Mechanisms of Signal Transduction:

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Characterization of the Condensin Component Cnap1 and Protein Kinase Melk as Novel E2F Target Genes Down-regulated by 1,25-Dihydroxyvitamin D_3*

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1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) has potent antiproliferative effects characterized by a hampered G_1/S transition. cDNA microarrays were used to monitor expression of 21,492 genes in MC3T3-E1 mouse osteoblasts at 1, 6, 12, 24, and 36 h after treatment with 1,25$(OH)_2D_3$. Statistical analysis revealed a cluster of genes that were strongly down-regulated by 1,25$(OH)_2D_3$ and which not only function in cell cycle regulation and DNA replication but also mediate checkpoint control, DNA repair, chromosome modifications, and mitosis. Because many of these genes were shown earlier to be regulated by the transcriptional repressor E2F4, the intergenic regions of these 1,25$(OH)_2D_3$-down-regulated genes were searched for the presence of E2F binding sites. This led to the characterization of two novel E2F target genes, chromosome condensation-related SMC-associated protein 1 (Cnap1) and maternal embryonic leucine zipper kinase (Melk). Transfection studies and site-directed mutagenesis confirmed Cnap1 and Melk to be *bona fide* E2F targets. Repression of Cnap1 and Melk by 1,25$(OH)_2D_3$ was confirmed not only in MC3T3-E1 cells but also in several other bone-unrelated cell types. This down-regulation as well as the antiproliferative effect of 1,25$(OH)_2D_3$ depended on the pocket proteins p107 and p130 because 1,25$(OH)_2D_3$ failed to repress these E2F target genes and lost its antiproliferative action in $p107^{-/-}$;$p130^{-/-}$ cells but not in $pRb^{-/-}$ cells.

Active complexes between cyclin D and cyclin-dependent kinases 4/6 regulate the transition through the G_1/S restriction point by phosphorylation of the retinoblastoma protein (pRb)[3] and other members of the pocket protein family, p107 and p130. The phosphorylation status of these pocket proteins determines their association with members of the E2F family of transcriptional regulators, which play a pivotal role in mediating gene expression during cell proliferation. These E2F proteins can be allocated to four subclasses. Upon release by their pocket protein pRb, E2Fs 1–3 function as transcriptional activators in late G_1 and in S phase. E2F4 and E2F5 act as transcriptional repressors in quiescent and early G_1 cells by associating with p107 or p130 (1, 2). In quiescent cells repression of the promoter activity of E2F target genes is associated with the recruitment of E2F4 and p130 and low levels of histone acetylation. By late G_1, these proteins are largely replaced by activator E2Fs in concert with histone acetylation and gene activation. It is, therefore, likely that two pathways, one controlled by pRb and the other by p130/p107, regulate distinct downstream events required for G_1 progression and G_1/S transition (2, 3). Recently, the transcriptional repressor E2F6 was proposed to make up the third subclass of E2F proteins (4, 5), whereas E2F7 and E2F8 form the last subclass and are thought to regulate a subset of E2F target genes during the cell cycle (6, 7).

1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the active metabolite of vitamin D_3, acts on bone and mineral homeostasis and also inhibits proliferation and induces differentiation of various normal and malignant cells (8). However, the exact molecular mechanism behind this growth-inhibitory effect is unknown. 1,25$(OH)_2D_3$ has a cell cycle-specific effect leading to an accumulation of cells in the G_1 phase of the cell cycle (9). It has been shown previously that 1,25$(OH)_2D_3$ reduces the activity of the cyclin D1-cyclin-dependent kinase 4/6 complex, which may contribute to its antiproliferative effect (10).

In the present study a cDNA microarray was performed to examine the expression profile of 21,492 genes in MC3T3-E1 cells treated with 1,25$(OH)_2D_3$ for different times up to 36 h. Statistical analysis revealed a cluster of down-regulated genes involved in cell cycle regulation and in DNA replication but also in checkpoint control, DNA repair, chromosome transactions, and mitosis. Approximately 30% of the genes in this cluster are known E2F targets, and *in silico* promoter analysis demonstrated an additional 20% of the genes to contain E2F binding sites in their promoter. Four of these genes were selected for further analysis, namely Cnap1, Melk, retroviral integration site 2 (Ris2), and enhancer of Zeste homolog 2 (Ezh2). Expression of these genes was growth-regulated as were the promoter activities of Cnap1 and Melk. Mutational analysis revealed that the identified E2F binding sites were required for transactivation by E2F family members. Rather than being key genes responsible for the antiproliferative effect of 1,25$(OH)_2D_3$, these genes are suggested to be part of the general mechanism by which the pocket proteins translate the effect of 1,25$(OH)_2D_3$ and regulate a large number of E2F target genes. Because $p107^{-/-}$;$p130^{-/-}$-cells no longer responded to the antiproliferative activity of 1,25$(OH)_2D_3$, we suggest that 1,25$(OH)_2D_3$ exerts this growth-inhibitory effect by means of the repressive activity of p107/p130·E2F complexes rather than by affecting pRb-related E2F activity, as previously suggested.

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[3] The abbreviations used are: pRb, retinoblastoma protein; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; MEF, murine embryonic fibroblast; QRT-PCR, quantitative real-time PCR; wt, wild type.

TABLE ONE

Identification of genes downregulated by 1,25(OH)$_2$D$_3$ treatment and their expression profile

Some genes may be classified into different functional categories and therefore appear more than once in TABLE ONE.

Name	Accession no.	Gene expression in MC3T3-E1 cells treated with 10^{-8} M 1,25(OH)$_2$D$_3$ relative to gene expression in vehicle-treated cells					Function
		1 h	6 h	12 h	24 h	36 h	
Cell Cycle Regulation							
Ccna2	BG069688	1.06	1.09	1.08	0.52	0.43	Cyclin-dependent protein kinase regulator activity
Ccna2	BG073518	1.00	0.86	0.78	0.49	0.43	
Ccnb1	AA396324	1.07	1.15	0.99	0.52	0.39	Cyclin-dependent protein kinase regulator activity (G_2/M)
Ccnb1	BG078426	1.01	1.07	1.13	0.54	0.40	
Cdc20	BG078638	1.01	1.18	0.89	0.54	0.38	Cell division cycle protein; key regulator of the cell cycle
Cdc2a	M38724/X16461	1.02	0.92	0.89	0.49	0.50	Cell division cycle protein; kinase activity (G_2/M)
Cdc2a	BG064846	0.94	1.05	0.91	0.45	0.34	
DNA replication							
Ask pending	BG082035	1.05	1.14	0.67	0.67	0.51	Regulation of S phase of mitotic cell cycle; kinase activity
Cdc45I	BG063139	1.00	1.04	0.59	0.56	0.41	Initiation of DNA replication
Cdc6	AA048426	1.02	0.84	0.76	0.40	0.29	Cell division cycle 6 homolog ~DNA replication; recruitment of Mcm proteins
Cdc6	AA189836	1.12	0.83	0.82	0.72	0.43	
Cdc6	BG077012	1.01	0.80	0.64	0.35	0.23	
Chaf1a	BG070452	0.93	0.95	0.74	0.36	0.27	Chromatin assembly factor 1 subunit A and B involved in DNA replication and repair
Chaf1b	BG072835	0.67	1.12	1.02	0.52	0.36	
CTF18	BG063423	1.03	1.10	0.69	0.62	0.39	Chromosome transmission fidelity factor
Fen1	BG063590	0.95	0.95	0.83	0.43	0.34	Flap structure-specific endonuclease 1 ~DNA replication
Fignl1	BG078212	0.97	0.90	0.96	0.49	0.42	Fidgetin-like 1; ATP and nucleotide binding
FoxM1	AA066741	1.01	1.05	0.72	0.47	0.36	Transcription factor; essential for DNA replication and mitosis
FoxM1	BG087468	0.92	1.15	0.90	0.52	0.32	
Lig1	U19604/M36067	0.94	0.89	0.79	0.48	0.37	Ligase 1, DNA- and ATP-dependent; involved in DNA replication
Lig1	U19604/M36067	0.96	0.96	0.83	0.55	0.38	
Lig1	W66626	1.19	0.90	1.10	0.58	0.36	
Lig1	BG079173	0.93	0.98	0.89	0.42	0.36	
Mcm2	AA011839	0.99	1.01	0.81	0.57	0.44	Minichromosome maintenance deficient mitotins; proteins involved in initiation of DNA replication
Mcm2	BG074668	0.89	0.72	0.73	0.55	0.44	
Mcm3	BG065055	0.98	0.93	0.86	0.43	0.28	
Mcm4	AA259788	1.03	0.92	0.85	0.38	0.31	
Mcm5	BG064865	0.92	0.96	0.86	0.41	0.27	
Mcm7	BG074721	0.90	1.02	0.94	0.73	0.28	
PCNA	BG064598	0.96	0.92	0.88	0.44	0.40	Proliferating cell nuclear antigen; regulator of DNA replication
PCNA	AA116947	0.91	0.95	0.85	0.51	0.43	
Pol ϵ	BG069732	0.88	0.88	0.92	0.46	0.47	Polymerase (DNA directed) ϵ; involved in DNA replication and repair
Pol ϵ2	BG071480	0.95	1.03	0.85	0.45	0.55	
Prim1	AA259900	0.89	0.88	0.65	0.51	0.40	Part of DNA-polα-primase complex; ~DNA replication
Rfc3	BG068309	0.85	0.90	0.68	0.54	0.50	Replication factor C (activator 1); ~DNA replication
Ris2	BG064684	1.00	0.92	0.77	0.59	0.50	Retroviral integration site 2; DNA replication factor
Rpa2	BG075372	0.89	0.88	0.73	0.53	0.38	Replication protein A2
Rrm2	BG076613	0.95	0.84	0.53	0.34	0.28	Ribonucleotide reductase M2; catalyzes formation of deoxyribonucleotides from ribonucleotides
Rrm2	BG078138	0.95	0.75	0.56	0.35	0.30	
Tk1	AA041834	0.95	1.15	0.62	0.31	0.23	Thymidine kinase 1; involved in DNA metabolism

TABLE ONE—*CONTINUED*

Name	Accession no.	Gene expression in MC3T3-E1 cells treated with 10^{-8} M $1,25(OH)_2D_3$ relative to gene expression in vehicle-treated cells					Function
		1 h	6 h	12 h	24 h	36 h	
Tk1	BG077745	0.83	0.96	0.69	0.39	0.31	
Umps	BG063291	0.98	1.02	0.84	0.59	0.55	Uridine monophosphate synthetase
Checkpoints							
Bub1b	BG069421	1.00	1.08	0.98	0.53	0.42	Essential for spindle checkpoint activation
Mad2l1	AA002895	0.98	1.01	0.83	0.55	0.40	Mitotic arrest deficient-like 1 (yeast); component of mitotic spindle assembly checkpoint
Mad2l1	BG067860	1.02	0.97	0.88	0.52	0.36	
Tlk1	AA466288	1.01	0.79	0.69	0.39	0.33	Tousled-like kinase 1; ~chromatin modification
DNA repair							
Chaf1a	BG070452	0.93	0.95	0.74	0.36	0.27	Chromatin assembly factor 1 subunit A and B involved in DNA replication and repair
Chaf1b	BG072835	0.87	1.12	1.02	0.52	0.36	
Exo1	NM_012012	1.01	0.91	0.80	0.69	0.45	Exonuclease 1; 5′–3′ exonuclease activity
Fen1	BG063590	0.95	0.95	0.83	0.43	0.34	Flap-structure specific endonuclease 1; ~DNA replication
PCNA	BG064598	0.96	0.92	0.88	0.44	0.40	Proliferating cell nuclear antigen
PCNA	AA116947	0.91	0.95	0.85	0.51	0.43	Proliferating cell nuclear antigen
Pol ϵ	BG069732	0.88	0.88	0.92	0.46	0.47	Polymerase (DNA directed) ϵ; involved in DNA replication and repair
Pol ϵ2	BG071480	0.95	1.03	0.85	0.45	0.55	
Rad51	D13473	0.92	0.87	0.91	0.54	0.36	RAD51 homolog (*Saccharomyces cerevisiae*); involved in homologous recombination and repair of DNA; interacts also with BRCA1 and BRCA2
Rad51	D13473	0.94	0.93	0.84	0.61	0.37	
Rad51	BG072904	1.03	1.12	0.89	0.46	0.37	
Rad51ap1	AA386769	0.91	0.83	0.76	0.55	0.53	RAD51-associated protein 1
Rfc3	BG068309	0.85	0.90	0.68	0.54	0.50	Replication factor C (activator 1); ~DNA replication
Rpa2	BG075372	0.89	0.88	0.73	0.53	0.38	Replication protein A2
Chromatin assembly, modification, condensation, segregation							
Cenpa	BG072056	0.98	1.20	0.90	0.55	0.43	Centromere autoantigen A; involved in chromosome organisation and biogenesis
Cenpa	BG082881	1.10	1.15	0.95	0.61	0.51	
Cenph	AA198524	0.90	0.88	0.87	0.52	0.42	Centromere autoantigen H; kinetochore protein involved in chromosome segregation
Cenph	BG071683	0.98	0.86	0.77	0.38	0.41	
Chaf1a	BG070452	0.93	0.95	0.74	0.36	0.27	Chromatin assembly factor 1 subunit A and B involved in DNA replication and repair
Chaf1b	BG072835	0.87	1.12	1.02	0.52	0.36	
Cnap1	BG082566	0.96	1.11	0.87	0.47	0.40	Chromosome condensation-related SMC-associated protein 1
Espl1	BG071861	0.91	1.10	0.93	0.38	0.48	Extra spindle poles like-1 (*S. cerevisiae*)
Ezh2	BG074931	1.02	0.91	0.96	0.43	0.42	Enhancer of Zeste homolog
H2afz	BG065110	0.98	0.98	1.01	0.58	0.44	H2A histone family, member Z; involved in chromosome organization and biogenesis
H2afz	BG065111	0.98	0.94	1.00	0.57	0.45	
Hmgb3	BG078700	0.97	0.96	0.96	0.49	0.54	High mobility group box 3
Hmgn2	BG078806	0.95	0.95	1.05	0.50	0.45	High mobility group nucleosomal binding domain 2
Nasp	BG076805	1.00	0.89	0.82	0.58	0.44	Nuclear autoantigenic sperm protein (histone binding)
Nusap1	AA265789	0.83	0.94	0.77	0.47	0.43	Nucleolar- and spindle-associated protein 1
Pcnt2	BG071845	0.92	1.03	0.93	0.49	0.56	Pericentrin2 ~spindle assembly, microtubule organizing center
Smc2like1	BG077844	1.05	1.01	0.98	0.44	0.41	SMC2 structural maintenance of chromosomes 2-like 1
Suv39h1	AA050907	0.93	0.97	1.03	0.63	0.54	Suppressor of variegation 3–9 homolog 1 (*Drosophila*); involved in chromatin modification
Suv39h1	BG087679	0.89	1.04	0.86	0.42	0.39	
Tlk1	AA466288	1.01	0.79	0.69	0.39	0.33	Tousled-like kinase 1; ~chromatin modification

TABLE ONE—*CONTINUED*

Name	Accession no.	Gene expression in MC3T3-E1 cells treated with 10^{-8} M $1,25(OH)_2D_3$ relative to gene expression in vehicle-treated cells					Function
		1 h	6 h	12 h	24 h	36 h	
Mitosis							
Anillin	BG063979	0.95	0.90	0.88	0.49	0.41	Actin binding protein involved in cytokinesis
Cdca5	BG068799	0.99	0.86	0.88	0.55	0.34	Cell division cycle associated 5; ~cytokinesis
Cdca8	BG078299	1.00	1.04	0.89	0.56	0.49	Cell division cycle associated 8; ~cytokinesis
Ect2	AA267000	1.03	0.94	1.15	0.54	0.37	Oncogene involved in regulation of cytokinesis
FoxM1	AA066741	1.01	1.05	0.72	0.47	0.38	Transcription factor; essential for DNA replication and mitosis
FoxM1	BG087468	0.92	1.15	0.90	0.52	0.32	
Incenp	BG076909	1.01	1.04	0.66	0.44	0.39	Inner centromere protein; involved in cytokinesis
Kif20a	AA177197	1.15	1.20	0.99	0.57	0.41	Kinesin family member 20A; microtubule associated complex
Kif22	AA008189	1.02	1.13	0.80	0.50	0.33	Kinesin family member 22; microtubule associated complex
Kif23	BG068324	1.04	1.05	0.99	0.41	0.42	Kinesin family member 23; microtubule associated complex
Kif23	BG068666	1.14	1.03	1.06	0.40	0.44	
Melk	BG076892	0.93	0.93	0.72	0.40	0.28	Maternal embryonic leucine zipper kinase
Nek2	AA268349	1.03	1.11	0.92	0.51	0.38	Nima (never in mitosis gene a)-related expressed kinase 2 involved in centrosome separation and cytokinesis
Nek2	BG065826	0.99	1.05	1.07	0.60	0.53	
Prc1	AA254552	1.03	1.00	0.89	0.47	0.40	Protein regulator of cytokinesis
Racgap1	AA140523	1.12	1.01	1.05	0.58	0.39	Rac GTPase-activating protein 1; regulates cytokinesis
Spag5	AA086796	1.00	1.10	0.85	0.58	0.42	Sperm assoc. antigen 5; localizes to mitotic spindles
Suv39h1	AA050907	0.93	0.97	1.03	0.63	0.54	Suppressor of variegation 3–9 homolog 1 (*Drosophila*); involved in chromatin modification
Suv39h1	BG087679	0.89	1.04	0.86	0.42	0.39	
Miscellaneous							
Aaas	BG083343	0.86	0.97	0.69	0.43	0.38	Alias Aladin; involved in nucleocytoplasmic transport
Exosc8	BG088541	0.94	0.97	0.87	0.44	0.44	Exosome component 8; involved in (r)RNA processing
Kpna2	BG066442	0.95	0.99	1.08	0.43	0.37	Karyopherin (importin) a2 involved in nuclear transport
Lsm3	AA270652	0.91	0.96	0.95	0.72	0.56	U6 small RNA processing; involved in mRNA processing
Nup37	BG081608	1.01	1.01	0.85	0.58	0.58	Nucleoporin 37; involved in protein transport
Nup43	BG082571	0.98	0.95	0.76	0.47	0.45	Nucleoporin 43; involved in protein transport
Nup93	BG063793	0.97	1.00	0.96	0.65	0.58	Nucleoporin 93; involved in protein transport
Nurim	AA270364	0.90	0.92	0.65	0.60	0.50	Nuclear envelope membrane protein
Nurim	BG071534	0.88	1.00	0.61	0.32	0.32	Nuclear envelope membrane protein
Odc	BG069647	0.76	0.94	0.76	0.48	0.45	Ornithine decarboxylase; ~polyamine biosynthesis
Pbk	AA036322	0.98	0.96	1.00	0.42	0.35	PDZ binding kinase; protein kinase activity
Pbk	AA415579	0.93	0.93	0.95	0.56	0.46	
Pbk	BG063624	1.05	1.05	1.05	0.38	0.30	
Stathmin	AA265396	0.90	1.00	0.79	0.39	0.29	Regulation of microtubule filament system
Tacc3	W85166	1.07	0.97	1.05	0.55	0.46	Transforming, acidic coiled-coll-containing protein 3
Tacc3	BG068759	0.98	1.05	1.15	0.74	0.40	
Tacc3	AA190123	1.01	1.12	1.14	0.51	0.41	
Tacc3	BG083765	0.99	1.09	1.08	0.49	0.43	
Tagnl2	BG077550	0.87	0.99	0.72	0.61	0.51	Transgelin 2; actin-associated protein, function unknown
Tcf19	BG069294	0.92	0.97	0.68	0.58	0.38	Transcription factor 19; ~DNA dependent transcription
Timm50	BG083100	0.86	1.09	0.79	0.55	0.65	Translocase of inner mitochondrial membrane 50 homolog; determines sensitivity to apoptotic signals

TABLE ONE—*CONTINUED*

Name	Accession no.	Gene expression in MC3T3-E1 cells treated with 10^{-8} M $1,25(OH)_2D_3$ relative to gene expression in vehicle-treated cells					Function
		1 h	6 h	12 h	24 h	36 h	
Timm50	BG071069	0.80	1.18	0.71	0.53	0.56	
Xpo1	AA105546	0.96	1.00	0.94	0.45	0.43	Exportin 1; involved in protein nucleus export
Riken clones							
1700021F05Rik	AA245492	0.90	0.97	0.71	0.45	0.28	
2410015N17Rik	BG063758	0.94	1.04	0.63	0.50	0.45	
2610005B21Rik	BG071704	0.99	1.05	0.99	0.44	0.47	
2610019103Rik	W89966	0.99	1.03	0.89	0.61	0.42	Proliferation associated nuclear element 1
2610040C18Rik	BG071555	0.93	0.92	0.82	0.42	0.45	~Chromatin structure and dynamics
2610528A17Rik	AA511242	1.00	0.94	0.83	0.51	0.51	
2610528M18Rik	BG074710	0.97	0.98	0.98	0.44	0.42	
2810417H13Rik	BG076569	1.09	0.92	0.98	0.48	0.44	
2810417H13Rik	BG076724	1.15	0.86	0.81	0.34	0.29	
2810417H13Rik	BG073230	0.98	0.93	0.73	0.36	0.38	
2810475A17Rik	BG078065	0.93	0.98	1.01	0.52	0.50	Membrane protein
F730047E07Rik	AA288248	0.97	0.98	0.96	0.59	0.50	
ESTs							
EST	AU018687	0.90	0.97	0.67	0.62	0.35	
EST	AW538220	1.08	0.98	0.87	0.60	0.46	
EST	BG064704	0.86	0.86	0.74	0.58	0.48	
EST	BG068885	1.10	1.05	0.85	0.60	0.45	
EST	BG074658	0.94	0.97	0.94	0.72	0.41	

EXPERIMENTAL PROCEDURES

Cell Culture—MC3T3-E1 cells (Riken Cell Bank, Tsukuba, Japan) and GR cells were cultured as previously described (11). Wild type, pRb, p107, and p130 nullizygous as well as p107 p130 double nullizygous murine embryonic fibroblasts (wt, $pRb^{-/-}$, $p107^{-/-}$, $p130^{-/-}$, and $p107^{-/-}$;$p130^{-/-}$ murine embryonic fibroblasts (MEFs)) were cultured in Dulbecco's modified Eagle's medium with 4.5 mg/ml glucose with 10% fetal bovine serum, 2 mM glutaMAX-I, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

Proliferation Assays—Antiproliferative effects of $1,25(OH)_2D_3$ were measured by [^{3}H]thymidine incorporation or by analysis of cell cycle distribution as previously described (11, 12).

Total RNA Extraction—Total RNA for microarray analysis was extracted with TRizol LS reagent (Invitrogen). Total RNA for quantitative RT-PCR analysis was isolated with the RNeasy kit (Qiagen, Hilden, Germany).

Construction of Microarrays—The mouse gene set consisted of 5 separate microarrays containing in total 21,492 cDNA fragments. The clone set was composed from the 8000 collection of Incyte (Mouse Gem I, Incyte, Wilmington, DE) and from the 15,000 collection of National Institute of Aging (HGMP Resource Centre, Cambridge, UK). A complete description of the array content and the printing procedures can be downloaded from ArrayExpress (www.ebi.ac.uk/arrayexpress) with accession number A-MECP-146.

RNA Labeling and Hybridization—Antisense RNA amplification, RNA labeling, and hybridization were performed as previously described (11). All protocols can be downloaded from www.microarrays.be or via ArrayExpress (www.ebi.ac.uk/arrayexpress) with accession number P-MEXP578-582.

Scanning and Microarray Data Analysis—Array slides were scanned using a Generation III scanner (Amersham Biosciences) with wavelength settings at 532 nm (Cy3 signal) and 635 nm (Cy5 signal). Image analysis was performed with ArrayVision (Imaging Research Inc., St. Catharines, Ontario, Canada). Spot intensities were measured as artifact-removed total intensities (ARVol). Spot intensities were normalized using a Loess-fit (13) for removing nonlinear dye related variation followed by a global analysis of variance normalization (14). The obtained expression data were clustered with the AQBC algorithm (15).

Subsequently, the intergenic regions of all the genes in the resulting clusters were selected using the Ensembl mart data base release 18.1 (16). The intergenic region is defined as the region upstream of the transcription start, limited to 2 kilobases, and the 5′-untranslated region, limited to the first intron. These intergenic regions (both direct and indirect strands) were then screened with a position-specific weight matrix of E2F, downloaded from the jaspar data base (jaspar.cgb.ki.se) (17, 18). The screening was performed using the MotifScanner algorithm with prior set to 0.2 and a mouse-specific zero-order background model (19).

Plasmids—A TK-TATA luciferase reporter vector served as a control vector for the same reporter construct in which six artificial E2F binding sites were cloned (20). The pGL3-Basic reporter vector (Promega, Madison, WI) was used as control for pGL3-Basic vectors in which both short and long fragments of the intergenic regions of mouse Melk and Cnap1 were cloned. Site-directed mutagenesis of the E2F binding sites in these promoter regions was performed by use of the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. The sequences of the primers used are available upon request.

Expression plasmids pcDNA-HA-E2F1, -E2F2, -E2F3, and -E2F4 were kind gifts of Dr. J. Nevins (Duke University Medical Center, Durham, NC). The cytomegalovirus-hemagglutinin-E2F5 expression plasmid was a kind gift of Dr. J Magae (Institute of Research and Innovation, Chiba, Japan). The β-galactosidase expression vector pcDNA3.1(-)/Myc-His/*lacZ* and the pcDNA3.1/Myc-His vector were obtained from Invitrogen.

Transfection Assays—Exponentially growing MC3T3-E1 cells were transfected with FuGENE 6 (Roche Diagnostics) in 24-well dishes (2×10^5 cells/well) with 100 ng of luciferase reporter vector (or representative control vector), 50 ng of the different E2F constructs (or the empty pcDNA3.1/Myc-His), and 10 ng of pcDNA3.1(−)/Myc-His/*lacZ*. Cells were lysed 48 h after transfection (with reporter lysis buffer, Roche Diagnostics), and luciferase activity was measured with the luciferase assay system (Promega) and normalized to β-galactosidase activity, measured with the Galacto-Light Plus System (Applied Biosystems, Foster City, CA).

To measure growth-dependent induction of the Cnap1 and Melk promoter activities, growth-arrested MC3T3-E1 cells (48 h in α-minimal essential medium with 0.1% fetal bovine serum) were transfected with 100 ng of luciferase reporter vector (or control vector) and 10 ng of pcDNA3.1(-)/Myc-His/*lacZ*. The next day cells were released into the cell cycle by the addition of α-minimal essential medium with 15% fetal bovine serum.

The effect of $1,25(OH)_2D_3$ treatment on the promoter activities of Cnap1 and Melk was determined by transfection of MC3T3-E1 cells and wt or $p107^{-/-}$;$p130^{-/-}$ MEFs with 100 ng of luciferase reporter vector (or control vector) and 10 ng of pcDNA3.1(−)/Myc-His/*lacZ*. The day after transfection MC3T3-E1 cells were stimulated with 10^{-8} M $1,25(OH)_2D_3$, and luciferase and β-galactosidase activities were assessed after a 24-h incubation period with $1,25(OH)_2D_3$. wt and $p107^{-/-}$;$p130^{-/-}$ MEFs were stimulated for 48 h with 10^{-7} M $1,25(OH)_2D_3$.

Quantitative Real-time PCR—cDNA production, PCR reactions, and subsequent quantification was performed as described previously (21). PCR primers and fluorogenic probes (6-carboxyfluorescein as reporter and 6-carboxytetramethylrhodamine as quencher dye) for mouse Ezh2, Ris2, Cnap1, Melk, VDR, CYP24, and β-actin were purchased from Eurogentec (Seraing, Belgium). Sequences of primers and probes are available upon request.

Chromatin Immunoprecipitation Reactions—Chromatin immunoprecipitation assays were based on a previously described protocol (22) with minor modifications. In brief, 10^6 MC3T3-E1 cells were cross-linked with formaldehyde (1%) for 10 min. After lysis of the cells, samples were sonicated with a Branson Sonifer 250 to generate DNA fragments with an average length of 500 bp. Subsequently, samples were incubated overnight with 10 μg of anti-E2F1-antibody (sc-193x, Santa Cruz Biotechnology, Santa Cruz, CA) or irrelevant antibody ("mock", rabbit anti-mouse immunoglobulins, Dako, Denmark) at 4 °C with rotation. After collection and elution of immunocomplexes, cross-links were reversed, and DNA was recovered with a QIAquick spin kit (Qiagen) and eluted in 30 μl. 4 μl of recovered DNA was used for PCR analysis. PCR products were analyzed by standard gel electrophoresis. The sequences of the primers used are available upon request.

Statistics—Statistical analysis was performed with the software program NCSS (NCSS, Kaysville, UT). All results are expressed as the means and S.E. of at least three independent experiments. Analysis of variance analyses were followed by a Bonferroni multiple comparison test or a Student's *t* test. $p < 0.05$ was accepted as significant.

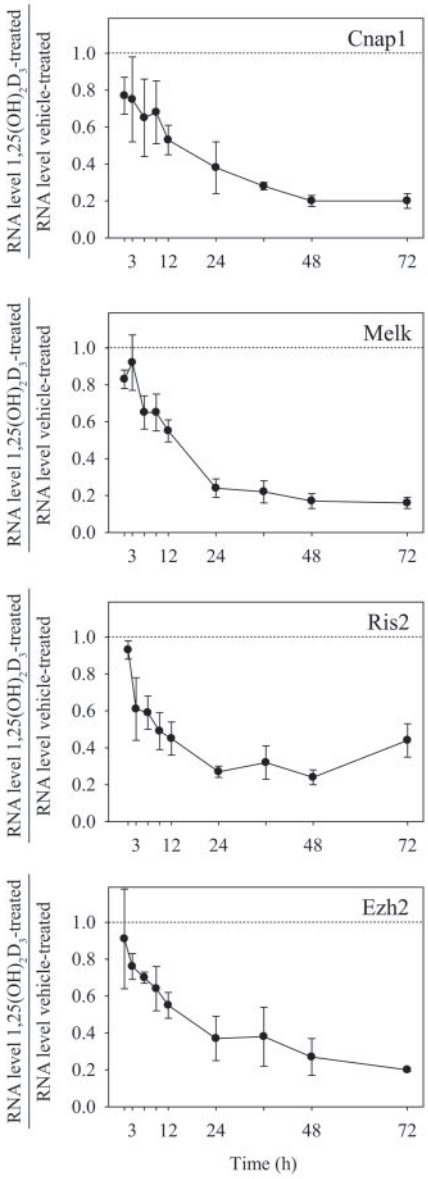

FIGURE 1. **QRT-PCR analyses on MC3T3-E1 cells.** MC3T3-E1 cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle for 1, 3, 6, 9, 12, 24, 36, 48, or 72 h. At each of these time points gene expression was determined by QRT-PCR analysis, normalized to β-actin levels, and expressed as a ratio between $1,25(OH)_2D_3$-treated and corresponding vehicle-treated samples. The *dotted line* indicates the 1:1 ratio. Data represent the mean and S.E. of three independent experiments. All samples used for QRT-PCR were independent from those used for cDNA microarray analysis. For all reported genes, the overall down-regulation by $1,25(OH)_2D_3$ was found to be significant according to the Bonferroni multiple-comparison test ($p < 0.05$).

RESULTS

Genes Down-regulated after Treatment with $1,25(OH)_2D_3$ Cluster into Distinct Functional Groups—Analysis of microarray data led to the identification of a cluster of 94 different genes, which were similarly down-regulated by $1,25(OH)_2D_3$ (TABLE ONE). Down-regulation started at 12–24 h after treatment, and the degree of down-regulation at 36 h ranged from 1.5- to 4.3-fold. $1,25(OH)_2D_3$ not only decreased the expression of genes that are involved in cell cycle regulation and DNA replication but also that of genes that mediate checkpoint control, DNA repair, chromosome transactions, and mitosis.

Approximately 30% of the genes in this cluster were known E2F targets. Therefore, the remaining genes in this cluster were screened for E2F binding sites in their promoter. An additional 20% of the genes was found to contain E2F binding sites. Four of these genes were selected for further study based on the highly conserved E2F binding sites in their promoter (Cnap1, Ezh2, and Ris2), on the one hand, and on their overexpression in undifferentiated cancers, on the other hand (Cnap1, Ezh2, and Melk) (23).

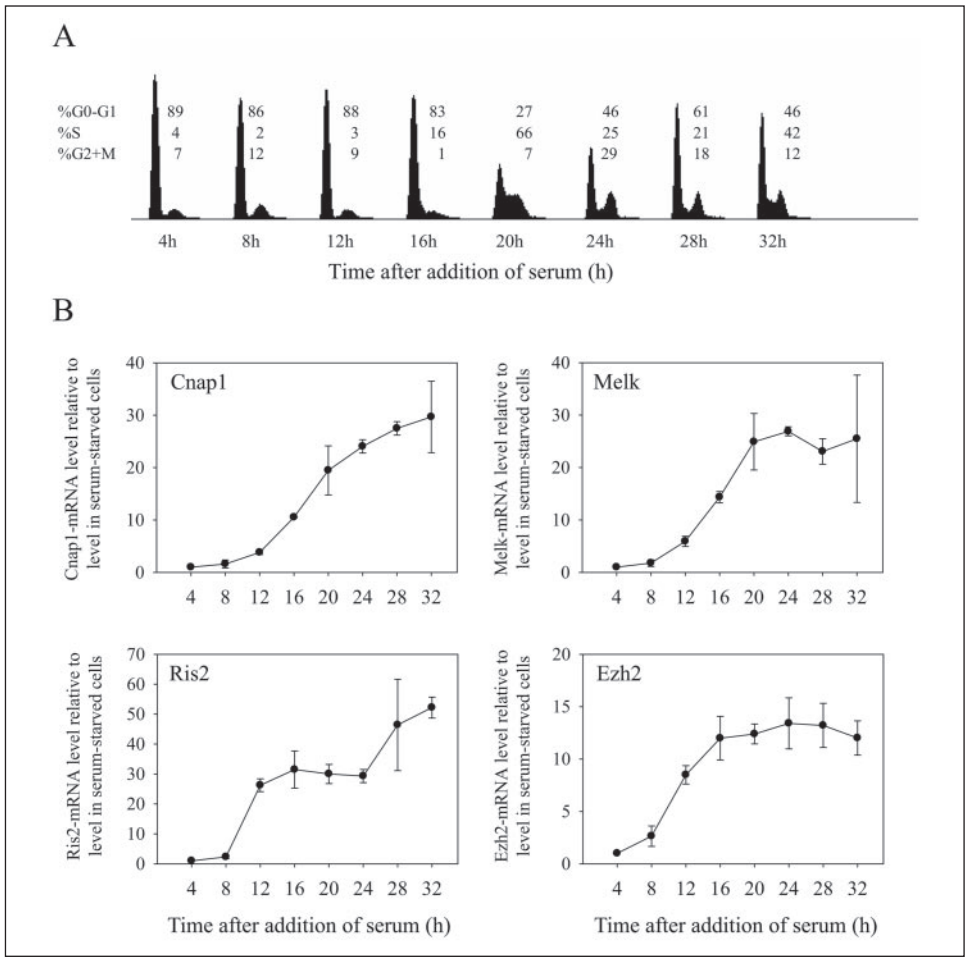

FIGURE 2. **Induction of gene expression after serum addition to serum-starved MC3T3-E1 cells.** *A*, time schedule of cell cycle re-entry after serum addition to serum-starved MC3T3-E1 cells as determined by fluorescence-activated cell sorter analysis. *B*, Cnap1, Melk, Ris2, and Ezh2 expression, normalized to β-actin, were measured by QRT-PCR after the addition of serum to MC3T3-E1 cells that were synchronized in the G_1 phase of the cell cycle by serum starvation. Data represent the mean and S.E. of three independent experiments. For all reported genes, the overall induction of gene expression after serum addition was found to be significant according to the Bonferroni multiple-comparison test ($p < 0.05$).

Expression Analysis of Cnap1, Melk, Ris2, and Ezh2 in 1,25(OH)$_2$D$_3$-treated Cells—Quantitative real-time PCR (QRT-PCR) experiments were performed in MC3T3-E1 cells to monitor the expression profile of these genes at different time points up to 72 h after treatment with a single dose of 1,25(OH)$_2$D$_3$ (10^{-8} M) (Fig. 1). The expression of all 4 genes decreased as soon as 6 h after treatment. A maximal 5-fold reduction was observed at 48–72 h after treatment.

Growth-dependent Expression of Cnap1, Melk, Ris2, and Ezh2—To determine whether the expression of Cnap1, Melk, Ris2, and Ezh2 was growth-regulated, MC3T3-E1 cells were serum-starved for 48 h and subsequently stimulated to re-enter the cell cycle by the addition of serum (Fig. 2). As shown in Fig. 2*B*, mRNA transcripts of all four genes strongly increased after the addition of serum and peaked at the G_1/S transition (16–20 h after re-feeding, Fig. 2*A*), which suggested that the regulation of these genes was growth-dependent.

Growth-dependence of Cnap1 and Melk Promoter Activities—Regulation of promoter activity was only investigated for Cnap1 and Melk because during the course of our studies Ezh2 and Cdt1 (human homolog of Ris2) were reported to be E2F-regulated (24, 25). As demonstrated in Fig. 3*B*, promoter constructs of Cnap1 and Melk, which carry an E2F-responsive site close to the transcription start site (Fig. 3*A*), were markedly up-regulated after re-stimulation of transfected serum-starved MC3T3-E1 cells. An artificial reporter construct with six E2F binding sites showed the same pattern of induction when transfected serum-starved MC3T3-E1 cells were re-fed with serum.

E2F Binds to the Promoter Regions of Cnap1 and Melk and Enhances Their Transcriptional Activities—Exponentially growing MC3T3-E1 cells were cotransfected with the abovementioned promoter constructs for Cnap1 and Melk and with expression plasmids for different members of the E2F family to investigate whether exogenous expression of E2Fs could enhance transcriptional activity of these promoter constructs. E2F1, -2, -3, and -4 were able to transactivate reporter constructs that were driven by either six artificial E2F binding sites (Fig. 4*A*) (7–10-fold induction) or by promoter regions of Cnap1 (Fig. 4*B*, *left panel*) (1.5–3.7-fold induction) or Melk (Fig. 4*C*, *left panel*) (1.5–2.6-fold induction). E2F5 did not enhance transcriptional activity of these promoter constructs. Comparable results were obtained with truncated reporter constructs that still carried the consensus E2F-responsive region (Fig. 4, *B–C*, *middle panels*). Mutation of the newly identified E2F binding sites within these truncated promoter constructs completely abolished their responsiveness to E2F (Fig. 4, *B--C*, *right panels*). The basal activities of the different reporter constructs were substantially higher than that of the pGL3-Basic reporter vector (Fig. 4*D*). Remarkably, mutation of the E2F-binding site in the Cnap1 reporter vector significantly increased the basal activity of this vector.

Chromatin immunoprecipitation assays demonstrated that Cnap1 and Melk were direct targets for E2F1 in vivo in living cells (Fig. 5). A promoter fragment of osteopontin, which contained no consensus E2F binding sites, was included as a negative control. No binding of E2F1 to the osteopontin promoter region was observed. Cell division cycle 6 homolog (Cdc6), previously identified as a direct target of E2F, was used as a positive control, and clear binding of E2F1 to its promoter region could be demonstrated.

1,25(OH)$_2$D$_3$-induced Down-regulation of Promoter Activities of Cnap1 and Melk Is Mediated by the E2F-responsive Region within Their Promoters—In exponentially growing, transfected MC3T3-E1 cells, 1,25(OH)$_2$D$_3$ clearly decreased the promoter activities of Cnap1 and

Melk. $1,25(OH)_2D_3$ even inhibited their promoter activities when added after exogenous overexpression of E2F transcription factors (data not shown). Mutation of the E2F-binding site in the Cnap1 promoter completely abolished the repressive effect of $1,25(OH)_2D_3$ (Fig. 6). When the E2F-binding site in the Melk promoter construct was mutated, the repressive effect of $1,25(OH)_2D_3$ was smaller in comparison with its effect on the wild type construct (from a 50% reduction to a 30% reduction in transcriptional activity) but was not completely abrogated.

The Pocket Proteins, p107 and p130, Are Essential Mediators of the $1,25(OH)_2D_3$-induced Down-regulation of Cnap1, Melk, Ris2, and Ezh2—The role of the pocket protein family in the antiproliferative effect of $1,25(OH)_2D_3$ and in the $1,25(OH)_2D_3$-induced down-regulation of E2F target gene transcription was assessed by the use of wt, $pRb^{-/-}$, $p107^{-/-}$, and $p130^{-/-}$ single knock-out MEFs as well as $p107^{-/-}$;$p130^{-/-}$ double knock-out MEFs.

$1,25(OH)_2D_3$ significantly reduced the growth of wt MEFs with a maximal inhibition at concentrations of 10^{-7}–10^{-6} M (Fig. 7*A*). Compared with the antiproliferative effect observed in MC3T3-E1 cells, this growth inhibition was rather mild. Therefore, gene expression was studied after treatment with a higher concentration of $1,25(OH)_2D_3$ (10^{-7} M) and at later time points (24 till 72 h after treatment). Down-regulation of Cnap1, Melk, and Ris2 expression levels started at 24 h after treatment with $1,25(OH)_2D_3$ and reached a maximal 1.7-fold reduction after 48–72 h (Fig. 7*B*). Down-regulation of gene expression was modest but occurred at the transcriptional level as treatment with $1,25(OH)_2D_3$ led to a significant decrease of Cnap1 and Melk promoter activities (Fig. 7*C*).

The antiproliferative activity of $1,25(OH)_2D_3$ was minimally affected by loss of either p107 or p130 (data not shown), whereas $1,25(OH)_2D_3$ failed to inhibit the proliferation of $p107^{-/-}$;$p130^{-/-}$ MEFs (Fig. 8*A*). These data confirmed that loss of either p107 or p130 is compensated by the remaining pocket protein (1, 3, 26, 27). Therefore, the role of the pocket proteins in the antiproliferative effect of $1,25(OH)_2D_3$ was investigated by the use of $p107^{-/-}$;$p130^{-/-}$ double knock-out MEFs. In these cells $1,25(OH)_2D_3$ did not affect the expression of Cnap1, Melk, Ris2, and Ezh2 (Fig. 8*B*). Correspondingly, $1,25(OH)_2D_3$ failed to repress the promoter activities of Cnap1 and Melk in $p107^{-/-}$;$p130^{-/-}$ MEFs (Fig. 8*C*). Nevertheless, $p107^{-/-}$;$p130^{-/-}$ MEFs (Fig. 8*D*) as well as wt MEFs (Fig. 7*D*) contain a functional VDR and are responsive to $1,25(OH)_2D_3$, judged by the huge induction of 24-hydroxylase (CYP24), a primary and direct $1,25(OH)_2D_3$-target gene.

$pRb^{-/-}$ MEFs remained responsive to the antiproliferative effects of $1,25(OH)_2D_3$ but to a lower extent than wt MEFs (Fig. 9*A*). Still, the expression of Cnap1, Melk, and Ris2 was significantly down-regulated in $pRb^{-/-}$ MEFs (Fig. 9*B*).

An overall statistical analysis of the regulation of Cnap1, Melk, and Ris2 in the different cell types revealed significant differences between wt and $p107^{-/-}$;$p130^{-/-}$ MEFs on the one hand and between $pRb^{-/-}$ and $p107^{-/-}$;$p130^{-/-}$ MEFs on the other hand. However, no significant differences were found between wt and $pRb^{-/-}$ MEFs.

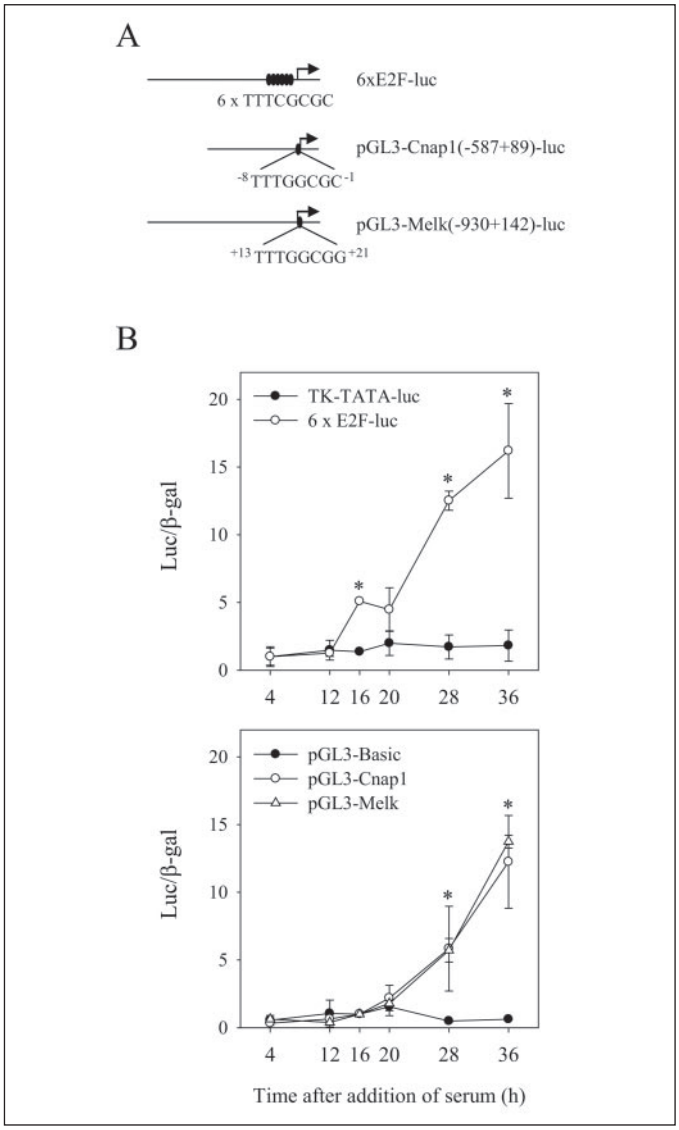

FIGURE 3. **Growth-regulated activity of Cnap1 and Melk promoter constructs.** *A*, schematic overview of reporter vectors used in transient transfection experiments. A luciferase reporter vector with six artificial E2F binding sites was used as a positive control. Part of the intergenic regions of Cnap1 (676 bp) or Melk (1028 bp) was cloned in the pGL3-Basic luciferase reporter vector. *Arrows* indicate transcription start sites, whereas *black ovals* represent the E2F binding sites. *B*, serum-starved MC3T3-E1 cells were transiently transfected with the luciferase reporter constructs or their corresponding empty control vectors. Luciferase activity was measured at different times after serum addition and normalized against β-galactosidase activity. Reported data are the mean and S.E. of at least three independent experiments. *, $p < 0.05$, 6xE2F-luc *versus* pTK-TATA-luc (*upper panel*) and pGL3-Cnap1 and pGL3-Melk *versus* pGL3-basis (*lower panel*) (Student's *t* test).

DISCUSSION

Statistical analysis of the present microarray study, in which the effect of $1,25(OH)_2D_3$ on MC3T3-E1 cells was investigated, revealed a cluster of genes that were strongly down-regulated after treatment with $1,25(OH)_2D_3$ (TABLE ONE). This gene cluster contained many DNA replication genes as well as genes required for normal cell cycle progression. Despite the fact that $1,25(OH)_2D_3$ impedes the progression from G_1 to S, it also down-regulated the expression of a large number of genes that are normally regulated at G_2 in the cell cycle and encodes proteins required for chromatin modifications as well as proteins that function in mitosis.

Promoter analyses of the genes in the abovementioned cluster revealed the presence of E2F binding sites in the promoter regions of genes that, until now, were not fully characterized as E2F targets. Four functionally different genes, Cnap1, Melk, Ris2, and Ezh2, were selected, and their down-regulation by $1,25(OH)_2D_3$ was confirmed by QRT-PCR analyses. Down-regulation was not only observed in normal cells (epidermal keratinocytes) but also in malignant cells (GR mouse mammary carcinoma cells) (data not shown), which suggested the general nature of this phenomenon.

Ris2, the murine homolog of human Cdt1, plays an important role in the initiation of DNA replication (28) and was during the preparation of

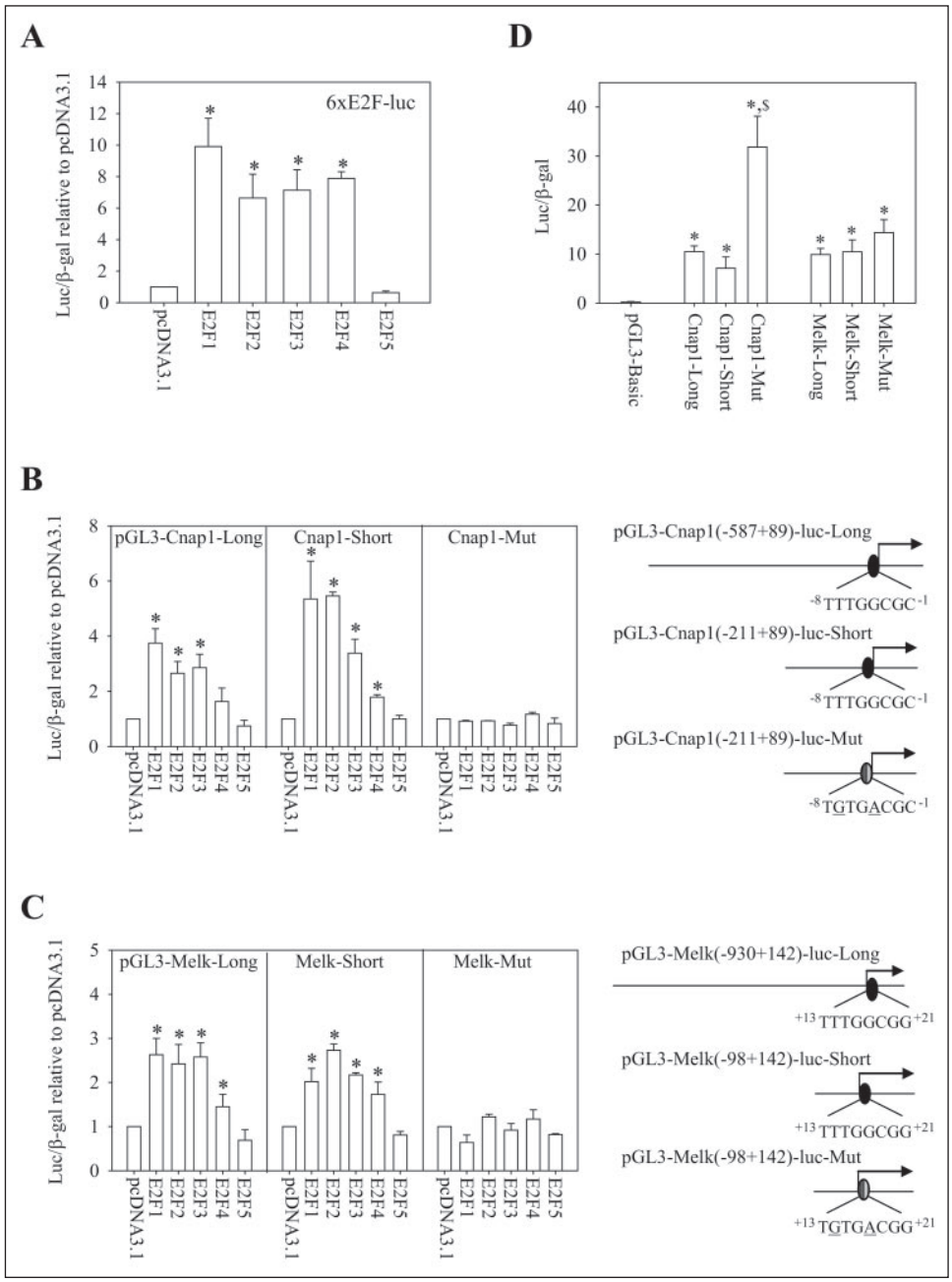

FIGURE 4. **E2F controlled Cnap1 and Melk promoter activity in MC3T3-E1 cells through the identified E2F binding sites.** *A*, *B*, and *C*, exponentially growing MC3T3-E1 cells were transfected with an E2F expression vector or corresponding empty control expression vector, a β-galactosidase expression vector, and a luciferase reporter construct with six E2F binding sites (*A*) or with luciferase reporter constructs that carried a long Cnap1/Melk fragment (*B* and *C*, *left panels*) or shorter Cnap1/Melk fragments in which the E2F binding sites were either intact (*B*/*C*, *middle panels*) or mutated (*B* and *C*, *right panels*). Cells were lysed 48 h after transfection. Luciferase activities, normalized for β-galactosidase activities, of the different reporter constructs were standardized against their respective empty control reporter vectors. Furthermore, induction by the different E2F expression plasmids was expressed relative to the pcDNA3.1. *Bars* represent the means and S.E. of at least three independent experiments. *, $p < 0.05$, E2F-overexpression *versus* empty vector control pcDNA3.1 (Student's *t* test). *D*, basal activities of the different reporter constructs in exponentially growing MC3T3-E1 cells. *, $p < 0.05$, different reporter constructs *versus* pGL3-Basic according to Bonferroni multiple comparison test; §, $p < 0.05$, mutated reporter constructs *versus* their wild type counterparts.

our present study shown to be regulated by the pRb/E2F pathway (25). Cnap1 is an essential component of the highly conserved condensin complex required for mitotic chromosome condensation (29) and for the correct attachment between chromosome kinetochores and microtubules of the mitotic spindle (30). The cell cycle-regulated protein Ser/Thr kinase Melk is involved in pre-mRNA processing (31) and is hypothesized to play a key role during preimplantation embryonic development (32). The histone methyltransferase Ezh2 belongs to the Polycomb group (PcG) genes that modify chromatin structure and play an important role in maintaining the silent state of HOX genes during embryonic development (33). Recent work suggested Ezh2 to be controlled by E2F transcription factors (24). A recent large scale meta-analysis of cancer microarray data has resulted in the identification of a transcriptional profile common to various types of undifferentiated cancer (23). Interestingly, Cnap1 and Melk as well as Ezh2 were significantly overexpressed in undifferentiated cancer relative to well differentiated cancer and might be involved in the mechanisms by which cancer cells progress, avoid differentiation, or dedifferentiate.

The specific roles of Cnap1, Melk, Ezh2, and Ris2 in the $1,25(OH)_2D_3$-induced down-regulation are as yet unknown. Rather than being key genes in the antiproliferative effect of $1,25(OH)_2D_3$, they are suggested to be part of the general mechanism by which the pocket proteins, pRb, p107, and p130, translate the effect of $1,25(OH)_2D_3$ and regulate a large number of E2F target genes.

All four genes were highly expressed in proliferating cells and significantly repressed in serum-arrested cells. Cnap1 and Melk promoter activity was induced upon the addition of serum to transfected, serum-starved cells. This cell growth-regulated promoter activity and gene expression were shown to be dependent on E2F transcription factors through binding to an E2F recognition motif close to the transcription start site because mutation of these sites abolished the induction by E2F. Remarkably, E2F4 acted as a (weak) transcriptional activator in these

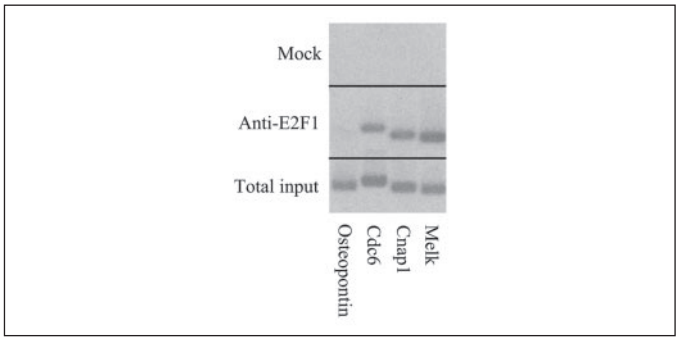

FIGURE 5. **Direct association of E2F1 with the promoters of Cdc6, Cnap1, and Melk.** Chromatin immunoprecipitation assays were performed with chromatin from exponentially growing MC3T3-E1 cells using irrelevant antibodies (*Mock*) or an antibody against E2F1. Enriched DNA was amplified with primers, which span the part of the intergenic regions of Cdc6, Cnap1, and Melk that contain the E2F binding motif. Osteopontin, which does not have an E2F-binding site in its intergenic region, was included as a negative control. Results shown are representative for at least three independent experiments.

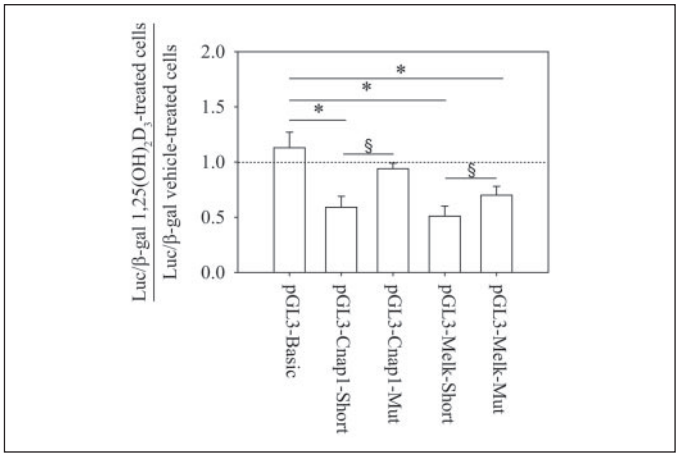

FIGURE 6. **Effect of $1,25(OH)_2D_3$ on the promoter activities of Cnap1 and Melk.** Exponentially growing MC3T3-E1 cells transfected with the truncated reporter constructs, which carried an intact or a mutated E2F-binding site, were incubated for 24 h with 10^{-8} M $1,25(OH)_2D_3$. Luciferase activities (*Luc*), normalized to β-galactosidase (*β-Gal*) activity, were expressed as ratios between $1,25(OH)_2D_3$-treated and corresponding vehicle-treated samples. *Bars* represent the means and S.E. of three independent experiments. The *dotted line* indicates a 1:1 ratio. *, $p < 0.05$, different reporter constructs *versus* pGL3-Basic according to Bonferroni multiple comparison test; §, $p < 0.05$, mutated reporter constructs *versus* their wild type counterparts.

settings. In this context it is noteworthy that E2F4 has been detected on the promoter of E2F target genes in late G_1 and S and might, therefore, also act as a transcriptional activator (34). The physiologic importance of E2F transcription factors in the regulation of these genes was confirmed by chromatin immunoprecipitation assay experiments, which showed *in vivo* binding of E2F1 to the promoter regions of Cnap1 and Melk in living cells. The hypothesis that $1,25(OH)_2D_3$ mediated its repressive effects through interaction with the E2F pathway was confirmed by the finding that $1,25(OH)_2D_3$ was no longer able to down-regulate the Cnap1 promoter construct in which the E2F-recognition site was mutated. In line with this, mutation of the E2F-binding site in the Cnap1 reporter construct significantly increased the basal activity of this construct, which may indicate that this site also mediated transcriptional suppression. The repression of the mutated Melk promoter construct by $1,25(OH)_2D_3$ was still apparent but significantly lower than that of the construct with the intact E2F binding motif, which suggested that another binding site may be involved. It is possible that the Melk promoter, in analogy with the human Cdc2 promoter, contains a binding element that specifically interacts with a subset of E2F4·p130 complexes but does not interact with S-phase-specific E2F complexes (35).

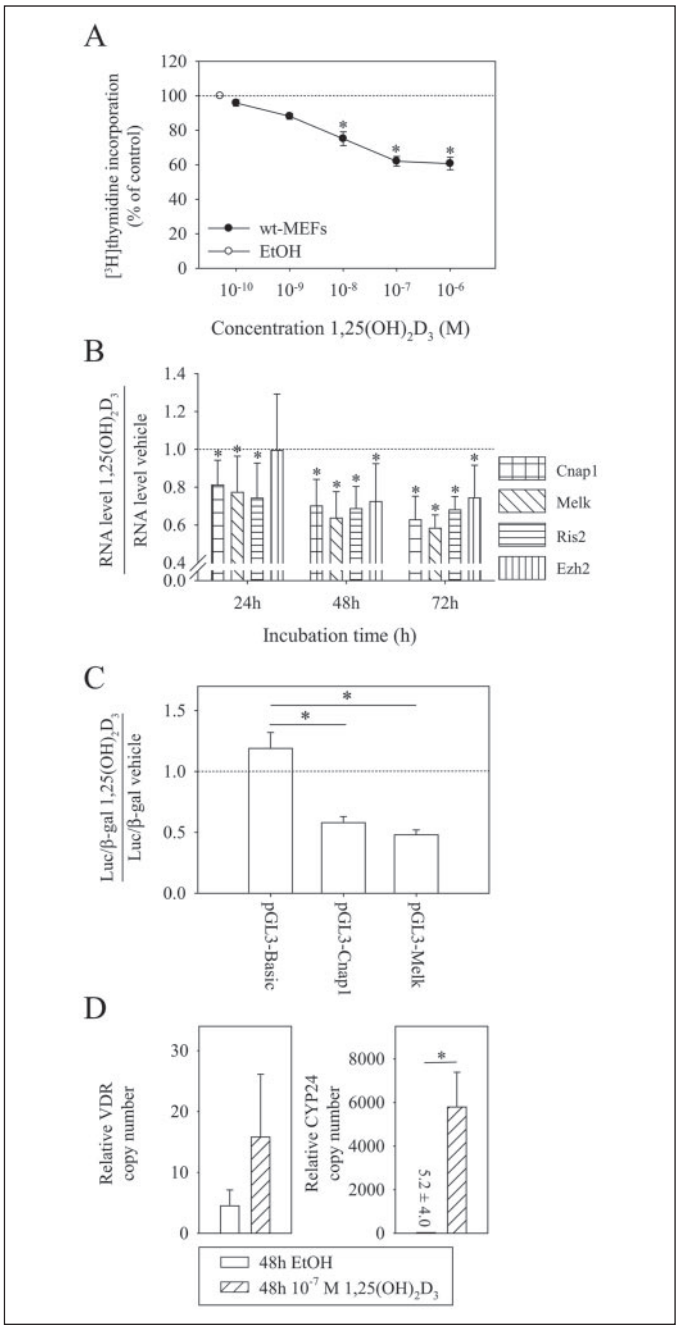

FIGURE 7. **Effect of $1,25(OH)_2D_3$ on wt MEFs.** *A*, proliferation of wt MEFs after 72 h of incubation with $1,25(OH)_2D_3$ as measured by [^{3}H]thymidine incorporation. *B*, analysis of Cnap1, Melk, Ris2, and Ezh2 expression by QRT-PCR analysis. wt MEFs were treated with 10^{-7} M $1,25(OH)_2D_3$, and gene expression was studied after a 24-, 48-, and 72-h incubation period. *C*, analysis of Cnap1 and Melk promoter activities in wt MEFs. Exponentially growing wt MEFs were transfected with luciferase reporter constructs (that contain long promoter fragments) or the corresponding empty control vector and treated with 10^{-7} M $1,25(OH)_2D_3$ for 48 h. Luciferase activities, normalized to β-galactosidase activity, were expressed as ratios between $1,25(OH)_2D_3$-treated and corresponding vehicle-treated samples. *D*, VDR (*left panel*) and CYP24 (*right panel*) mRNA levels, determined by QRT-PCR analysis in wt MEFs that were treated for 48 h with 10^{-7} M $1,25(OH)_2D_3$. All data represent mean and S.E. of at least three independent experiments. *, $p < 0.05$, $1,25(OH)_2D_3$-treated *versus* vehicle-treated (Student's *t* test).

Close inspection of this down-regulated gene cluster revealed a remarkable overlap with a group of genes of which the promoter was bound by the transcriptional repressor E2F4 in primary fibroblasts (36). Although a subset of these genes can also be bound by E2F1, it is generally accepted that p107 and p130 redundantly repress a subset of E2F targets distinct from the subset of genes controlled by pRb (26). Promot-

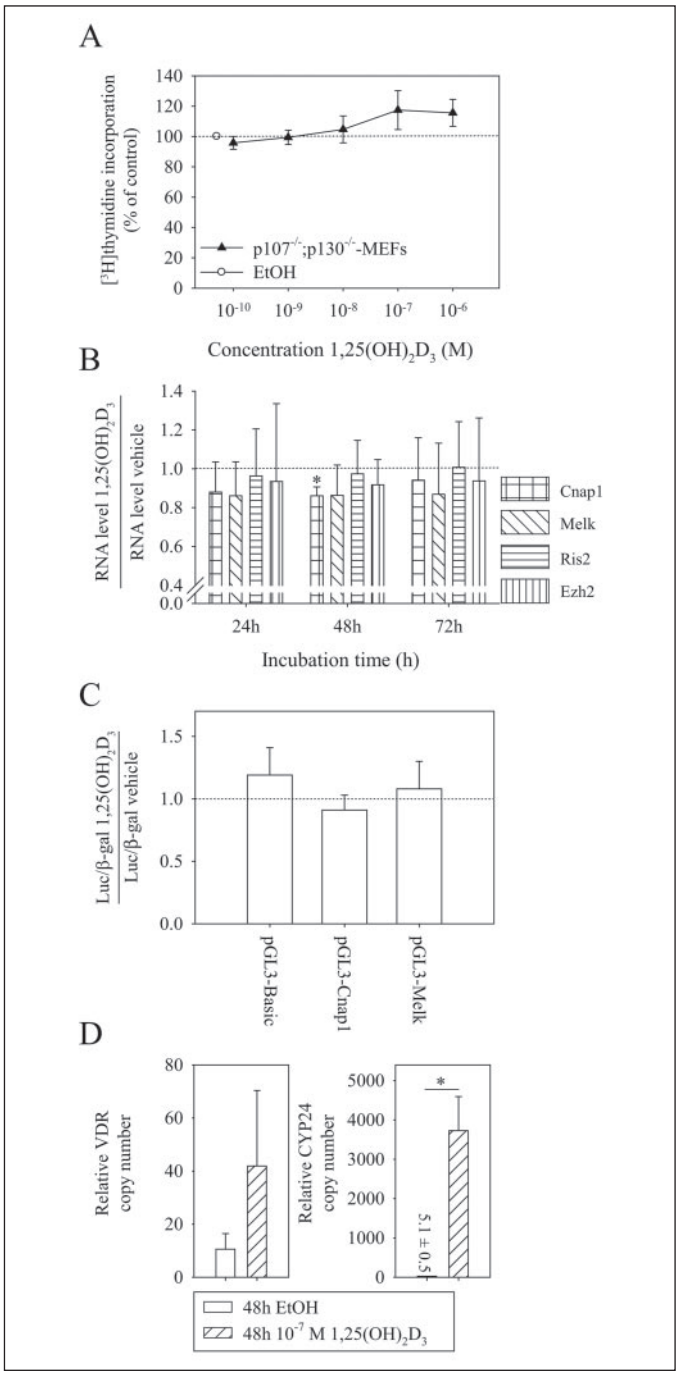

FIGURE 8. **Effect of $1,25(OH)_2D_3$ on $p107^{-/-}$;$p130^{-/-}$ MEFs.** *A*, proliferation of $p107^{-/-}$;$p130^{-/-}$ MEFs after 72 h of incubation with $1,25(OH)_2D_3$ as measured by [^{3}H]thymidine incorporation. *B*, analysis of Cnap1, Melk, Ris2, and Ezh2 expression by QRT-PCR analysis. $p107^{-/-}$;$p130^{-/-}$ MEFs were treated with 10^{-7} M $1,25(OH)_2D_3$, and gene expression was studied after a 24-, 48-, and 72-h incubation period. *C*, analysis of Cnap1 and Melk promoter activities in $p107^{-/-}$;$p130^{-/-}$ MEFs. Exponentially growing $p107^{-/-}$;$p130^{-/-}$ MEFs were transfected with luciferase reporter constructs (that contain long promoter fragments) or the corresponding empty control vector and treated with 10^{-7} M $1,25(OH)_2D_3$ for 48 h. Luciferase activities, normalized to β-galactosidase activity, were expressed as ratios between $1,25(OH)_2D_3$-treated and corresponding vehicle-treated samples. *D*, VDR (*left panel*) and CYP24 (*right panel*) mRNA levels, determined by QRT-PCR analysis in $p107^{-/-}$;$p130^{-/-}$ MEFs that were treated for 48 h with 10^{-7} M $1,25(OH)_2D_3$. All data represent the mean and S.E. of at least three independent experiments. *, $p < 0.05$, $1,25(OH)_2D_3$-treated *versus* vehicle-treated (Student's *t* test).

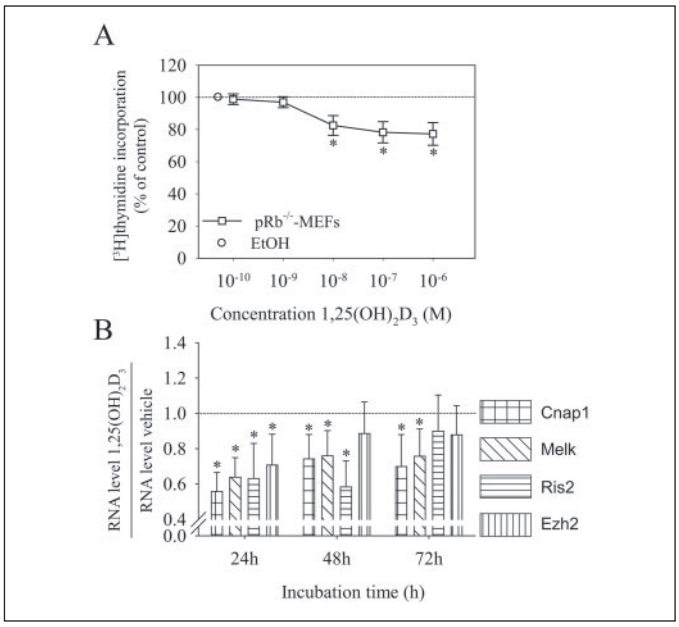

FIGURE 9. **Effect of $1,25(OH)_2D_3$ on $pRb^{-/-}$ MEFs.** *A*, proliferation of $pRb^{-/-}$ MEFs after 72 h of incubation with $1,25(OH)_2D_3$ as measured by [^{3}H]thymidine incorporation. *B*, analysis of Cnap1, Melk, Ris2, and Ezh2 expression by QRT-PCR analysis. $pRb^{-/-}$ MEFs were treated with 10^{-7} M $1,25(OH)_2D_3$, and gene expression was studied after a 24-, 48-, and 72-h incubation period. All data represent the mean and S.E. of at least three independent experiments. *, $p < 0.05$, $1,25(OH)_2D_3$-treated *versus* vehicle-treated (Student's *t* test).

ers of E2F-regulated genes in quiescent cells are bound by corepressor complexes that, next to E2F4 and the pocket proteins, p107 or p130, also contain histone deacetylase (HDAC1). In $p107^{-/-}$;$p130^{-/-}$-deficient cells, HDAC1 complexes are not recruited to E2F binding sites. Moreover, p107/p130 deficiency triggers a dramatic loss of E2F4 nuclear localization as well as transcriptional derepression (27). Transcriptional derepression of Cnap1, Melk, Ris2, and to a lesser extent, Ezh2 in $p107^{-/-}$;$p130^{-/-}$ MEFs suggested these $1,25(OH)_2D_3$-down-regulated genes to be physiological targets of the transcriptional repressor complex between E2F and these pocket proteins (data not shown). This raised the question of whether these repressor complexes could be key mediators of the growth-inhibitory activity of $1,25(OH)_2D_3$. The inability of $1,25(OH)_2D_3$ to inhibit the proliferation of $p107^{-/-}$;$p130^{-/-}$ MEFs and to down-regulate the expression of these p107/p130 target genes strongly suggested that $1,25(OH)_2D_3$ exerts its antiproliferative effect by the recruitment of E2F·p107/p130 transcriptional repressor complexes to the promoters of E2F-responsive genes. $p107^{-/-}$;$p130^{-/-}$ MEFs did express the vitamin D receptor and remained sensitive to direct $1,25(OH)_2D_3$ signaling (induction of CYP24), which indicated that the growth-inhibitory pathway was selectively abrogated. Because the pocket proteins p107 and p130 probably compensate for one another in single knock-out cells, it was not possible to elucidate the role of the individual pocket proteins in the antiproliferative effect of $1,25(OH)_2D_3$. However, when protein levels of p107 and p130 were determined in different cell lines with varying responsiveness to $1,25(OH)_2D_3$, we found that the cell response to $1,25(OH)_2D_3$ correlated well with the protein levels of p107 but not with p130 levels (data not shown). These findings suggested that treatment with $1,25(OH)_2D_3$, analogously to all-*trans* retinoic acid and the estrogen antagonist ICI 182780, led to increased nuclear levels of E2F4, p107 and p130, to decreased phosphorylation of the pocket proteins, to enhanced complex formation between the pocket proteins and E2F family members and finally to a repressed transcription of E2F target genes (37, 38). A parallel pathway, leading to a decreased phosphorylation of pRb and a subsequent decrease in free activator E2F family members, is likely to contribute to the observed growth inhibitory effect. Indeed, previous

findings illustrate that treatment of cells with $1,25(OH)_2D_3$ results in the appearance of the growth-suppressive hypophosphorylated form of pRb (10). Yet, pRb proved not to be the major mediator of the growth-inhibitory effect of $1,25(OH)_2D_3$ because $1,25(OH)_2D_3$ significantly reduced the proliferation of $pRb^{-/-}$ MEFs. The effect of $1,25(OH)_2D_3$ on $pRb^{-/-}$ cells was smaller than that on their wild type counterparts, but the expression of Cnap1, Melk, and Ris2 was significantly down-regulated after treatment with $1,25(OH)_2D_3$. The finding that also Y79 retinoblastoma cells remain sensitive to $1,25(OH)_2D_3$ signaling reinforces this hypothesis (39).

In conclusion, this elaborate microarray analysis revealed the ability of $1,25(OH)_2D_3$ to regulate not only the expression of genes involved in the G_1/S transition but also that of genes, which function in later stages of the cell cycle and that are implicated in chromosome transactions and regulation of mitosis. The transcriptional repressor complex between E2F family members and the pocket proteins p107 and p130 fulfilled a crucial role in establishing the growth-inhibitory effects of $1,25(OH)_2D_3$ because the antiproliferative capacity of $1,25(OH)_2D_3$ was specifically abolished in $p107^{-/-}$;$p130^{-/-}$ MEFs and not in $pRb^{-/-}$-cells. Additional evidence for this interplay was provided by the finding that $1,25(OH)_2D_3$-induced down-regulation of Cnap1 and Melk promoter activity was mediated by the E2F recognition motifs within their promoters. This experimental approach led to the recognition of the crucial role of the E2F pathway in the regulation of two genes, Cnap1 and Melk, which are highly expressed in undifferentiated cancer cells.

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