

Vitamin D receptor regulates autophagy in the normal mammary gland and in luminal breast cancer cells

Luz E. Tavera-Mendoza^{a,b,1}, Thomas Westerling^{a,b,1}, Eric Libby^c, Andriy Marusyk^d, Laura Cato^{a,b}, Raymundo Cassani^e, Lisa A. Cameron^f, Scott B. Ficarro^g, Jarrod A. Marto^g, Jelena Klawitter^h, and Myles Brown^{a,b,2}

^aDivision of Molecular and Cellular Oncology, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215; ^bCenter for Functional Cancer Epigenetics, Dana-Farber Cancer Institute, Boston, MA 02215; ^cSanta Fe Institute, Santa Fe, NM 87501; ^dDepartment of Cancer Imaging and Metabolism, Moffitt Cancer Center, University of South Florida, Tampa, FL 33612; ^eInstitut National de la Recherche Scientifique, Centre Énergie, Matériaux, Télécommunications, University of Quebec, Montreal, QC H5A 1K6, Canada; ^fConfocal and Light Microscopy Core, Dana-Farber Cancer Institute, Boston, MA 02215; ^gBlais Proteomics Center, Dana-Farber Cancer Institute, Boston, MA 02215; and ^hDepartment of Anesthesiology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045

Contributed by Myles Brown, January 18, 2017 (sent for review September 8, 2016); reviewed by Mitchell A. Lazar and Donald P. McDonnell

Women in North America have a one in eight lifetime risk of developing breast cancer (BC), and a significant proportion of these individuals will develop recurrent BC and will eventually succumb to the disease. Metastatic, therapy-resistant BC cells are refractory to cell death induced by multiple stresses. Here, we document that the vitamin D receptor (VDR) acts as a master transcriptional regulator of autophagy. Activation of the VDR by vitamin D induces autophagy and an autophagic transcriptional signature in BC cells that correlates with increased survival in patients; strikingly, this signature is present in the normal mammary gland and is progressively lost in patients with metastatic BC. A number of epidemiological studies have shown that sufficient vitamin D serum levels might be protective against BC. We observed that dietary vitamin D supplementation in mice increases basal levels of autophagy in the normal mammary gland, highlighting the potential of vitamin D as a cancer-preventive agent. These findings point to a role of vitamin D and the VDR in modulating autophagy and cell death in both the normal mammary gland and BC cells.

vitamin D | vitamin D signaling | autophagy | breast cancer | oncology

Breast cancer (BC) is the most common nonskin cancer diagnosed in North American women, accounting for 29% of newly diagnosed cancers in the United States (1). Despite considerable recent progress in the development of BC therapeutics, a significant proportion of patients with BC will eventually develop resistance to therapy and relapse (2), making BC the second most common cause of cancer-related deaths in US women. Several molecular subtypes of BC have been identified: luminal A and B (accounting for 50–60% of BC), basal-like or triple-negative (10–20% of BC cases), and human epidermal growth factor receptor 2 (HER2)-enriched (10–15% of cases) (3). In the United States alone, nearly 41,000 BC-related deaths are estimated for 2015 (1). The risk for developing BC is modulated by a number of factors (age, early menarche, family cancer history, ethnicity, and late menopause). However, modifiable risk factors, resulting from the patient's behavior and environment, can also impact the relative risk for developing BC. These risk factors include postmenopausal hormone use, alcohol and tobacco consumption, exercise, obesity and weight gain, and dietary components such as vitamin D.

Epidemiological data indicate that circulating vitamin D levels (serum levels of ≥ 45 ng/mL; achieved with a daily intake of $\sim 4,500$ international units (IU) in winter) protect against BC (4) (1). Furthermore, a prospective cohort study showed that sufficient vitamin D serum levels in patients with luminal BC correlate with better response to therapy as well as improved disease-free survival (5). However, because of the lack of randomized clinical trials on the use of vitamin D to prevent cancer (6), the Institute of Medicine stated in their most recent controversial report (7–9) that no evidence exists to justify an increase in the recom-

mended levels of vitamin D supplementation (600 IU) and called for additional mechanistic studies of vitamin D in cancer models (10). Because most women in North America are deficient in vitamin D (11), elucidating the functional mechanistic roles of vitamin D and of the vitamin D receptor (VDR) in BC models and prevention is of paramount importance.

Vitamin D is a secosteroidal prohormone, and arguably not a true vitamin, because it can be synthesized at sufficient levels in skin, given adequate skin exposure to UV B radiation from sunlight; in fact, humans naturally can make up to 140 ng/mL in their skin per day (12). The “vitamin” misnomer originates from its discovery as the key antirachitic compound in cod liver oil in 1922. Cod liver oil had been used effectively to prevent and treat rickets, a childhood disease characterized by defective calcification of bones (13). Since its discovery, vitamin D is still best known for its role as a key regulator of the homeostasis of calcium and a requirement for bone health. However, growing experimental and epidemiological data point to a wide range of biological properties of vitamin D. The VDR is expressed in a wide variety of tissues unrelated to calcium homeostasis, and vitamin D has pleiotropic effects that range from immune system modulation, to nervous and muscular system regulation, and (notably) to arrest of cancer cell proliferation (14). However, numerous studies have suggested that vitamin D serum levels required to achieve most of these physiological benefits are higher (30–60 ng/mL) than levels required for achieving bone health (~ 20 ng/mL) (4, 15).

Significance

Epidemiological evidence suggests that vitamin D can protect women from developing breast cancer (BC). This study reveals that vitamin D and its receptor regulate autophagy in both normal mammary epithelial cells and luminal BCs, and suggests a potential mechanism underlying the link between vitamin D levels and BC risk. In addition, this work suggests that vitamin D receptor ligands could be exploited therapeutically for the treatment of a significant subset of BCs.

Author contributions: L.E.T.-M. and M.B. designed research; L.E.T.-M., A.M., L.C., L.A.C., S.B.F., and J.K. performed research; A.M., L.A.C., and J.K. contributed new reagents/analytic tools; L.E.T.-M., T.W., E.L., L.C., R.C., S.B.F., J.A.M., and M.B. analyzed data; and L.E.T.-M. and M.B. wrote the paper.

Reviewers: M.A.L., Perelman School of Medicine at the University of Pennsylvania; and D.P.M., Duke University School of Medicine.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

¹L.E.T.-M. and T.W. contributed equally to this work.

²To whom correspondence should be addressed. Email: myles_brown@dfci.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1615015114/-DCSupplemental.

Vitamin D modulates its biological effects by directly regulating target gene expression through the VDR, a ligand-regulated transcription factor and a member of the nuclear receptor superfamily. Whether synthesized in the skin or ingested, vitamin D requires two hydroxylation steps to become the biologically active hormone, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], a form that signals through the VDR. The hormone-bound VDR modulates target gene transcription in response to vitamin D. In cancer models, such changes in gene expression lead to modulation of the cell cycle; arrest of cell proliferation; cell differentiation; and regulation of programmed cell death, including apoptosis and autophagy (16, 17).

Autophagy (macroautophagy) or “self-eating” is a conserved lysosomal event that consists of autophagosome formation by encapsulation of intracellular components by a double membrane; the autophagosomes are then delivered to and fuse with lysosomes, forming autolysosomes, wherein their contents are digested and recycled back into the cytosol (18, 19). Autophagy is multifunctional: It serves as a mechanism for clearing lipids, toxic protein aggregates, and damaged or defective organelles. It is also a prosurvival mechanism in response to starvation and hypoxia. Additionally, it is a mechanism of cell death. The physiological consequences of induction of autophagosomes can vary, depending on whether the induction process is acute or chronic. The role of autophagy in cancer appears to be context-specific: In some cases, autophagy is antineoplastic, whereas in other

instances, it seems to promote cancer growth and survival (20–22). However, in normal tissue, autophagy generally serves to protect the tissue from damage and cancer initiation (18). In various cell models, 1,25(OH)₂D₃ can modulate autophagy (17, 23). We have found that 1,25(OH)₂D₃ regulates cell death by modulating autophagy in luminal-like BC-cell models, and also in normal mammary gland in mice. This mechanistic work underlines the importance of 1,25(OH)₂D₃ signaling in normal mammary gland and the role of the VDR as a fine-tuned modulator of autophagy.

Results

Vitamin D Induces Autophagy Specifically in Luminal-Like BC Cells.

Antiproliferative functions of vitamin D have been reported in a variety of cancer cell models, including BC cells (24). We treated different BC-cell lines corresponding to the different molecular subtypes of BC with 1,25(OH)₂D₃ and observed that it has antiproliferative properties in luminal-like models, but not in mesenchymal basal-like BC or immortalized basal cell models (Fig. 1A). Also, following vitamin D treatment, 1,25(OH)₂D₃-sensitive cells appeared granular by phase-contrast microscopy, suggesting the presence of autophagy (Fig. S14, arrows). Because autophagy induction has been reported following 1,25(OH)₂D₃ treatment in MCF-7 cells (24), we tested for potential induction of autophagy by staining the cellular acid compartments with LysoTracker Red. Fluorescence microscopy

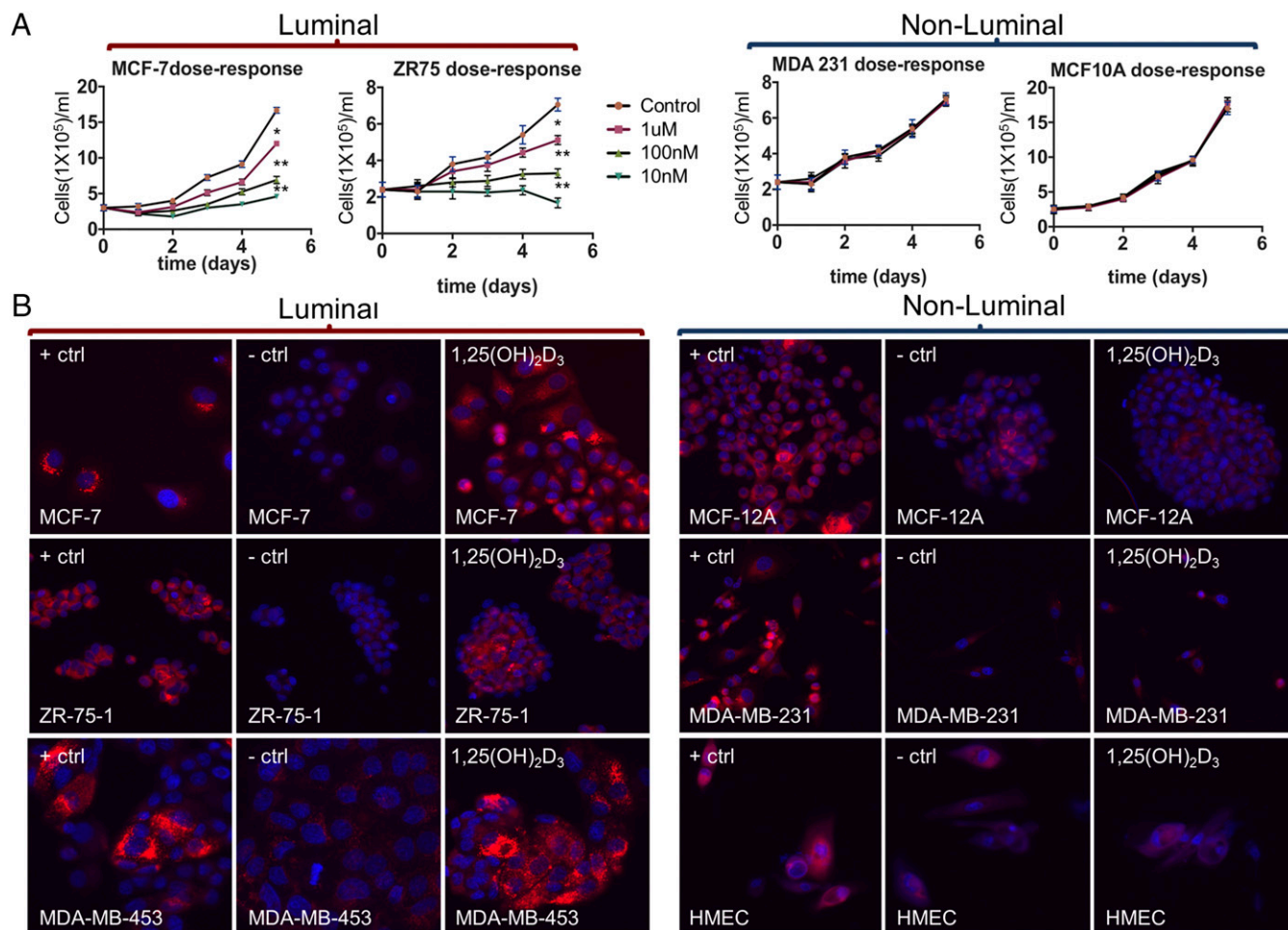


Fig. 1. Vitamin D increases the acid compartments and inhibits proliferation specifically in luminal-like BC cells. (A) Cell counts of luminal-like and non-luminal-like BC-cell lines with 10 nM, 100 nM, and 1 μM 1,25(OH)₂D₃. **P* ≤ 0.05 and ***P* ≤ 0.01 as determined by one-way ANOVA. (B) LysoTracker Red staining of live luminal and nonluminal cell lines. (Magnification: 20×.) The positive control is 8 h of serum starvation, the negative control is carrier control, and treatment is 5 d of 100 nM 1,25(OH)₂D₃. ctrl, control.

confirmed that only the luminal-like models exhibited increased acidic compartments, consistent with autophagy (Fig. 1B). Importantly, our luminal-like panel included the estrogen receptor (ER)-negative cell line MDA-MB-453, indicating that this $1,25(\text{OH})_2\text{D}_3$ -mediated effect is ER-independent. We transfected MCF-7 luminal-like model cells with an EGFP-LC3 expression vector and confirmed the presence of increased punctae following $1,25(\text{OH})_2\text{D}_3$ treatment (Fig. S1B). Sorted MCF-7 EGFP-LC3 cells treated with $1,25(\text{OH})_2\text{D}_3$ showed significantly ($P \leq 0.05$) higher GFP intensity relative to control cells (Fig. S1B). Western blotting was used to document increased expression and cleavage of the autophagy marker LC3 following $1,25(\text{OH})_2\text{D}_3$ treatment, along with a modest increase in Beclin1 (Fig. S1C). Transmission electron microscopy (TEM) confirmed that the increased acidic structures (with a dark appearance following citrate counterstain) were autolysosomes (Fig. 2). Taken together, these data confirm that $1,25(\text{OH})_2\text{D}_3$ induces autophagy in luminal-like BC-cell models.

A Vitamin D-Induced Autophagy Signature Is Present in the Normal Mammary Gland and Is Lost During BC Progression. Expression analysis was used to identify genes differentially regulated by $1,25(\text{OH})_2\text{D}_3$ in MCF-7 cells. Gene ontology analysis of the top 800 regulated genes showed metabolism and cell death to be significantly present ($1.07\text{E-}13$ and $4.32\text{E-}11$), along with other biological processes reported to be regulated by vitamin D, such as cellular differentiation and regulation of proliferation (Fig. S1E). We built the $1,25(\text{OH})_2\text{D}_3$ -induced autophagy network by using the Dijkstra algorithm to calculate the shortest direct path connecting our regulated gene set using MetaCore software (Fig. S1G). The resulting $1,25(\text{OH})_2\text{D}_3$ -specific autophagy signature present in treated $1,25(\text{OH})_2\text{D}_3$ MCF-7 cells was compared against the expression profiles of patients with invasive ductal and lobular breast carcinoma relative to the normal mammary gland [The Cancer Genome Atlas (TCGA) breast dataset]. We found that the induced autophagy profile was present in the normal mammary gland, but was significantly lost ($P \leq 0.05$) upon BC progression (Fig. S2A). Moreover, expression of some of the genes from this signature was also modestly, yet significantly, correlated with long-term survival of all patients with BC (Fig. S2B). The interaction between these genes was graphed with the use of Ingenuity Pathway Analysis (Fig. S2C). Interestingly, this

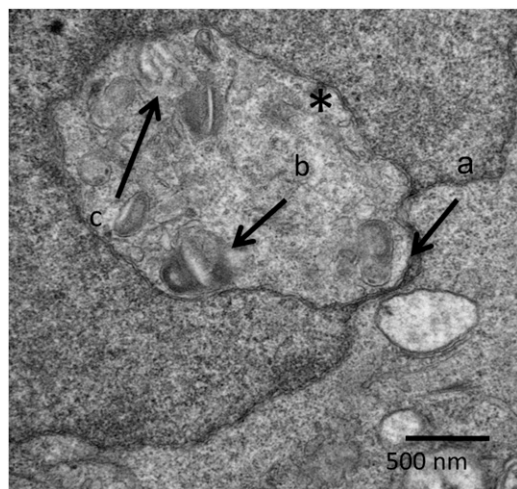


Fig. 2. The vitamin D-induced acidic compartment are autolysosomes. Autolysosome formation following $1,25(\text{OH})_2\text{D}_3$ treatment exhibiting defining autolysosomal features: double membrane (a), lysosome (b), partially digested organelle (mitochondria) (c), and cytoplasm (*).

signature was also present in human breast stroma (Finak Breast; 59 samples), not just in the ductal or luminal cell compartment, and it was also significantly lost upon cancer progression in stroma (Fig. S2D).

Motivated by these findings, we fed a vitamin D-supplemented diet ad libitum to mice (11 IU/g and 23 IU/g vitamin D_3 added; TestDiet) to determine whether autophagy levels in the normal mammary gland could be modulated by chronic, orally administered noncalcemic doses of vitamin D (Fig. S3A). A vitamin D-supplemented diet significantly ($P \leq 0.05$) modulated basal levels of autophagy in wild-type mice in a dose-responsive manner in the mammary gland at doses that do not induce hypercalcemia (Fig. 3 and Fig. S3). These results also suggest that the levels of dietary vitamin D in the control diet, although sufficient to maintain bone homeostasis, are not sufficient to induce autophagy in the mammary gland.

We also characterized the mammary glands of VDR knockout (VDRKO) and wild-type littermate virgin mice synchronized and vaginally staged (25) in all four stages of the estrous cycle: proestrus, estrus, metestrus, and diestrus (Fig. S4). As previously reported for apoptosis (26), autophagy was found to cycle according to the estrous phase in the mammary glands of wild-type virgin mice. In VDRKO (B6.129S4-Vdr^{tm1Mbd/J}) mice, the periodicity and amplitude of the autophagy cycle were significantly ($P \leq 0.05$) disrupted (Fig. S5).

We next examined the biological function and cellular localization of the autophagy genes regulated by $1,25(\text{OH})_2\text{D}_3$ (Ingenuity Pathway Analysis software) in MCF-7 cells. We noted that over 55% of the proteins encoded by these genes are involved in autophagosome formation (Fig. S6). Because autophagosome accumulation is toxic to cells (27, 28), we hypothesized that inducing autophagosome formation with $1,25(\text{OH})_2\text{D}_3$ and then blocking autophagosome degradation would be detrimental to cell survival. Therefore, we tested whether adding the 4-aminoquinoline compound chloroquine (CHQ), an inhibitor of autophagosome acidification, which is the last step in autophagosome degradation, would augment the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$ in MCF-7 cells. We found that CHQ significantly ($P \leq 0.001$) synergizes with the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$ in MCF-7 cells (Fig. 4A, Left). TEM demonstrated that cells treated with both $1,25(\text{OH})_2\text{D}_3$ and CHQ were saturated with vacuoles as expected (Fig. 4B). Next, to assess the potential additional therapeutic benefit of an autolysosome inhibitor to mice fed a vitamin D-supplemented diet, we treated mice harboring MCF-7 xenografts with a combination of the 4-aminoquinoline hydroxychloroquine (HCQ; 10 mg/kg i.p. every 3 to 7 d) and a vitamin D-supplemented diet ad libitum, and then compared them with MCF-7 xenograft-bearing mice treated with HCQ or vitamin D individually and with controls. The size of the xenografts was substantially smaller in animals treated with the combination therapy relative to animals receiving the individual therapies and significantly smaller ($P \leq 0.05$) compared with control animals (Fig. 4A, Right). These findings suggest that the induction of autophagy by vitamin D, together with an inhibitor of autolysosomal acidification, may be an effective therapeutic strategy in luminal BCs.

Absence of VDR Results in Higher Levels of Autophagy than Seen with Vitamin D Treatment Alone. Because the hormonal form of vitamin D mediates its effects through transcriptional regulation via the VDR, we silenced the VDR in MCF-7 cells by RNA interference, and used LysoTracker Red to monitor autophagy. Surprisingly, VDR knockdown led to much higher levels of autolysosome formation than did $1,25(\text{OH})_2\text{D}_3$ treatment, and there was no appreciable effect of the addition of $1,25(\text{OH})_2\text{D}_3$ in the setting of VDR knockdown. Importantly, the induction of autolysosome formation by silencing VDR was rescued by transfection of a small interfering VDR-resistant construct (Fig. 5A). TEM demonstrated that the total volume of autolysosomes following

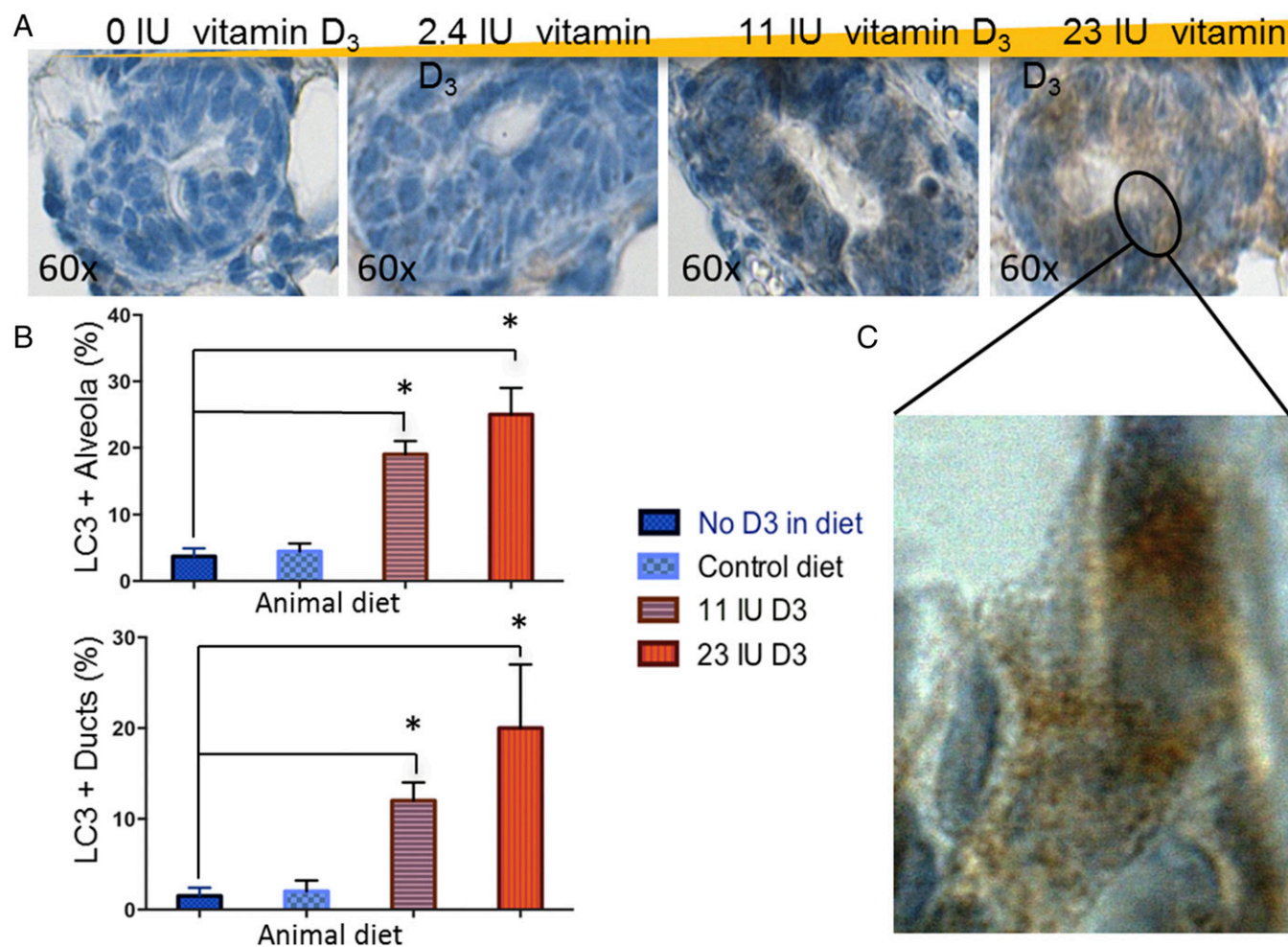


Fig. 3. Vitamin D modulation of autophagy in mouse mammary gland. (A) Immunohistochemistry of mouse mammary gland fed with increasing doses of vitamin D-supplemented diet stained for cleaved LC3. (Magnification: 60 \times .) (B) LC3 + mammary gland alveolae and ducts significantly increase in the vitamin D-supplemented diet. * $P \leq 0.05$ as determined by the stratified random approach (Braun-Blanquet) sampling method followed by Kruskal-Wallis one-way analysis of variance. (C) Punctate LC3 staining pattern. (Magnification: 100 \times .)

VDR knockdown had increased by ~ 100 -fold relative to cells treated with $1,25(\text{OH})_2\text{D}_3$ ($P \leq 0.01$) (Fig. 5B). The increased net volume of autophagolysosomes was accompanied by an enhanced autophagic flux. Autophagic flux was determined by stably expressing mCherry-EGFP-LC3B in MCF-7 cells (29) for $1,25(\text{OH})_2\text{D}_3$ treatment and VDR knockdown (Fig. 5C). Finally, we compared the levels of autophagy observed in the mammary glands of VDRKO mice at estrus with the levels of autophagy in wild-type littermates fed a vitamin D-supplemented diet and in control mice. LC3B quantification revealed significantly ($P \leq 0.05$) higher levels of autophagy in the mammary glands of the VDRKO mice compared with control mice following vitamin D supplementation (Fig. 5D). Collectively, these results show that the absence of VDR induces higher levels of autophagy than does $1,25(\text{OH})_2\text{D}_3$ treatment alone, and suggest that the VDR acts as a constitutive repressor of autophagy and that this repression is partially relieved upon $1,25(\text{OH})_2\text{D}_3$ stimulation.

VDR Directly Regulates Autophagy Gene Expression in MCF-7 BC Cells.

To identify VDR direct target genes, we performed ChIP sequencing (ChIP-Seq) of endogenous VDR in MCF-7 cells in the presence and absence of $1,25(\text{OH})_2\text{D}_3$. Model-based analysis of ChIP-Seq was used to call the VDR peaks, and it revealed 2,278 VDR-binding sites in the absence of $1,25(\text{OH})_2\text{D}_3$: a total of 7,418 sites seen only following 4 h of ligand stimulation and 660 sites that remained unchanged in the presence or absence of

$1,25(\text{OH})_2\text{D}_3$ (Fig. 6A). Consistent with other VDR and nuclear receptor cistromes, most of the VDR-binding sites were detected in enhancers located in intergenic regions, and rarely ($\sim 5\%$) near promoters (Fig. S7A). A motif-based sequence analysis tool (Tomtom) was used to identify enriched motifs, which revealed significant enrichment of the consensus VDR-RXR binding motif (DR3) in both the presence ($P = 2.76 \text{ e-}9$) and absence ($P = 1 \text{ e-}30$) of $1,25(\text{OH})_2\text{D}_3$ (Fig. S7A). Also, other specific transcription factor-binding motifs were differentially enriched in peaks found in the control-specific group, in the $1,25(\text{OH})_2\text{D}_3$ stimulation-specific group, and in common peaks (Fig. S7B). We found evidence of constitutive VDR binding in the first intron of the key autophagy gene *MAP1LC3B* (Fig. 6B). Interestingly, although VDR was found constitutively bound at this site, we could demonstrate by directed ChIP-quantitative PCR for HDAC3 and p300 that there was an exchange of coregulators induced by $1,25(\text{OH})_2\text{D}_3$ (Fig. 6B). Furthermore, addition of $1,25(\text{OH})_2\text{D}_3$ activated transcription of the LC3B gene (*MAP1LC3B*). Treatment with the HDAC inhibitor Trichostatin A (TSA) also significantly induced *MAP1LC3B* expression, and the combined treatment of $1,25(\text{OH})_2\text{D}_3$ with TSA synergized to induce *MAP1LC3B* expression to similar levels as found following VDR knockdown (Fig. 6C). These experiments demonstrate a constitutive repression of the *MAP1LC3B* gene by VDR that is partially relieved upon $1,25(\text{OH})_2\text{D}_3$ stimulation and that this repression is mediated, in part, by HDAC-associated corepressors.

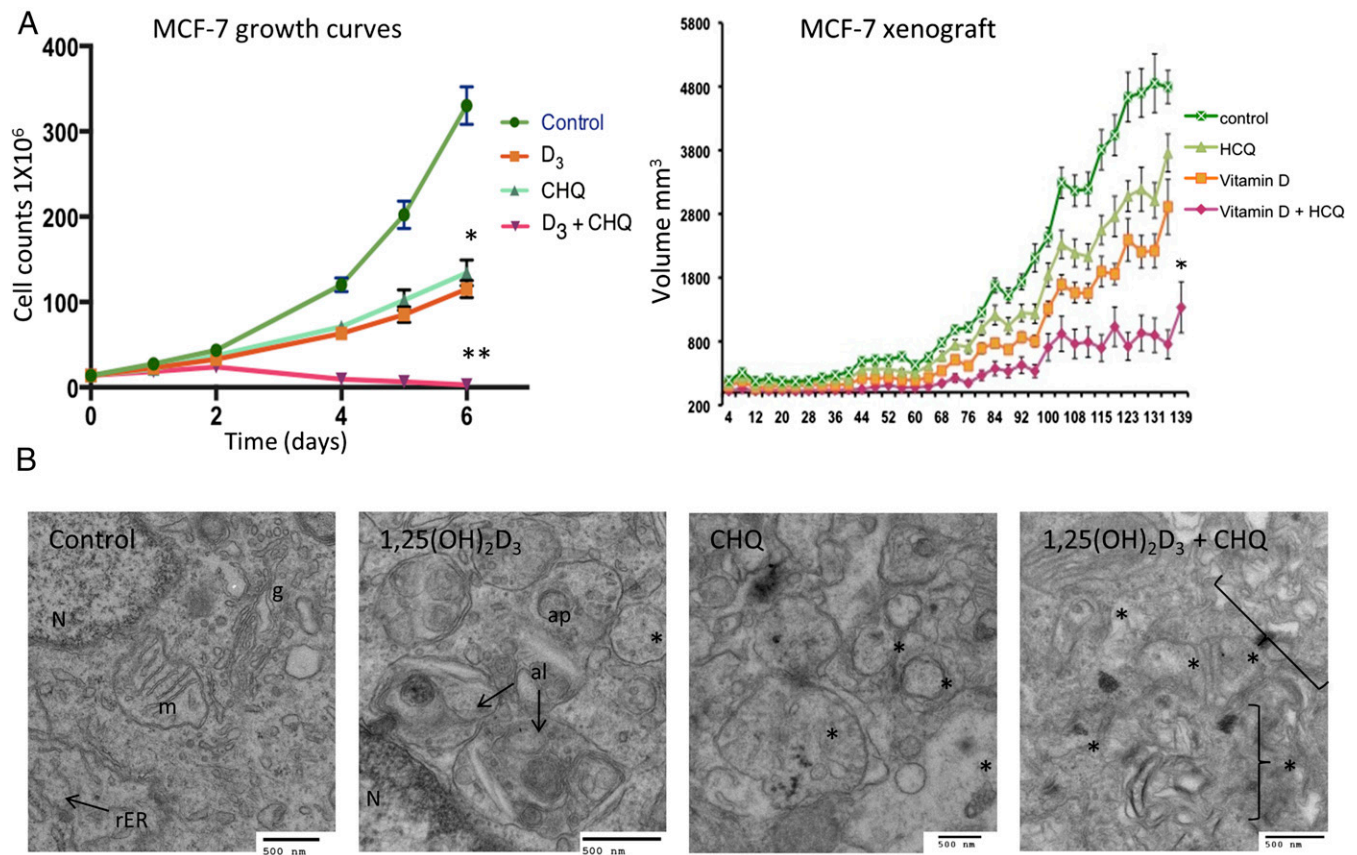


Fig. 4. 1,25(OH)₂D₃ shows antiproliferative benefit with 4-aminoquinoline compounds in vitro and in vivo. (A) MCF-7 cell counts treated with control, 10 nM 1,25(OH)₂D₃, and 500 nM CHQ alone and combined. MCF-7 side-flank xenograft measurements from mice treated with control, mice fed add libitum vitamin D-supplemented diet (23 IU/kg), mice treated with HCQ (10 mg/kg i.p. three times per week), or mice fed the vitamin D diet and treated with HCQ. **P* ≤ 0.05 and ****P* ≤ 0.01 as determined by one-way ANOVA. (B) TEM of MCF-7 cells treated with a combination of 1,25(OH)₂D₃ and CHQ show a higher vacuole accumulation than control or either treatment alone. al, autolysosomes; ap, autophagosomes; g, Golgi apparatus; m, mitochondria, N, nucleolus; rER, rough endoplasmic reticulum. Undigested vacuoles are shown by an asterisk.

This mode of gene regulation was not recapitulated at all vitamin D target genes, such as *CYP24A1* (Fig. S7C). In addition, we identified the genes located near VDR-bound enhancers (± 30 kb from promoter regions) and performed pathway analysis on these genes. Interestingly, the pathway analysis of the genes found near VDR enhancers consistently yielded apoptotic and metabolic (autophagy) pathways to be significantly enriched in the three groups of VDR peaks: control, 1,25(OH)₂D₃-stimulated, and common peaks (Fig. S7D).

To investigate the mechanisms involved in the increased autophagy levels upon VDR knockdown further, we performed gene expression analysis comparing knockdown control with no-ligand, knockdown control treated with 1,25(OH)₂D₃ for 16 h, and VDR knockdown (Fig. 6D and Dataset S1). This analysis revealed that 1,271 of the total 1,689 genes induced by 1,25(OH)₂D₃ are down-regulated following VDR knockdown. Likewise expression of 2,581 of the total 3,450 genes repressed by 1,25(OH)₂D₃ is enhanced following VDR knockdown. This result indicates, as expected, that regulation of most genes by 1,25(OH)₂D₃ is dependent on the VDR. Interestingly, this analysis shows that the largest group of regulated genes is up-regulated in the absence of the VDR, consistent with a constitutive gene repression mechanism mediated by the VDR that we observed in LC3B. Therefore, the constitutive repression of autophagy by VDR we observe is a widespread mechanism of gene regulation. Similar to 1,25(OH)₂D₃-induced genes (45%), nearly 40% of the genes up-regulated following VDR knockdown contain at least one VDR-binding site within

30 kb, suggesting direct VDR regulation (Fig. 6E, Fig. S7E, and Dataset S2). Interestingly, upon VDR knockdown, the 1,25(OH)₂D₃-induced autophagy signature is lost and only four autophagy-related genes remain present: *FOS*, *CXCR4*, *VEGFA*, and *MAP1LC3B* (Fig. S7F); however, only LC3B was directly up-regulated by 1,25(OH)₂D₃ [with vitamin D response elements (VDREs) located ± 30 kb from the gene] and further up-regulated following VDR knockdown. Given that our phenotype suggests constitutive repression of autophagy by the VDR, and as a proof of principle for constitutive VDR repression, we did ChIP-Seq of HDAC3 and confirmed VDR overlap in the absence of 1,25(OH)₂D₃ (Fig. S7G). Next, we performed rapid immunoprecipitation mass spectrometry (RIME) of endogenous proteins in the presence of 1,25(OH)₂D₃ to identify the proteins associated with VDR when it is bound to DNA (Dataset S3). Ingenuity Pathway Analysis of the VDR interactome revealed highly significant (*P* ≤ 0.01 × 10⁻⁵) enrichment of proteins involved in transcriptional repression and DNA methylation, as well as chromatin remodeling and gene silencing (Fig. 6F and Fig. S7H), providing a mechanistic insight on VDR constitutive repression of gene expression. These results indicate a role of the ligand-bound VDR as regulator of autophagy in normal mammary gland and BC cells by regulated expression of autophagy-related genes by both activation of gene transcription and relief of constitutive repression (Fig. 7A) of the key autophagy gene LC3B, which is highly expressed in the absence of VDR (Fig. 7B) and de-repressed in the presence of 1,25(OH)₂D₃ (Fig. 7C).

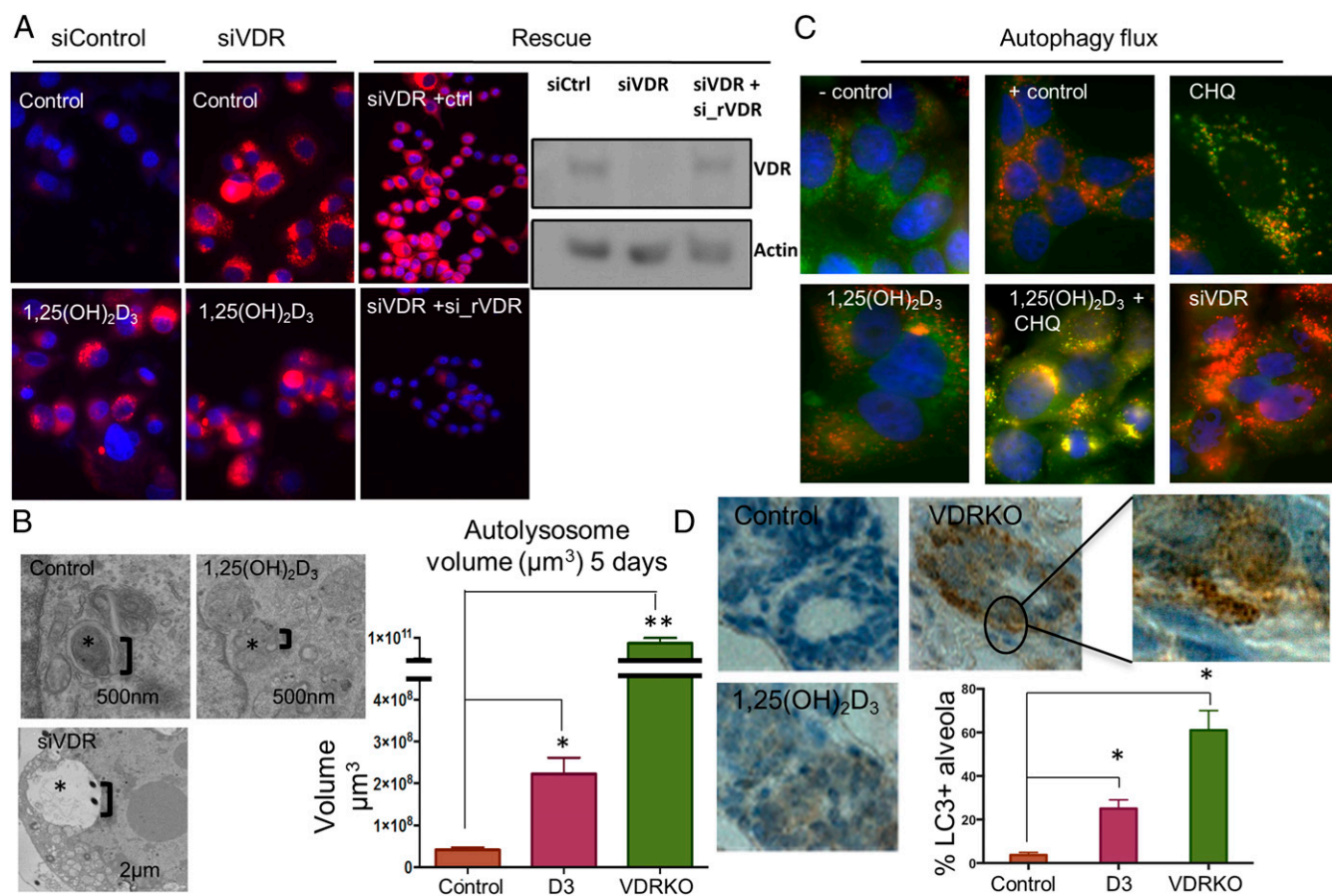


Fig. 5. Absence of VDR results in higher autophagy levels than the levels seen with vitamin D treatment alone. (A) Live MCF-7 cells transfected with small interfering VDR (siVDR) and stained with LysoTracker Red show a higher level of acidic compartment than MCF-7 cells treated with 1,25(OH)₂D₃. (Magnification: 20 \times .) This effect can be rescued by cotransfecting an siVDR-resistant construct. (B) TEM of control, 1,25(OH)₂D₃, and VDR knockdown in MCF-7 cells showing significant differences on autolysosome volumes. * $P \leq 0.05$ as determined by Kruskal–Wallis one-way analysis of variance. (C) MCF-7 cells stably expressing mCherry-EGFP-LC3B exhibit higher levels of autophagy flux upon VDR knockdown than MCF-7 cells following 1,25(OH)₂D₃ treatment. (Magnification: 40 \times .) (D) Immunohistochemistry of mammary gland alveolae immunostained against cleaved LC3 (40 \times magnification) in control mice, mice fed a vitamin D-supplemented diet, and the VDRKO mice (punctate stain, 60 \times magnification) * $P \leq 0.05$ and ** $P \leq 0.01$ as determined by the stratified random approach (Braun–Blanquet) sampling method followed by Kruskal–Wallis one-way analysis of variance.

In summary, our results show that in response to 1,25(OH)₂D₃, luminal-like BC cells enter autophagy and create a transcription signature that is present in normal breast and lost during cancer progression. We have shown that vitamin D in the diet can increase basal levels of autophagy in the normal breast, and that modulation of autophagy by vitamin D can provide a potential therapeutic opportunity as a combinational therapy with autophagy inhibitory chemotherapeutic drugs.

Discussion

Our study shows that 1,25(OH)₂D₃ modulates autophagy via the VDR, by direct regulation of transcription. Moreover, treatment with 1,25(OH)₂D₃ leads to an autophagy-related gene expression signature, which is present in normal mammary gland but is no longer detected as the cancer progresses, offering a potential mechanism for the epidemiological observation that serum vitamin D levels ≥ 45 ng/mL are protective against BC. Mechanistically, the VDR constitutively represses autophagy; upon 1,25(OH)₂D₃ stimulation, basal levels of autophagy increase by de-repression of the key autophagy gene *MAP1LC3B* (LC3B).

Vitamin D-Induced Autophagy Is Antisurvival in Luminal-Like Models.

The role of autophagy in cancer is context-dependent, and extensive reports document a prosurvival role of autophagy in

cancer. However, in some systems, autophagy also reportedly inhibits cancer growth (20). Our data indicate that 1,25(OH)₂D₃-induced autophagy plays an antisurvival role. Upon stimulation with 1,25(OH)₂D₃, we observed increased levels of autophagy, exclusively in BC-cell models (luminal-like models), which also exhibit an antiproliferative response. The landscape of gene expression following 1,25(OH)₂D₃ treatment in our model, as well as in many others (30), also reflects inhibition of proliferation, cell cycle arrest, and cell death, as opposed to a proliferative or prosurvival gene transcription profile.

VDR-Mediated Mechanisms of 1,25(OH)₂D₃-Induced Autophagy.

Vitamin D induces autophagy in macrophages, neurons, and models of skin and BC (31, 32). Høyer-Hansen et al. (24) described a 1,25(OH)₂D₃-induced autophagy mechanism involving AMP-activated protein kinase (AMPK) activation triggered by activation of calcium/calmodulin-dependent protein kinase 2 (CAMKK2) activation in MCF-7 cells. We also observed increased activation of AMPK that was not accompanied by a change in protein levels. In contrast, however, no changes were detected in levels of CaMKK transcript, its protein levels, or its activation upon 1,25(OH)₂D₃ treatment. Unexpectedly, levels of autophagy in the absence of VDR were much higher than with 1,25(OH)₂D₃ treatment in MCF-7 cells that expressed VDR. Mirroring these in vitro

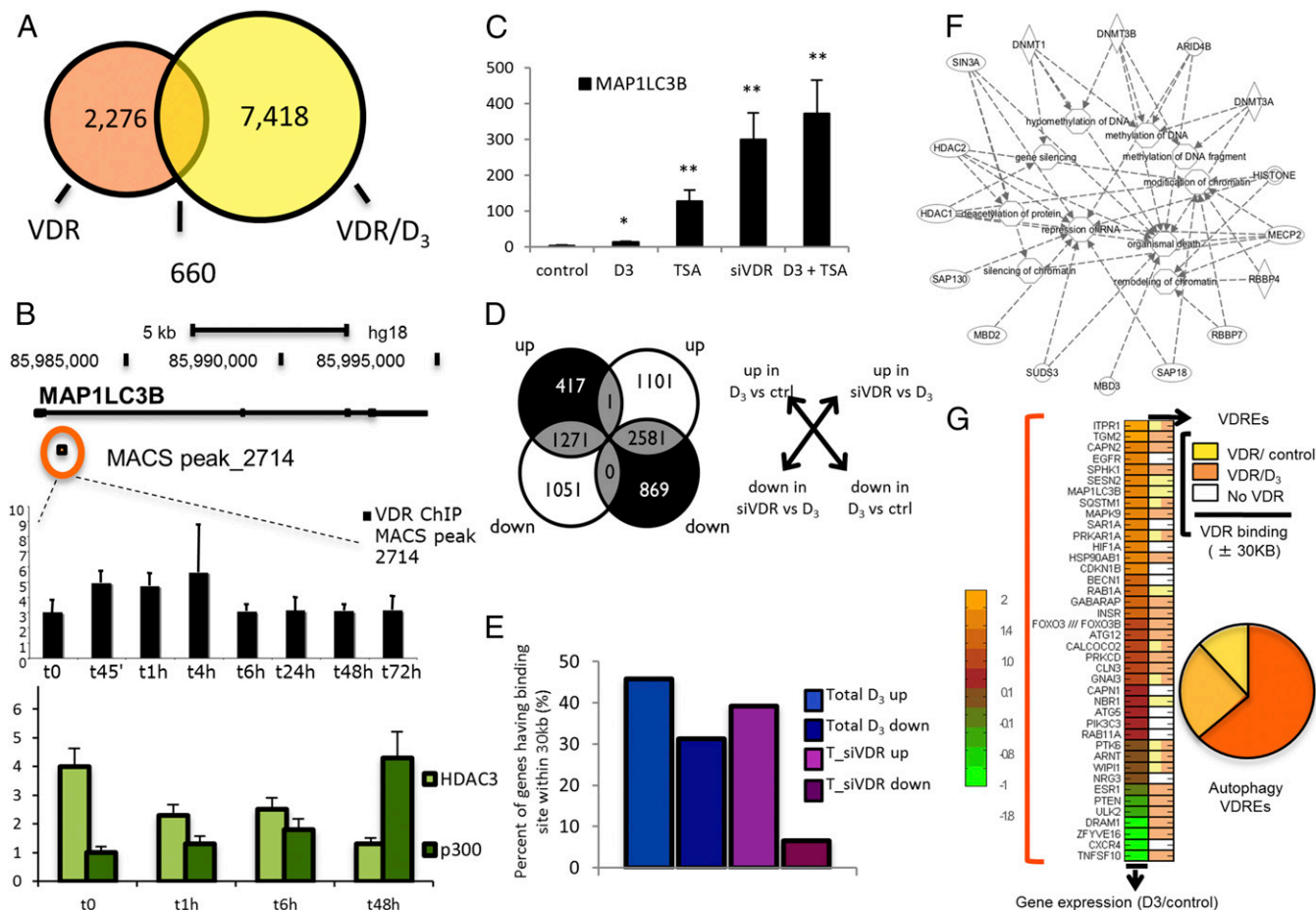


Fig. 6. Autophagy regulation by the VDR. (A) MCF-VDR ChIP-Seq of control and 4 h of 1,25(OH)₂D₃ treatment (0.05 false discovery rate). (B) Time course of directed ChIP-quantitative PCR on a VDRE located in the first intron of autophagy gene MAP1LC3B (LC3B). (C) LC3B expression following control, 1,25(OH)₂D₃, TSA, combined 1,25(OH)₂D₃ and TSA, and siVDR knockdown. **P* ≤ 0.05 and ***P* ≤ 0.01 as determined by one-way ANOVA. (D) Venn diagram of expression analysis comparison of genes significantly up-regulated (Top) or down-regulated (Bottom) in the presence of 1,25(OH)₂D₃ relative to control (black) and VDR knockdown relative to 1,25(OH)₂D₃ (*P* ≤ 0.01 and Benjamini–Hochberg correction). (E) ChIP-Seq and expression array integration indicating the percentage of expressed genes with VDREs (direct targets). (F) Pathway analysis of the VDR protein corepressor interactome (RIME) in the presence of 1,25(OH)₂D₃ in MCF-7 cells. (G) Integration of 1,25(OH)₂D₃-induced autophagy genes with ChIP-Seq data.

findings, we found that *in vivo*, VDR KO mice have higher basal levels of autophagy in their mammary glands than do their wild-type littermates. Vitamin D supplementation also increases basal autophagy levels in the mammary gland, consistent with our *in vitro* findings. Collectively, the above findings suggest a constitutive VDR-mediated repression of autophagy that is partially relieved upon stimulation with 1,25(OH)₂D₃.

VDR: Master Transcription Regulator of Autophagy in the Mammary Gland. Canonically, upon ligand binding, VDR regulates transcription as an RXR heterodimer that is bound to DNA. In the presence of ligand, this VDR-RXR heterodimer mediates gene activation by recruiting coactivators of transcription, but in the absence of ligand, VDR instead binds corepressors (33). However, gene expression studies show a very similar number of genes being up-regulated and down-regulated across a number of different cancer models upon 1,25(OH)₂D₃ treatment. In addition, Meyer and Pike (34, 35) used ChIP-Seq studies in a colon cancer model (LS180) to show that the recruitment of corepressors NCoR and SMRT overlaps with VDR binding in 1,25(OH)₂D₃-activated enhancers. To confirm the presence of corepressors associated with DNA-bound VDR after 1,25(OH)₂D₃ stimulation, we performed RIME of endogenous protein experiments. Our RIME data show that VDR interacts with corepressor complexes

following 1,25(OH)₂D₃ treatment, including the characterized VDR corepressors SIN3/NurD/CoREST, PRC2, TFIIIC, and SWI/SNF, as well as coactivator complexes INO80, CBP, and SRC3/ncp3 and WTAP-SFRS (a complete list of the VDR interactome is provided in [Dataset S3](#)). Although we did not find p300 or HDAC3 in the VDR interactome (perhaps due to stringent analysis cut-offs and extensive washing in the RIME protocol), ChIP-Seq HDAC3 confirmed VDR overlap in both the presence and absence of 1,25(OH)₂D₃. Canonical pathway analysis of our data revealed that in the presence of vitamin D, the DNA-bound VDR is significantly associated with protein groups that are involved not only with transcription and translation initiation but also with DNA methylation and transcription repression. Our RIME studies offer a global view of DNA-bound 1,25(OH)₂D₃-activated VDR functions as sophisticated transcription node fine-tuning gene expression regulation via multiple concurrent mechanisms.

VDR Constitutively Represses Key Autophagy Gene LC3B. We focused on autophagy regulator genes that were activated by 1,25(OH)₂D₃ but showed higher activation upon VDR knockdown: Candidates were narrowed down with regard to the VDR enhancers found in the absence and presence of 1,25(OH)₂D₃. We identified LC3B, the most extensively studied member of the Atg8 protein family

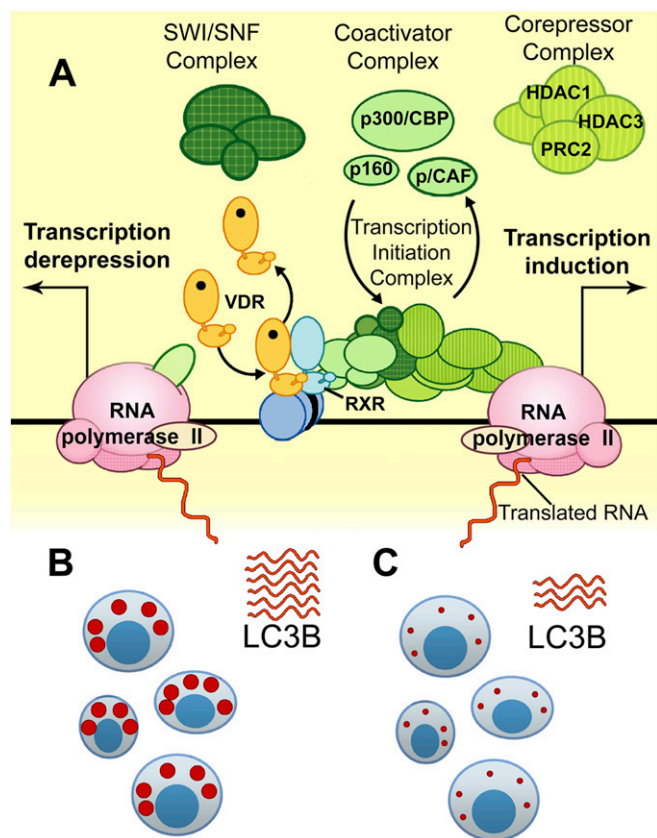


Fig. 7. Model for VDR regulation of autophagy in luminal-like models. (A) Upon $1,25(\text{OH})_2\text{D}_3$ binding, the VDR activates gene transcription to target genes to vitamin D by derepression (Left) or induction of transcription (Right). (B) In the absence of VDR, there is a full de-repression of autophagy and 100-fold higher expression levels of MAP1LC3B than with $1,25(\text{OH})_2\text{D}_3$ alone. (C) In the presence of VDR, $1,25(\text{OH})_2\text{D}_3$ induces a constitutive de-repression of autophagy gene LC3B and regulation of transcription by the VDR, resulting in autophagy induction following vitamin D treatment.

(36), which plays a key role in autophagosome biogenesis. LC3B is essential for autophagy, and has a critical function in autophagosome elongation and autophagy flux. LC3B is believed to control autophagosome size on account of its hemifusion and membrane tethering activities (37, 38). Overexpression of LC3B alone increases both formation and elongation of autophagosomes by more than 60% in human HeLa cells. Furthermore, LC3 proteins serve as docking sites for adaptor proteins, and thereby play a key role in the selective recruitment of autophagy cargoes into autophagosomes (37). MCF-7 treatment with $1,25(\text{OH})_2\text{D}_3$ results in LC3B induction. Moreover, VDR knockdown results in a 100-fold up-regulation of LC3B; this much higher up-regulation can be partially mimicked by cotreatment with $1,25(\text{OH})_2\text{D}_3$ and the HDAC class I and II inhibitor TSA, suggesting that LC3B is modulated by a constitutive de-repression of the VDR at the *MAP1LC3B* VDRE. As with $1,25(\text{OH})_2\text{D}_3$, LC3B not only plays a role in autophagy but is also heavily involved in the immune response, particularly during phagocytosis (39). Ramagopalan et al. (40) used ChIP-Seq to characterize the VDR-binding elements in lymphoblastic cell models (GM10855 and GM10861), and also identified VDR *cis*-regulatory sites near the *MAP1LC3B* gene. These observations support the possibility that VDR modulation of LC3 plays a wider role than in autophagy in the mammary gland, but also in innate and adaptive immunity, all of which are among the broad spectrum of biological functions of vitamin D.

Induction of Autophagy as a $1,25(\text{OH})_2\text{D}_3$ -Mediated Mechanism That Protects Against BC. Serum levels of vitamin D correlate with a positive prognosis in patients with BC (5). We show that $1,25(\text{OH})_2\text{D}_3$ induces an autophagy gene expression signature: Comparison against TCGA human BC data reveals that this signature is present in normal mammary gland and lost upon cancer progression. Interestingly, we have shown that dietary supplementation of vitamin D can increase basal levels of autophagy in the normal mammary gland in mice. Modulation of autophagy, although still premature, is an attractive venue in BC therapeutics (41), given that autophagy plays a role in the different stages of BC (42).

Vitamin D and its analogs are known to reduce the growth of luminal BC xenografts *in vivo*. As a proof of concept that vitamin D cotreatment amplifies potential therapeutic properties of autophagy-modulating drugs, we treated mice harboring MCF-7 xenografts with vitamin D alone, HCQ alone, or a combination of the two. We observed added benefit from the combination compared with the use of either compound alone, showing added therapeutic gain from concurrent induction of autophagy and inhibition of the last step of autolysosome acidification. However, HCQ is not highly specific in inhibiting autophagy. Further studies are indicated, with better inhibitors of autophagy, such as Lys05, along with low-calcemic vitamin D analogs, to determine the therapeutic potential of vitamin D analogs and autophagy-modulating drugs. Retinoids constitute another interesting non-calcemic venue that is actively under investigation for prevention of mammary cancers (43), which could potentially be enhanced by autophagy manipulation. Autophagy induction, along with inhibition for cancer therapeutics, is currently under study for melanoma and other solid tumors (44). Our results suggest that vitamin D serum levels and intake should be monitored in patients as they are recruited to clinical trials for autophagy-modulating therapeutics.

In terms of cancer risk, epidemiological studies show that vitamin D reduces the risk of developing BC, but only when serum levels are ≥ 45 ng/mL (4). Although levels up to 140 ng/mL can be synthesized in skin (12), the overall incidence of vitamin D deficiency (serum levels ≤ 20 ng/mL) is strikingly high in the United States: 41.6% of Americans show this deficiency, with the highest rate seen in African Americans (81.2%) (7, 11). Several factors contribute to these low levels of vitamin D: geographical location with respect to the equator, an indoor lifestyle, and sunscreen protection. Because there is naturally very little vitamin D in food, including in vitamin D-supplemented products (45), we rely mostly on supplementation for the fulfillment of our vitamin D needs. Our findings show that only vitamin D diet supplementation at higher levels than required for bone health increases basal autophagy in the mouse mammary gland. Notably, mouse mammary tissue in which autophagy is compromised exhibits hyperproliferative and preneoplastic changes: These changes are likely due to a compromised ability to cope with metabolic stress and overall cell damage, resulting in DNA instability and further damage, which, in turn, may accelerate tumor progression (20, 42). Further studies are needed to determine whether vitamin D supplementation induces changes in basal autophagy in human breast tissue, and whether it produces the cancer-protective gene signature that we report.

Materials and Methods

Cell Lines. The cell lines MCF-7, ZR-75-1, MDA-MB-453, MCF-12A, MDA-MB-231, and HMEC were purchased from the American Type Culture Collection (ATCC) and authenticated. Cells were cultured and kept in recommended media and conditions (ATCC).

Antibodies and Reagents. The $1,25(\text{OH})_2\text{D}_3$ (D1530; Sigma) treatments were done at 100 nM concentrations unless specified otherwise in *Results*. HCQ sulfate (H0915) and CHQ salt (C6628) were purchased from Sigma. Antibodies for LC3 immunohistochemistry cleaved LCA3A (AP1805a; Abgent). For Western

blotting, LC3B (2775; Cell Signaling), Beclin-1 (3738; Cell Signaling), and VDR (D-6) (sc-13133; Santa Cruz Biotechnology) were used. For ChIP applications, we used VDR (C-20) (sc-1008; Santa Cruz Biotechnology), p300 (N-15) (sc-534; Santa Cruz Biotechnology), and HDAC3 (ab7030; Abcam) antibodies.

Microscopy, Data Analysis, and Animal Studies. Information on microscopy, data analysis, and animal studies is provided in *SI Materials and Methods*. All animal studies were approved by the Institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute.

MCF-7 VDR ChIP-Seq and RIME. MCF-7 cells grown in full media were treated with 100 nM 1,25(OH)₂D₃ for 4 h before VDR immunoprecipitation (C-20; Santa Cruz Biotechnology). Four independent immunoprecipitations and libraries were prepared for ChIP-Seq, and three independent

RIME samples were prepared. VDR ChIP-Seq samples were prepared according to the laboratory protocol of Brown and co-workers (46), and RIME sample preparation and analysis were performed according to Mohammed et al. (47).

ACKNOWLEDGMENTS. We thank Ana Tavera Mendoza for illustration (Fig. 7) design and drawing; Christine Unitt for help with LC3 immunohistochemistry; Louise M. Trakimas for TEM assistance; Melinda Baker (Thomson Reuters) for invaluable advice in creating the autophagy pathway; the Dana-Farber Animal Facility staff (Michael Terrasi, Antonia Garcia, Daisy Moreno, Carmen Da Silva, Elsy Moreno, and Catherine A. Sypher) for assistance with animal experiments and overall excellence in animal care; and Sonal Jhaveri-Schneider for editing help. This work was supported by National Cancer Institute Grant P01 CA080111 (to M.B.).

- American Cancer Society (2014) *Cancer Facts and Figures 2014* (American Cancer Society, Atlanta).
- DeSantis C, Ma J, Bryan L, Jemal A (2014) Breast cancer statistics, 2013. *CA Cancer J Clin* 64(1):52–62.
- American Cancer Society (2013) *Breast Cancer Facts & Figures 2013-2014* (American Cancer Society, Atlanta).
- Garland CF, et al. (2007) Vitamin D and prevention of breast cancer: Pooled analysis. *J Steroid Biochem Mol Biol* 103(3-5):708–711.
- Goodwin PJ, Ennis M, Pritchard KI, Koo J, Hood N (2009) Prognostic effects of 25-hydroxyvitamin D levels in early breast cancer. *J Clin Oncol* 27(23):3757–3763.
- Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ (2014) The role of vitamin D in reducing cancer risk and progression. *Nat Rev Cancer* 14(5):342–357.
- Holick MF (2012) Evidence-based D-bate on health benefits of vitamin D revisited. *Dermatoendocrinol* 4(2):183–190.
- White JH (2013) Vitamin D and human health: More than just bone. *Nat Rev Endocrinol* 9(10):623.
- Giovannucci E (2011) Vitamin D, how much is enough and how much is too much? *Public Health Nutr* 14(4):740–741.
- Ross AC, et al. (2011) The 2011 Dietary Reference Intakes for Calcium and Vitamin D: What dietetics practitioners need to know. *J Am Diet Assoc* 111(4):524–527.
- Forrest KYZ, Stuhldreher WL (2011) Prevalence and correlates of vitamin D deficiency in US adults. *Nutr Res* 31(1):48–54.
- Vieth R (1999) Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. *Am J Clin Nutr* 69(5):842–856.
- Deluca HF (2014) History of the discovery of vitamin D and its active metabolites. *Bonekey Rep* 3:479–487.
- Lin R, White JH (2004) The pleiotropic actions of vitamin D. *BioEssays* 26(1):21–28.
- Mohr SB, et al. (2012) Does the evidence for an inverse relationship between serum vitamin D status and breast cancer risk satisfy the Hill criteria? *Dermatoendocrinol* 4(2):152–157.
- Welsh J (2012) Vitamin D and cancer: Integration of cellular biology, molecular mechanisms and animal models. *Scand J Clin Lab Invest Suppl* 243(243):103–111.
- Hoyer-Hansen M, Nordbrandt SP, Jäättelä M (2010) Autophagy as a basis for the health-promoting effects of vitamin D. *Trends Mol Med* 16(7):295–302.
- White E (2012) Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer* 12(6):401–410.
- Ladoire S, et al. (2012) Immunohistochemical detection of cytoplasmic LC3 puncta in human cancer specimens. *Autophagy* 8(8):1175–1184.
- White E (2015) The role for autophagy in cancer. *J Clin Invest* 125(1):42–46.
- Mathew R, White E (2011) Autophagy in tumorigenesis and energy metabolism: Friend by day, foe by night. *Curr Opin Genet Dev* 21(1):113–119.
- Mathew R, White E (2011) Autophagy, stress, and cancer metabolism: What doesn't kill you makes you stronger. *Cold Spring Harb Symp Quant Biol* 76:389–396.
- Verway M, Behr MA, White JH (2010) Vitamin D, NOD2, autophagy and Crohn's disease. *Expert Rev Clin Immunol* 6(4):505–508.
- Hoyer-Hansen M, et al. (2007) Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-β, and Bcl-2. *Mol Cell* 25(2):193–205.
- Caligioni CS (2009) Assessing reproductive status/stages in mice. *Curr Protoc Neurosci* Appendix 4(48):Appendix 41.
- Fata JE, Chaudhary V, Khokha R (2001) Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17β-estradiol during the estrous cycle. *Biol Reprod* 65(3):680–688.
- Khoh-Reiter S, et al. (2015) Contribution of membrane trafficking perturbation to retinal toxicity. *Toxicol Sci* 145(2):383–395.
- Fields J, et al. (2015) HIV-1 Tat alters neuronal autophagy by modulating autophagosome fusion to the lysosome: Implications for HIV-associated neurocognitive disorders. *J Neurosci* 35(5):1921–1938.
- Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. *Cell* 140(3):313–326.
- Lin R, Wang TT, Miller WH, Jr, White JH (2003) Inhibition of F-Box protein p45(SKP2) expression and stabilization of cyclin-dependent kinase inhibitor p27(KIP1) in vitamin D analog-treated cancer cells. *Endocrinology* 144(3):749–753.
- Bristol ML, et al. (2012) Dual functions of autophagy in the response of breast tumor cells to radiation: Cytoprotective autophagy with radiation alone and cytotoxic autophagy in radiosensitization by vitamin D 3. *Autophagy* 8(5):739–753.
- Jang W, et al. (2014) 1,25-Dihydroxyvitamin D₃ attenuates rotenone-induced neurotoxicity in SH-SY5Y cells through induction of autophagy. *Biochem Biophys Res Commun* 451(1):142–147.
- Teichert A, et al. (2009) Quantification of the vitamin D receptor-coregulator interaction. *Biochemistry* 48(7):1454–1461.
- Meyer MB, Pike JW (2013) Corepressors (NCoR and SMRT) as well as coactivators are recruited to positively regulated 1α,25-dihydroxyvitamin D₃-responsive genes. *J Steroid Biochem Mol Biol* 136:120–124.
- Molnár F (2014) Structural considerations of vitamin D signaling. *Front Physiol* 5:191–213.
- Wild P, McEwan DG, Dikic I (2014) The LC3 interactome at a glance. *J Cell Sci* 127(Pt 1):3–9.
- Weidberg H, et al. (2010) LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. *EMBO J* 29(11):1792–1802.
- Sou YS, et al. (2008) The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol Biol Cell* 19(11):4762–4775.
- Mehta P, Henault J, Kolbeck R, Sanjuan MA (2014) Noncanonical autophagy: One small step for LC3, one giant leap for immunity. *Curr Opin Immunol* 26:69–75.
- Ramagopalan SV, et al. (2010) A ChIP-seq defined genome-wide map of vitamin D receptor binding: Associations with disease and evolution. *Genome Res* 20(10):1352–1360.
- Cook KL, et al. (2014) Hydroxychloroquine inhibits autophagy to potentiate anti-tumor responsiveness in ER+ breast cancer. *Clin Cancer Res* 20(12):3222–3232.
- Karantza-Wadsworth V, White E (2007) Role of autophagy in breast cancer. *Autophagy* 3(6):610–613.
- Atigadda VR, et al. (2015) Conformationally defined retinoids and their efficacy in the prevention of mammary cancers. *J Med Chem* 58(19):7763–7774.
- Rangwala R, et al. (2014) Combined MTOR and autophagy inhibition: Phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. *Autophagy* 10(8):1391–1402.
- Tavera-Mendoza LE, White JH (2007) Cell defenses and the sunshine vitamin. *Sci Am* 297(5):62–65, 68–70, 72.
- Bailey ST, Shin H, Westerling T, Liu XS, Brown M (2012) Estrogen receptor prevents p53-dependent apoptosis in breast cancer. *Proc Natl Acad Sci USA* 109(44):18060–18065.
- Mohammed H, et al. (2013) Endogenous purification reveals GREB1 as a key estrogen receptor regulatory factor. *Cell Reports* 3(2):342–349.
- Tavera-Mendoza L, et al. (2006) Convergence of vitamin D and retinoic acid signalling at a common hormone response element. *EMBO Rep* 7(2):180–185.
- N'Diaye EN, et al. (2009) PLIC proteins or ubiquilins regulate autophagy-dependent cell survival during nutrient starvation. *EMBO Rep* 10(2):173–179.
- Jackson WT, et al. (2005) Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol* 3(5):e156.
- Moser AR, Hegge LF, Cardiff RD (2001) Genetic background affects susceptibility to mammary hyperplasias and carcinomas in Apc(min)/+ mice. *Cancer Res* 61(8):3480–3485.
- Mueller-Dombois D, Ellenberg H (1974) *Aims and Methods of Vegetation Ecology* (The Blackburn Press, New York).
- Hu M, et al. (2009) Role of COX-2 in epithelial-stromal cell interactions and progression of ductal carcinoma in situ of the breast. *Proc Natl Acad Sci USA* 106(9):3372–3377.
- Liu T, et al. (2011) Cistrome: An integrative platform for transcriptional regulation studies. *Genome Biol* 12(8):R83.