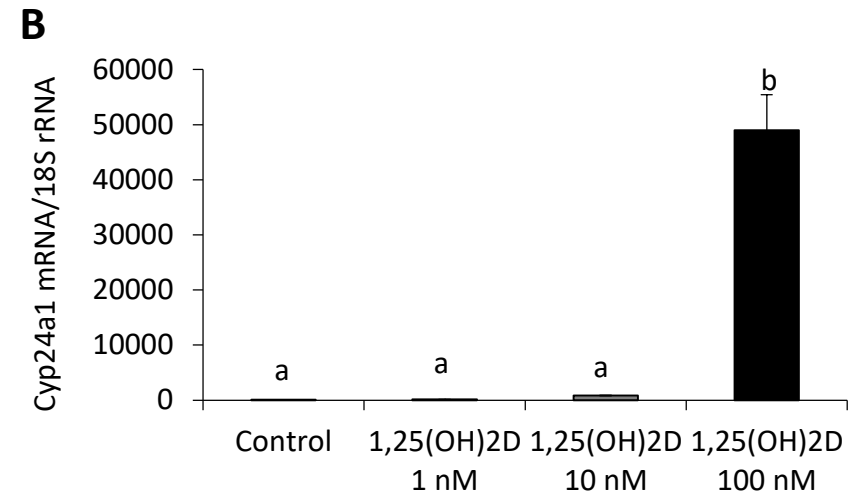
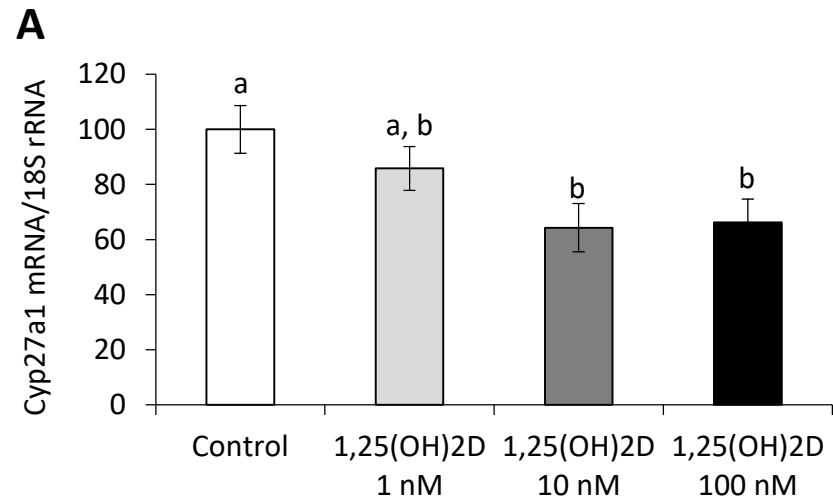
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587 11:191-202
- 588

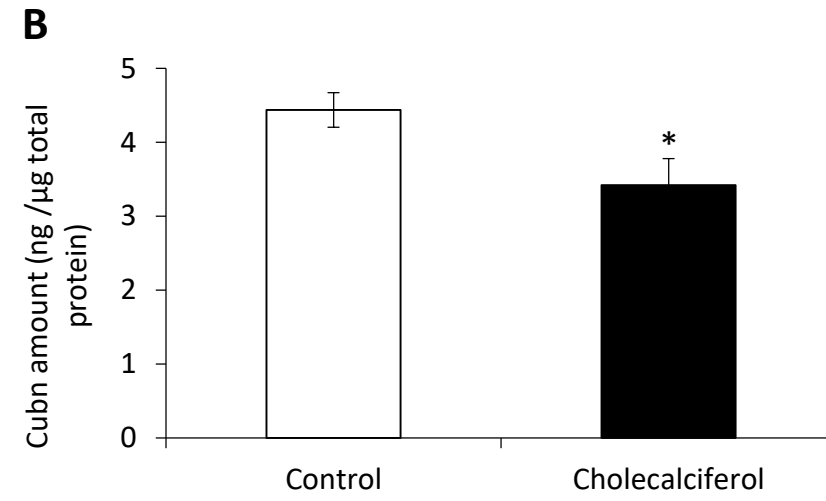
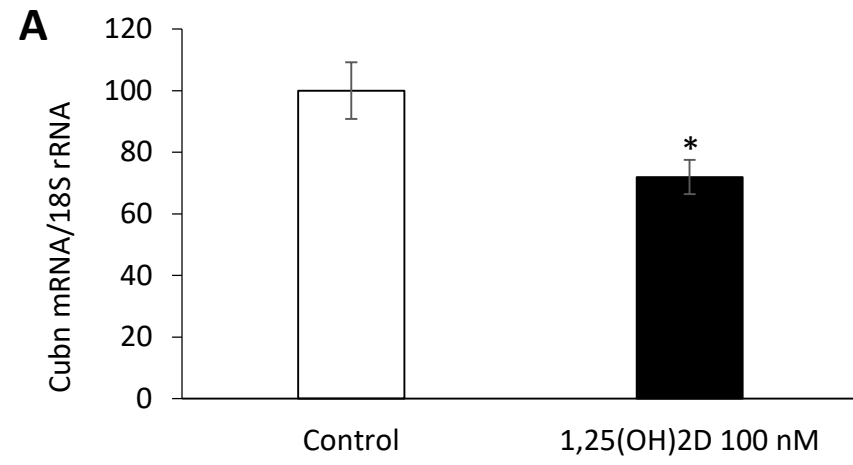
**Figure 1**



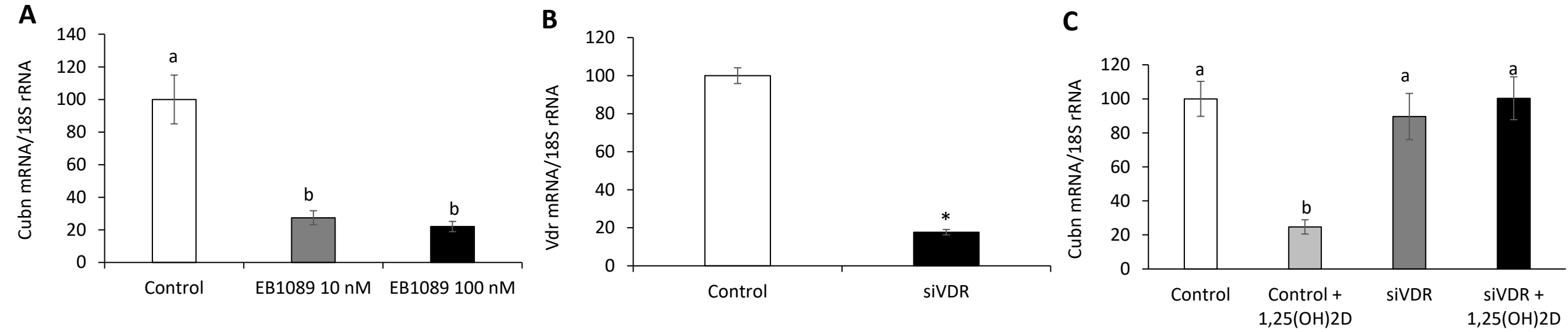
**Figure 2**



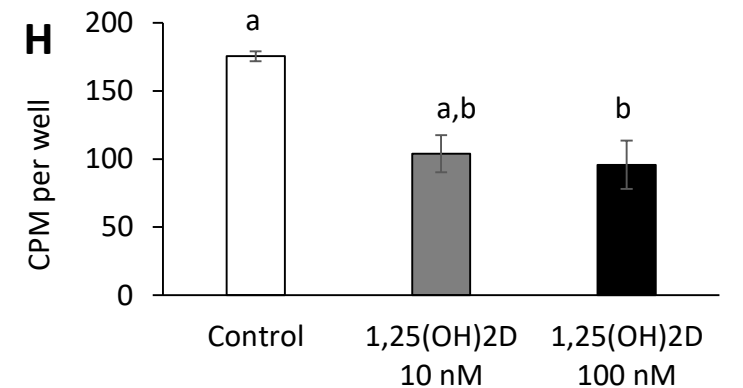
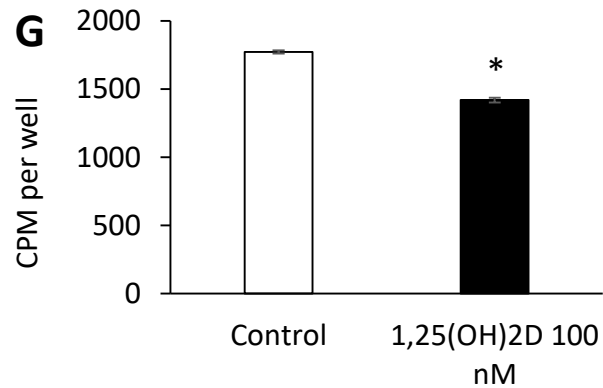
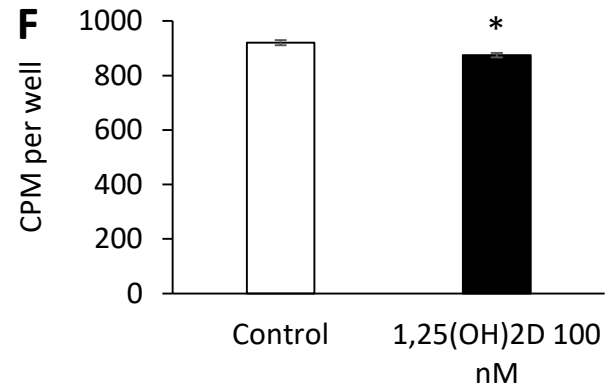
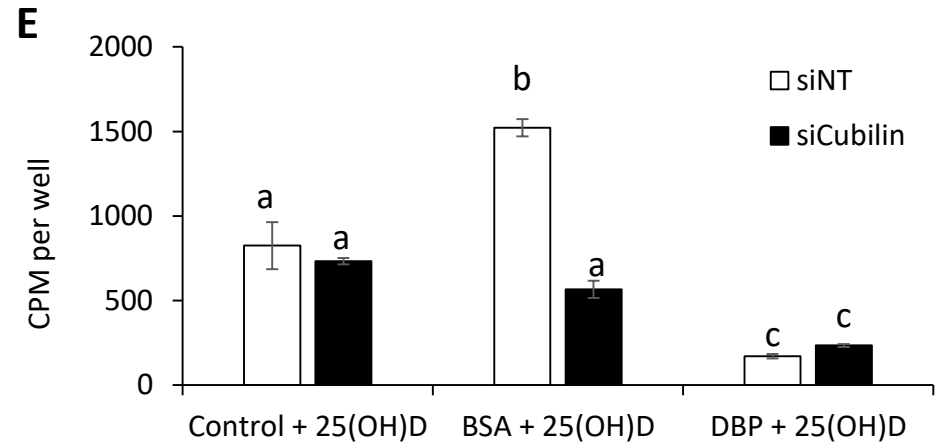
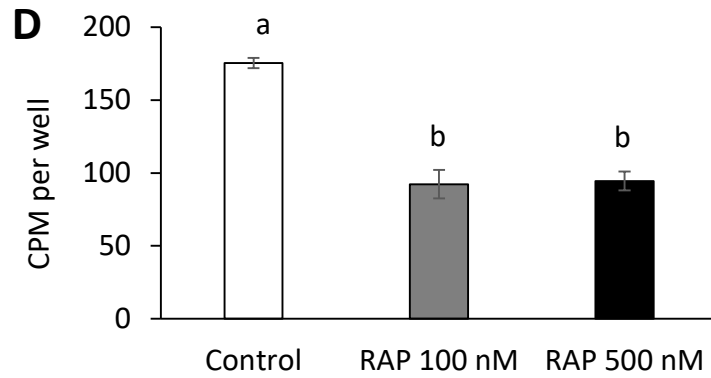
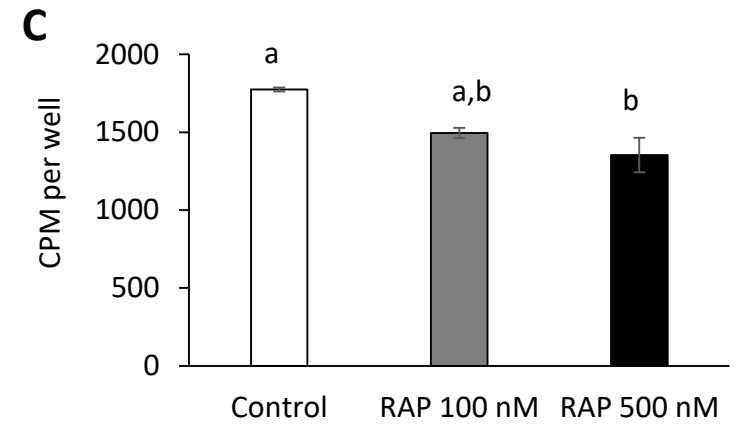
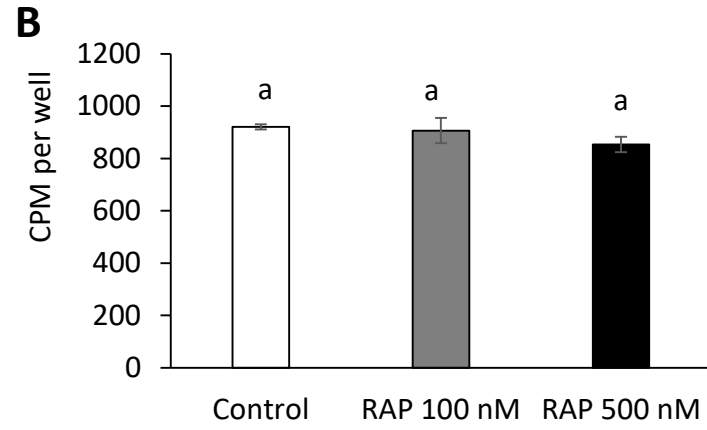
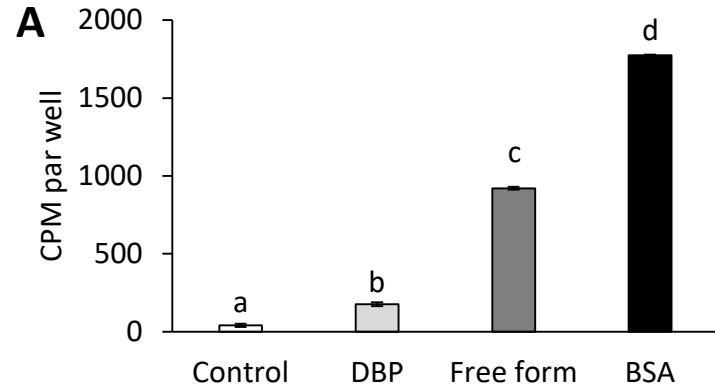
**Figure 3**



**Figure 4**



**Figure 5**



## Supplemental tables

**Supplemental table 1:** Primers sequences

Gene	Forward sequence	Reverse sequence
<b>Cyp2r1</b>	TTTGTCTGGCAACATCTGCT	TGCCTCCAAGATCTAAACTGAAA
<b>Cyp3a11</b>	TGAATATGAAACTTGCTCTCACTAAAA	CCTTGTCTGCTTAATTTTCAGAGCT
<b>Cyp27a1</b>	CCTCACCTATGGGATCTTCATC	TTAAGGCATCCGTGTAGAGC
<b>Cyp2j6</b>	CCCTCTACCCAGAAGTCCAA	TTCTGGCCAATCACCTATC
<b>Cyp24a1</b>	AAGCCTACGCGCTGATGAT	CACGGGCTTCATGAGTTTC
<b>Cyp27b1</b>	AGTGGGGAATGTGACAGAGC	GGAGAGCGTATTGGATACCG
<b>Megalin</b>	GATGGATTAGCCGTGGACTG	TCCGTTGACTCTTAGCATCTGA
<b>Cubn</b>	GCCATCCAGATGCAACCT	GGTGCAGACAGGCAACAAG
<b>Vdr</b>	AACCCCTCATAAAGTTCCAGGT	CTGTACCCAGGTCGGTCT
<b>Gc</b>	CTACCTCAGAGGATTGCATGG	CTTTTTGGATAAGTTTTTGACAGATTTT
<b>Dab2</b>	GCAGTCGAACTTTCTGCATCTC	GGTGTTACTGGGACCGTACCT
<b>Amn</b>	AGACAGTCACGCCATCTCG	GAGGCCAGGACCAACTCC.
<b>18S rRNA</b>	CGCCGCTAGAGGTGAAATTCT	CATTCTTGCAAATGCTTTTCG

**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) <sub>2</sub> D	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.

	<b>Liver</b>		<b>Kidney</b>		<b>Epididymal WAT</b>	
	Control	Cholecalciferol	Control	Cholecalciferol	Control	Cholecalciferol
<b>Cyp27a1</b>	100 ± 6	109 ± 6			100 ± 10	66 ± 5 *
<b>Cyp2r1</b>	100 ± 8	92 ± 5			100 ± 8	83 ± 16
<b>Cyp2j6</b>	100 ± 6	109 ± 8			100 ± 8	96 ± 8
<b>Cyp3a11</b>	100 ± 9	76 ± 9			100 ± 33	103 ± 38
<b>Cyp24a1</b>			100 ± 11	2068 ± 171 *	100 ± 19	41 ± 10 *
<b>Cyp27b1</b>			100 ± 9	4 ± 0 *	100 ± 25	78 ± 22
<b>Vdr</b>	100 ± 10	79 ± 6	100 ± 4	178 ± 10 *	100 ± 16	77 ± 14
<b>Gc</b>	100 ± 6	115 ± 6	100 ± 9	67 ± 4 *	100 ± 44	36 ± 11
<b>Megalin</b>	100 ± 7	93 ± 7	100 ± 5	98 ± 3	100 ± 25	50 ± 9
<b>Cubilin</b>	100 ± 16	56 ± 4 *	100 ± 5	82 ± 4 *	100 ± 19	38 ± 10 *
<b>Dab2</b>	100 ± 8	106 ± 5	100 ± 4	82 ± 5	100 ± 5	73 ± 9
<b>Amn</b>	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 epididymal 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)<sub>2</sub>D incubation on gene expression of cholecalciferol metabolism in 3T3-L1 adipocytes.

<b>3T3-L1 adipocytes</b>			
Control	1,25(OH) <sub>2</sub> D 1 nM	1,25(OH) <sub>2</sub> D 10 nM	1,25(OH) <sub>2</sub> D 100 nM

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

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  $p < 0.05$ .