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The *Cables1* Gene in Glucocorticoid Regulation of Pituitary Corticotrope Growth and Cushing Disease

Audrey Roussel-Gervais,* Catherine Couture,* David Langlais, Shinobu Takayasu, Aurelio Balsalobre, Bo R. Rueda, Lawrence R. Zukerberg, Dominique Figarella-Branger, Thierry Brue, Jacques Drouin

Laboratoire de Génétique Moléculaire (A.R.-G., C.C., D.L., S.T., A.B., J.D.), Institut de Recherches Cliniques de Montréal, Montréal, Quebec H2W 1R7, Canada; Massachusetts General Hospital (B.R.R., L.R.Z., J.D.), Boston, Massachusetts 02114; Aix-Marseille Université (D.F.-B., T.B.), Centre National de la Recherche Scientifique, Center for Research in Neurobiology and Neurophysiology of Marseille, Unité Mixte de Recherche 7286, 13344 Marseille, France; and Assistance Publique-Hôpitaux de Marseille (D.F.-B., T.B.), Hôpital Conception, Service d'Endocrinologie, Diabète et Maladies Métaboliques, Centre de Référence des Maladies Rares d'Origine Hypophysaire, 13285 Marseille, France

Context: Cushing disease (CD) is due to pituitary corticotrope adenomas that produce unrestrained ACTH secretion and have lost the negative feedback exerted by glucocorticoids (GCs). GCs also restrain corticotrope proliferation, and the mechanisms of this inhibition are poorly understood.

Objective: The aim of the study was to identify cell cycle regulatory genes that are regulated by GCs and the glucocorticoid receptor and to assess regulatory genes that have a rate-limiting action on corticotrope proliferation and may be disregulated in CD.

Design: The mouse corticotrope tumor cells AtT-20 were used to identify GC-regulated genes that contribute to control of cell cycle progression. Surgery sections from patients with CD were used to assess expression of CABLES1 in corticotrope adenomas.

Methods: Gene expression profiling, small interfering RNA knockdowns, cell cycle analyses, and genetic manipulations were performed in AtT-20 cells. Sequencing of chromatin immunoprecipitation for pituitary-restricted transcription factors and RNA polymerase II were used to identify regulatory elements and genes that bind GR and are direct transcriptional targets. A panel of previously well-characterized corticotrope adenomas was used to correlate expression of CABLES1 with that of other markers.

Results: GCs altered expression of 3 positive and 3 negative regulators of cell cycle progression. Two *Myc* genes (*L-Myc* and *N-Myc*) and *E2F2* are repressed by GCs, whereas genes for the negative regulators of the cell cycle, *Gadd*45β, *Gadd45*γ, and *Cables1* are activated by GCs. *Cables1* small interfering RNA knockdown strongly stimulates AtT-20 cell proliferation and antagonizes the growth inhibition produced by GCs. The *Gadd45* and *Cables1* genes have the hallmarks of direct GC targets. CABLES1 is expressed in normal human pituitary cells, but expression is lost in ~55% of corticotrope adenomas, and this is strongly correlated with the loss of $p27^{Kip1}$ expression.

Conclusions: CABLES1 is a critical regulator of corticotrope proliferation that defines a pathway often inactivated in CD and links proliferation to GC resistance. (***J Clin Endocrinol Metab*** **101: 513–522, 2016)**

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* A.R.-G. and C.C. contributed equally to the study.

Abbreviations: CD, Cushing disease; ChIP-Seq, sequencing of chromatin immunoprecipitation; Dex, dexamethasone; EGF, epidermal growth factor; GC, glucocorticoid; GR, glucocorticoid receptor; phosphoserine 5 polymerase II; Ser5-Pol II; qPCR, quantitative PCR; RNA-Seq, RNA sequencing; siRNA, small interfering RNA; TSS, transcription start site; USP8, ubiquitin-specific protease 8.

Glucocorticoids (GCs) acting through their receptor, the glucocorticoid receptor (GR), have widespread effects on gene expression in most cells and tissues. Notably, their metabolic, anti-inflammatory, and immunosuppressive actions can be either beneficial or detrimental, depending on the context. Another important aspect of the critical actions of GCs is regulation of cell proliferation. Indeed, GCs regulate cell proliferation, either positively or negatively, depending on cells and tissues or on concentration. In many tissues, GCs stimulate growth at low concentrations, but block cell cycle progression at high concentrations (for reviews, see Refs. 1–3). Under pharmacological conditions such as treatment with the synthetic GC dexamethasone (Dex), a reversible block in the G_1 phase of the cell cycle leads to a relative enrichment of cells in the S phase. A few mechanisms and target genes have been proposed to account for this cell cycle arrest, including GC-dependent repression of positive regulators of cell cycle progression such as cyclin D3 and c-*myc* together with GC-dependent activation of cell cycle inhibitors such as $p21^{Cip1}$ and $p27^{Kip1}$. These seemingly direct effects of GCs on cell cycle regulators are complemented by antagonistic cross talk between GCs and p53 and with positive regulators of cytokine production such as the transcription factors AP1 and NF-κB (4–8). These cross talks and GC antiproliferative effects have been particularly studied in inflammation and immunity.

GCs are produced by the adrenal glands in response to pituitary ACTH, and they exert negative feedback on both pituitary corticotrope cells and on hypothalamic CRH neurons. This negative feedback is in part exerted on secretion of granule-stored ACTH in corticotropes and hypothalamic CRH as well as on the transcription of pituitary *POMC* and hypothalamic *CRH* genes (9). In the pituitary, GCs also block corticotrope cell cycle progression, and the loss of this repressor mechanism probably contributes to the development of corticotrope adenomas causing Cushing disease (CD).

Direct assessment of gene expression in these adenomas together with investigations of the mechanisms underlying GC repression of *POMC* have identified a number of misexpressed cell cycle regulators as well as oncogenes and tumor suppressors. Indeed, about 75% of corticotrope adenomas exhibit aberrant expression of cyclin E (10), whereas about 50% are marked by the loss of expression of the cell cycle inhibitor $p27^{Kip1}$ (CDKN1B) (11, 12). Other subsets of adenomas exhibit a loss of $p21^{Cip1}$ (CDKN1A) or of the tumor suppressor BRG1 (13). Although each of these misexpressed genes may represent independent "hits" that contribute to adenoma development or maintenance, the causal relationships with normal GC-dependent arrest of cell cycle progression and its disruption due to the GC resistance that characterizes corticotrope adenomas remain undefined. Recently, exome sequencing of corticotrope adenomas identified mutations in the deubiquitinase gene *USP8* in 40% to 60% of patients with CD (14–16). These ubiquitin-specific protease 8 (USP8) mutations are clustered around a 14–3-3 regulatory binding site, and the resulting gain of deubiquitinase activity may lead to persistent epidermal growth factor (EGF) signaling and increased *POMC* gene expression (15). The relationship between deregulated EGF signaling, GC resistance, and cell cycle progression remains unexplained.

We now report on the identification of GC-responsive cell cycle regulatory genes in a pituitary corticotrope model cell line, the AtT-20 cells, and in particular, on the characterization of one GC-activated cell cycle regulator, Cables1. Whereas gene profiling analyses of AtT-20 cells after GC treatment identified target genes previously associated with GC control of cell cycle progression such as *myc* and *Gadd45* genes, the rapid induction of *Cables1* gene expression is novel. Further, the *Cables1* locus includes putative enhancer elements that are targets of the pituitary-restricted transcription factors Pitx1 and Tpit as well as GR. We further report on the major effect of Cables1 expression on AtT-20 cell proliferation, which appears to result from a block in the G_1 phase with accumulation of cells in the S phase. Finally, Cables1 expression is lost in about 55% of corticotrope adenomas, and this loss strongly correlates with the loss of $p27^{Kip1}$ expression. Taken together, these data identify Cables1 as a major regulator of cell cycle progression in corticotrope cells and suggest that it may be a hub to integrate various cell cycle regulatory processes for control of proliferation.

Materials and Methods

Cell culture, transfections, and small interfering RNA (siRNA) knockdown

AtT-20 D16v cells were grown in DMEM supplemented with 10% fetal bovine serum as described previously (17). Control and Cables1 Smartpool siRNAs were obtained from Dharmacon and introduced into AtT-20 cells using Lipofectamine as described previously (18). Cables1 protein levels were assessed by Western blot using a rabbit antibody (sc-14794; Santa Cruz).

For RT-quantitative PCR (qPCR), RNA was extracted using RNeasy columns (QIAGEN), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qPCR was performed using PerfeCta reagent (Quanta) on an MX-3005 device (Stratagene). All quantifications were relative to TBP mRNA using the following primers: *Cables1*, 5′-AAGGTCGGAAGTGACCTCAGAAAG-3′ and 5′-CAAACTCCAAGGCCACTAACACTG-3′; *Gadd45β*, 5′-GACATTGACATCGTCCGGGTATCA-3′ and (TGTATG ACAGTTCGTGACCAGGAG-3′; *Gadd45γ*, 5′-CTGAATG

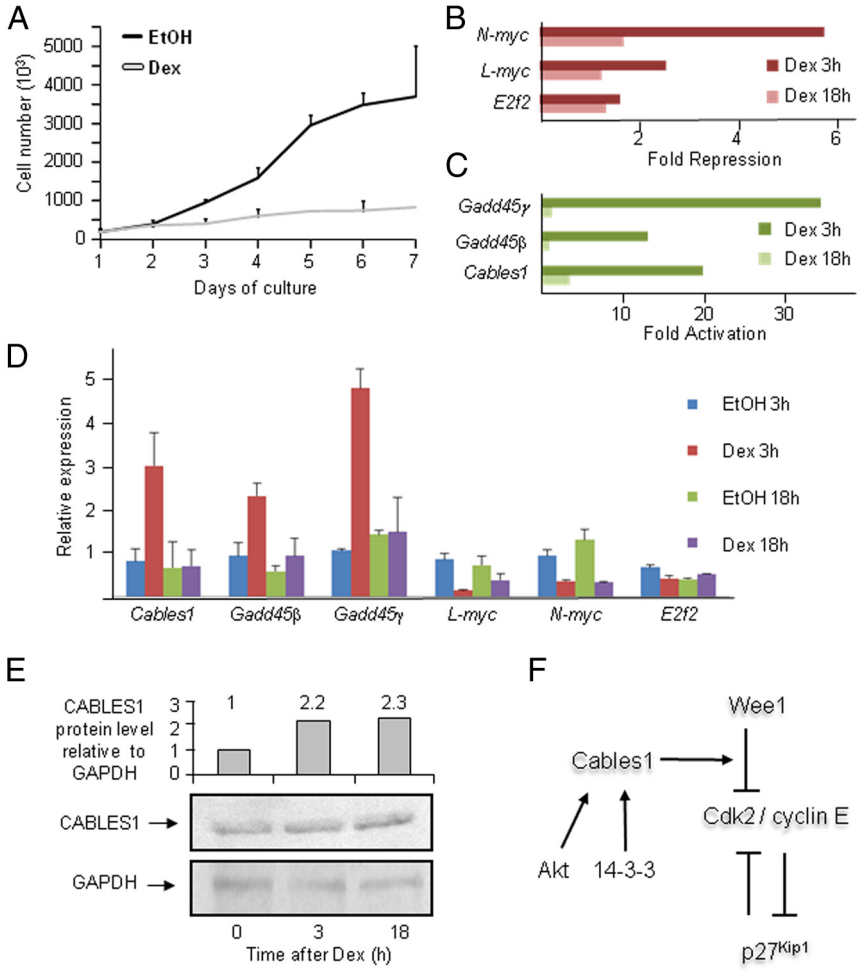


Figure 1. GC regulation of cell cycle regulatory genes. A, Inhibition of AtT-20 cell growth by the synthetic GC Dex (10^{-7} M). B and C, Expression microarray data for cell cycle regulatory genes affected by treatment of AtT-20 cells with Dex for 3 or 18 hours. RNAs from control and Dex-treated AtT-20 cells were analyzed using Affymetrix MOE 430 2.0 microarrays (17). Expression data are shown relative to untreated cells for positive regulators of cell cycle (B) and for inhibitors of cell cycle progression (C). D, RT-qPCR validation of Dex-regulated cell cycle regulatory genes after treatment of AtT-20 cells for 3 or 18 hours. Data are presented as means ± SEM of triplicate experiments. Comparisons to control (ethanol [EtOH]) cells: *Cables1*, 3 hours (P = 1.5E−05) and 18 hours (P = .66); *Gadd45β*, 3 hours (P = 6.2E−06) and 18 hours (P = .06); *Gadd45γ*, 3 hours (P = 1.02E−07) and 18 hours (P = .48); *L-myc*, 3 hours (P = .0004) and 18 hours (P = .07); and *N-myc*, 3 hours (P = .0006) and 18 hours (P = .009). E, CABLES1 protein levels after treatment of AtT-20 cells for 3 and 18 hours with Dex assessed by Western blotting and quantitated (top panel) relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). F, Summary of reported *Cables1* regulation discussed in text.

TGGACCCTGACAATGTG-3′ and 5′-TTCTCACAGCAGA ACGCCTGAATC-3′; *L-myc*, 5′-CCAGCGATTCTGAAGGTG AAGAGA-3′ and 5′-TGATGGTGACTGGCTTTCGGATGT-3′; *N-myc*, 5′-AGCGACTCAGATGATGAGGATGAC-3′ and 5′-TTACCGCCTTGTTGTTAGAGGAGG-3′; and *TBP*, 5′-CCGTGAATCTTGGCTGTAAACTTGACC-3′ and 5′-TCTT GGCTCCTGTGCACACCATTT-3′.

Genomic analyses

Expression profiling of AtT-20 cells after treatment with 10^{-7} M Dex for 3 or 18 hours was described previously (17), and the data are available using Gene Expression Omnibus (GEO) accession number GSE19042. Similarly treated AtT-20 cells were also analyzed by RNA-sequencing (RNA-Seq) technology. High throughput sequencing of chromatin immunoprecipitation (ChIP-Seq) was performed as described previously (19, 20) using homemade antibodies against Tpit and Pitx1 (described in Refs. 21, 22) or commercial antibodies for GR (sc-1004; Santa Cruz) and phosphoserine 5 RNA polymerase II (Ser5-Pol II) (2532; Abcam). Data analysis was performed as described previously (19, 20).

Fluorescence-activated cell sorting and immunohistochemistry

The distribution of cell cycle phases was determined with propidium iodide–stained AtT-20 cells using established procedures on a FACSstar instrument operated by the Institut de Recherches Cliniques de Montréal Cell Sorting Facility. Human corticotrope adenoma sections obtained after surgery of patients with CD were paraffin-embedded and assessed by immunohistochemistry as described previously (13) using Cables1 antibody (ab75535; Abcam). $p27^{kip1}$ expression data for this adenoma set was as described previously (12). The use of human CD samples in the present work was approved by the Human Ethics Committees of Institut de Recherches Cliniques de Montréal and of Assistance Publique-Hôpitaux de Marseille.

Results

GC-regulated cell cycle genes

GCs are potent inhibitors of pituitary corticotrope cell proliferation and, in particular, the growth of the corticotrope model cells (AtT-20) is inhibited by GCs. This inhibition is almost complete as shown by the almost complete failure of AtT-20 cells to proliferate in presence of the synthetic GC Dex (Figure 1A).

We used RNA expression profiling data to identify cell cycle regulatory genes that are regulated by GCs. Gene expression data were obtained after treatment of AtT-20 cells with Dex for either 3 or 18 hours; these data were published previously (17). The microarray data identified the *L-myc*, *N-myc*, and *E2F2* genes, 3 positive regulators of cell cycle that are repressed by Dex treatment at both time points (Figure 1B); the data also identified 3 negative regulators of cell cycle progression, *Cables1*, *Gadd45β*, and *Gadd45γ*, for their activation after 3 hours of Dex treatment (Figure 1C).

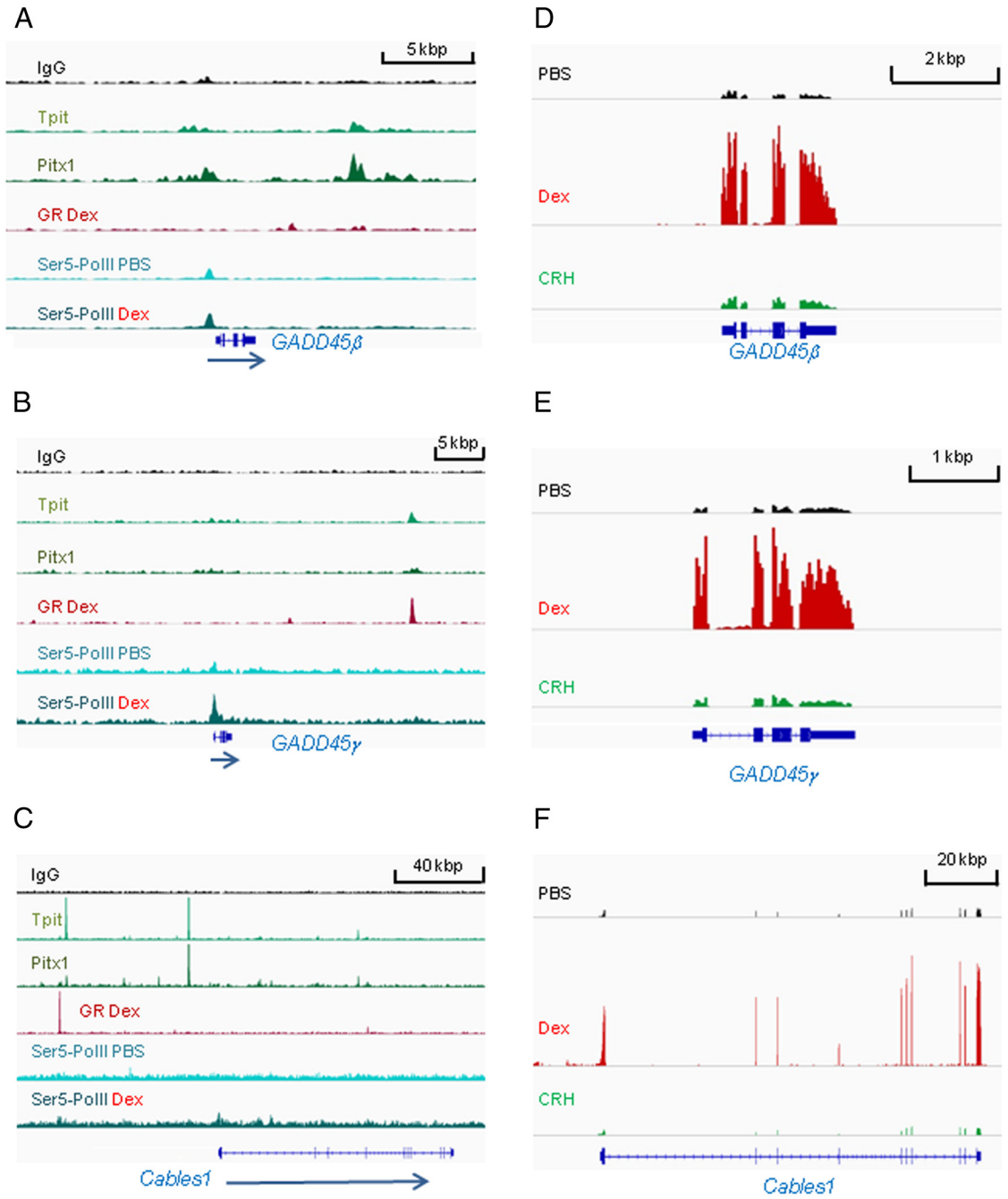


Figure 2. Genomic profiles of Dex-activated cell cycle regulatory genes reveal putative direct targets of GR action. A–C, ChIP-Seq data for control (IgG), Tpit, Pitx1, and Ser5-Pol II from untreated AtT-20 cells (PBS) and for GR and Ser5-Pol II after 30 minutes of Dex treatment. A, *Gadd45β* locus. B, *Gadd45γ* locus. C, *Cables1* locus. D–F, RNA-Seq profiles for the same loci in control (PBS) and AtT-20 cells treated with Dex or CRH showing increased transcripts after Dex, but not CRH, treatment.

These microarray data were confirmed by RT-qPCR analyses (Figure 1D).

In view of the well-documented role of *myc* genes in regulation of cell cycle progression, the repression of *L-myc* and *N-myc* expression by Dex supports the idea that these genes contribute to maintenance of the cell cycle in cultured AtT-20 cells and, consequently, that their repression by Dex would likewise contribute to inhibit proliferation. The role of the cell cycle inhibitors GADD45β and GADD45γ is also well established (23–25) and, moreover, it was shown that expression of GADD45γ is often lost in pituitary tumors and particularly in ACTH-producing corticotrope adenomas (26). The implication of Cables1 in pituitary cell cycle control has not been observed before, and we further assessed its importance for GC inhibition of corticotrope growth.

The transient effect of Dex on *Cables1* mRNA (Figure 1, C and D) led us to ascertain the effect of Dex on CA-

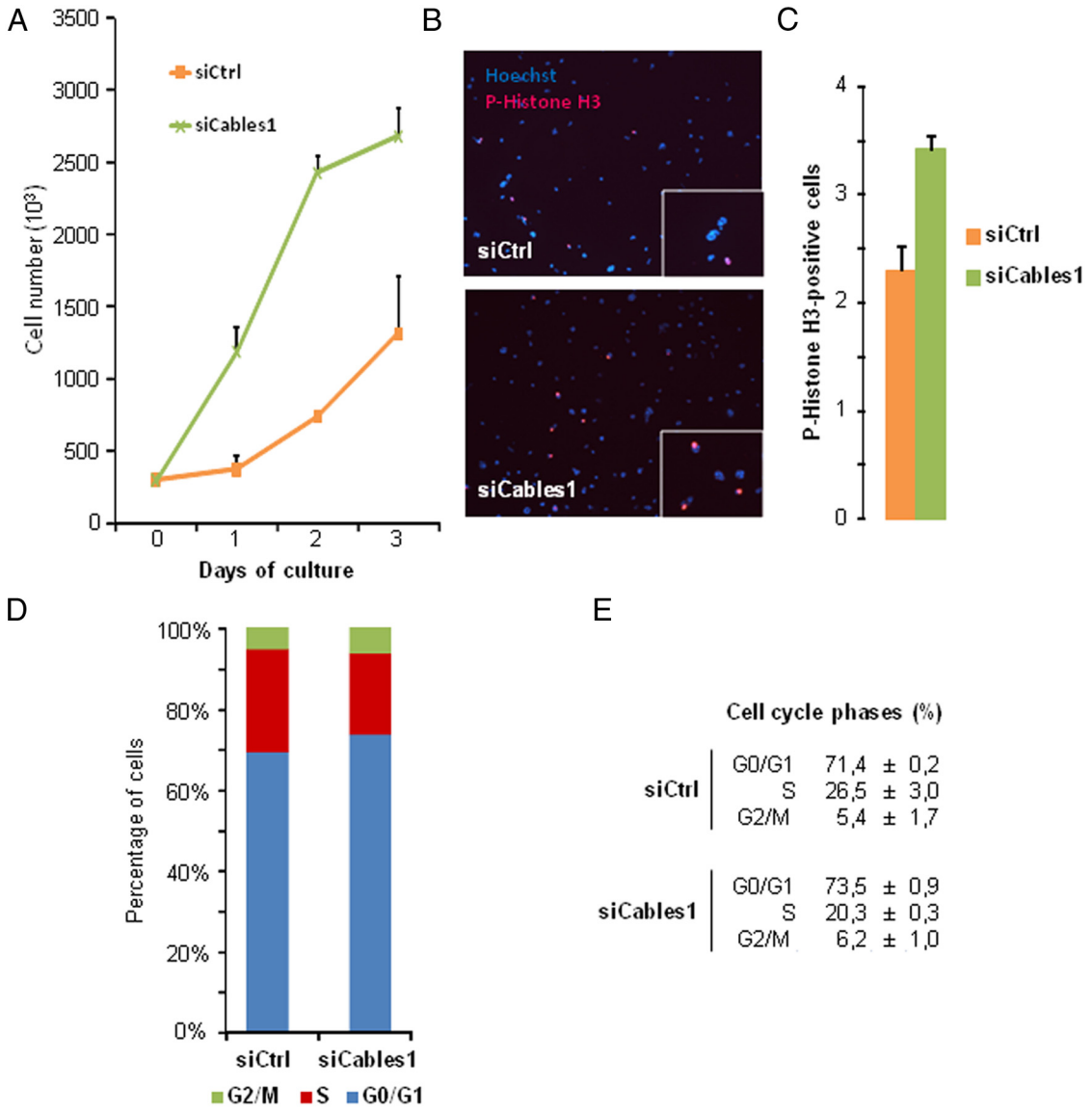


Figure 3. Increased proliferation of AtT-20 cells after knockdown of Cables1 expression. A, Treatment of AtT-20 cells with siRNAs targeting *Cables1* mRNA (siCables1), but not scrambled control siRNAs (siCtrl), accelerates cell growth. B and C, Increased number of AtT-20 cells in mitosis as revealed by immunohistochemistry against phospho-Ser10 histone H3 (red) (B) and by quantitation of the fraction of positive relative to total number of cells ($P = .05$) (C). D and E, Propidium iodide flow cytometry analysis of control and AtT-20 cells treated with siRNAs against Cables1 (D) reveal a shortened S phase after knockdown of Cables1 (E).

BLES1 protein expression at different times after Dex addition: we found that CABLES1 protein is increased about 2-fold at both 3 and 18 hours (Figure 1E) despite the 3-hour transient increase in mRNA (Figure 1D). CABLES1 was previously shown to inhibit cell cycle progression through its action on Cdk2 (27), and its expression is frequently lost in many cancers (28–31). Recent evidence indicated that CABLES1 can also stabilize $p21^{Cip1}$ protein (31) and that CABLES1 levels are controlled by Akt phosphorylation and recruitment of 14–3-3 (32). CABLES1 thus appears to integrate various signals for control of cell cycle progression (Figure 1F).

The rapid effects of Dex on expression of these cell cycle control genes suggests that GCs and their receptor (GR) may have a direct effect on the genes. We further supported this idea using ChIP-Seq data for GR and for the activated form of RNA polymerase II, Ser5-Pol II, that accumulates at the transcription start site (TSS) of regulated genes (33). For both *Gadd45* genes, the peak of Ser5-Pol II was greater after 30 minutes of Dex treatment than that for the control (Figure 2, A and B), consistent with a rapid action of GC on transcription as further supported by RNA-Seq data of AtT-20 cells treated with either Dex or CRH (Figure 2, D and E). Further, both have GR binding peaks downstream of the gene, at +4.4 and +8 kb for *Gadd45β* and at +7.3 and +19.3 kb downstream of the *Gadd45γ* gene, that may be responsible for GC regulation; the *Gadd45β* gene GR peaks at +8 kb and the *Gadd45γ*

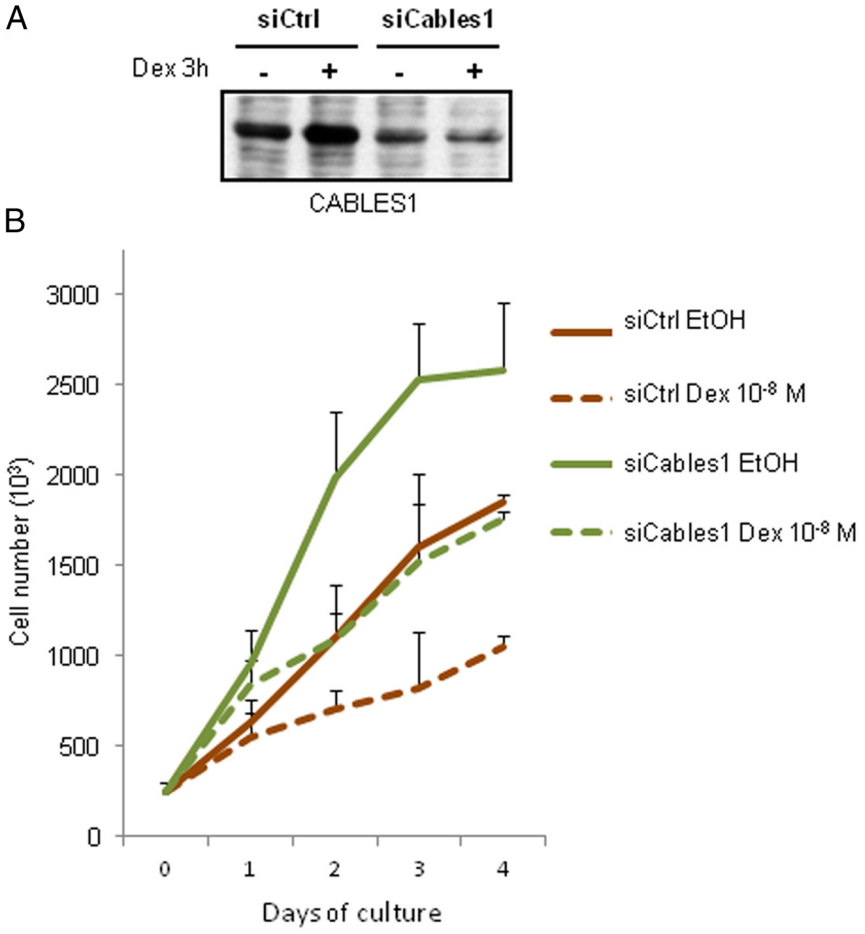


Figure 4. Knockdown of Cables1 antagonizes GC inhibition of AtT-20 cell growth. A, CABLES1 protein is increased by Dex treatment and decreased by *Cables1* siRNA as revealed by Western blotting. B, Growth inhibition produced by treatment of AtT-20 cells with Dex is reversed by knockdown of Cables1 expression using Cables1 siRNAs. EtOH, ethanol.

gene GR peaks at +19.3 kb, coincide with peaks of the pituitary-specific factors Tpit and/or Pitx1 (Figure 2, A and B). Further work is needed to determine the specific target sequences for GC activation, but the rapid (30-minute) increases in Ser5-Pol II at the TSS of these genes after Dex (Figure 2, A and B) clearly supports a direct transcriptional response to GC.

We also observed an increase in Ser5-Pol II at the TSS of the *Cables1* gene after 30 minutes of Dex (Figure 2C), in agreement with the RNA-Seq data showing increased Cables1 transcripts at 3 hours after Dex, but not CRH, treatment (Figure 2F). Interestingly, the ChIP-Seq data revealed 2 putative regulatory sequences that may contribute to *Cables1* expression in pituitary corticotropes as well as to GC activation. Indeed, a putative tissue-specific enhancer that binds both the pituitary-restricted factor Pitx1 and the POMC lineage control factor Tpit is located at −14.3 kb, whereas another putative enhancer at −72.3 kb exhibits strong peaks of Tpit and GR binding (Figure 2C). The latter represents a likely target of GR action for activation of *Cables1* expression in response to GC.

Cables1 is a critical regulator of AtT-20 cell growth

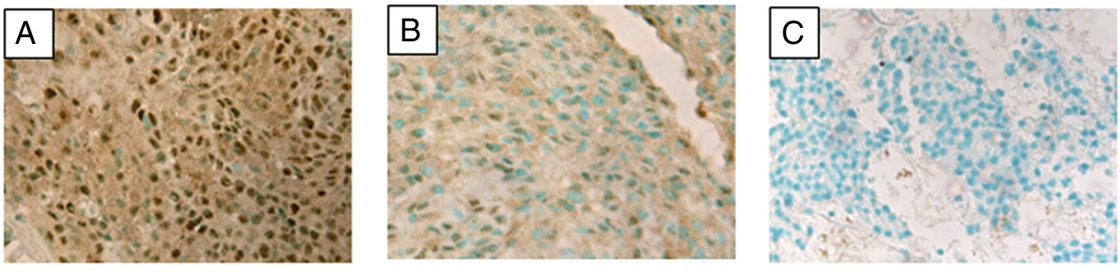
To assess the importance of *Cables1* for regulation of AtT-20 cell proliferation, we used siRNAs to knock down its expression. The growth of AtT-20 cells was greatly accelerated in cells in which *Cables1* expression is knocked-down in comparison to cells transfected with a control scrambled siRNA (Figure 3A). The effect on growth was ascertained by labeling cells in mitosis using immunohistofluorescence against phospho-histone-H3 (Figure 3B). Quantitation of these data indicated a significant increase in the number of mitotic cells in *Cables1* siRNA-treated cultures compared with that of the control (Figure 3C).

We further assessed the distribution of AtT-20 cells in different phases of the cell cycle in control and Cables1 siRNA-treated cells by FACS (Figure 3D). The shortening of the S phase (Figure 3E) in cells with reduced levels of CABLES1 (Figure 4A) is consistent with the purported role of CABLES1 as a negative regulator of the cdk2 complex and of the G_1/S phase checkpoint.

These data indicated that *Cables1* knockdown reduces the length of the S phase relative to those of other cell cycle phases (Figure 3, D and E). We further assessed the importance of Dex-induced *Cables1* expression in inhibition of AtT-20 cell growth by attempting to reverse this effect using *Cables1* siRNA. Quite strikingly, the growth inhibition produced by Dex was reversed by *Cables1* siRNA which, on its own, led to increased cell proliferation (Figure 4B). These data suggest that the regulation of *Cables1* mRNA by GCs may be a critical step for GC regulation of corticotrope cell growth.

Loss of *Cables1* expression in corticotrope adenomas from patients with CD

Given the importance of *Cables1* expression of the control of AtT-20 cell growth, it is interesting to assess CABLES1 expression in corticotrope adenomas that cause CD. CABLES1 protein expression is detectable in normal human pituitary and in a subset of corticotrope adenomas obtained after surgery (Figure 5A). CABLES1 expression



D

Cables1	number adenomas	%
+	11	35
Weak (and <50% positive cells)	3	10
-	17	55
total	31	100

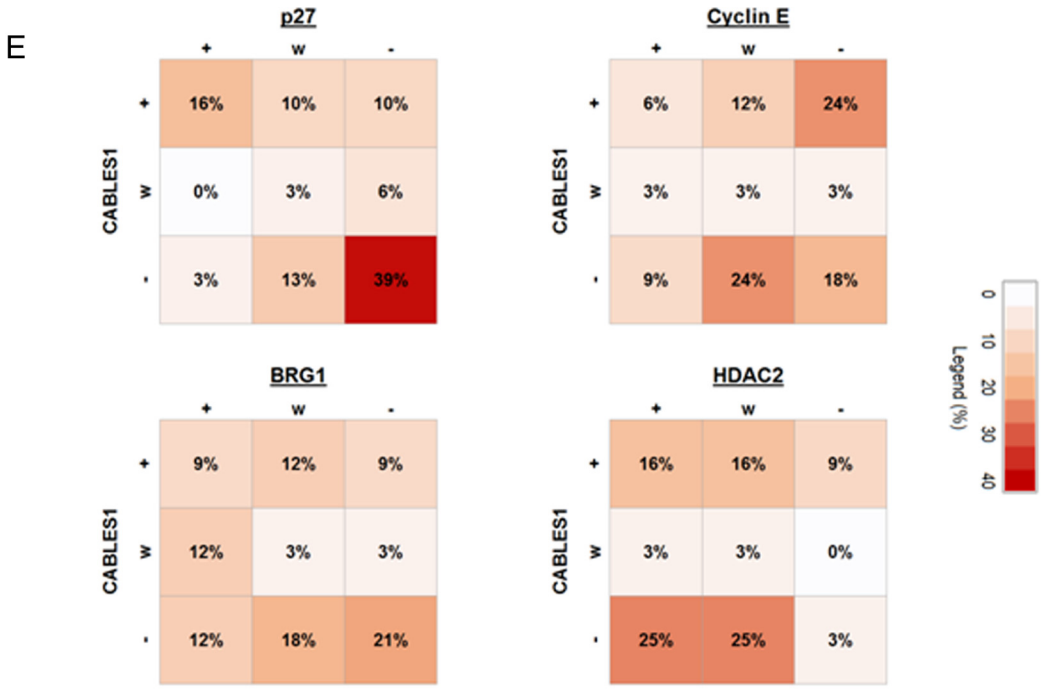


Figure 5. Loss of CABLES1 expression in corticotrope adenomas from patients with CD. A–C, CABLES1 protein is present in normal human pituitary (A) but is significantly reduced (B) or absent (C) in corticotrope adenoma samples as revealed by immunohistochemistry. D, Summary of CABLES1 expression in a panel of 31 corticotrope adenomas that was previously investigated for expression of other markers (12, 13). E, Loss of CABLES1 expression is strongly associated with loss of $p27^{Kip1}$ expression but not with expression of BRG1, HDAC2, or cyclin E. Square plots represent the relative proportions of adenomas scored for expression of indicated markers. +, positive; −, negative; w, weak expression in <50% of cells.

was assessed by immunohistochemistry in a panel of 31 corticotrope adenomas (12, 13); clinical data for the patients are provided in Supplemental Table 1. Thus, 3 samples were found to have weak CABLES1 expression (Figure 5B), and 17 adenomas had undetectable CABLES1 (Figure 5C). Overall, 55% of the corticotrope adenoma samples have completely lost CABLES1 expression, whereas about 40% retain expression (Figure 5D). Because Cables1 is an upstream regulator of $p27^{Kip1}$, it was interesting to assess the relation between the loss of CABLES1 expression in corticotrope adenomas with expression of $p27^{Kip1}$. Interestingly, the loss of CABLES1 expression is strongly associated with the loss of $p27^{Kip1}$ expression as observed for both the CABLES1-negative

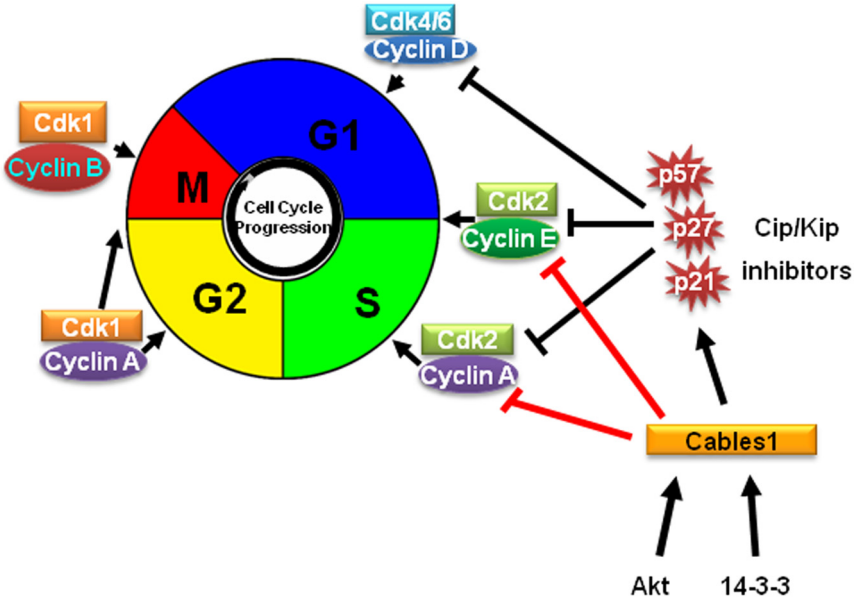


Figure 6. Summary of reported targets of Cables1 action for regulation of cell cycle progression.

and CABLES1-weak group of adenomas in contrast to the group of CABLES1-positive samples (Figure 5E). This unique correlation contrasts with the absence of correlation between CABLES1 protein levels and those of either cyclin E, BRG1, or HDAC2 (Figure 5E).

Discussion

The present study has identified multiple targets of GCs/GR for inhibition of corticotrope cell proliferation, namely, GC repression of positive regulators and activation of at least 3 negative regulators of proliferation. These multiple targets of GC action probably provide regulatory robustness to ensure efficient control of proliferation. Conversely, these multiple targets are likely to be somewhat redundant with each other, eg, the 2 Dex-repressed *Myc* genes. These basic observations support the idea that multiple genetic and/or regulatory disruptions will be needed to transform a normal Dex-repressed corticotrope into a Dex-resistant corticotrope adenoma cell.

As for a large number of Gc-activated genes, the activation of *Gadd45β*, *Gadd45γ*, and *Cables1* genes appears to reflect the direct action of GR on those genes as indicated by the presence of genomic GR recruitment sites at putative enhancers of these loci and by the rapid (30-minute) increase in the amount of activated RNA polymerase II (Ser5-Pol II) at the gene promoters (Figure 2, A–C). In contrast and in agreement with prior work on the mechanism of GR action (19), Dex repression of the *Myc* genes could not be associated with direct genomic binding sites of GR; the repressor activity of GR may be mediated by a secondary effector.

Despite the apparent redundancy of GC regulation of proliferation revealed through this study in AtT-20 cells, it is striking that knockdown by siRNA of CABLES1 protein results in a dramatic increase of proliferation (Figure 3). The reversal of this effect by Dex and of the Dex repression of proliferation by knockdown of *Cables1* is as dramatic and clearly indicates that in AtT-20 cells, CABLES1 is a major determinant for cell proliferation.

Cables1 thus has a dominant role in control of proliferation of these transformed cells that were established in culture more than 50 years ago from a mouse pituitary tumor (34). It is noteworthy that the AtT-20 cells were established in culture with the objective of maintaining the differentiated phenotype together with the ability to be immortalized. These cells are thus likely to have lost some of their normal mechanisms for control of proliferation, but not all, and they have, in particular, maintained sensitivity to GC control of proliferation.

In this context, it is possible that the dominant role of Cables1 on proliferation may be more prevalent in AtT-20 cells than in normal corticotropes. This view is supported by the relatively normal pituitary development and cell composition of *Cables1* knockout mice up to 1 year of age (data not shown). Normal mouse corticotropes may thus require more than loss of Cables1 to exhibit deregulated cell growth, and the situation may be similar in humans. The normal human pituitary exhibits CABLES1 expression by immunohistochemistry in most cells (Figure 5A), and this is consistent with mouse gene expression profiling data performed on isolated cells of each pituitary lineage (data not shown). It is thus significant that 55% of human corticotrope adenomas have lost expression of CABLES1 with an additional 10% that have weak expression (Figure 5D).

The loss of CABLES1 expression is strongly correlated with the loss of $p27^{Kip1}$ expression because all but one of the CABLES1-negative adenomas had either undetectable or weak $p27^{Kip1}$ expression (Figure 5E). This correlation is stronger than any correlation observed previously (12, 13) in the same panel of corticotrope adenomas for expression (or loss of expression) of BRG1 and HDAC2 and/or with the gain of cyclin E expression (Figure 5E). It

is thus unlikely to be coincidental and indeed, the loss of CABLES1 in mouse embryonic fibroblasts was shown to result in decreased $p27^{Kip1}$ together with $p16^{INK4}$ and cyclin D1 (35). Further, expression of CABLES1 is strongly correlated with $p21^{Cip1}$ in lung cancer (31).

A subgroup of $p27^{Kip1}$-negative adenomas retain CABLES1 expression but almost never the opposite, thus suggesting that the loss of CABLES1 may have an incremental effect on the loss of $p27^{Kip1}$ expression. Nonetheless, the loss of expression of either gene may represent independent events during the process of adenoma formation. The analysis of this small group of 31 adenomas did not reveal any other correlation: in particular, the study samples included 9 larger macroadenomas, and these did not show a significantly higher frequency of the loss of either CABLES1 or $p27^{Kip1}$. Similarly, we did not find significant associations between the loss of CABLES1 expression and the loss of BRG1 or HDAC2 or with the expression of cyclin E (Figure 5E).

The available data on Cables1 action (Figures 1F and 6) are consistent with the associated decreases in $p27^{Kip1}$ (35). In addition, Cables1 normally stabilizes $p21^{Cip1}$ through inhibition of its degradation by the proteasome pathway and $p21^{Cip1}$, which is expressed in adult but not fetal pituitary (36–38), was suggested to have tumor suppressor activity in some pituitary tumors (37). The loss of CABLES1 may thus contribute to deregulated cell growth by destabilization of $p21^{Cip1}$ and down-regulation of $p27^{Kip1}$.

It was recently found that Cables1 stability is regulated by Akt phosphorylation and 14–3-3 binding to 2 phosphorylated sites (32). The USP8 mutations recently identified in Cushing adenomas were associated with increased deubiquination and recycling of the EGF receptor (15). It is interesting to speculate whether the USP8 deubiquitinase may also affect CABLES1 stability or its action. However, the phosphorylated sites associated with 14–3-3 binding in EGF receptor and CABLES1 are not very similar; indeed whereas the EGF receptor is phosphorylated by Erk1/2, the CABLES1 14–3-3 binding sites are substrates for Akt (32). Further work is needed to assess these possibilities.

In summary, the loss of CABLES1 expression may be an important contributing factor for development of corticotrope adenomas, and it may link the tumorigenic process with GC regulation of cell cycle progression.

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Address all correspondence and requests for reprints to: Jacques Drouin, Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal (IRCM), 110, avenue des Pins Ouest, Montréal, QC H2W 1R7, Canada. E-mail: jacques.drouin@ircm.qc.ca.

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References

1. **Almawi WY, Melemedjian OK.** Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. *J Leukoc Biol*. 2002;71:9–15.
2. **Mattern J, Büchler MW, Herr I.** Cell cycle arrest by glucocorticoids may protect normal tissue and solid tumors from cancer therapy. *Cancer Biol Ther*. 2007;6:1345–1354.
3. **Dickmeis T, Foulkes NS.** Glucocorticoids and circadian clock control of cell proliferation: at the interface between three dynamic systems. *Mol Cell Endocrinol*. 2011;331:11–22.
4. **Yang-Yen HF, Chambard JC, Sun YL, et al.** Transcriptional interference between c-jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell*. 1990;62:1205–1215.
5. **McKay LI, Cidlowski JA.** Cross-talk between nuclear factor-κB and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol Endocrinol*. 1998;12:45–56.
6. **De Bosscher K, Vanden Berghe W, Haegeman G.** The interplay between the glucocorticoid receptor and nuclear factor-κB or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev*. 2003;24:488–522.
7. **Nissen RM, Yamamoto KR.** The glucocorticoid receptor inhibits NFκB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev*. 2000;14:2314–2329.
8. **Pascual G, Glass CK.** Nuclear receptors versus inflammation: mechanisms of transrepression. *Trends Endocrinol Metab*. 2006;17:321–327.
9. **Drouin J, Bilodeau S, Vallette-Kasic S.** Of old and new diseases: genetics of pituitary ACTH excess (Cushing) and deficiency. *Clin. Genet*. 2007;72:175–182.
10. **Jordan S, Lidhar K, Korbonits M, Lowe DG, Grossman AB.** Cyclin D and cyclin E expression in normal and adenomatous pituitary. *Eur J Endocrinol*. 2000;143:R1–R6.
11. **Dahia PL, Aguiar RC, Honegger J, et al.** Mutation and expression analysis of the p27/kip1 gene in corticotrophin-secreting tumours. *Oncogene*. 1998;16:69–76.
12. **Roussel-Gervais A, Bilodeau S, Vallette S, et al.** Cooperation between cyclin E and $p27^{Kip1}$ in pituitary tumorigenesis. *Mol Endocrinol*. 2010;24:1835–1545.
13. **Bilodeau S, Vallette-Kasic S, Gauthier Y, et al.** Role of Brg1 and HDAC2 in GR *trans*-repression of pituitary POMC gene and misexpression in Cushing disease. *Genes Dev*. 2006;20:2871–2886.
14. **Perez-Rivas LG, Theodoropoulou M, Ferraù F, et al.** The gene of the ubiquitin-specific protease 8 is frequently mutated in adenomas causing Cushing's disease. *J Clin Endocrinol Metab*. 2015;100:E997–E1004.
15. **Reincke M, Sbiera S, Hayakawa A, et al.** Mutations in the deubiquitinase gene USP8 cause Cushing's disease. *Nat Genet*. 2015;47:31–38.

16. **Ma ZY, Song ZJ, Chen JH, et al.** Recurrent gain-of-function USP8 mutations in Cushing's disease. *Cell Res*. 2015;25:306–317.
17. **Langlais D, Couture C, Balsalobre A, Drouin J.** Regulatory network analyses reveal genome-wide potentiation of LIF signaling by glucocorticoids and define an innate cell defense response. *PLoS Genet* 2008;4:e1000224.
18. **Rambaud J, Desroches J, Balsalobre A, Drouin J.** TIF1β/KAP-1 is a coactivator of the orphan nuclear receptor NGFI-B/Nur77. *J Biol Chem*. 2009;284:14147–14156.
19. **Langlais D, Couture C, Balsalobre A, Drouin J.** The Stat3/GR interaction code: predictive value of direct/indirect DNA recruitment for transcription outcome. *Mol Cell*. 2012;47:38–49.
20. **Budry L, Balsalobre A, Gauthier Y, et al.** The selector gene Pax7 dictates alternate pituitary cell fates through its pioneer action on chromatin remodeling. *Genes Dev*. 2012;26:2299–2310.
21. **Lamolet B, Pulichino AM, Lamonerie T, et al.** A pituitary cell-restricted T-box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. *Cell*. 2001;104:849–859.
22. **Lanctôt C, Gauthier Y, Drouin J.** Pituitary homeobox 1 (Ptx1) is differentially expressed during pituitary development. *Endocrinology*. 1999;140:1416–1422.
23. **Tront JS, Hoffman B, Liebermann DA.** Gadd45a suppresses Ras-driven mammary tumorigenesis by activation of c-Jun NH_2-terminal kinase and p38 stress signaling resulting in apoptosis and senescence. *Cancer Res*. 2006;66:8448–8454.
24. **Vairapandi M, Balliet AG, Hoffman B, Liebermann DA.** GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. *J Cell Physiol*. 2002;192:327–338.
25. **Ying J, Srivastava G, Hsieh WS, et al.** The stress-responsive gene GADD45G is a functional tumor suppressor, with its response to environmental stresses frequently disrupted epigenetically in multiple tumors. *Clin Cancer Res*. 2005;11:6442–6449.
26. **Zhang X, Sun H, Danila DC, et al.** Loss of expression of GADD45 gamma, a growth inhibitory gene, in human pituitary adenomas: implications for tumorigenesis. *J Clin Endocrinol Metab*. 2002;87:1262–1267.
27. **Wu CL, Kirley SD, Xiao H, et al.** Cables enhances cdk2 tyrosine 15 phosphorylation by Wee1, inhibits cell growth, and is lost in many human colon and squamous cancers. *Cancer Res*. 2001;61:7325–7332.
28. **Arnason T, Pino MS, Yilmaz O, et al.** Cables1 is a tumor suppressor gene that regulates intestinal tumor progression in Apc^{Min} mice. *Cancer Biol Ther*. 2013;14:672–678.
29. **Dong Q, Kirley S, Rueda B, Zhao C, Zukerberg L, Oliva E.** Loss of cables, a novel gene on chromosome 18q, in ovarian cancer. *Mod Pathol*. 2003;16:863–868.
30. **Kirley SD, D'Apuzzo M, Lauwers GY, Graeme-Cook F, Chung DC, Zukerberg LR.** The Cables gene on chromosome 18Q regulates colon cancer progression in vivo. *Cancer Biol Ther*. 2005;4:861–863.
31. **Shi Z, Li Z, Li ZJ, et al.** Cables1 controls p21/Cip1 protein stability by antagonizing proteasome subunit α type 3. *Oncogene* 2015;34:2538–2545.
32. **Shi Z, Park HR, Du Y, et al.** Cables1 complex couples survival signaling to the cell death machinery. *Cancer Res*. 2015;75:147–158.
33. **Hsin JP, Manley JL.** The RNA polymerase II CTD coordinates transcription and RNA processing. *Genes Dev*. 2012;26:2119–2137.
34. **Buonassisi B, Sato G, Cohen AI.** Hormone-producing cultures of adrenal and pituitary tumor origin. *Biochemistry*. 1962;48:1184–1190.
35. **Kirley SD, Rueda BR, Chung DC, Zukerberg LR.** Increased growth rate, delayed senescense and decreased serum dependence characterize cables-deficient cells. *Cancer Biol Ther*. 2005;4:654–658.
36. **Bilodeau S, Roussel-Gervais A, Drouin J.** Distinct developmental roles of cell cycle inhibitors $p57^{Kip2}$ and $p27^{Kip1}$ distinguish pituitary progenitor cell cycle exit from cell cycle reentry of differentiated cells. *Mol Cell Biol*. 2009;29:1895–1908.
37. **Chesnokova V, Zonis S, Kovacs K, et al.** $p21^{Cip1}$ restrains pituitary tumor growth. *Proc Natl Acad Sci USA*. 2008;105:17498–17503.
38. **Ikeda H, Yoshimoto T, Shida N.** Molecular analysis of p21 and p27 genes in human pituitary adenomas. *Br J Cancer*. 1997;76:1119–1123.