Characterization of the Condensin Component Cnap1 and Protein Kinase Melk as Novel E2F Target Genes Down-regulated by 1,25-Dihydroxyvitamin D₃

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1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) has potent antiproliferative effects characterized by a hampered G₁/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)₂D₃. Statistical analysis revealed a cluster of genes that were strongly down-regulated by 1,25(OH)₂D₃ 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)₂D₃-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)₂D₃ 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)₂D₃ depended on the pocket proteins p107 and p130 because 1,25(OH)₂D₃ 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 G₁/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 two subclasses. Upon release by their pocket protein pRb, E2Fs 1–3 function as transcriptional activators in late G1 and in S phase. E2F4 and E2F5 act as transcriptional repressors in quiescent and early G1 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 G1, 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 G₁ progression and G₁/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 D₃ (1,25(OH)₂D₃), the active metabolite of vitamin D₃, 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)₂D₃ has a cell cycle-specific effect leading to an accumulation of cells in the G₁ phase of the cell cycle (9). It has been shown previously that 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃, these genes are suggested to be part of the general mechanism by which the pocket proteins translate the effect of 1,25(OH)₂D₃ and regulate a large number of E2F target genes. Because p107⁻/⁻;p130⁻/⁻ cells no longer responded to the antiproliferative activity of 1,25(OH)₂D₃, we suggest that 1,25(OH)₂D₃ 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.
### TABLE ONE

**Identification of genes downregulated by 1,25(OH)2D3 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)2D3 relative to gene expression in vehicle-treated cells | Function                                                                                           |
|---------------|---------------|-----------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| **Cell Cycle Regulation**                                                                                                                                         |
| Ccn2          | BG069688      | 1.06 1.09 1.08 0.52 0.43                                                                                         | Cyclin-dependent protein kinase regulator activity                                                 |
| Ccn2          | BG073518      | 1.00 0.86 0.78 0.49 0.43                                                                                         | Cyclin-dependent protein kinase regulator activity                                                 |
| Ccnb1         | AA396324      | 1.07 1.15 0.99 0.52 0.39                                                                                         | Cyclin-dependent protein kinase regulator activity (G2/M)                                         |
| Ccnb1         | BG078426      | 1.01 1.07 1.13 0.54 0.40                                                                                         | Cell division cycle protein; key regulator of the cell cycle                                     |
| 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 (G2/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                                                                                         | Polymerase (DNA directed) ε; involved in DNA replication and repair                              |
| Pol ε         | BG069732      | 0.88 0.88 0.92 0.46 0.47                                                                                         |                                                                                                    |
| Pol ε2        | BG071480      | 0.95 1.03 0.85 0.45 0.55                                                                                         | Part of DNA-pole-primase complex; −DNA replication                                             |
| Prim1         | AA259900      | 0.89 0.88 0.65 0.51 0.40                                                                                         |                                                                                                    |
| 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                                                   |
| 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                                                                 |
|------------|---------------|-------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Tk1        | BG077745      | 0.83 0.96 0.69 0.39 0.31                                                                       | Uridine monophosphate synthetase                                          |
| Umps       | BG063291      | 0.98 1.02 0.84 0.59 0.55                                                                       |                                                                          |
| Checkpoints|               |                                                                                                |                                                                          |
| Bub1b      | BG069421      | 1.00 1.08 0.98 0.53 0.42                                                                       | Essential for spindle checkpoint activation                               |
| Mad2li     | AA002895      | 0.98 1.01 0.83 0.55 0.40                                                                       | Mitotic arrest deficient-like 1 (yeast); component of mitotic spindle assembly checkpoint |
| Mad2li     | 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                                                                       |                                                                          |
| 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                                                |
| Hmgm2      | 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                                                                       | Nuclear- and spindle-associated protein 1                                |
| Pcnt2      | BG071845      | 0.92 1.03 0.93 0.49 0.56                                                                       | Pericentri2 −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)$_2$D$_3$ relative to gene expression in vehicle-treated cells | Function                                                                 |
|--------------|---------------|-----------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| **Mitosis**  |               | **1 h** | **6 h** | **12 h** | **24 h** | **36 h** |                                                                                     |
| Anillin      | BG063979      | 0.95    | 0.90    | 0.88     | 0.49     | 0.41     | Actin binding protein involved in cytokinesis                                          |
| Cdc45        | BG068799      | 0.99    | 0.86    | 0.88     | 0.55     | 0.34     | Cell division cycle associated 5; cytokinesis                                         |
| Cdc8         | BG078299      | 1.00    | 1.04    | 0.89     | 0.56     | 0.49     | Cell division cycle associated 8; cytokinesis                                         |
| Ect2         | AA267600      | 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 associat. 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    | 1.07    | 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)2D3 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   |           |
| 2610085612Rik | 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 |
| 261052A17Rik | AA511242 | 1.00    | 0.94   | 0.83   | 0.51   | 0.51   |           |
| 261052818Rik | BG074710 | 0.97    | 0.98   | 0.98   | 0.44   | 0.42   |           |
| 2810417H13Rik | BG076569 | 0.91    | 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 | BG0674704 | 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).

**Total RNA Extraction**—Total RNA for microarray analysis was isolated with the RNeasy kit (Qiagen, Hilden, Germany).

**Construction of Microarrays**—The mouse gene set consisted of 7 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 with 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.
Cnap1 and Melk Are E2F Target Genes

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 p107α/p107β 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 p107α/p107β 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 moiety) were designed for the detection of the different E2F constructs (or the empty vector) and were synthesized by 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 using 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.

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 repair 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).
Expression Analysis of Cnap1, Melk, Ris2, and Ezh2 in 1,25(OH)2D3-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)2D3 (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. 2B, mRNA transcripts of all four genes strongly increased after the addition of serum and peaked at the G1/S transition (16–20 h after re-feeding, Fig. 2A), 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. 3B, promoter constructs of Cnap1 and Melk, which carry an E2F-responsive site close to the transcription start site (Fig. 3A), 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. 4A) (7–10-fold induction) or by promoter regions of Cnap1 (Fig. 4B, left panel) (1.5–3.7-fold induction) or Melk (Fig. 4C, 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. 4D).

Chromatin immunoprecipitation assays demonstrated that Cnap1 and Melk are 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)2D3-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)2D3 clearly decreased the promoter activities of Cnap1 and

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 G1 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).
Cnap1 and Melk Are E2F Target Genes

Melk. 1,25(OH)\textsubscript{2}D\textsubscript{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)\textsubscript{2}D\textsubscript{3} (Fig. 6). When the E2F-binding site in the Melk promoter construct was mutated, the repressive effect of 1,25(OH)\textsubscript{2}D\textsubscript{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)\textsubscript{2}D\textsubscript{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)\textsubscript{2}D\textsubscript{3} and in the 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced down-regulation of E2F target gene transcription was assessed by the use of wt, pRb\textsuperscript{-/-}, p107\textsuperscript{-/-}, and p130\textsuperscript{-/-} single knock-out MEFs as well as p107\textsuperscript{-/-} p130\textsuperscript{-/-} double knock-out MEFs.

1,25(OH)\textsubscript{2}D\textsubscript{3} significantly reduced the growth of wt MEFs with a maximal inhibition at concentrations of 10\textsuperscript{-7}–10\textsuperscript{-8} M (Fig. 7A). 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)\textsubscript{2}D\textsubscript{3} (10\textsuperscript{-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)\textsubscript{2}D\textsubscript{3} and reached a maximal 1.7-fold reduction after 48–72 h (Fig. 7B). Down-regulation of gene expression was modest but occurred at the transcriptional level as treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} led to a significant decrease of Cnap1 and Melk promoter activities (Fig. 7C).

The antiproliferative activity of 1,25(OH)\textsubscript{2}D\textsubscript{3} was minimally affected by loss of either p107 or p130 (data not shown), whereas 1,25(OH)\textsubscript{2}D\textsubscript{3} failed to inhibit the proliferation of p107\textsuperscript{-/-} p130\textsuperscript{-/-} MEFs (Fig. 8A). 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)\textsubscript{2}D\textsubscript{3} was investigated by the use of p107\textsuperscript{-/-} p130\textsuperscript{-/-} double knock-out MEFs. In these cells, 1,25(OH)\textsubscript{2}D\textsubscript{3} did not affect the expression of Cnap1, Melk, Ris2, and Ezh2 (Fig. 8B). Correspondingly, 1,25(OH)\textsubscript{2}D\textsubscript{3} failed to repress the promoter activities of Cnap1 and Melk in p107\textsuperscript{-/-} p130\textsuperscript{-/-} MEFs (Fig. 8C). Nevertheless, p107\textsuperscript{-/-} p130\textsuperscript{-/-} MEFs (Fig. 8D) as well as wt MEFs (Fig. 7D) contain a functional VDR and are responsive to 1,25(OH)\textsubscript{2}D\textsubscript{3}, judged by the huge induction of 24-hydroxylase (CYP24), a primary and direct 1,25(OH)\textsubscript{2}D\textsubscript{3}-target gene.

The PPAR\gamma agonist ciglitazone and p107\textsuperscript{-/-} MEFs remained responsive to the antiproliferative effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} but to a lower extent than wt MEFs (Fig. 9A). Still, the expression of Cnap1, Melk, and Ris2 was significantly down-regulated in pRb\textsuperscript{-/-} MEFs (Fig. 9B).

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

DISCUSSION

Statistical analysis of the present microarray study, in which the effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} on MC3T3-E1 cells was investigated, revealed a cluster of genes that were strongly down-regulated after treatment with 1,25(OH)\textsubscript{2}D\textsubscript{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)\textsubscript{2}D\textsubscript{3} impedes the progression from G\textsubscript{1} to S, it also down-regulated the expression of a large number of genes that are normally regulated at G\textsubscript{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)\textsubscript{2}D\textsubscript{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 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 \( \beta \)-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).
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)2D3-induced down-regulation are as yet unknown. Rather than being key genes in the antiproliferative effect of 1,25(OH)2D3, 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)2D3 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
settings. In this context it is noteworthy that E2F4 has been detected on the promoter of E2F target genes in late G1 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)2D3 mediated its repressive effects through interaction with the E2F pathway was confirmed by the finding that 1,25(OH)2D3 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)2D3 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).

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-
Cnap1 and Melk Are E2F Target Genes

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 Ezh2 by p107−/−;p130−/− MEFs suggested these 1,25(OH)2D3-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)2D3. The inability of 1,25(OH)2D3 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)2D3 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)2D3 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)2D3. However, when protein levels of p107 and p130 were determined in different cell lines with varying responsiveness to 1,25(OH)2D3, we found that the cell response to 1,25(OH)2D3 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)2D3, 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

![FIGURE 8. Effect of 1,25(OH)2D3 on p107−/−;p130−/− MEFs. A, proliferation of p107−/−;p130−/− MEFs after 72 h of incubation with 1,25(OH)2D3 as measured by [3H]thymidine incorporation. B, analysis of Cnap1, Melk, Ris2, and Ezh2 expression by QRT-PCR analysis. 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)2D3 for 48 h. Luciferase activities, normalized to β-galactosidase activity, were expressed as ratios between 1,25(OH)2D3-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)2D3. All data represent the mean and S.E. of at least three independent experiments. *p < 0.05, 1,25(OH)2D3-treated versus vehicle-treated (Student’s t test).](image)

![FIGURE 9. Effect of 1,25(OH)2D3 on pRb−/− MEFs. A, proliferation of pRb−/− MEFs after 72 h of incubation with 1,25(OH)2D3 as measured by [3H]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)2D3, 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)2D3-treated versus vehicle-treated (Student’s t test).](image)
findings illustrate that treatment of cells with 1,25(OH)2D3 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)2D3 because 1,25(OH)2D3 significantly abolishes the antiproliferative capacity of 1,25(OH)2D3 to regulate not only the expression of genes involved in the G1/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)2D3 because the antiproliferative capacity of 1,25(OH)2D3 was specifically abolished in p107−/−;p130−/− MEFs. This finding that also Y79 retinoblastoma cells remain sensitive to 1,25(OH)2D3 signaling reinforces this hypothesis (39).

In conclusion, this elaborate microarray analysis revealed the ability of 1,25(OH)2D3 to regulate not only the expression of genes involved in the G1/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)2D3 because the antiproliferative capacity of 1,25(OH)2D3 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)2D3-induced down-regulation of Cnap1 and Melk promoter activities 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 these two genes, Cnap1 and Melk, which are highly expressed in undifferentiated cancer cells.

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