Supporting Information

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SI Materials and Methods

Antibodies and Reagents. GFP (B2), GST (B14), melanocortin 1 receptor (MC1R) (N19), and CyclinB1 (H-433) were purchased from Santa Cruz Biotechnologies and used at dilutions ranging from 1:500–1,000. P-cdc25B S323, P-cdc2 [cyclin dependent kinase 1 (CDK1)] T14, and Y15 (western) antibodies were purchased from Abcam and used at dilutions ranging from 1:250–1:1,000. Cdc25C, P-cdc25C S216, cdc25B, P-Erk1/2 T202/Y204, and P-cdc2 (CDK1) Y15 (FACS) were purchased from cell signaling and used at 1:500–1:1,000 for western and 1:50 for FACS where applicable. DMSO, Forskolin, 3-isobutyl-1-methylxanthine (IBMX), and α-melanocyte–stimulating hormone (MSH) were purchased from Sigma.

Cell Lines and Plasmids. MM200, MM370, MM415, and MM485 cell lines were part of a panel of cell lines generously given by Nick Hayward (Queensland Institute of Medical Research, Queensland, Australia). A375 cells were obtained from the ATCC. Melanoma lines were cultured in RPMI or DMEM supplemented with 10% FBS.

The FG12 GFP, H59, H60, and H61 plasmids were generously given by Marisol Soengas (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain). MC1R 155T, cdc25B, and cdc25C cDNAs were purchased from OpenBiosystems. The wild-type allele of MC1R was generated by site-directed mutagenesis of threonine 155 to isoleucine using the following primer pair:

F CGCACTGCGCTACCACAGCATCGTGACCCTGCCG-CGGGCGCGGC

R GCCGCGCCCGCGGCAGGGTCACGATGCTGTGGTA-GCGCAGTGCG

The pLVX tet on and tight-puro vectors were purchased from Clontech. MC1R wild type, Cdc25B, and Cdc25C were first GFP-tagged using the gateway cloning system, and then subcloned into the pLVX tight-puro vector. Stable, inducible MM485 and MM370 cell lines were generated by infection with pLVX tet on, selection with G418, infection with pLVX tight-puro-derived vectors, and selection with puromycin.

The cells used in the cAMP experiments were derived from the MM485 pLVX tet on MC1R-GFP cell line described above. This cell line was transfected with the glosensor plasmid (Promega) and selected with hygromycin.

cAMP Assay. MM485 pLVX tet on MC1R-GFP were plated at 40,000 cells per well in 96-well plates in the presence or absence of doxycycline. Forty-eight hours after plating, cells were equilibrated with glosensor reagent at 4% in optimem (phenol-free) with 10% FBS and 15 mM Hepes for 1 h at 37 °C and 1 h at room temperature. Plates were read on the Spectramax FL. Following 20 min of equilibration to machine temperature, stimuli were added in triplicate (Forskolin to final concentration of 20 μM, IBMX at 100 μM, and MSH at 100 nM). Kinetic reads were performed and data were analyzed in prism.

Flow Cytometry. Cells were fixed by one of three different protocols. For cells that were nontransduced or transduced with GFP-tagged constructs, cells were fixed in 70% EtOH. Briefly, cells were trypsinized, spun down, resuspended in 1.5 mL of PBS, and fixed by drop-wise addition of 3 mL of ice-cold 95% EtOH with 5% PBS. Cells that expressed GFP alone could not be processed by EtOH fixation because GFP leaks out of the cells and transduced populations could not be differentiated from nontransduced populations. Instead, these cells were fixed by resuspension in 2% PFA in PBS overnight at room temperature. Cells that were stained for P-CDK1 were fixed for 10 min with 4% PFA, then permeabilized with ice-cold methanol and stored at −20 °C until processing. For DNA content analysis, cells were washed two times in PBS and resuspended in PBS with 1 mg/mL propidium iodide with RNaseA. PFA-fixed (2%) cells were permeabilized with 0.3% triton for 5 min before PBS washes. P-H3 staining was performed similarly to DNA content staining except cells were washed once in PBS, once in PBS-T (PBS+ 0.1% BSA+ 0.2% Tween-20) and incubated with anti-PH3 conjugated to Alexa-647 (Molecular Probes) diluted 1:100 in PBS-T (PBS + 0.1% BSA and 0.2% Tween-20) for 1 h on ice in the dark. P-CDK1 staining was performed by washing PFA-fixed, MeOH-permeabilized cells twice with PBS-B (PBS+0.2% BSA), incubation with anti-PCDK1 Y15 (Cell Signaling) for 30 min at room temperature in the dark, washing once with PBS-B, incubating with APC-conjugated anti-rabbit (Jackson Immunoresearch) diluted 1:400 in PBS-B for 30 min at room temperature in the dark, and twice washing with PBS-B and resuspension in PBS+1 mg/mL PI and RNaseA.

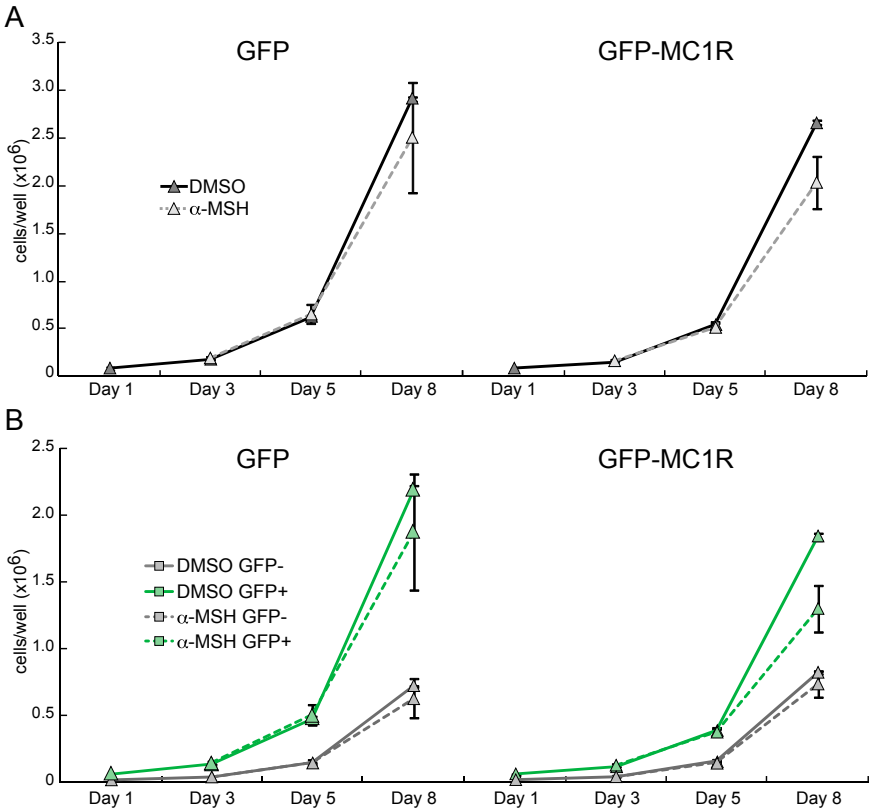

Fig. S1. MC1R overexpression slows growth of melanoma cell lines. (*A*) Before analysis by flow cytometry, samples from Fig. 1 (days 1–8) were trypsinized and counted in duplicate. These graphs represent the average number of cells per well with error bars representing one SD from the mean. (*B*) The total number of GFP-positive and GFP-negative cells was ascertained by multiplying the total cell number by the fraction of GFP-positive cells indicated in Fig. 1*A*.

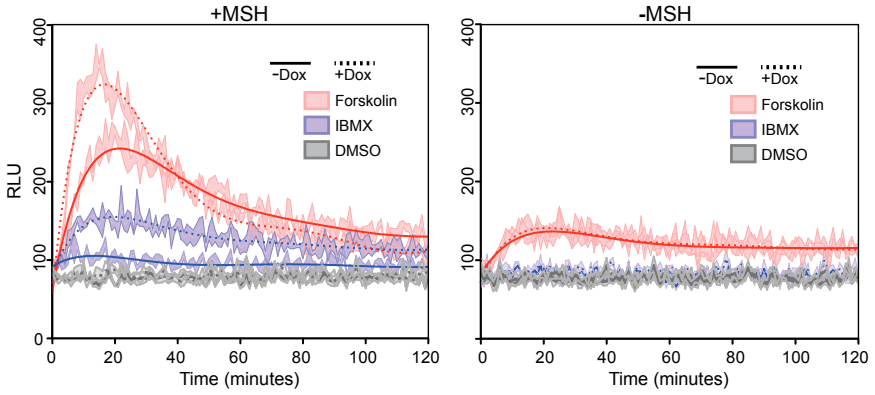

Fig. S2. MC1R overexpression increases cAMP signaling. MM485 tet on MC1R-GFP glosensor cells were plated in 96-well plates and treated in triplicate with combinations of doxycycline, MSH, Forskolin, IBMX, and DMSO. The lines in the graphs are sixth order polynomial fits of the data. The shaded region around each line represents the SE. No cAMP signal was observed in the absence of IBMX or Forskolin, presumably because changes in cAMP were below the limits of detection of the assay. cAMP signal was observed in all forskolin-treated conditions, but was enhanced by addition of MSH and further enhanced by the combination of MSH treatment and overexpression of MC1R. cAMP induction was seen in IBMX-treated cells only in the presence of MSH and overexpressed MC1R. These trends were reproduced in multiple independent experiments. This indicates that the overexpressed GFP-tagged MC1R is functional and that it is MSH-sensitive. Additionally, this indicates that the presence of functional MC1R and IBMX can induce cAMP to levels comparable to those found in forskolin-treated cells. This matches the functional results shown in Fig. 2*C*.

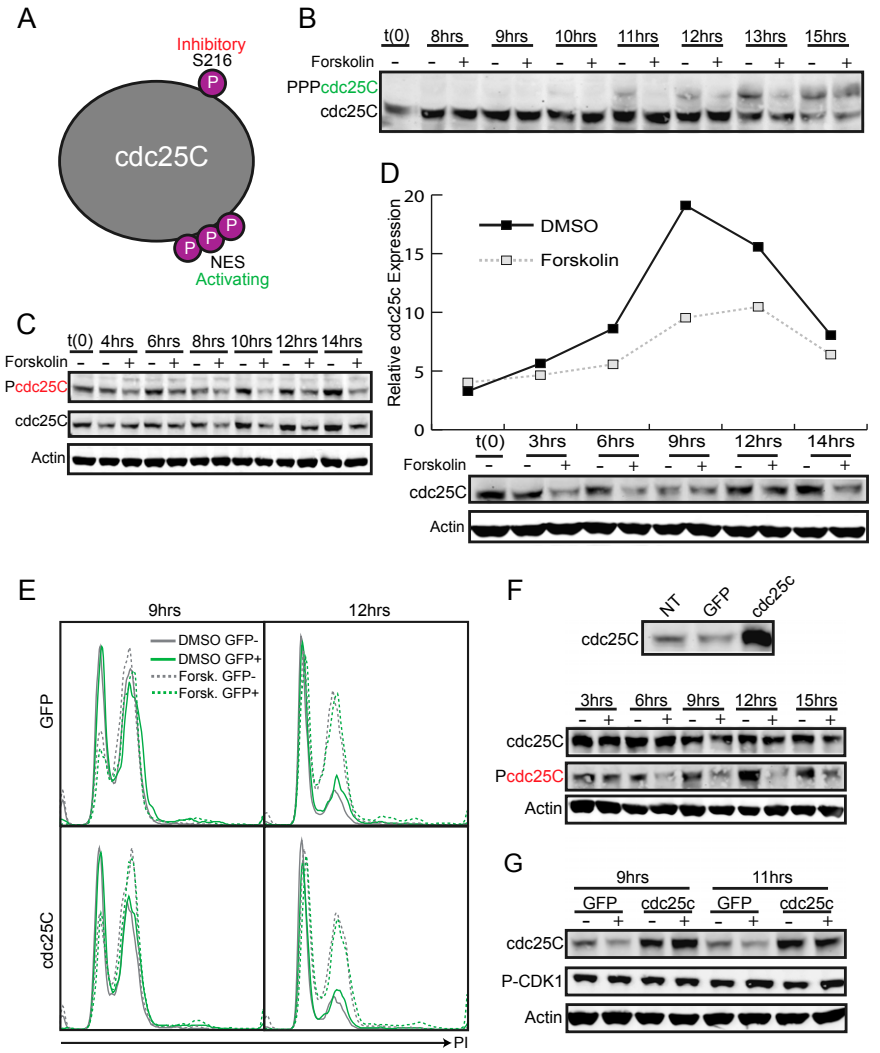

Fig. S3. Cdc25C does not mediate the forskolin-induced delay in mitotic entry. (*A*) Key activating and inhibitory phosphorylation sites on cdc25C. (*B*) MM370 cells were synchronized and released into nocodazole plus DMSO or forskolin (50 μM). Lysates were collected at indicated times postrelease, separated by SDS/PAGE and visualized with a cdc25C antibody. The higher molecular weight band corresponds to the polyphosphorylated, active form of cdc25C. (*C*) MM370 cells were synchronized and released in the presence of DMSO or forskolin. Lysates were collected at indicated time points and blots probed with antibody for cdc25C phospho-Ser216. (*D*) MM370 cells were synchronized and released in the presence of DMSO or forskolin. RNA and protein was isolated from cells harvested at indicated time points. RNA was quantified by QPCR and normalized to GAPDH, and protein was visualized as described. (*E*) MM370 cells were transfected with FG12 GFP or FG12 GFP cdc25C, synchronized and released into DMSO or Forskolin. Cells were collected at indicated time points and cell cycle was analyzed by DNA content analysis. Cdc25C expression caused no changes in cell cycle profile of DMSO or forskolin-treated cells. (*F*) Lysates were taken from duplicate wells from the experiment described above, and levels were assessed by immunoblot. Cdc25C levels were significantly increased by the transfection, but phosphorylation levels were not, indicating that the protein was not being inactivated. (*G*) A duplicate experiment was performed with MM370 cells lentivirally transduced with FG12 GFP or GFP cdc25C. Lysates were taken at indicated time points and protein levels were assessed by immunoblot.

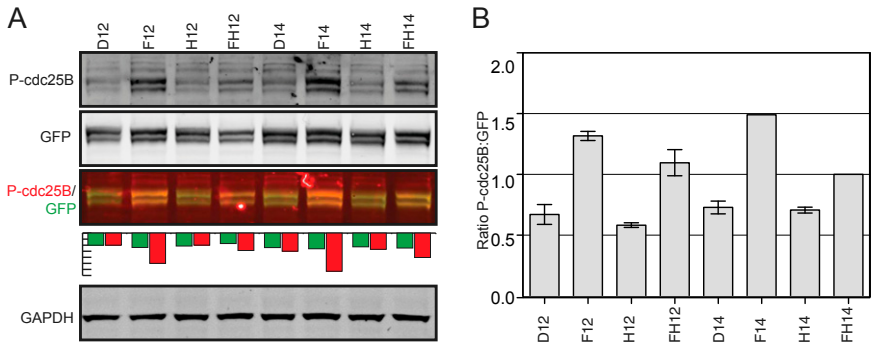

Fig. S4. Protein kinase A (PKA) inhibition blocks phosphorylation of cdc25B. (*A*) Cells were synchronized, released and treated as previously described with the PKA inhibitor H-89 added as an additional condition. H-89 treatment decreases both the levels of cdc25B-GFP and the ratio of phosphorylated:total cdc25B-GFP. Western blots represent one representative experiment. The graph represents the amounts of total cdc25B-GFP (green) and P-cdc25B (red) as quantified by licor and normalized to the D12 treatment condition. (*B*) Graph with error bars showing the results of duplicate experiments. The ratio of P-cdc25B:total cdc25B-GFP was normalized to FH14 for each experiment and this normalized ratio was used to calculate means and SDs (error bars).

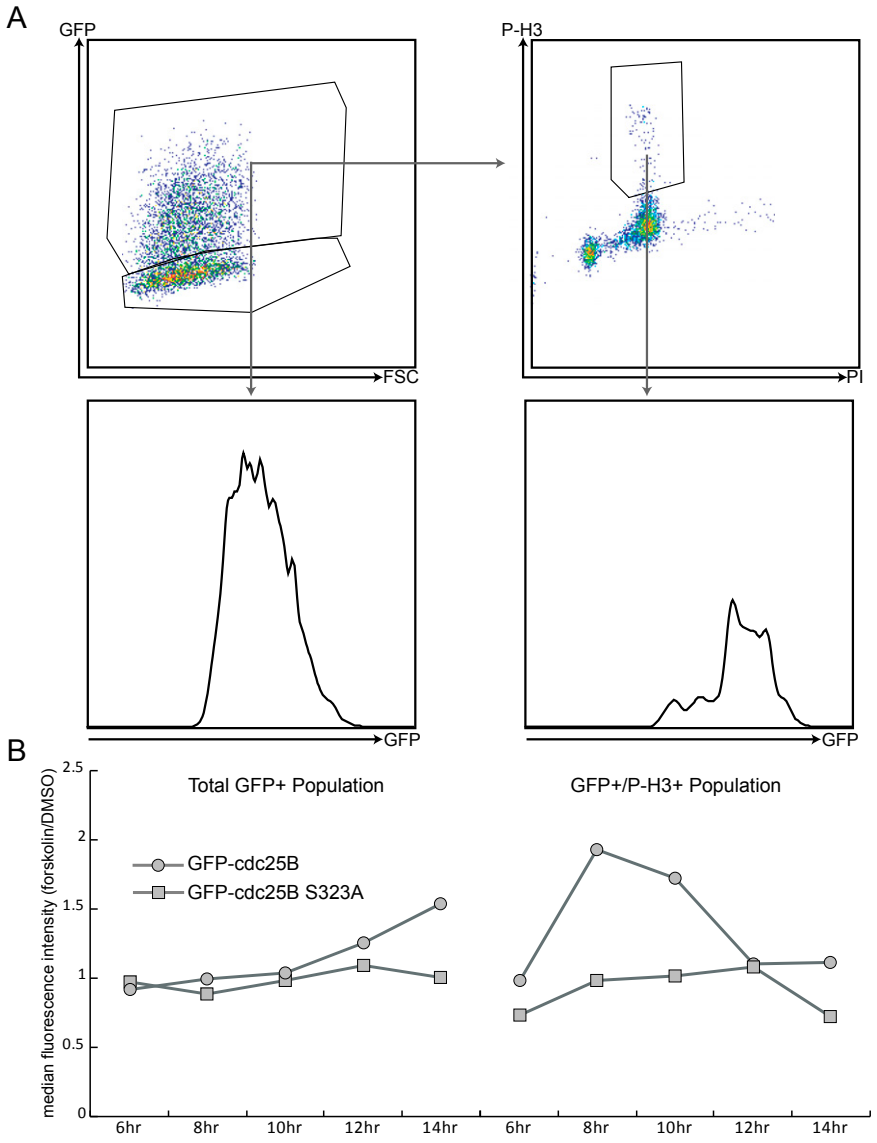

Fig. S5. Mitotic cells in forskolin-treated wells are associated with increased expression of cdc25B but not cdc25B S323A. (*A*) Detailed gating strategy for comparison of GFP intensity. Changes in GFP intensity between DMSO and Forskolin-treated cells were assessed in GFP-positive cells and compared with changes in GFP intensity in GFP- and P-H3–double positive cells. (*B*) These graphs depict the ratio of GFP median fluorescence intensity between forskolin and DMSO-treated cells in total GFP populations and GFP+/P-H3+ populations. Similar curves were seen for mean fluorescence intensity. The total median fluorescence intensity is not affected by forskolin treatment for both wild-type and mutant GFP-cdc25B (*Left*). In the P-H3+ population, there is a twofold increase in GFP-cdc25B intensity in the forskolin-treated cells at 8 and 10 h postrelease. This increased intensity is not seen for GFP-cdc25B S323A.

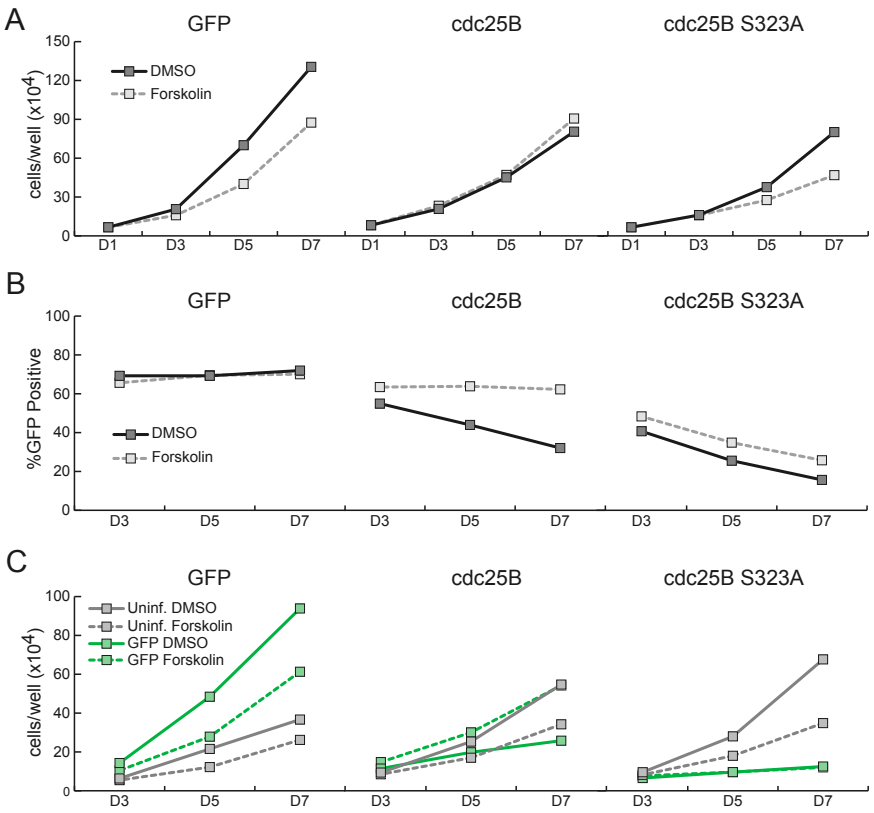

Fig. S6. Cdc25B activity does not rescue forskolin-induced growth inhibition. (*A*) MM370 cells stably expressing inducible GFP, GFP-cdc25B, or GFP-cdc25B S323A were diluted with the parental cell line and cultured in the presence of doxycycline and DMSO or forskolin (50 μM). Every other day, cells were trypsinized, counted and analyzed for percentage of GFP-positive cells by flow cytometry. Panel A depicts the total cells/well. (*B*) Cells from *A* were analyzed by flow cytometry to determine the percentage of GFP-positive cells. In the GFP-only controls, the percentage of GFP-positive cells did not change during the course of the experiment. The percentage of GFP-positive, cdc25B-expressing cells dropped over time unless the activity of cdc25B is inhibited by cAMP activity. (*C*) Total cell number of both uninfected controls and GFP-positive cells were calculated by multiplying the total cell number (*A*) by the fraction of GFP-negative and -positive cells (*B*). Expression of active cdc25B causes decreased cell number. Forskolin treatment of GFP-cdc25B-expressing cells stabilizes the percentage of GFP-positive cells over the course of the assay confirming that cdc25B activity is negatively regulated by cAMP signaling. GFP-cdc25B S323A cells decrease in number relative to the uninfected cells regardless of treatment condition although there is a relatively higher percentage in the forskolin-treated wells because the proliferation of the uninfected cells is inhibited at the same time. Because cdc25B activity is deleterious to the cells, these data are not able to address the question of whether cdc25B inhibition and G2/M delay are entirely responsible for the decreased proliferation seen after forskolin treatment; however, they do offer further evidence that cdc25B activity can be regulated by phosphorylation of S323 following cAMP signaling.