

1,25-Dihydroxyvitamin D₃ Controls a Cohort of Vitamin D Receptor Target Genes in the Proximal Intestine That Is Enriched for Calcium-regulating Components^{*[5]}

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Background: Vitamin D₃ regulates intestinal calcium absorption to maintain mineral homeostasis.

Results: Genome-wide analyses reveal vitamin D₃ target genes and their regulatory components in intestine.

Conclusion: A gene network involved in vitamin D₃-mediated calcium uptake in the intestine is defined.

Significance: The network of genes provides a basis for understanding molecular mechanisms of vitamin D₃-mediated active calcium transport in the intestine.

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) plays an integral role in calcium homeostasis in higher organisms through its actions in the intestine, kidney, and skeleton. Interestingly, although several intestinal genes are known to play a contributory role in calcium homeostasis, the entire caste of key components remains to be identified. To examine this issue, *Cyp27b1* null mice on either a normal or a high calcium/phosphate-containing rescue diet were treated with vehicle or 1,25(OH)₂D₃ and evaluated 6 h later. RNA samples from the duodena were then subjected to RNA sequence analysis, and the data were analyzed bioinformatically. 1,25(OH)₂D₃ altered expression of large collections of genes in animals under either dietary condition. 45 genes were found common to both 1,25(OH)₂D₃-treated groups and were composed of genes previously linked to intestinal calcium uptake, including *S100g*, *Trpv6*, *Atp2b1*, and *Cldn2* as well as others. An additional distinct network of 56 genes was regulated exclusively by diet. We then conducted a ChIP sequence analysis of binding sites for the vitamin D receptor (VDR) across the proximal intestine in vitamin D-sufficient normal mice treated with vehicle or 1,25(OH)₂D₃. The residual VDR cistrome was composed of 4617 sites, which was increased almost 4-fold following hormone treatment. Interestingly, the majority of the genes regulated by 1,25(OH)₂D₃ in each diet group as well as those found in common in both groups contained frequent VDR sites that likely regulated their expression. This study revealed a global network of genes in the intestine that both represent direct targets of vitamin D action in mice and are involved in calcium absorption.

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃)² orchestrates calcium homeostasis through its regulatory actions on gene expression in the intestine, kidney, and bone (1). These actions via the intracellular vitamin D receptor (VDR) lead to enhanced calcium and phosphate absorption by the intestine, elevated reabsorption of calcium and phosphate from the kidney, and orchestrated bone remodeling within the skeleton (1). The biological activity of the hormone is maintained directly through its blood levels, which are controlled by renal *CYP27B1* (2), whose expression in the proximal tubules of the kidney is regulated at the transcriptional level by parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and 1,25(OH)₂D₃ (3, 4). As the elaboration of these hormones is modulated directly by the calcium content in the blood, the activities of 1,25(OH)₂D₃ in the intestine, kidney, and bone as well as other target tissues are linked directly to the levels of calcium and phosphate that are present in the extracellular compartment. Interestingly, although the vast majority of these fundamental principles of calcium homeostasis has been defined through studies in animal models as well as in humans over several decades (5, 6), the phenotypes of a number of genetically modified murine models are currently providing important new insights. These models include strains which contain crippling genomic mutations in either the *Cyp27b1* (7, 8) or the *Vdr* genes (9–12) that closely replicate the molecular defects observed and characterized in humans. Importantly, the pathophysiological consequences in these two models have turned out to be remarkably similar.

Although many of the biological processes and the genes that are expressed and associated with vitamin D action in bone and kidney are well defined, neither the components nor the processes responsible for mediating the transepithelial transport of calcium from the gut lumen to the blood are fully understood. Initial studies suggested that the soluble calcium-binding protein calbindin D9K (*S100g*) and its renal counterpart calbindin D28K (*Calb1*) might participate in the transepithelial transport

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^[5] This article contains supplemental Table S1.

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² The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; TSS, transcription start site; VDR, vitamin D receptor; seq, sequencing; PTH, parathyroid hormone; bw, body weight; RPKM, reads/kb of exon per million mapped; VDRE, vitamin D response element.

of calcium in the intestine and kidney, respectively (13). Deletion studies, however, have revealed that neither protein is essential for vitamin D-mediated calcium uptake as the loss of the genes for calbindin D9K or calbindin D28K expression did not alter intestinal sensitivity to 1,25(OH)₂D₃ (14–16). Rather, these proteins were eventually deemed to be calcium-sensitive buffer components likely involved in the shuttling of calcium to organelles within the cytoplasm (17). Interestingly, although both *S100g* and *Calb1* were identified early on as direct targets of vitamin D action (18, 19), the molecular details through which their expression is enhanced by 1,25(OH)₂D₃ is currently unclear. Studies also identified the basolateral membrane protein *Pmca1* (*Atp2b1*) in the enterocyte as a potential component of the transport process (20). *Pmca1* utilizes ATP to actively extrude calcium across the epithelial basolateral membrane and into the blood (20). Thermodynamic principles associated with calcium gradients were used to argue for its role as a principal component of the vitamin D-dependent intestinal calcium uptake process (21, 22). Unfortunately, deletion of this gene on a global scale is embryonically lethal (23); thus, the consequence of deleting this gene specifically in intestinal epithelial cells has yet to be determined. In addition, although *Atp2b1* was also observed to be modulated by 1,25(OH)₂D₃ (24–26), this regulation is modest, and the mechanism, like those of the calbindins, remains currently undefined. Finally, *Trpv6*, a member of the transient receptor potential vanilloid family of genes and located in the brush border membranes of the intestinal epithelia (27), has been identified as a potential mediator of calcium uptake into the enterocyte. While retaining many of the characteristics necessary for a true transepithelial calcium transporter, genetic deletion of this gene was similarly unable to prevent the induction of calcium uptake through the intestine by 1,25(OH)₂D₃, although deletion of this gene did compromise overall calcium uptake by this tissue (28, 29). In contrast to *Atp2b1* and *S100g*, however, contemporary studies of *Trpv6* have identified the locations of enhancer elements upstream of the gene's transcription start site (TSS) that mediate up-regulation by 1,25(OH)₂D₃ (30, 31). Despite this, mice containing simultaneous deletion of both *S100g* and *Trpv6* gene expression in the intestinal tract of mice genetically still retained significant sensitivity to 1,25(OH)₂D₃ (15). These studies collectively suggest that *Atp2b1* represents the only known yet genetically untested component of this group of three candidates.

It is currently proposed that *Trpv6* mediates the uptake of calcium across the brush border of epithelial cells, and calbindin D9K serves as an intracellular shuttle to move calcium to the basolateral membrane where *Pmca1* actively extrudes the ion through this barrier into the blood. It is formally possible that a single specific but as yet unidentified component that is a key to vitamin D-dependent calcium uptake by the intestine will be identified. We hypothesize, however, that the actions of 1,25(OH)₂D₃ may not be restricted to the regulation of a single gene but rather facilitate the differential up-regulation of a network of genes across the intestinal tract that contributes collectively to the efficient uptake of calcium from the gut to maintain calcium homeostasis but may also contain redundant components. This network could include genes involved in both the

intracellular handling of calcium as well as its transepithelial transport. Accordingly, the activity of vitamin D in the intestine likely involves not only *Trpv6*, calbindin D9K, and *Pmca1* but additional players that participate in the process of calcium flux as well.

The actions of 1,25(OH)₂D₃ are mediated by the VDR, a relatively small nuclear protein that binds its cognate ligand with high affinity and selectivity, relocates to the regulatory regions of target genes, and interacts directly with consensus DNA sequences composed of hexameric half-sites separated by 3 bp (32–34). In most instances, this association is facilitated by a partner protein termed retinoid X receptor, which both increases the affinity of the VDR complex for DNA and also plays an active role in modulating events essential for gene activation (35). Additional studies now demonstrate that the ability of the VDR, other steroid receptors of which the VDR is a member, and indeed most transcription factors to regulate gene expression involves their capacity to recruit large, highly complex molecular machines, which function at numerous levels to alter the unique restraints imposed at target gene loci by chromatin structure (36, 37). This recruitment is facilitated through an interaction between the steroid-regulated activation domain of the VDR and leucine-rich LXXLL motifs that are located in at least one component of each of these coregulatory complexes (38). Most of these machines contain unique enzymatic activities that represent the cornerstone of their ability to alter the chromatin environments surrounding individual target genes (39). Principles of vitamin D action have been defined at a number of genes, including human and mouse *TRPV6*, where VDR occupies at least two separate sites upstream of the TSS of the genes through multiple VDREs and serves to recruit coregulators as summarized above (30, 31). Interestingly, although early studies have suggested that VDREs are largely located within several kilobases of gene promoters, more recent unbiased genome-wide studies using ChIP-seq analyses now support the concept that the majority of genes are regulated by multiple enhancers routinely located many kilobases distal to the TSSs of target genes (40). *In vivo* ChIP-seq studies in the intestine and other tissues will likely reinforce these particular features at additional 1,25(OH)₂D₃ target genes.

In this report, we utilize *Cyp27b1*^{−/−} mice maintained on either a normal or a high calcium and phosphate rescue diet to explore global transcriptomic responses of the intestine to a single acute dose of 1,25(OH)₂D₃. We identified a single cohort of 45 genes whose expression was responsive to 1,25(OH)₂D₃ irrespective of diet or of their calcemic consequences. This group of genes contained many if not all of the genes known to be involved in intestinal calcium uptake, including *Trpv6*, *S100g*, *Atp2b1*, and *Cldn2* as well as genes such as *Lrrc26*, *Mctp2*, and *Slc30a1* that may be involved in intracellular calcium metabolism. Surprisingly, the rescue diet alone modulated a more limited yet distinct network of genes that did not contain members associated with calcium metabolism. Subsequent ChIP-seq analysis of intestinal epithelial cells derived from vitamin D sufficient mice revealed an important residual VDR cisrome that was substantially increased in response to exogenous 1,25(OH)₂D₃. Our results define a cohort of genes composed, in part, of all known 1,25(OH)₂D₃-regulated genes

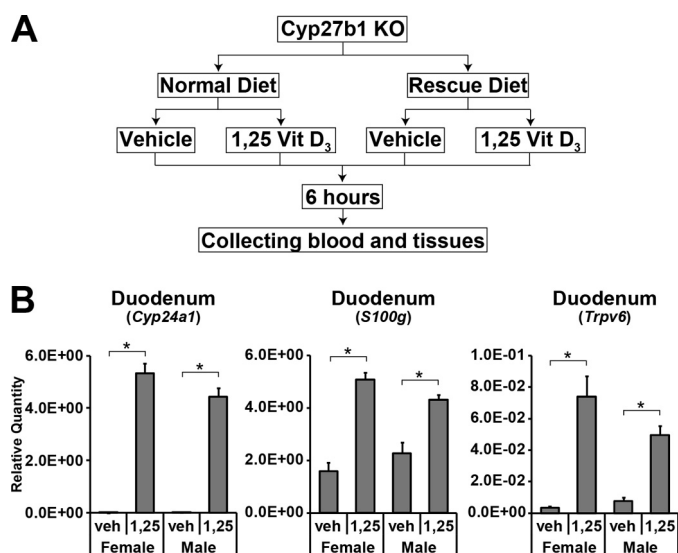


FIGURE 1. Study overview. A, *Cyp27b1*^{−/−} mice at weaning were fed either a normal diet containing 0.47% calcium and 0.3% phosphate or a rescue diet containing 2% calcium, 1.5% phosphate, and 20% lactose and injected with either vehicle or 1,25(OH)₂D₃ (2 ng/g bw) at 8 weeks of age. The mice were sacrificed 6 h later to harvest blood and tissues for subsequent analyses of serum components and specific gene expression. B, regulation of candidate gene expression by 1,25(OH)₂D₃ in male and female mice is similar. Expression of the indicated genes in duodenum of either female or male wild type mice (*n* = 5) were compared by RT-PCR. Data were normalized using *Gapdh* and are presented as the mean ± S.E. Significant differences between two experimental groups were identified by Student's unpaired *t* test (*, *p* < 0.05). veh, vehicle-treated sample; 1,25, 1,25(OH)₂D₃-treated sample; Vit, vitamin.

involved in the calcium absorption and likely identify a network that collectively regulates this process in the intestine.

Experimental Procedures

Animal Study—*Cyp27b1*^{−/−} mice in which the coding region of the *Cyp27b1* gene was replaced with a cassette containing the *lacZ* and neomycin resistance genes (41) were used as a model to explore the global effects of 1,25(OH)₂D₃ on gene expression. As summarized in Fig. 1A, *Cyp27b1*^{−/−} mice weaned at 3 weeks of age were divided into two groups of 16 mice each. One group was fed a highly purified vitamin D-deficient diet (42) containing 0.47% calcium, 0.3% phosphate, and vitamins A, E, and K (normal diet), and the other group was fed the same diet containing 20% lactose, 2% calcium, 1.25% phosphate (rescue diet) (43). At 8 weeks of age, eight mice per group were administered 1,25(OH)₂D₃ (2 ng/g body weight (bw)) or vehicle by intraperitoneal injection. Mice were euthanized at 6 h, and both tissues and blood were collected for further analyses. Wild type mice, littermates from C57BL/6 mice (Harlan), were fed a standard rodent chow diet (Harlan Teklad, catalog no. 5008). At 8 weeks of age, five mice were similarly treated with 1,25(OH)₂D₃ (10 ng/g bw) or vehicle control, and the duodena were collected 6 h later for gene expression analysis. For chromatin immunoprecipitation-sequencing analysis, the proximal half of the small intestine (duodenum and jejunum) was collected from wild type mice after 1 h of treatment with 1,25(OH)₂D₃ (10 ng/g bw) or vehicle control. Because no significant difference in gene expression response to 1,25(OH)₂D₃ was observed between females and males (Fig. 1B), male mice were used for all animal studies. Mice were exposed

to a 12-h light-dark cycle. All animal studies were reviewed and approved by Research Animal Care and Use Committee of University of Wisconsin-Madison.

Serum Analysis—Blood was collected via cardiac puncture; aliquots were used to obtain both serum and plasma. Serum calcium and phosphate concentrations were obtained using QuantiChrom calcium assay kit (BioAssay Systems) and QuantiChrom phosphate assay kit (BioAssay Systems), according to the manufacturer's directions. EDTA/plasma was utilized to obtain measurements of PTH and FGF23 concentrations using a mouse Intact PTH ELISA kit (Immunotopics) and an FGF-23 ELISA kit (Kainos Laboratories), respectively, according to the manufacturer's directions. A Student's unpaired *t* test was used to identify significant differences (*p* < 0.05).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Isolated mouse tissues were soaked in TRI Reagent (Molecular Research Center) and then frozen in liquid nitrogen. Tissues were thawed on ice and then homogenized using a PowerGen model 125 homogenizer (Fisher) to prepare total RNA preparations. RNAs were subjected to DNase I treatment and to reverse transcription using High Capacity cDNA reverse transcription kit (Applied Biosystems). Double-stranded cDNAs were used to measure gene expression by quantitative real time PCR using TaqMan primers (Applied Biosystems) described in supplemental Table S1. Significant differences were identified using a Student's unpaired *t* test (*p* < 0.05).

RNA Sequencing (RNA-seq) Analysis—Six independent duodenal RNA samples from each experimental group were subjected to RNA-seq analysis. RNA samples were first depleted of rRNA using a RiboMinus eukaryote kit (Invitrogen) and then used to prepare RNA-seq libraries using ScriptSeqTM version 2 RNA-Seq library preparation kits (Epicenter). Both rRNA depletion (RNA 6000 nano kit) and final library quality (high sensitivity DNA kit) were assessed using an Agilent Bioanalyzer (Agilent Technologies). The prepared libraries were subjected to next generation sequencing using an Illumina HiSeq 2000 system (100-bp read length); over 100 million reads per sample were obtained. The reads were mapped to the mouse July 2007 (NCBI37/mm9) assembly using TopHat, resulting in more than 85% mapped reads per sample. Reads were aligned with reference sequences from the NCBI Entrez Genome Project database using ArrayStar Version 5 with Q-Seq module (DNASTAR). The reference library contained 30,795 RNAs (27,037 mRNAs, 42 tRNAs, 8 rRNAs, 566 ncRNAs, including 426 miRNAs and 3142 miscRNAs); gene expression was normalized and quantified as reads/kb of exon per million mapped (RPKM). To identify significant differences in gene expression between two experimental groups, Student's *t* test was applied using ArrayStar version 5 with the Q-Seq module (*p* < 0.05). The Database for Annotation, Visualization, and Integrated Discovery (DAVID; from NIAID, National Institutes of Health) was used for Gene Ontology (GO) analysis (44). Genes were mapped to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for further insight.

Chromatin Immunoprecipitation Sequencing (ChIP-seq) Analysis—Intestinal epithelial cells were isolated using EDTA as reported previously (45). In brief, collected small intestines

were cleaned with cold PBS, sliced into small pieces and cross-linked with 1.5% formaldehyde. The tissues were then incubated in cold PBS containing 2 mM EDTA on ice for 30 min and washed with cold PBS. Epithelial cells were isolated by vortexing the tissues with cold PBS five times for 1.5 min with 30-s intervals on ice and then by passing the mixture through a 70- μ m cell strainer (Falcon). The isolated cells were centrifuged at 2000 rpm for 5 min, washed with cold PBS, and subjected to chromatin immunoprecipitation using either a control IgG antibody or anti-VDR antibody (Santa Cruz Biotechnology, sc-1008) as described previously (46). ChIP-seq analysis was performed using three independent ChIP samples per experimental group, and the data were processed as reported previously (47).

Functional Genomic Data—All RNA-seq and ChIP-seq data are deposited in the Gene Expression Omnibus (accession number GSE69180).

Results

Acute Effects of 1,25(OH)₂D₃ on Systemic Parameters and Tissue-specific Gene Expression Targets in Normocalcemic and Hypocalcemic *Cyp27b1*^{−/−} Mice—*Cyp27b1*^{−/−} mice were utilized to identify a 1,25(OH)₂D₃-regulated network of genes that was involved in transepithelial calcium uptake by intestine. Accordingly, 21-day-old male *Cyp27b1*^{−/−} mice as documented in Fig. 1 were weaned onto a vitamin D-deficient diet containing either normal calcium and phosphate (normal diet) or high calcium and phosphate plus 20% lactose (rescue diet) with the expectation that this regimen would result in mice that were either hypocalcemic and hypophosphatemic or normocalcemic and normophosphatemic, respectively. At 8 weeks of age, half of the animals from each group were treated with a single i.p. injection of vehicle, and the other half received a single injection of 1,25(OH)₂D₃ (2 ng/g bw) as described under “Experimental Procedures.” Animals were sacrificed 6 h post-treatment both to maximize peak transcriptional response to 1,25(OH)₂D₃ and to minimize the potential effects of transepithelial calcium flux in the intestine as a confounding inducer of gene expression; both blood and tissues were then harvested for further experimental examination. Because a single injection of 1,25(OH)₂D₃ increases serum calcium and phosphate levels similarly as a function of time in mice maintained on either diet, we hypothesized that genes induced in both of these groups of animals were likely to represent a viable 1,25(OH)₂D₃-sensitive network that was involved in intestinal calcium uptake. As documented in Fig. 2 and shown previously (48, 49), animals maintained on the normal diet were indeed hypocalcemic and hypophosphatemic, and those on the rescue diet were normocalcemic and normophosphatemic. Importantly, although a trend of increased serum calcium and phosphate levels was noted, treatment with 1,25(OH)₂D₃ did not raise the level of either mineral significantly at the 6-h time point. Finally, measurement of circulating PTH and FGF23 levels in the two groups revealed that both exhibited high levels of PTH and low levels of FGF23, although PTH and FGF23 levels in the rescue diet group were marginally reduced and elevated, respectively. Treatment with 1,25(OH)₂D₃ did not reduce PTH levels in animals maintained on the normal diet but strikingly reduced PTH

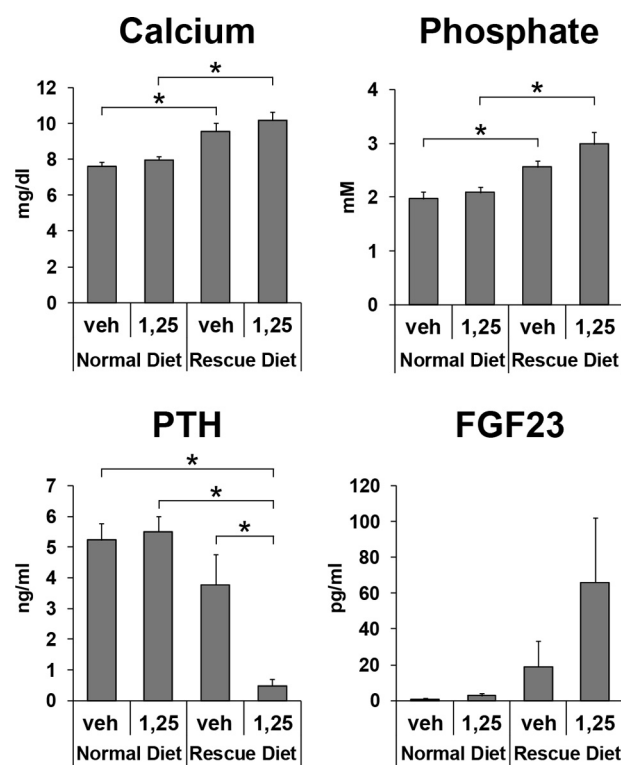


FIGURE 2. Analysis of systemic parameters. Calcium, phosphate, PTH, and FGF23 content was assessed in serum or EDTA/plasma obtained from *Cyp27b1*^{−/−} mice within each experimental group. Data are presented as the mean \pm S.E. ($n = 8$). Student's unpaired *t* test was used to identify significant differences between two groups (*, $p < 0.05$). veh, vehicle-treated sample; 1,25, 1,25(OH)₂D₃-treated sample.

levels in those maintained on the rescue diet. Hormone treatment, however, was effective in raising FGF23 levels in both dietary groups; although these increases were more dramatic in animals with normal calcium and phosphate levels, neither was statistically significant. These results demonstrate that dietary calcium and phosphate levels alter mineral homeostasis in *Cyp27b1*^{−/−} mice and influence systemic response to 1,25(OH)₂D₃ in a fashion similar to that seen in VDR null mice (49–51).

In addition to these systemic parameters, we also assessed the effects of 1,25(OH)₂D₃ on selected target gene regulation in specific segments of the intestinal tract and in kidney and calvarial bone. As can be seen in Fig. 3, 1,25(OH)₂D₃ strongly induced the expression of *Cyp24a1* in the jejunum, colon, and kidney under both dietary conditions. Additional known targets of 1,25(OH)₂D₃ action were also induced in calvaria including the *Vdr*, *Spp1*, and *Fgf23*. These acute effects of 1,25(OH)₂D₃ at the systemic level as well as on tissue-specific gene expression reveal an anticipated effect of 1,25(OH)₂D₃ on *Cyp27b1*^{−/−} mice influenced only modestly by diet-induced calcium and phosphate status. Importantly, it is worth noting that although changing levels of PTH or FGF23 can influence gene expression in bone and kidney, the absence of PTH receptor in the intestine precludes a role for PTH in this specific organ.

RNA-seq Analysis of Transcriptomes in the Duodenum of *Cyp27b1*^{−/−} Mice—To explore more comprehensively the effects of 1,25(OH)₂D₃ on the expression of genes involved in

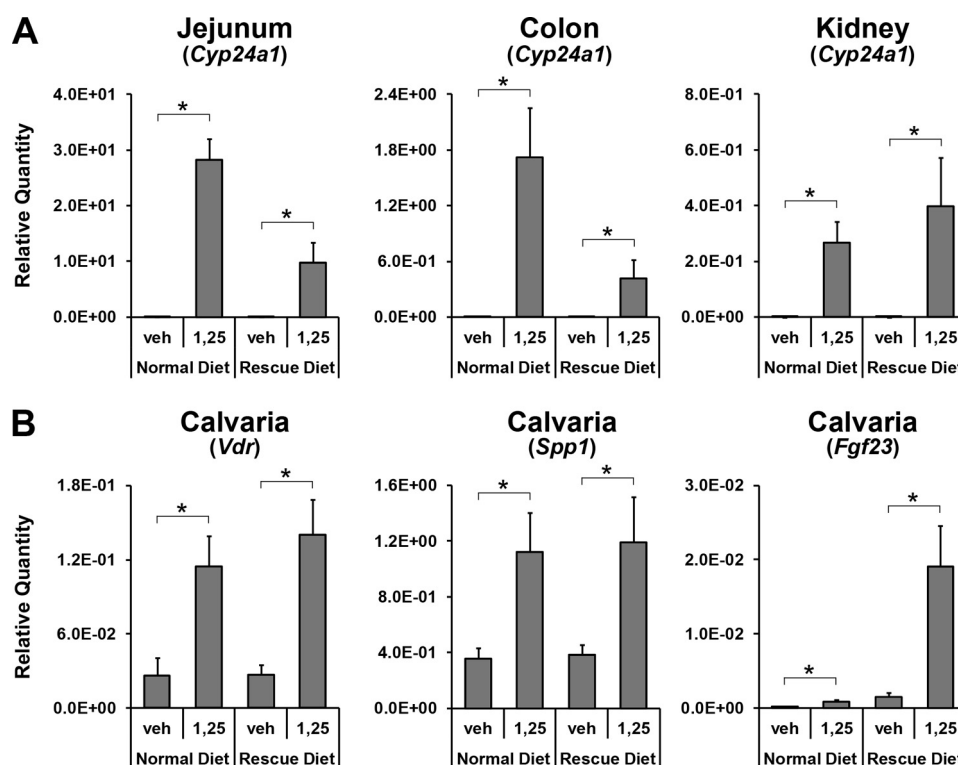


FIGURE 3. 1,25(OH)₂D₃-target gene expression in tissues. RNA was isolated and examined by RT-PCR for expression of *Cyp24a1* in jejunum, colon, and kidney (A) and *Vdr*, *Spp1*, and *Fgf23* in calvaria (B) as indicated. Data were normalized to *Gapdh* and presented as the mean \pm S.E. ($n = 8$). Student's unpaired *t* test was used to identify significant differences between two groups (*, $p < 0.05$). veh, vehicle-treated sample; 1,25, 1,25(OH)₂D₃-treated sample.

calcium uptake in the intestine, we performed RNA-seq analysis on RNA isolated from the duodenum of each of the *Cyp27b1*^{-/-} mice derived from the four groups outlined above. Data sets containing a minimum of 10⁸ reads per sample were mapped to the mouse mm9 genome assembly using TopHat as described under “Experimental Procedures” and were normalized and quantified using ArrayStar as RPKM. Estimates for the expression of some 30,000 genes were obtained. A representative example of the results from a single mouse sample for the *Cyp24a1* gene obtained from each group is illustrated in Fig. 4A. These data clearly document the striking capacity of 1,25(OH)₂D₃ to raise the expression level of this gene in mice fed either of the two diets. The low basal levels of *Cyp24a1* expression known to occur in the intestine result in a significant fold up-regulation by 1,25(OH)₂D₃. A significant up-regulation of transcripts was also seen for *Trpv6*, *S100g*, and *Atp2b1* by 1,25(OH)₂D₃ but not for *Vdr* under both dietary conditions as summarized in Fig. 4B for all *Cyp27b1*^{-/-}-derived mouse samples in each group. When these data are contrasted directly with an RT-PCR analysis of the same mouse samples and in samples from wild type mice, all three data sets are consistent with respect to both basal as well as inducible response to 1,25(OH)₂D₃, thereby validating the extensive genome-wide RNA-seq analyses obtained from the *Cyp27b1*^{-/-} mice.

1,25(OH)₂D₃ Regulates a Common Cohort of Genes That Is Independent of Diet and Enriched for Genes Involved in Calcium Metabolism—Based on the reliability of these RNA-seq data, we next identified genes as illustrated in Fig. 5A that were differentially expressed in response to 1,25(OH)₂D₃ in mice on the normal diet (group 1) versus those on the rescue diet (group

2) as well as those that were differentially expressed as a result of the diet alone (group 3). As summarized in Fig. 5B, 599 genes were modulated by 1,25(OH)₂D₃ in mice fed the normal diet (group 1, $p < 0.05$) of which the majority (546, 91%) were up-regulated, and 119 genes were modulated by the hormone in mice fed the rescue diet (group 2, $p < 0.05$) of which 77 (65%) were up-regulated. Only 56 genes were differentially expressed as a result of maintenance on the rescue diet (group 3). Specific genes associated with each of these groups are listed in [supplemental Table S1](#). Interestingly, as summarized in Fig. 5C and Table 1, a cohort of 45 genes found to be regulated by 1,25(OH)₂D₃ in group 1 was also similarly regulated by the hormone in group 2, suggesting that the regulation of these genes by 1,25(OH)₂D₃ was fully independent of the dietary or calcemic/phosphatemic status of the mice. Importantly, this collection contained the classic genes, including *Trpv6*, *Atp2b1*, and *S100g* believed to be involved in calcium uptake, that as shown in Fig. 4 were regulated not only in *Cyp27b1*^{-/-} but also in wild type mice. As documented in Fig. 6, additional genes were also found to be induced by 1,25(OH)₂D₃ as well in wild type mice. Given the common calcemic response to 1,25(OH)₂D₃ manifested in both groups of mice, we hypothesize that this cohort of genes includes, at least in part, a network of genes involved in comprehensive calcium and perhaps phosphate transport in the intestine. Surprising, only 9 of the 599 genes regulated by 1,25(OH)₂D₃ in group 1 and 1 of the 119 genes similarly regulated in group 2 overlapped the 56 genes that were differentially expressed in group 3 (Table 1 and Fig. 5C). Moreover, none of the genes in this group or those that overlapped groups 1 or 2 include genes such as *Trpv6*, *Atp2b1*,

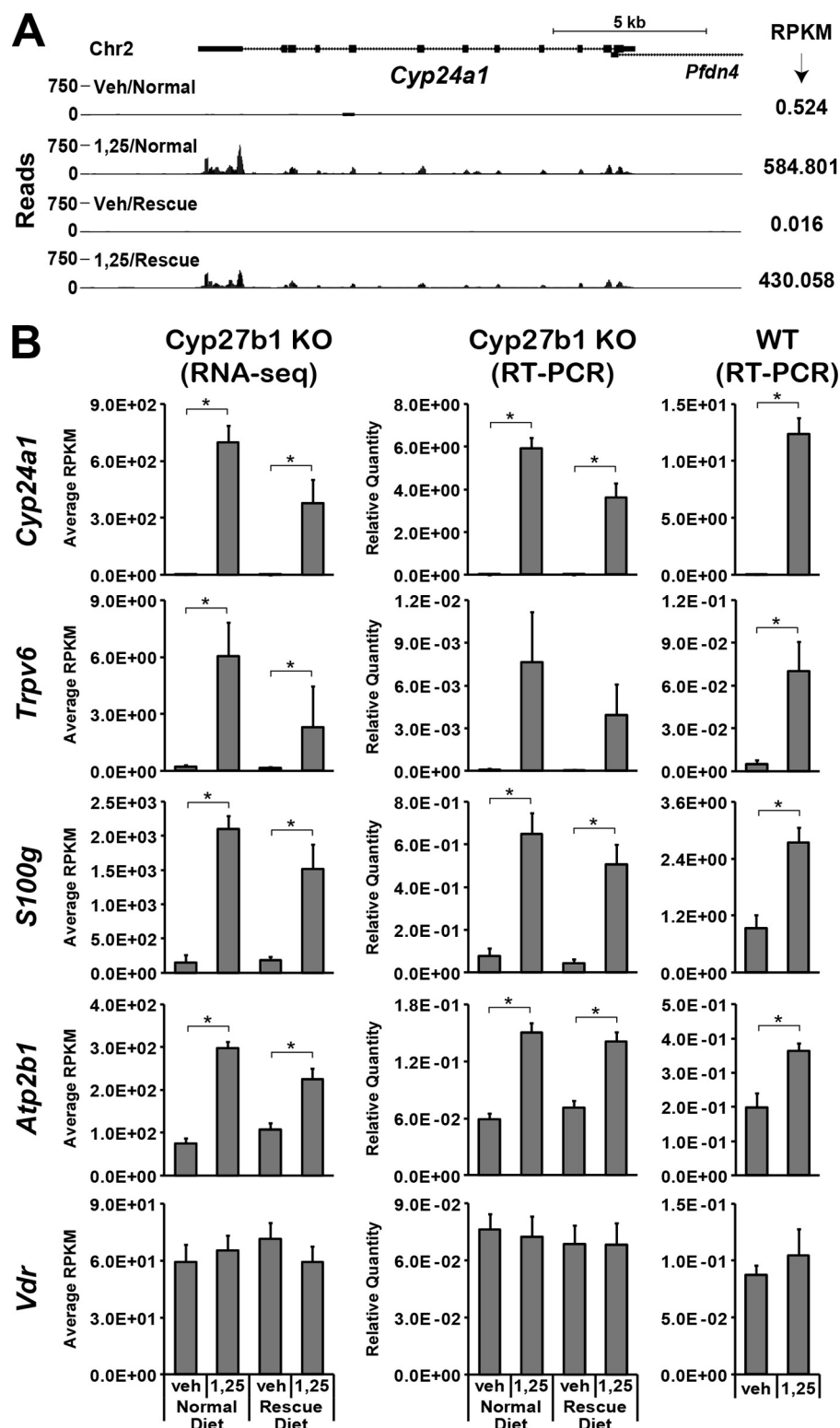


FIGURE 4. 1,25(OH)₂D₃ target gene expression in duodenum. *A*, genomically mapped reads and the RPKM of a representative sample from each experimental group at the mouse *Cyp24a1* gene are presented as examples. *B*, quantified 1,25(OH)₂D₃ target gene expression in the duodenum of each experimental group were measured by RNA-seq and are shown as the mean of RPKM \pm S.E. (left column; $n = 6$). The expression levels of the indicated genes in the duodenum of each experimental group of *Cyp27b1*^{-/-} mice (*Cyp27b1* KO; middle column; $n = 8$), and wild type mice (WT; right column; $n = 5$) were measured by RT-PCR and are presented as the mean \pm S.E. The gene expression quantified by RT-PCR was normalized by *Gapdh*. Significant differences between two experimental groups in RNA-seq were identified by Student's *t* test applied by ArrayStar Version 5 with Q-Seq module (*, $p < 0.05$). For RT-PCR data, Student's unpaired *t* test was used to identify significant differences between two groups (*, $p < 0.05$). veh, vehicle-treated sample; 1,25, 1,25(OH)₂D₃-treated sample.

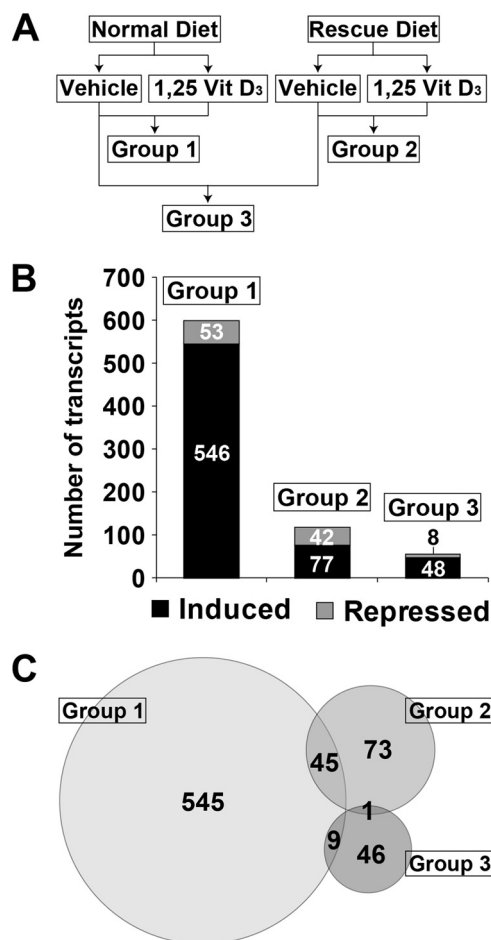


FIGURE 5. Identification of differentially expressed genes by 1,25(OH)₂D₃ or diet. A, genes we examined by RNA-seq were categorized according to their expression patterns. The genes altered by 1,25(OH)₂D₃ in normal and rescued diet groups were designated as groups 1 and 2, respectively. Genes that were changed by diet were designated as group 3. B, number of differentially expressed genes is shown in the graph. The genes in groups 1 and 2 are genes either induced (black) or repressed (gray) by 1,25(OH)₂D₃, and those in group 3 are genes expressed either more (induced; black) or less (repressed; gray) in rescue diet group than normal diet group. C, differentially expressed gene groups were compared with each other using a Venn diagram depiction. Each number represents the number of genes included only in a group or in both groups. No genes common to all three groups were identified. 1,25 Vit D₃, 1,25(OH)₂D₃.

S100g, or *Cldn2* that are believed to be involved in calcium transport. This result suggests that the mechanisms through which 1,25(OH)₂D₃ normalizes calcium and phosphate uptake into the blood and those through which the rescue diet normalizes calcium and phosphate are largely distinct. It is possible, however, that the effects of the rescue diet are not gene regulation-dependent.

GO Analysis of Genes Regulated by 1,25(OH)₂D₃ or Dietary Calcium and Phosphate Levels—We next performed GO analysis on these gene sets responsive to 1,25(OH)₂D₃ under the two dietary conditions as documented in their entirety in supplemental Table S1. As can be seen in Table 2, the top five GO terms in group 1 (18–25% of total genes) and group 2 (23–34% of total genes), as well as those that overlap between groups 1 and 2 were functionally related to ion, cation, metal, and transitional metal-binding proteins and integral/intrinsic plasma membrane proteins. Surprisingly, GO terms of genes in group 3

TABLE 1
Common genes between groups

Transcript ID	Gene Symbol	Fold Change		P Value	
		Group 1	Group 2	Group 1	Group 2
Common Genes to Group 1 and 2					
NM_009996.3	<i>Cyp24a1</i>	1157.57	2815.24	7.77E-07	1.29E-05
NM_022413.4	<i>Trpv6</i>	27.62	14.63	3.13E-04	1.46E-02
NM_021306.2	<i>Ecel1</i>	22.40	4.18	1.17E-02	1.77E-03
XR_104626.1	<i>Gm2061</i>	21.99	7.28	7.44E-05	1.45E-02
NM_020258.4	<i>Slc37a2</i>	14.96	6.33	4.14E-06	5.22E-04
NM_001145960.1	<i>Slc37a2</i>	14.96	6.33	4.14E-06	5.22E-04
NM_009789.2	<i>S100g</i>	14.38	8.23	1.37E-02	1.43E-03
NM_001033286.2	<i>Slc30a10</i>	9.40	4.33	1.35E-04	1.41E-02
NM_145978.1	<i>Pdlim2</i>	8.66	7.74	1.25E-05	4.09E-03
XR_104625.1	<i>9630028B13Rik</i>	6.39	4.08	5.18E-05	4.31E-03
NM_010359.1	<i>Gstm3</i>	5.51	3.08	6.65E-03	3.64E-02
NM_146117.2	<i>Lrrc26</i>	4.46	3.16	3.22E-03	1.08E-02
NM_026482.2	<i>Atp2b1</i>	3.98	2.10	5.28E-05	4.43E-02
NM_001013369.1	<i>Pf4n4</i>	3.53	2.83	5.52E-03	4.30E-03
NM_172428.2	<i>Ccdc134</i>	3.27	2.49	1.56E-03	1.55E-03
NM_001163314.1	<i>Pgap1</i>	3.00	2.72	4.90E-04	2.12E-06
NM_033073.3	<i>Krt7</i>	2.86	2.08	2.32E-03	1.09E-02
NM_007520.2	<i>Bach1</i>	2.70	1.92	1.08E-04	1.39E-02
NM_016675.3	<i>Cldn2</i>	2.68	2.11	1.56E-02	1.53E-02
XM_003084445.1	<i>LOC100504348</i>	2.60	2.62	7.01E-05	2.11E-05
NM_177667.4	<i>Ttc22</i>	2.58	2.58	6.33E-04	3.67E-02
NM_010180.2	<i>Fbhl1</i>	2.52	1.80	1.28E-03	2.72E-02
NM_009579.3	<i>Slc30a1</i>	2.46	1.93	5.46E-05	4.81E-03
NM_031396.2	<i>Cnnm1</i>	2.41	2.16	6.16E-03	4.12E-02
NM_001033273.2	<i>S031439G07Rik</i>	2.31	1.80	5.34E-05	5.38E-04
NM_026955.2	<i>2200002K05Rik</i>	2.23	1.97	3.41E-03	4.34E-03
NM_028104.3	<i>Ppp1r14d</i>	2.21	1.85	1.86E-03	2.80E-02
NM_008814.3	<i>Pdx1</i>	2.16	1.58	8.39E-03	2.78E-02
NM_001024703.1	<i>Mctp2</i>	2.15	2.03	2.57E-03	9.99E-03
NM_025998.3	<i>Nkain1</i>	2.14	2.19	3.13E-02	1.43E-02
NM_027238.2	<i>Ttc39b</i>	2.13	1.97	2.68E-02	1.27E-02
XR_105729.1	<i>9130004J05Rik</i>	1.87	1.65	2.35E-03	1.62E-04
NM_133819.3	<i>Ppp1r15b</i>	1.71	1.42	1.70E-03	2.29E-02
NM_001033135.3	<i>Rnf149</i>	1.70	1.68	3.05E-02	4.61E-03
NM_011145.3	<i>Ppard</i>	1.70	1.80	4.58E-02	1.13E-02
NM_008982.5	<i>Ptprrj</i>	1.69	1.69	1.57E-02	3.20E-02
NM_022885.2	<i>Slc30a5</i>	1.69	1.30	1.56E-02	1.46E-02
NM_199449.2	<i>Zhx2</i>	1.68	1.54	2.77E-03	2.16E-03
NM_025414.3	<i>Myo19</i>	1.68	1.72	1.13E-02	8.96E-03
NM_008684.2	<i>Neo1</i>	1.63	1.40	1.19E-02	9.37E-03
NM_018864.3	<i>Impa1</i>	1.62	1.34	3.70E-02	2.30E-03
NM_025824.3	<i>Bzw1</i>	1.60	1.53	4.42E-02	4.20E-05
NM_001085495.2	<i>Arfgef2</i>	1.52	1.43	4.40E-03	2.75E-03
NM_009566.4	<i>Zfp92</i>	0.55	0.55	3.34E-02	2.60E-02
NM_033269.4	<i>Chrm3</i>	0.53	0.71	3.74E-03	4.82E-02
Common Genes to Group 1 and 3					
NM_001177730.1	<i>Nr1h3</i>	4.93	4.95	1.92E-03	3.68E-03
NR_033210.1	<i>BC005764</i>	3.05	3.76	1.76E-02	1.26E-02
NM_008290.2	<i>Hsd17b2</i>	3.00	1.79	2.83E-04	4.34E-02
NM_197999.2	<i>2210023G05Rik</i>	1.82	1.84	1.45E-02	2.15E-02
NM_025797.3	<i>Cyb5</i>	1.46	1.29	7.70E-04	4.31E-02
NM_010044.2	<i>Dffa</i>	1.44	1.40	1.19E-02	3.61E-02
NM_013778.2	<i>Akr1e13</i>	1.38	1.57	1.38E-02	4.11E-03
NM_007661.3	<i>Cdk11b</i>	1.29	1.34	2.23E-02	4.66E-02
NM_001043322.1	<i>Fmn1</i>	0.80	0.81	8.19E-03	4.84E-03
Common Genes to Group 2 and 3					
NM_173740.3	<i>Maoa</i>	0.58	1.90	3.93E-02	2.47E-03

appeared to be involved in biological processes distinct from those involved in ion transport, including components of mitochondria, regulation of organelle organization, transcription factor binding and processes associated with oxidative reduction. To investigate these biological pathways further, we mapped regulated genes to KEGG pathways as documented in supplemental Table S1. As anticipated, a pathway involved in intestinal calcium uptake (ko04978; mineral absorption) composed, in part, of *Trpv6*, *S100g*, and *Atp2b1* was identified from genes expressed in groups 1 and 2 as well as those in common between the two groups. Of course, pathways unique to groups 1 and 2 were also identified, suggesting that dietary calcium and phosphate levels likely impact both basal levels of gene expression in the intestine as well as the expression of genes uniquely responsive to 1,25(OH)₂D₃ under these two conditions. Analyses of pathways associated with genes in group 3 suggest a minimal differential effect of diet on these gene regulatory pathways.

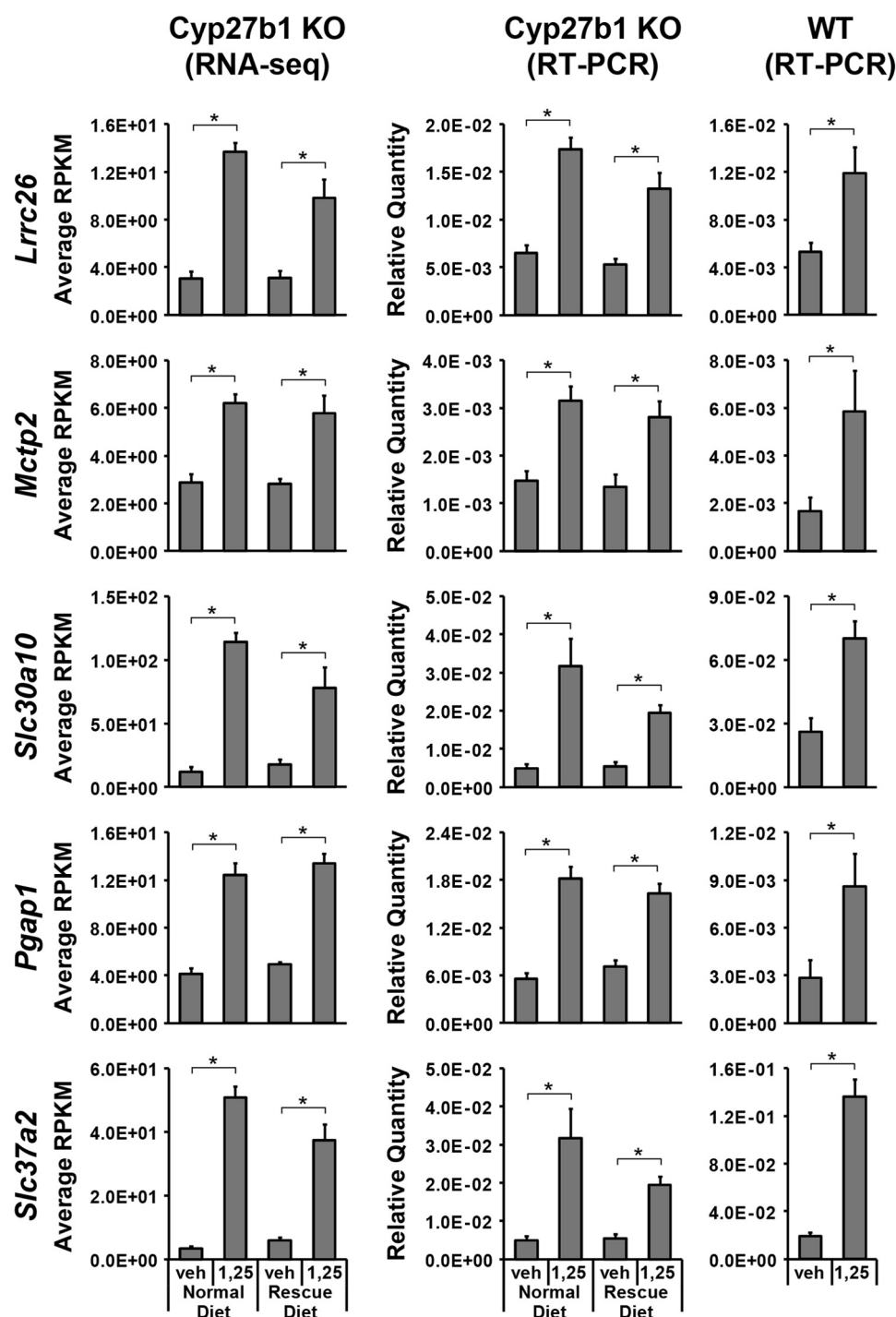


FIGURE 6. Expression of the genes identified as 1,25(OH)₂D₃-regulating genes by RNA-seq of duodenum from both normal and rescue diet-fed mice. Among the genes identified to be regulated by 1,25(OH)₂D₃ in duodenum of *Cyp27b1*^{-/-} mice (Cyp27b1 KO) regardless of diet condition through RNA-seq, the expression of the indicated genes are presented as the mean of RPKM \pm S.E. (left column; $n = 6$) and compared with the expression levels measured by RT-PCR using the same RNA samples and shown as the mean \pm S.E. (middle column; $n = 8$). The expression levels of the genes in duodenum of wild type (WT) mice were also measured by RT-PCR and are shown as the mean \pm S.E. (right column; $n = 5$). The gene expression quantified by RT-PCR was normalized by *Gapdh*. Significant differences between two experimental groups in RNA-seq were identified by Student's *t* test applied by ArrayStar Version 5 with Q-Seq module (*, $p < 0.05$). For RT-PCR data, Student's unpaired *t* test was used to identify significant differences between two groups (*, $p < 0.05$). veh, vehicle-treated sample; 1,25, 1,25(OH)₂D₃-treated sample.

Differential Regulation of VDR Target Genes in Intestinal Segments—Individual intestinal segments display unique as well as overlapping biological functions with respect to nutrient absorption (52). To examine whether a subset of genes belonging to groups 1 and 2 were differentially expressed across these

segments, we isolated RNA from the jejunum, ileum, and colon and contrasted the expression of a subset of genes common to groups 1 and 2 in these segments with that seen in the duodenum (Fig. 4) and with each other. As documented in Figs. 4, 6, and 7, although the basal levels of expression differed among all

TABLE 2Top GO terms of top five annotation clusters for the genes modulated by either 1,25(OH)₂D₃ or dietary condition

	Category	GO Term	<i>p</i> Value	Total No. of Genes (Induced/Repressed)
Group 1	Metabolic Function	GO:0043167~ion binding	1.2E-04	147 (140/7)
	Metabolic Function	GO:0043169~cation binding	9.3E-05	146 (139/6)
	Metabolic Function	GO:0046872~metal ion binding	1.4E-04	144 (136/8)
	Cellular Compartment	GO:0005886~plasma membrane	4.0E-03	105 (101/4)
	Metabolic Function	GO:0046914~transition metal ion binding	7.7E-03	95 (91/4)
Group 2	Cellular Compartment	GO:0031224~intrinsic to membrane	1.9E-02	40 (27/13)
	Cellular Compartment	GO:0016021~integral to membrane	1.9E-02	39 (27/12)
	Metabolic Function	GO:0043167~ion binding	6.7E-02	28 (20/8)
	Metabolic Function	GO:0046872~metal ion binding	8.6E-02	27 (20/7)
	Metabolic Function	GO:0043169~cation binding	9.4E-02	27 (20/7)
Group 3	Cellular Compartment	GO:0005739~mitochondrion	3.5E-02	7 (7/0)
	Biological Process	GO:0055114~oxidation reduction	1.1E-02	6 (6/0)
	Metabolic Function	GO:0008134~transcription factor binding	2.2E-02	4 (3/1)
	Biological Process	GO:0033043~regulation of organelle organization	4.0E-02	3 (1/2)
	Biological Process	GO:0032535~regulation of cellular component size	4.3E-02	3 (2/1)
Common to Group 1 & 2	Cellular Compartment	GO:0016021~integral to membrane	3.7E-02	19 (18/1)
	Cellular Compartment	GO:0031224~intrinsic to membrane	5.3E-02	19 (18/1)
	Metabolic Function	GO:0046872~metal ion binding	1.7E-02	16 (15/1)
	Metabolic Function	GO:0043169~cation binding	1.9E-02	16 (15/1)
	Metabolic Function	GO:0043167~ion binding	2.1E-02	16 (15/1)

the genes examined, key genes such as *S100g*, *Trpv6*, and *Atp2b1* were induced by 1,25(OH)₂D₃ in all segments of the intestine. In contrast, although *Lrrc26*, *Mctp2*, and *Slc30a10* were similarly regulated in each of the segments (Figs. 6 and 7), *Pgap1* and *Slc37a2* were found to be regulated largely in the duodenum and jejunum, less so in the ileum, and not regulated in the colon. Given the resolution of *Vdr* gene rescue studies in the intestine (proximal intestine includes the duodenum and jejunum, and the distal intestine includes ileum and colon) (53, 54), none of these genes can be ruled out as viable components of the vitamin D-dependent calcium-regulating network. This result suggests that the contribution of individual biological components that regulate calcium absorption within different segments of the intestine may also differ.

Properties of the VDR Cistrome in Vivo—The actions of 1,25(OH)₂D₃ to modulate the expression of genes comprising a functional calcium and phosphate regulation network as well as networks for additional functional activities in the intestine are mediated by the VDR, the transcription factor responsible for all of the actions of the hormone. Having identified the 1,25(OH)₂D₃-regulated transcriptomes in the intestines of mice under normal and rescue diet conditions, we conducted a ChIP-seq analysis of the VDR in proximal intestinal epithelial

cells to assess the locations of the *cis*-acting mechanisms through which these genes were regulated. As VDR binding is predominantly ligand-dependent (55), we utilized vitamin D-sufficient wild type mice treated with either vehicle or 1,25(OH)₂D₃ to assess both residual VDR binding in the normal vitamin D-sufficient state as well as the ability of exogenous 1,25(OH)₂D₃ treatment to perturb and increase VDR occupancy at these residual sites, and/or to promote increased recruitment of the VDR at newly occupied sites. As summarized in Fig. 8A and [supplemental Table S1](#), VDR was observed at 4617 sites across the genome in intestinal epithelial cells from vitamin D-replete mice, supporting the idea that VDR was indeed active at direct targets of action in the normal mouse intestine. Exogenous treatment with 1,25(OH)₂D₃ strikingly increased this number of sites almost 4-fold to 17,839, of which 4314 (93%) sites overlapped those identified under vehicle conditions alone. Importantly, *de novo* motif finding analysis, as documented in Fig. 8B, revealed that over 31–32% of these sites contained a DNA sequence typical of a consensus VDRE which is composed of two directly repeated hexameric half-sites separated by 3 bp (55). In addition, as seen in Fig. 8C, whereas many of these sites were located near the TSSs, the vast majority were located within intergenic regions or within introns many kilo-

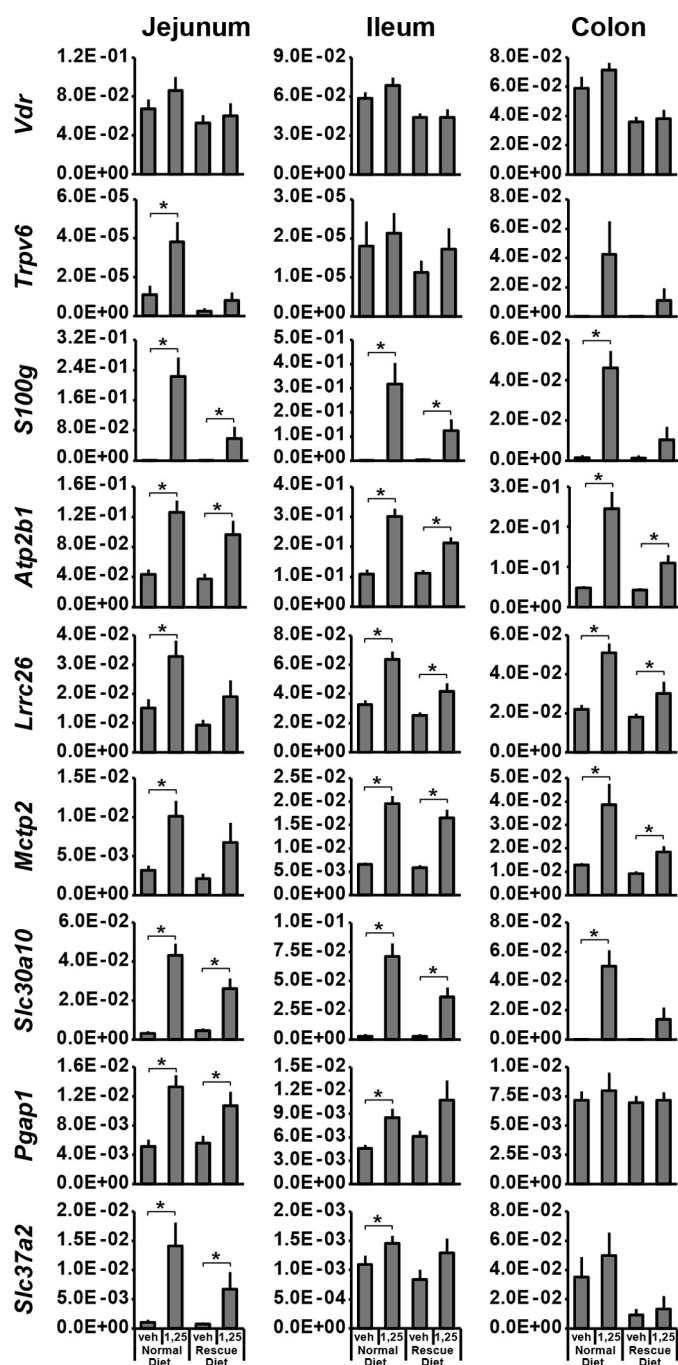


FIGURE 7. **Differential gene expression in intestinal segments.** The expression of the indicated genes in jejunum, ileum, and colon of each experimental group was measured by RT-PCR. y axis represents relative quantity of each gene to *Gapdh*. Data are presented as the mean \pm S.E. ($n = 8$). Student's unpaired *t* test was used to identify significant differences between two groups (*, $p < 0.05$). veh, vehicle-treated sample; 1,25, 1,25(OH)₂D₃-treated sample.

bases from this genetic landmark, an observation that has now been made for almost all transcription factors. These results suggest that although the VDR occupancy is fully active in the intestines of normal mice, the potential for additional gene modulation by 1,25(OH)₂D₃ via increased VDR levels at existing genetic targets or through the accumulation of VDR at *de novo* sites in response to added 1,25(OH)₂D₃ is also evident, likely revealing the full complement of direct sites of action by the VDR, highlighting a potential rheostatic effect.

Association of VDR Cistromic Sites with 1,25(OH)₂D₃-regulated Genes—VDR cistromic sites were linked through nearest neighbor analysis via the GREAT algorithm to 5011 and 10,643 potential target genes, respectively (Fig. 8A) (47). A thorough examination of these sites relative to all genes expressed in the mouse intestine is likely to reveal distinct and perhaps unexpected associations. Simple genetic proximity does not provide definitive evidence that a gene and an adjacent enhancer or enhancers are linked, however, due to the three-dimensional nature of regulatory domains and the discovery that regulatory regions are frequently located distal to the genes they regulate. As a result of this, we chose to explore the relationship between the genes that were shown to be regulated by 1,25(OH)₂D₃ in the *Cyp27b1*^{-/-} mice and components of the cistromic VDR sites. As summarized in Fig. 8D, 500 of the 599 genes (83%) regulated by 1,25(OH)₂D₃ and contained in group 1 and 91 of the 119 genes (76%) identified in group 2 were present in the cohort of potential target genes identified via GREAT. Perhaps more importantly, 39 of the 45 genes (87%) that were common to groups 1 and 2 and that represent the proposed network of genes involved in calcium and phosphate regulation genes were found in this group of GREAT-identified targets as well. In contrast, only 23 of 56 genes (41%) in group 3 were observed in the gene collection identified by GREAT. This result suggests that a high percentage of the gene subsets regulated by 1,25(OH)₂D₃ in *Cyp27b1*^{-/-} mice fed either the normal or rescue diet as well as those regulated by 1,25(OH)₂D₃ in common with both dietary conditions all represent direct genetic targets of VDR action. Interestingly, as documented in Fig. 8B, response elements for the transcription factors hepatocyte nuclear factor 4 (HNF4A and HNF4G) and Gata4 are also enriched in or near the binding sites for the VDR, suggesting that they may bind to these 1,25(OH)₂D₃-regulated regions and contribute to expression as well.

VDR Binding Near S100g, Atp2b1, and Cldn2 Resolve Sites of Potential Regulation by 1,25(OH)₂D₃—Early efforts to identify the locations of the regulatory regions of genes such as *S100g* and *Calb1* as well as *Atp2b1* and *Cldn2* and to prove that these genes were direct targets of vitamin D activation were largely unsuccessful (56, 57), primarily because existing technologies limited exploration almost exclusively to the promoter regions of genes. This limitation was not a condition of the unbiased analysis documented here, however, enabling us to explore the loci of these and other genes for VDR-binding sites located within their vicinity. Fig. 9 depicts ChIP-seq data tracks, which document VDR binding at the loci for both *Cyp24a1* and *Trpv6*, two genes whose regulatory components are now well known, and at the loci for *S100g*, *Atp2b1*, and *Cldn2* as well. As can be seen in the Fig. 9A, *Cyp24a1* is marked by both proximal elements located near the gene's promoter that were identified very early (58, 59) and by a cluster of recently identified distal sites located +35 to +37 kb downstream of the gene (60, 61). *Trpv6*, however, is characterized by a collection of upstream sites at -2 and -4 kb that was also identified recently (31). Importantly, extensive functional analysis has confirmed that each of these sites are directly linked to the regulation of *Cyp24a1* and *Trpv6* by 1,25(OH)₂D₃, respectively (31, 60). Residual or induced binding sites for the VDR in vitamin D-suf-

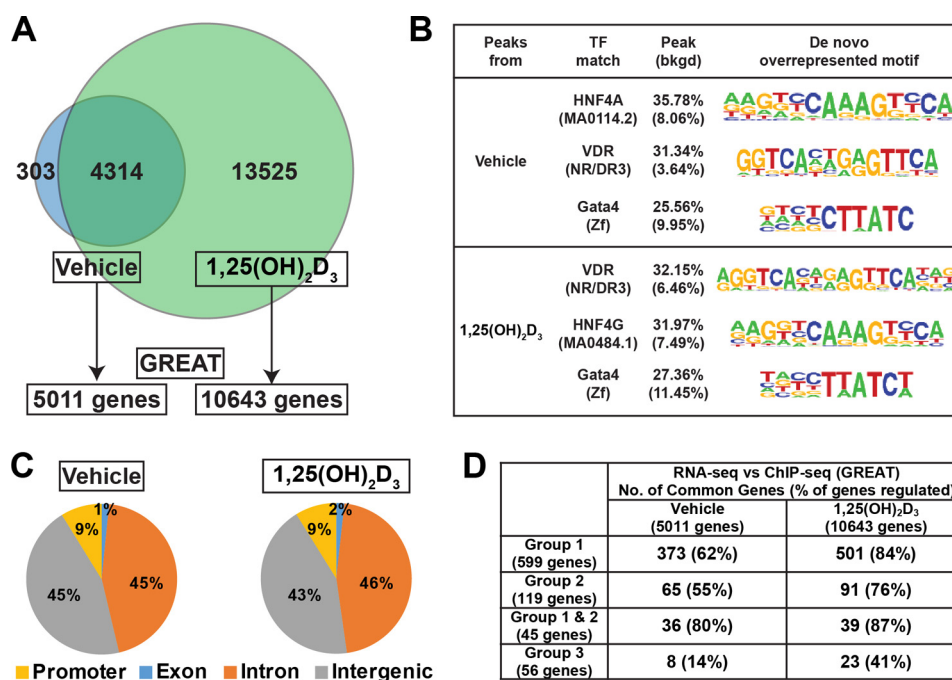


FIGURE 8. Properties of the intestinal VDR cistrome. A, VDR-binding sites in the proximal small intestine from vehicle- or 1,25(OH)₂D₃-treated wild type mice were compared with each other using a Venn diagram depiction. Each number indicates the number of VDR-binding peaks in each experimental group or both groups. The numbers of the genes associated with the VDR-binding sites in each experimental group were identified through GREAT algorithm. B, *de novo* motif for VDR-binding peaks was assessed by HOMER, and the top three over-represented motifs in each experimental group are presented. C, locations of the VDR-binding peaks in each experimental group were defined as exon, intron, intergenic (> -500 bp or any distance downstream of 3'-untranslated region), and promoter (within -500/+500 bp). D, regulated genes from RNA-seq analysis (groups 1–3 and common genes to groups 1 and 2) were compared with the genes associated with VDR bindings through GREAT algorithm. The number of genes common to the genes identified by both RNA-seq and ChIP-seq analyses and the percentage of the regulated genes are presented.

ficient mice can also be seen upstream of the *S100g* promoter at -3, -6, and -23 kb and upstream of the *Atp2b1* promoter at -0.3, -17, and -35 kb as well (Fig. 9B). Importantly, occupancy at some of these sites is increased in response to exogenous 1,25(OH)₂D₃ treatment. Interestingly, as seen in the Fig. 9C, VDR-binding sites within the locus for *Cldn2* are similarly observed not only upstream of the promoter at -6 kb but downstream of the gene at +5 kb and +13 kb as well. Although these sites together with those identified in the *S100g* and *Atp2b1* gene loci likely represent sites of active 1,25(OH)₂D₃ regulation for their cognate genes, additional experimental evidence will be required to confirm this hypothesis.

VDR-binding Sites Are Enriched Near Components of the Proposed Gene Network Involved in Vitamin D-dependent Calcium Absorption and Deficient at Genes Regulated by Diet—Table 3 summarizes sites of VDR occupancy in vitamin D-sufficient normal mice at genes that comprise the proposed network involved in calcium absorption both in the absence and presence of exogenously added 1,25(OH)₂D₃. 39 of these 45 genes (87%) contain sites present within several hundred kilobases of their associated TSSs that are statistically valid in the three replicates and most contain sites within 50 kb. With a few exceptions, all of these sites represent newly identified regions that are likely to contribute in some manner to the associated gene's regulation by 1,25(OH)₂D₃. The presence of VDR sites at or near these genes supports their inclusion as direct targets of vitamin D action in the intestine *in vivo* and hypothetically as comprising a network of genes that includes those involved in the metabolism and transport of calcium across the intestinal

epithelial cell and into the blood. In contrast, only 23 of the 56 genes (41%) regulated by diet contain VDR-binding sites, although as expected those few genes that overlap the cohorts in groups 1 and 2 (9 and 1 genes, respectively) exhibited a much higher frequency of binding sites for the VDR (supplemental Table S1).

Discussion

Despite many decades of effort, the components and mechanisms that facilitate vitamin D-dependent calcium absorption from the gut remain to be resolved. In this report, we examined the effects of 1,25(OH)₂D₃ on the intestinal transcriptome of *Cyp27b1*^{-/-} mice that were maintained on diets that resulted in either a hypocalcemic or a normocalcemic state. As 1,25(OH)₂D₃ can induce hypercalcemia in both of these conditions, we reasoned that genes that were regulated by 1,25(OH)₂D₃ under these conditions likely represented potential candidates responsible for vitamin D-regulated calcium absorption. We discovered an overlapping set of 1,25(OH)₂D₃-induced target genes in the duodenum that was comprised of several previously described mediators of intestinal calcium absorption, a number of genes whose functions included participation in cellular calcium uptake, at least one gene known to be involved in passive paracellular movement of calcium, and a variety of additional genes that facilitate the regulation of intracellular calcium. Genes that are involved in other vitamin D functions were also found. We hypothesize that in contrast to a single gene target whose identity has long been the subject of vitamin D investigation, this group may actually contain a com-

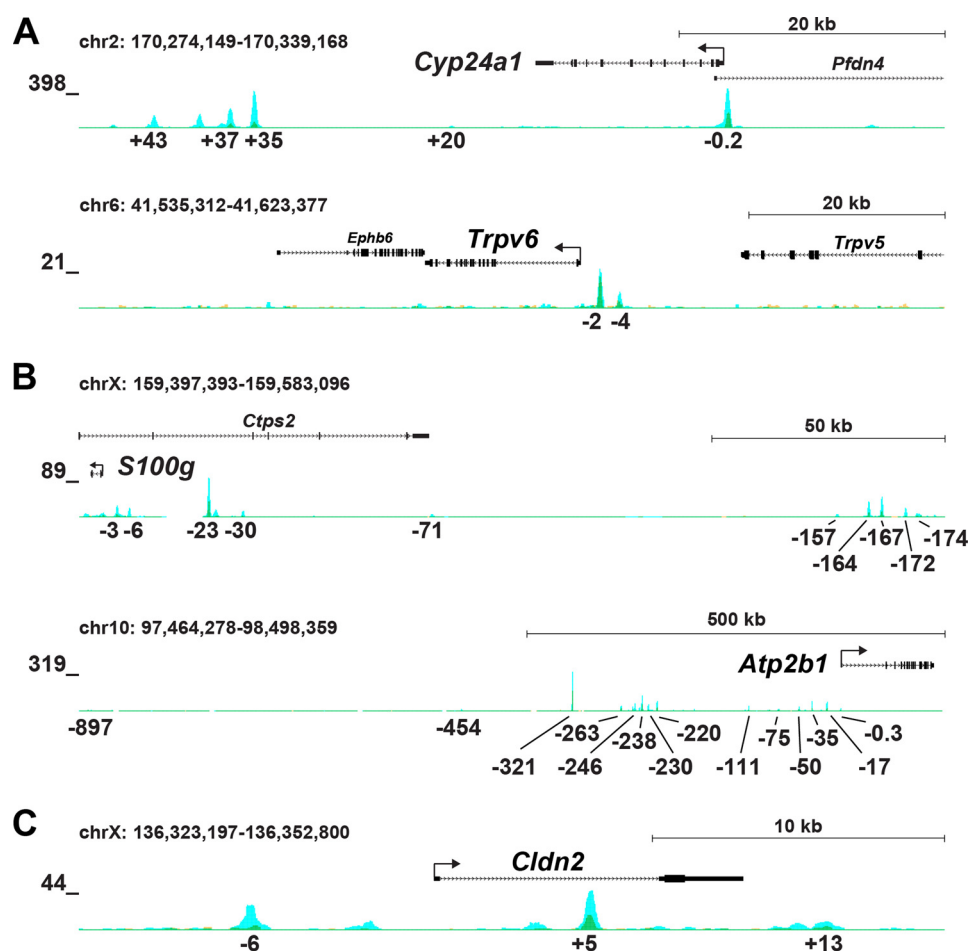


FIGURE 9. ChIP-seq analysis of the VDR at regulated target genes. Representative ChIP-seq tracks of triplicate samples at the gene locus for *Cyp24a1* and *Trpv6* (A), *S100g* and *Atp2b1* (B), and *Cldn2* (C) are presented as tag density tracks normalized to input and 10⁷ tags. Exons and introns are shown in boxes and lines, respectively. Transcriptional direction of each gene is indicated by an arrow at the TSS and genomic location and scale are provided above. Maximum height of tag sequence density for the data track is indicated on the y axis. VDR bindings in vehicle- and 1,25(OH)₂D₃-treated samples are shown in yellow and blue, respectively. Overlapped VDR binding is shown in green. Peak regions that are statistically significant and associated with the genes through GREAT algorithm are indicated by the approximate distance (kilobase) from TSS below.

prehensive network of vitamin D-sensitive genes that are involved not only in epithelial calcium uptake but in the dynamic maintenance of intracellular calcium metabolism during the transport process as well. Surprisingly, the small subset of genes that was involved in rescuing the hypocalcemic phenotype associated with vitamin D deficiency via dietary calcium alone contained only a few of the genetic components found in this vitamin D-sensitive collection. Additional mechanistic studies using ChIP-seq analysis to localize the *cis*-regulatory components of these genes revealed that the majority of the genes that belonged to this calcium absorption network were direct targets of vitamin D action by virtue of VDR occupancy at regulatory sites adjacent to these genes. To the contrary, aside from the few genes that were modulated by both dietary calcium and 1,25(OH)₂D₃, those that were regulated exclusively by diet were deficient in binding sites for the VDR. We conclude that these studies have identified a collection of genes that participates in the overall vitamin D-regulated calcium absorption process.

Several concepts support the idea that a network of genes rather than a single element whose deletion might mimic vitamin D deficiency may be involved in vitamin D-dependent cal-

cium absorption. First, the uptake of calcium by the intestine is composed of a complex process involving both vitamin D-independent and -dependent processes (62, 63). Surprisingly, the latter makes only a small, albeit critical, contribution to the maintenance of organismic calcium homeostasis by the intestine. Second, this idea is reinforced by the fact that although numerous investigations over many years have identified multiple components that are regulated by 1,25(OH)₂D₃ and that contribute to calcium uptake, they have failed to identify a single component that upon deletion fully recapitulates the state of vitamin D deficiency. *Trpv6* expression, for example, exerts a significant impact on calcium transport independent of its regulation by 1,25(OH)₂D₃, yet it fails upon deletion to block intestinal response to 1,25(OH)₂D₃ (15, 29). Overexpression of calbindin D9K, however, has no impact on intestinal calcium absorption, and *Pmca1* has not been examined. Third, the proposed mechanism through which calcium is acquired from the diet involves at least three components, each of which contributes to the unique process that is postulated to orchestrate the transepithelial uptake of calcium into the blood. Some level of redundancy may be present with regard to these components as well, particularly those associated with a calbindin D9K-like

TABLE 3

VDR-binding sites in common genes to groups 1 and 2

Gene symbol	Peak center location (approximate distance from transcription start site; kilobase)	
	Vehicle	1,25(OH) ₂ D ₃
<i>Cyp24a1</i>	-0.3, +35, +37, +43	-0.2, +20, +35, +37, +43
<i>Trpv6</i>	-2	-4, -2
<i>Ecel1</i>	+12	-21, +0.7, +4, +12
<i>Slc37a2</i>	+9	-14, +1, +9
<i>S100g</i>	-167, -164, -23, -3	-175, -172, -167, -164, -157, -71, -30, -23, -6, -3
<i>Slc30a10</i>	-6, -4, +29, +48, +54, +61, +157	-30, -4, -0.1, +8, +15, +21, +29, +32, +42, +48, +54, +61, +133, +157, +161
<i>Pdlim2</i>	-2, +1	-29, -2, +1
<i>Gstm3</i>	-9, -5	-9, -5, -0.1, +19
<i>Lrrc26</i>	-3	-3
<i>Atp2b1</i>	-335, -321, -263, -238, -220, -35, -17	-896, -629, -454, -321, -263, -249, -246, -238, -230, -220, -176, -111, -87, -86, -76, -75, -50, -43, -35, -17, -0.3, +60
<i>Pfdn4</i>		-3
<i>Ccdc134</i>	-9, +11	-41, -19, -9, +11
<i>Pgap1</i>	-214, -55, -43, -24	-355, -214, -200, -121, -95, -55, -44, -43, -24, +12
<i>Krt7</i>	+72	-14, +80
<i>Bach1</i>	-96, -68, -18, +42	-126, -101, -96, -95, -68, -59, -28, -18, +27, +42, +374, +498
<i>Cldn2</i>	-6, +5	-6, +5, +13
<i>Ttc22</i>	+7	-0.7, +7
<i>Fbln1</i>	+1, +10, +26	+1, +10, +25, +26, +27, +34, +39
<i>Slc30a1</i>	-67, -66, -60, -26, -15	-67, -66, -60, -31, -26, -15, -5, +58
<i>Cnm1</i>	+13, +59	+13, +59, +72, +74
<i>5031439G07Rik</i>	-0.5	-24, -14, -0.5, +59
<i>2200002K05Rik</i>	+0.7, +7	-27, +730, +6
<i>Ppp1r14d</i>	-0.2, +1	-0.2, +1
<i>Pdx1</i>		-34, -9, +29, +30
<i>Mctp2</i>	-213, +8	+8, +14
<i>Nkain1</i>	+5, +21, +71	-80, -58, +5, +13, +20, +54, +68, +71
<i>Ttc39b</i>	+7, +20	-4, -71, +7, +9, +20, +41, +82
<i>Ppp1r15b</i>	-80, -73, -7, -0.2	-80, -73, -48, -47, -12, -7, -0.2, +23, +60, +81, +100
<i>Rnf149</i>	+3	-30, +3
<i>Ppard</i>	-2, +35, +48, +55	-2, +35, +39, +48, +55
<i>Ptpri</i>	-61, +84, +92, +106	-88, -69, -61, -2, +15, +43, +68, +84, +92, +101, +105, +139
<i>Slc30a5</i>	-0.9, -10	-928, -920, -905, -891, -865, -689, -648, -479, -314, -279, -258, -23, -10, -5, +3
<i>Zfx2</i>	-66, -2, +11	-85, -66, -2, +11, +67, +144, +195, +196
<i>Neo1</i>	-235, -118	-242, -235, -148, -118, -104, -71, -47, -0.5, +47
<i>Impa1</i>	+16	+0.2, +16
<i>Bzw1</i>	-92, -7, +29	-92, -88, -86, -47, -39, -34, +29
<i>Arfgef2</i>	+29	+17, +29
<i>Chrm3</i>		-85, +311, +587

capability to bind calcium. Fourth, the regulation of *Cldn2* by 1,25(OH)₂D₃ suggests that vitamin D may contribute to an ancillary mechanism of calcium uptake that is diffusion-mediated and fully independent of that which mediates the transepithelial uptake of calcium (64). Fifth, a number of genes regulated by 1,25(OH)₂D₃ independent of calcium homeostasis and identified in this study are also involved in calcium regulation. Many of these genes retain membrane calcium transport activity despite the fact that their primary functional activity may be associated with the transport of other ions and/or nutritional components (65–72). A subset of these genes also participates in the regulation of intracellular calcium metabolism (71, 73), activities that may be critical to the maintenance of calcium homeostasis in the presence of large quantities of calcium transiting the enterocyte. Finally, although many of the genes identified here are expressed and regulated by vitamin D across all segments of the intestine, others are regulated by vitamin D only in a subset of these segments. Recent studies have suggested that calcium absorption may not represent the sole purview of the duodenum but rather a functional component of more distal segments of the intestine as well (54). Thus, it is possible that the mechanisms that underlie the uptake of calcium throughout the length of the intestine may manifest both distinct as well as overlapping components. Accordingly, the

relative contribution of each participating process may differ and be determined uniquely by additional factors such as *in situ* calcium gradients and by the relative presence of the individual components involved in the calcium uptake process. Further delineation of the contribution of each of the individual participants identified in this study will be necessary to gain a full appreciation of the actions of vitamin D in the intestine.

Interestingly, genes involved in rescuing calcium homeostasis and normalizing blood calcium and phosphate in the *Cyp27b1*^{-/-} mouse through the diet were not associated directly with the genes linked to calcium absorption following 1,25(OH)₂D₃ treatment, and those that were modulated by diet did not retain functions linked to calcium metabolism. This observation was surprising in view of the fact that a previous study has suggested that *Trpv6* and *S100g*, for example, are both regulated following exposure to a high calcium rescue diet (11), although a suppression rather than an induction was seen. The nutritional status, strain, and age were also different. It is possible that the RNA-seq analysis conducted here was unable to detect these changes or that they were below our statistical cutoff, although it is notable that our analysis was restricted to the duodenum. It is also possible, however, that normalization of calcium by the rescue diet does not involve the up-regulation of genes, but rather simple diffusion via paracellular pathways

or activation through other pathways that already exist within the intestine itself. It is also worth noting that although the rescue diet has been utilized to normalize deranged mineral levels in the vitamin D-deficient state and in genetic models of *CYP27B1*^{−/−} and *VDR*^{−/−}, this process takes considerable time in mice *in vivo*, and the levels of hormones such as PTH and FGF23 can remain elevated for several months or more (49, 74). Importantly, although receptors for these two hormones are not found in the intestine, they are present in kidney and bone, and thus may also participate in these tissues in an attempt to accommodate less efficient uptake of calcium from the gut. Clearly, a better understanding of the mechanisms that underlie the ability of the rescue diet to normalize mineral levels in the blood is necessary.

Our studies using ChIP-seq analysis provide the first examination of the distribution of the VDR at genomic sites under a normal vitamin D-sufficient state and show that acute exogenous administration of 1,25(OH)₂D₃ not only increases the level of occupancy at these sites, but reveals a 4-fold increase in the accumulation of VDR-binding sites as well. This suggests the possibility that the regulation of genes by changing levels of systemic 1,25(OH)₂D₃ is graded by virtue of both an increase in VDR occupancy at sites of regulation but also through *de novo* occupancy of the VDR at additional sites linked to the same gene target. Thus, regulation of gene expression may involve the accumulation of focal as well as dispersed VDR binding activity within the gene loci. Nevertheless, the extent to which VDR occupancy at these additional sites in response to high blood levels of 1,25(OH)₂D₃ contributes to the expression of the genes to which they are linked remains to be determined. Interestingly, although many of these sites are unique to genes expressed in the intestine, the overall properties of the VDR cistrome are generally similar to those identified in other cell types (40, 75). For example, most sites are enriched for VDREs, are located within intronic and intergenic regions at considerable linear distances from the genes they regulate, and comprise a population of several distinct regulatory elements that function to control the expression of a single gene target. Although it will be necessary through direct experimentation to confirm the precise gene targets of many of these distally located enhancers, our previous work with several of the 1,25(OH)₂D₃-regulated genes identified in this study has unequivocally validated this concept. Thus, for example, we have shown that several VDR-binding sites discovered distal to the *Cyp24a1* and *Trpv6* gene loci are functionally linked directly with those specific genes (31, 60). Perhaps most importantly, VDR binding is highly enriched within loci for genes that comprise both the calcium-regulating network as well as additional genes that are regulated by 1,25(OH)₂D₃ yet deficient at genes that are regulated by diet alone, providing additional evidence that these genes represent direct targets of vitamin D action. Indeed, the identification of potential regulatory sites within loci for genes such as *S100g* and *Atp2b1* not only resolve the question of whether they represent direct targets of vitamin D action but also identify the locations of the possible regulatory sites as well. Interestingly, although an early transgene containing only a reporter linked to proximal segments of the rat *S100g* promoter was neither targeted to the intestinal tract nor responsive to

1,25(OH)₂D₃, a larger 4.4-kb construct containing a hormone-regulated hypersensitive site ~3 kb upstream of the *S100g* gene TSS displayed both features in transgenic mice (56). This early analysis is consistent with the observed VDR binding activity seen in this study upstream of the mouse *S100g* at −3 kb, although additional sites of VDR binding were evident further upstream (−6 kb and −23 kb) as well.

The identification of a number of genes that belong to the network prompted us to explore the properties of many of these genes to determine their functional roles. Moreover, many of these genes have been genetically deleted in mice, providing the opportunity to assess whether they yield a vitamin D deficiency-type phenotype, although the lack of a complete vitamin D deficiency phenotype in mice containing deletions of *S100g*, *Trpv6*, or their combination and the more complex contributions of a gene network suggest that individual deletions may not reveal this type of deficiency phenotype (14, 29). A number of genes regulated by 1,25(OH)₂D₃ in addition to *S100g*, *Trpv6*, *Atp2b1*, and *Cldn2* have been suggested to be involved in calcium regulation, although their primary function may be different. For example, ZnT10 (*Slc30a10*) was recently reported as a manganese efflux transporter (72); *Cnnm1* encodes a copper-binding protein (70); IMPase 1 (*Impa1*) is involved in the inositol monophosphate pathway participating in calcium signaling (65); *Lrrc26* encodes a channel accessory subunit involved in voltage-dependent gating in membrane hyperpolarization facilitating calcium influx (71); *Mctp2* encodes a protein with a C2 calcium binding domain that may play a role as a potential exocytotic calcium sensor (69); *Pdlim2* is involved in cell attachment and migration (76), and R-PTP-J (*Ptprj*), a J-type PTP is involved in the Ras pathway to reduce the increase of intracellular calcium (67). The induction of the zinc transporters *Slc30a1* and *Slc30a5* by 1,25(OH)₂D₃ may also be important as it has been suggested that calcium and zinc compete for common ion channels (77) and that dietary calcium levels may affect the intestinal absorption of zinc (78, 79). Indeed, it has been reported that 1,25(OH)₂D₃ induces zinc transport in the human intestinal colon cancer cell line Caco-2 (80) and in pig intestinal tissue (77). Zinc transporter 1 (ZnT1) encoded by *Slc30a1* is expressed in various tissues, including intestine, and is localized to the basolateral membrane of enterocytes in rats, suggesting a possible role for this protein in zinc transfer into blood (66). Zinc transporter 5 (ZnT5) encoded by *Slc30a5* was shown to be expressed in mouse small intestine (81), and its expression at the apical membrane of human small intestine has been shown (82). Studies of ZnT5 null mice reveal a highly complex phenotype that includes osteopenic defects caused by impairment of osteoblast maturation (68). Several additional zinc transporters encoded by *Slc30a2* and *Slc39a4* and abundantly expressed in the intestine (83–85) were also induced by 1,25(OH)₂D₃ in the normal diet-fed group, although those encoded by *Slc30a1* and *Slc30a5* were induced in both normal and rescue diet-fed mice. Taken together with our data, these studies suggest that the induction of the expression of the zinc transporters by the 1,25(OH)₂D₃/VDR system may increase intestinal absorption of either zinc, calcium, or both. These and additional genes may all contribute in variable ways across the movement of calcium across the intestinal mucosa. It is also

possible that some of the genes found within this network are involved indirectly in calcium regulation and are therefore not annotated as such. Finally, it is also likely that subsets of these 1,25(OH)₂D₃-regulated genes are linked to activities known to be controlled in the intestine by this hormone independent of calcium and phosphate homeostasis.

In conclusion, we show herein that 1,25(OH)₂D₃ induces a network of genes independent of dietary calcium and phosphate status that is composed of genes that are known to regulate the transepithelial uptake of calcium, participate in paracellular uptake of calcium, and are involved in intracellular metabolism of calcium as well. The majority of the components of this network of genes represent unequivocal direct targets of 1,25(OH)₂D₃ action by virtue of the presence of 1,25(OH)₂D₃-activated VDR at sites adjacent to these genes that likely represent regulatory enhancers. We hypothesize that components of this complex network of genes may contribute in an overlapping manner to the overall calcium absorption process across the intestinal tract.

Author Contributions—S. M. L. and J. W. P. conceived the study and wrote the paper. S. M. L. performed the experiments with animals and organized all data. E. M. R. provided technical assistance for the mouse work. M. B. M. processed and analyzed RNA-seq and ChIP-seq data. N. A. B. provided technical assistance for ChIP and preparation of the sequencing library. L. A. P. and H. F. D. contributed to interpretation of data. All authors reviewed the results and approved the final version of the manuscript.

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