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The Aurora B kinase activity is required for the maintenance of the differentiated state of murine myoblasts

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Reversine is a synthetic molecule capable of inducing dedifferentiation of C2C12, a murine myoblast cell line, into multipotent progenitor cells, which can be redirected to differentiate in nonmuscle cell types under appropriate conditions. Reversine is also a potent inhibitor of Aurora B, a protein kinase required for mitotic chromosome segregation, spindle checkpoint function, cytokinesis and histone H3 phosphorylation, raising the possibility that the dedifferentiation capability of reversine is mediated through the inhibition of Aurora B. Indeed, here we show that several other well-characterized Aurora B inhibitors are capable of dedifferentiating C2C12 myoblasts. Significantly, expressing drug-resistant Aurora B mutants, which are insensitive to reversine block the dedifferentiation process, indicating that Aurora B kinase activity is required to maintain the differentiated state. We show that the inhibition of the spindle checkpoint or cytokinesis *per se* is not sufficient for dedifferentiation. Rather, our data support a model whereby changes in histone H3 phosphorylation result in chromatin remodeling, which in turn restores the multipotent state.
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Lineage-restricted cells can be reprogramed to a state of pluripotency by several different manipulations, including somatic cell nuclear transfer[1] embryonic stem cell (ES) fusion [2] or even by cotransfection of four specific transcription factors. [3–5] In particular, C2C12 myoblasts can be reprogramed to dedifferentiate into multipotent progenitor cells that can be redirected to other cell types under appropriate stimuli, including the ectopic expression of *Msx1* gene,[6] or by exposure to chemical compounds such as reversine.[7]

Reversine, a purine derivative, was identified by virtue of its ability to increase the plasticity of C2C12 myoblasts. This compound interferes with the normal differentiation pathway causing C2C12 cells to dedifferentiate to a pluripotent state. More recently, it was demonstrated that reversine can differentiate human fibroblasts into skeletal muscle cells *in vitro* and *in vivo,*[8] suggesting that reversine is capable of also reprograming primary somatic cells to a state of increased plasticity. We recently discovered that reversine is also a potent Aurora kinase inhibitor.[9] The Auroras are serine/threonine kinases required for multiple aspects of mitosis in eukaryotic cells. Aurora A promotes centrosome maturation and spindle assembly,[10] whereas Aurora B is required for histone H3 phosphorylation, chromosome segregation,[11] the spindle assembly checkpoint and cytokinesis.[12]

Evidence is emerging that the role of Aurora B is not restricted to mitosis and cell division. A key substrate of Aurora B is serine 10 (ser10) of histone H3, and although this phosphorylation event was originally thought to be involved in chromosome condensation, it now appears that it dissociates HP1 proteins from methylated histone H3 (K9me3) at the onset of mitosis.[13,14] Indeed, histone H3 (ser10) is an important element in the combinatorial histone code that is associated with both active and inactive chromatin.[15] At the onset of mitosis, phospho-Ser10-H3 is responsible for the ejection of HP1 proteins from their binding site on chromatin, probably through a steric hindrance.[13,14] Furthermore, it was demonstrated that the modifications of histone H3 and consequently the redistribution of HP1s are linked to chromatin reorganization and to cellular dedifferentation.[16] Consistent with a role for Aurora B in chromatin structure, it was recently shown that Aurora B is required to remodel chromatin during postmitotic cell differentiation of mesenchymal stem cells and in the transition of B cell to plasma cells.[17]

The ability of reversine to both dedifferentiate C2C12 cells and to inhibit Aurora B raised the possibility that its dedifferentiation capability is mediated through Aurora B. Here, we present the evidence that in addition to reversine, several other Aurora B inhibitors can dedifferentiate C2C12 myoblasts. By using drug-resistant Aurora B mutants, we

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Abbreviations: ES, embryonic stem cells; ap2, adipocyte fatty acid-binding protein; LPL, lipoprotein lipase ; ALP, alkaline phosphatase; NMMII, nonmuscle myosin II heavy chain ; MEK, mitogen-activated extracellular signal-regulated kinase; ADM, adipogenic-differentiating medium; ODM, osteogenic-differentiating medium; MRFs, muscle regulatory factors; Id(s), inhibitor of differentiation(s); Myf5, myogenic factor 5; Lys, lysine; Ser, serine; p, phospho; me, methyl
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provide compelling evidence that the effects of reversine are mediated through Aurora B as opposed to an off-target effect. Furthermore, we demonstrate that reversine remodels the chromatin associated with several genes that are induced or repressed during the phenomenon of dedifferentiation. Altogether, our data suggest a novel role for the Aurora B kinase in gene regulation and in the maintenance the differentiated state.

Results and Discussion

Aurora kinase inhibitors reprogram C2C12 myoblasts. Reversine inhibits several proteins including MEK1, nonmuscle myosin II (NMMII), Aurora A and B.[9] On the basis of several indirect lines of evidence, it was suggested that reversine induces dedifferentiation by the simultaneous inhibition of MEK1 and of NMMII.[18] However, as reversine is a potent Aurora inhibitor, we wanted to determine whether the inhibition of Aurora A and/or B may be involved in the dedifferentiation process. To do this, we asked whether a panel of other Aurora kinase inhibitors could dedifferentiate C2C12 cells, reasoning that structurally diverse inhibitors should have different spectra of off-target effects.[19] We chose to use hesperadin, which is relatively selective for Aurora B,[20] VX-680, which is a dual Aurora A/B inhibitor[21] and MLN-8054, which is relatively selective for Aurora A.[22] Significantly, hesperadin, VX-680 and MLN-8054 induced dedifferentiation of C2C12 myoblasts (Figure 1). C2C12 cells were treated with compounds for 72 h as indicated; after this, the drug-containing media were removed and the cells were cultured in drug-free medium suitable for the development of adipocytes (adipogenic-differentiating medium; ADM) and osteoblasts (osteogenic-differentiating medium; ODM), respectively. After 7 days, cells were stained with Oil Red O reagent to evaluate the presence of adipocytes or for alkaline phosphatase to evaluate the presence of osteoblasts (Figure 1a). In addition, to have a quantitative analysis of the dedifferentiation phenomenon, we used a fluorescent substrate system to evaluate the amount of lipid droplets in the case of adipocytes and the alkaline phosphatase activity in the case of osteoblasts (Figure 1b, see Materials and Methods also).

Furthermore, we decided to investigate also the expression of specific markers of both osteoblast and adipocyte differentiation. In particular, after the treatment with 500 nM

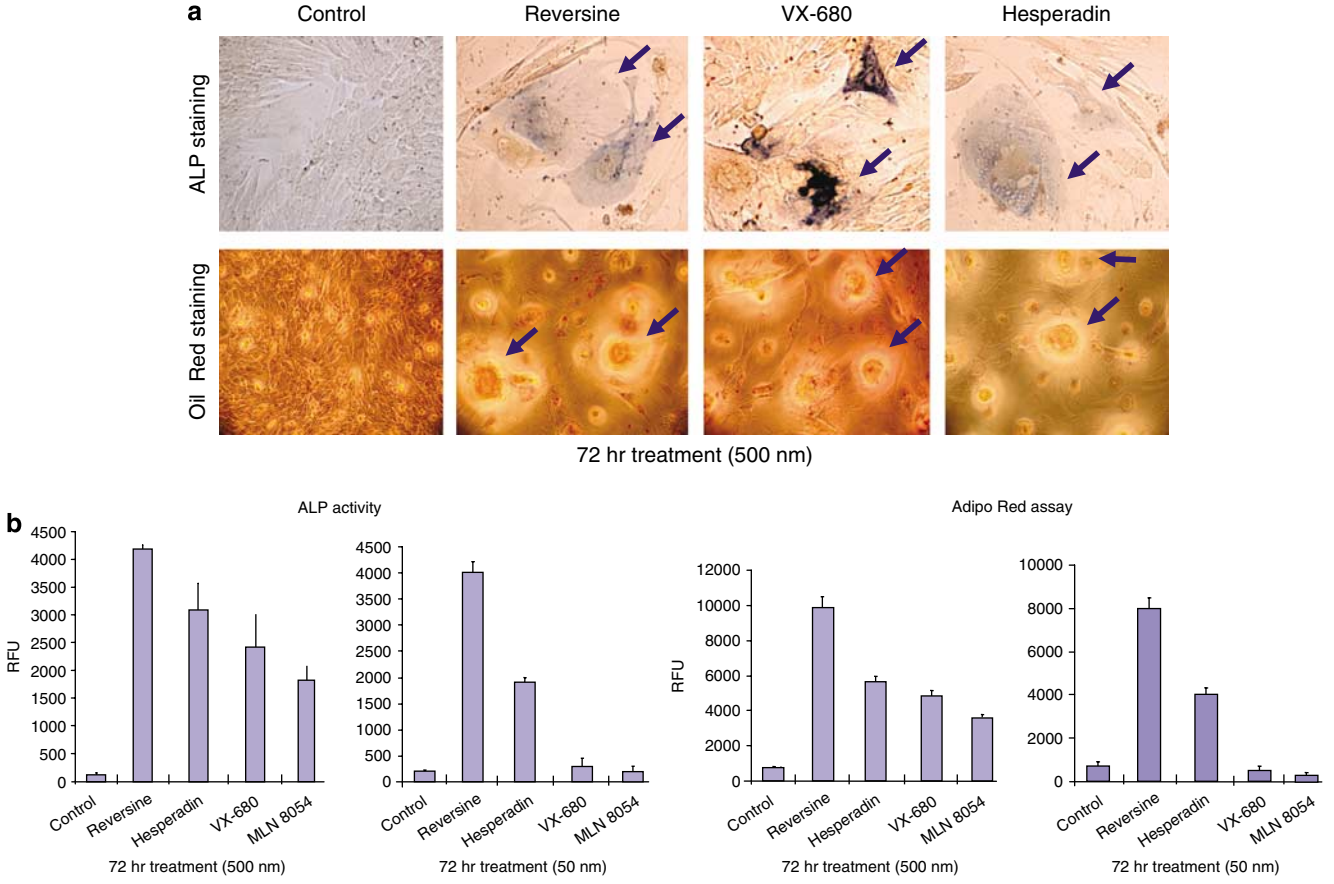

Figure 1 Aurora kinases inhibitors induce C2C12 dedifferentiation. (**a**) C2C12 cells were treated for 72 h with 500 nM of reversine, hesperadin and VX-680. Alkaline phoshatase and Oil Red O stainings were performed after 7 days of exposure to osteogenic-differentiating (ODM) and to adipogenic-differentiating media (ADM), respectively. The arrows indicate cells positive for Alkaline phosphatase (upper panel) and for lipid droplets content (lower panel) **(b)** C2C12 cells were treated for 72 h with 500 or 50 nM of reversine, hesperadin, VX-680 and MLN-8054. Then, cells were cultured in ODM and ADM media as indicated. ALP activity of C2C12 cells after the treatment was assayed using a fluorescent substrate system and the results were expressed in relative fluorescence units (RFUs). Quantitative analysis of adipocyte differentation was assayed with AdipoRed reagent to evaluate the lipids droplets content. The histograms show the RFUs measured with excitation at 485 nm and an emission at 572 nm

of each compound and the exposure for 1 week to differentiating media, we analyzed the expression of osteopontin and collagen type 1 for the osteoblasts and the expression of the adipocyted fatty acid-binding protein (ap2) and of lipoprotein lipase (LPL) for the adipocytes. In Figure 2, we show that the treatment with the Aurora kinase inhibitors caused a relevant increase of these markers strengthening the notion that reversine-treated myoblasts can be induced to differentiate into other cell types.

Among the Aurora kinase inhibitors tested, reversine and hesperadin were very potent inducers of dedifferentiation, whereas MLN-8054 and VX-680 were only active at significantly higher concentration (Figure 1b). Interestingly, reversine and hesperadin are low nanomolar inhibitors of Aurora B kinase (S Santaguida and A Musacchio, unpublished observations, and Figure 3), and their effect on cultured cells is mainly because of the inhibition of Aurora B kinase. By contrast, when using VX-680 and MLN-8054, on cultured cells, the predominant phenotype from these drugs is the appearance of monopolar spindles, a hallmark of Aurora A inhibition (A Musacchio and S Santaguida, unpublished observations, and Manfredi *et al*.[22] and Girdler *et al*.[23] Thus, the spectrum of inhibitor potencies suggests that Aurora B kinase rather than A might be the target of reversine in the dedifferentiation program.

Importantly, although reversine inhibits MEK1 and NMMII,[18] hesperadin and VX680 are not particularly potent against these two enzymes (Supplementary Figure 1), indicating that the inhibition of MEK1 and NMMII is not necessary for C2C12 dedifferentiation.

Drug-resistant Aurora B kinase mutants block the dedifferentiation capabilities of reversine . The fact that multiple inhibitors of Aurora B can dedifferentiate C2C12 cells is consistent with the notion that Aurora B activity is required to maintain the myoblasts in the differentiated state. However, although unlikely, we cannot rule out the possibility that all these inhibitors share a common 'off-target' enzyme. Therefore, to exclude this possibility, we set out to express drug-resistant Aurora B mutants in C2C12 cells and ask whether this expression blocked reversine-induced dedifferentiation. We reasoned that if the dedifferentiation

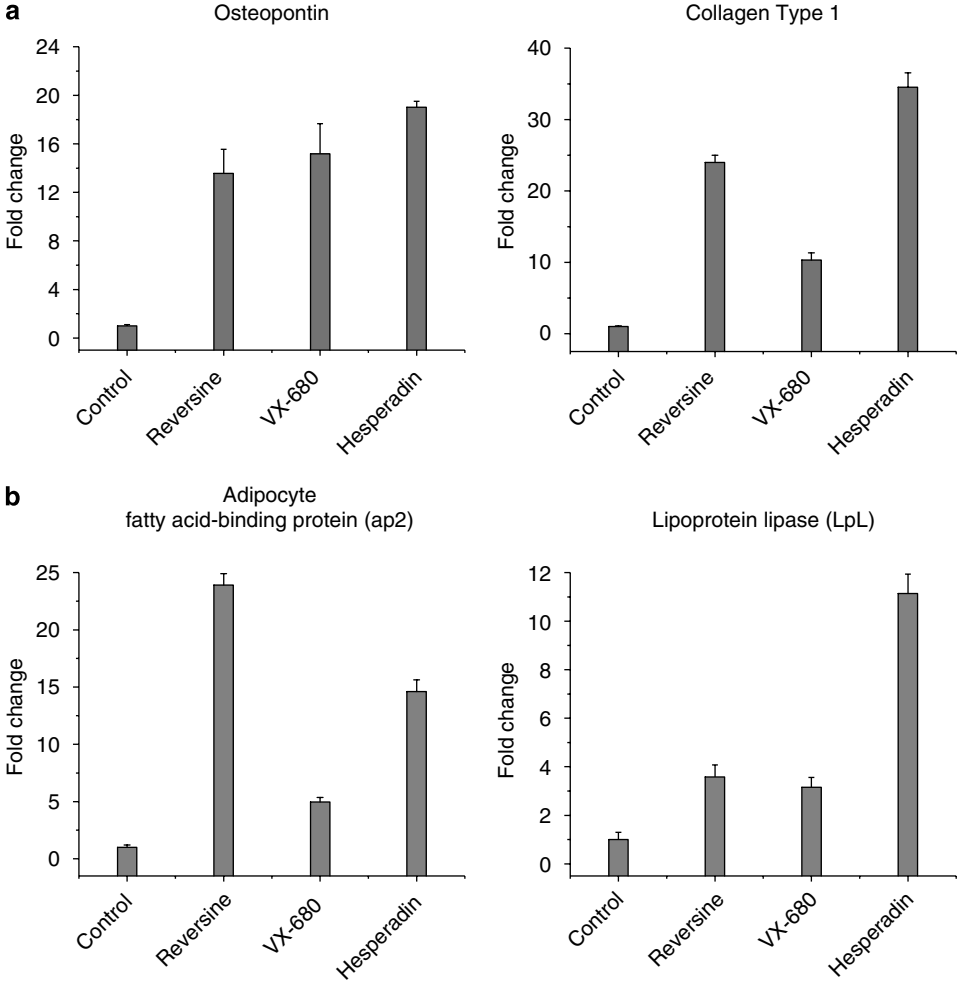

Figure 2 The use of Aurora kinases inhibitor induces the initiation of osteogenic and adipogenic program. (**a**) qRT-PCR for osteogenic (osteopontin and collagen type 1) and (**b**) adipogenic (adipocyte fatty acid-binding protein and lipoprotein lipase) genes was performed on C2C12 cells treated with 500 nM reversine, VX-680 and hesperadine, and then cultured in osteogenic-differentiating (ODM) and adipogenic-differentiating media (ADM) (as described in Materials and Method section). Genes mRNA levels were normalized using GAPDH. Untreated C2C12 cells were used to calculate the fold enrichment

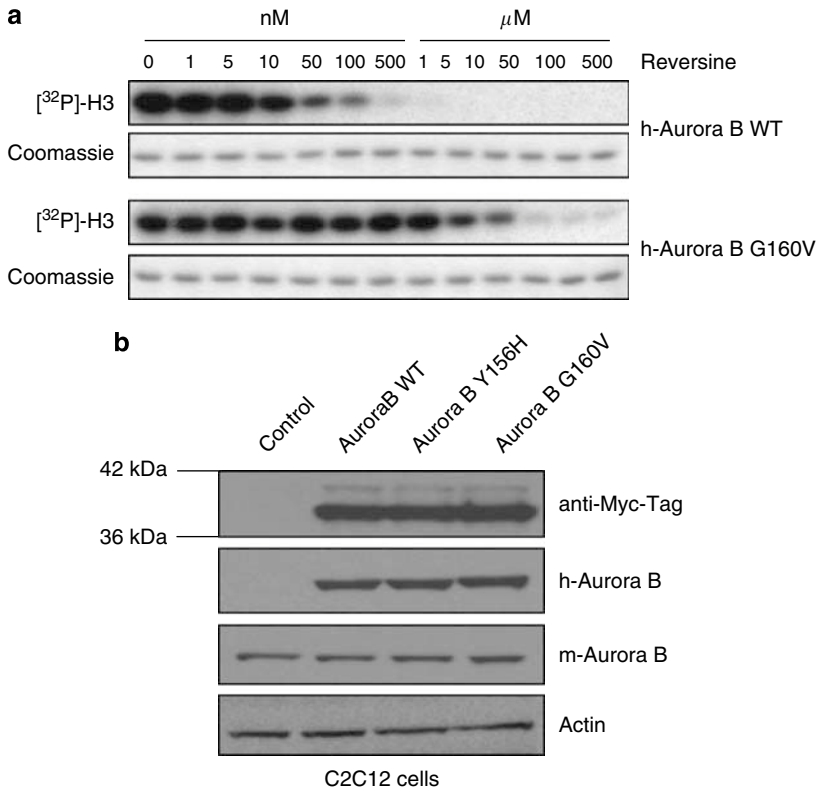

Figure 3 The Aurora B G160V mutant is resistant to reversine. Human Aurora B:INCENP IN-box wild-type (WT; upper panels) and G160V mutant (lower panel) were incubated at 30°C for 1 h with histone H3 as a substrate, the indicated growing concentrations of reversine, and [γ-^{32}P]-ATP. DMSO was used as control. The incorporation of radioactive phosphate on histone H3 was visualized by autoradiography. (**a**) C2C12 cells were transfected with Aurora B WT, Aurora B Y156H and Aurora B G160V. The presence of ectopic gene expression was assessed after 48 h post-trasfection by western blot analysis using both an anti-Myc-tag antibody and anti-Aurora B antibody. An antiactin antibody was used as loading control. (**b**) C2C12 cells were transfected with Aurora B WT, Aurora B Y156H and Aurora B G160V as indicated. After 48 h, cells were treated with reversine 50 nM for further 72 h and typical western blot was performed to evaluate the protein levels of ectopic genes

capability of reversine was mediated through the inhibition of Aurora B, the ectopic expression of a drug-resistant Aurora B mutant should maintain the myoblast state despite the presence of reversine. By contrast, if reversine effects were through another target, the expression of the drug-resistant Aurora B mutants should have no effect.

Recently, we have described two mutations in the active site of Aurora B, namely G160V and Y156H, which render it resistant to ZM447439, hesperadin, VX-680 and MLN-8054.[23] On the basis of the 3D structure of the complex between reversine and Aurora B,[9] we reasoned that these mutants should also be resistant to reversine. To prove this prediction, we tested the ability of increasing concentrations of reversine to inhibit the activity of recombinant wild-type (WT) human Aurora B:INCENP IN-box complex, or of equivalent complexes bearing the G160V (Figure 3a) or the Y156H mutation (data not shown), using histone H3 as an *in vitro* substrate. In agreement with our prediction, the G160V and Y156H mutations conferred a ~100-fold or higher resistance to reversine compared with the WT enzyme (Figure3a, and data not shown).

Next, we used these drug-resistant mutants to directly test the role of Aurora B in the process of dedifferentiation induced by reversine. We transiently transfected C2C12 cells at 30% of confluence with plasmids encoding either for WT human Aurora B or the Y156H and G160V mutants. After 48 h, western blot analysis showed expression of the exogenous Aurora B proteins (Figure 3b). In a parallel experiment, 48-h post-transfection, 50 nM reversine was added for 72 h. We observed that reversine efficiently dedifferentiated the non-transfected controls as shown by the change of cell morphology (Figure 4a). Significantly, C2C12 cells transfected with the drug-resistant mutants did not acquire the round morphology typical of the controls (Figure 4a). In particular, a myotube formation assay was performed to better investigate whether the mutant G160V was sufficient to inhibit the dedifferentiation capabilities of reversine. After 72 h of reversine treatment, the cells were kept in the same plate for 8 days replacing the medium each 48 h to permit the myotubes formation. We observed that only the control cells and the cells previously transfected with the resistant mutant were able to form myotubes after reversine exposure. We found that after the transfection with the mutant, about 70% of cells gave rise myotubes. Not trasfected cells and cells trasfected with Aurora B WT lost completely the myoblast shape, and were not able to form myotubes (Figure 4a).

Furthermore, we also investigated whether the presence of Aurora B, resistant to reversine, would inhibit the whole dedifferentiation–redifferentiation process induced by reversine. After 72 h of reversine treatment, cells were transferred into the appropriate differentiation medium and cultured for 1 week. Remarkably, in the presence of both mutants,

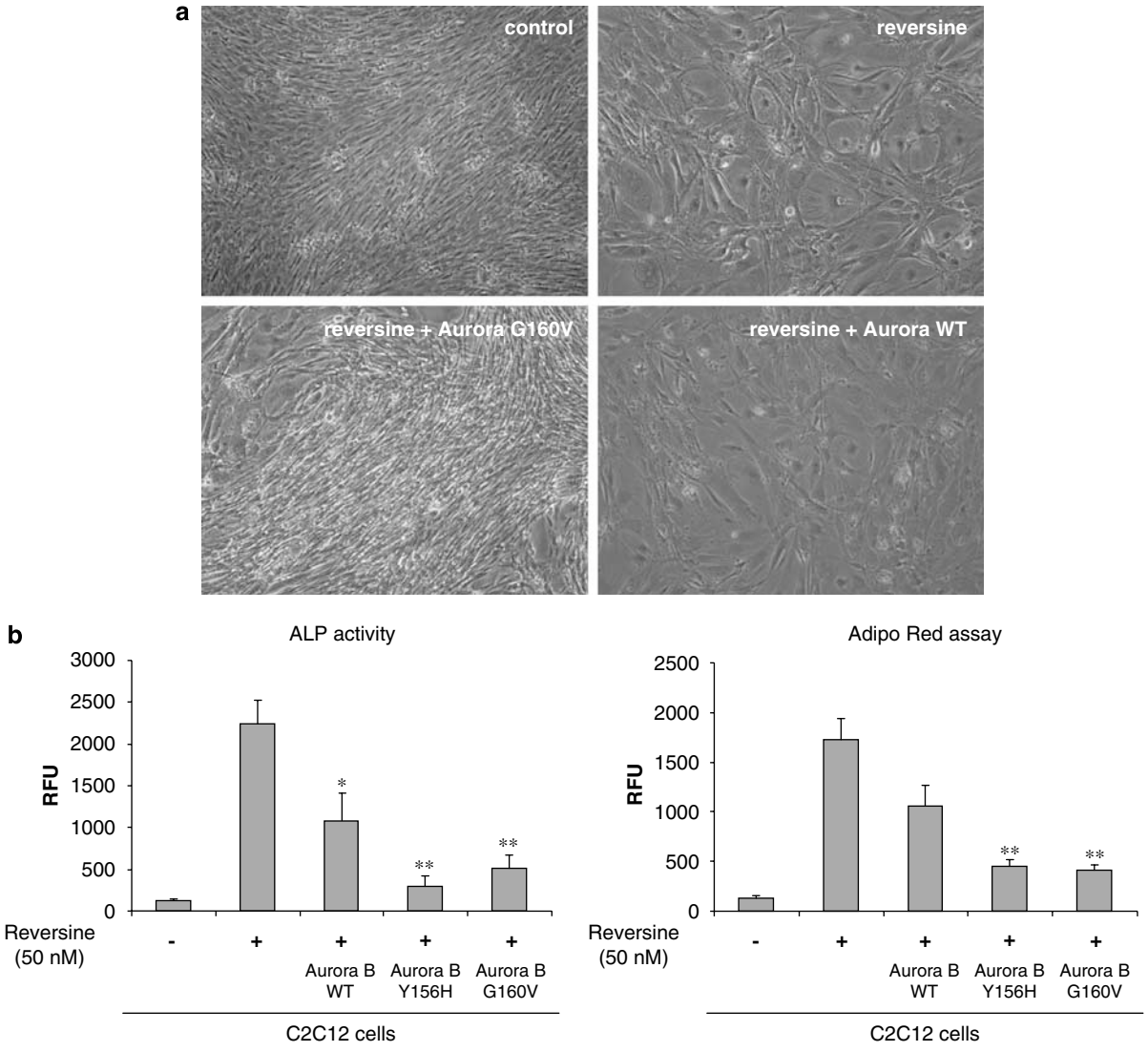

Figure 4 Drug-resistant Aurora B kinase inhibits the dedifferentiation process showing positive evidence of differentiation. C2C12 cells were transfected with Aurora B wild type (WT) and Aurora B G160V. After 48 h, cells were treated with reversine 50 nM for further 72 h and then cultured in growth medium for additional 8 days. (**a**) Myotubes formation was observed for control cells, as well for the Aurora B G160V tranfected cells. C2C12 cells treated with reversine (not transfected) and cells transfected with Aurora B WT did not show any evidence of terminal differentiation. (**b**) C2C12 cells were transfected with Aurora B WT, Y156H and G160V variants as indicated. After the treatment (72 h), the cells were cultured in osteogenic-differentiating (ODM) and adipogenic-differentiating media (ADM) for 1 week. ALP activity and lipid droplets content were measured to evaluate the presence of osteoblasts and adipocytes, respectively using a fluorescent substrate system. Data are expressed as relative fluorescence units (RFUs; see Materials and Methods also). Values of RFUs were statistically different with ($^{*}P<0.05$) and ($^{**}P<0.01$)

reprograming of C2C12 myoblasts induced by reversine was drastically reduced (Figure 4b). The simplest interpretation of these results is that Aurora B kinase activity is essential for the action of reversine. We observed that cells transfected with WT Aurora B displayed only a partial abrogation of the reversine effect, presumably because of a gene dosage effect.

Reversine induces polyploidy and spindle checkpoint override at high concentration. The results presented so far show that Aurora B kinase is a key player in the nuclear reprograming induced by reversine. Aurora B is involved in multiple mitotic functions, and therefore inhibiting its kinase activity causes a variety of phenotype including chromosome segregation failure, spindle checkpoint override and impairment of cytokinesis. Taken together, these effects result in polyploidy.[24] In principle, it is conceivable that it is the polyploidy itself, rather than the inhibition of Aurora B *per se,* which is responsible for the dedifferentiation process. However, we feel that this is unlikely: other agents, which cause polyploidy, including the MEK1 inhibitor U0126 and the NMMII inhibitor blebbistatin do not cause reprogramming (data not shown). Moreover, reversine induces reprograming at concentrations as low as 20–50 nM, whereas significant polyploidy is only observed at higher concentration of reversine (Figure 5a). Similar considerations are true also for the effect on the spindle check point: SP600125, a potent

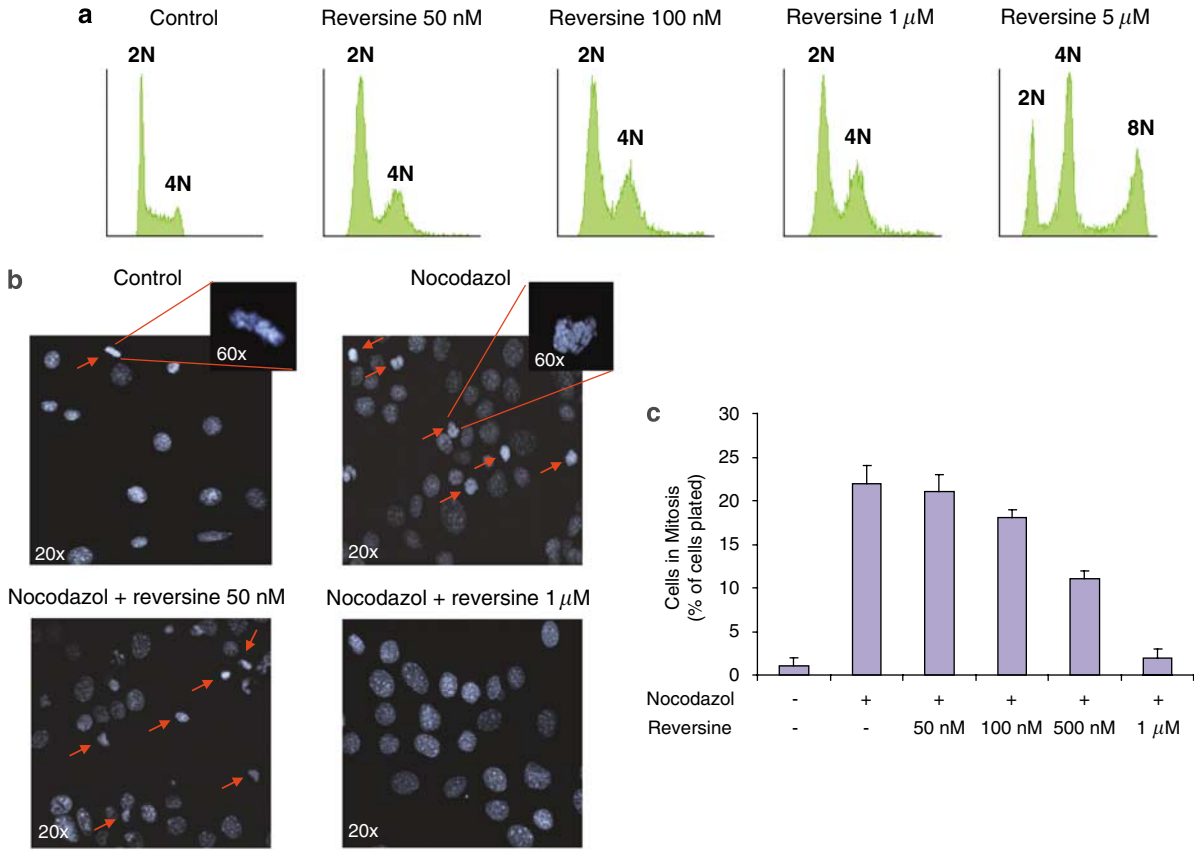

Figure 5 Reversine causes polyploidy and spindle checkpoint overriding at high concentrations. (**a**) C2C12 cells were treated for 72 h with different concentration of reversine as indicated, thus cultured in ODM for additional 7 days. After this period, the cell-cycle analysis of C2C12 cell was performed using propidium Iodide with FACS Canto II. (**b–c**) C2C12 cells were treated with 500 nM nocodazol in presence of increasing concentrations of reversine (as indicated) for 4 h. After this time, the cells were stained with DAPI to analyze the nucleus status and the cells blocked in mitosis were counted. Red arrows in the panel b indicate cells in mitosis

spindle checkpoint overrider does not induce reprograming, and even reversine must be used at concentrations above 1 μM to cause spindle checkpoint overriding in C2C12 myoblasts (Figure 5b and c).

On balance, therefore, we suspect that the role of Aurora B in maintaining the differentiated state of C2C12 cells is not linked to its mitotic functions. More likely, the direct involvement of Aurora B on reversine-induced dedifferentiation is based on its role in chromatin remodeling. Consequently, we turned our attention to another key function of Aurora B, namely histone H3 (Ser10) phosphorylation (phospho-Ser10-H3) and a possible role in chromatin remodeling. As predicted for a potent Aurora B inhibitor, reversine does indeed inhibit phospho-ser10-H3 in C2C12 cells (Figure 6a). Furthermore, the inhibitory effect of reversine is significantly reduced following the expression of the drug-resistant Aurora B mutants (Figure 6a) confirming that Aurora B is the bona fide serine 10 kinase, at least in C2C12 cells.

Reversine induces remodeling of chromatin at the level of muscle differentiation genes. Myogenesis is a complex process regulated by muscle regulatory factors (MRFs) and their inhibitors (Ids proteins).[25] As reported by several groups,[26–28] ectopic expression of MRFs, such as *MyoD* or *myogenin*, converts nonmuscle cells into muscle cells, suggesting that these factors play a crucial role both in the induction of muscle differentiation and in the maintenance of the differentiated muscle phenotype. Reversine treatment affects the expression of MRFs; in Figure 6b–c, we show an evident downregulation of the mRNA levels coding for *MyoD*, *myogenin* and myogenic factor-5 (*Myf5*) after reversine treatment. Interestingly, we also observed that the expression of *Id1*, a known inhibitor of myogenesis,[25] increased considerably after the treatment (Figure 6b, lower right panel). The treatment of myoblasts with the other Aurora inhibitors also caused a relevant dowregulation of *MyoD* and *Myf5*, although, on the other hand, appeared the upregulation of the *Id1* expression (Figure 6c), suggesting a common mechanism of action for the three drugs, most likely triggered by the inhibition of Aurora B kinase. In fact, reversine treatment in the presence of Aurora B kinase-resistant mutants did not lead to the downregulation of *MyoD* expression compared with the untreated cells of with cells trasfected with Aurora B WT (Figure 6b).

Apparently, Aurora B inhibitors and reversine can cause not only the repression but also, with similar kinetics, the activation of genes. To elucidate the putative common molecular event that is at the core of both activation and repression of genes, we analyzed by chromatin immunoprecipitation (ChIP) histone modifications at the level of the

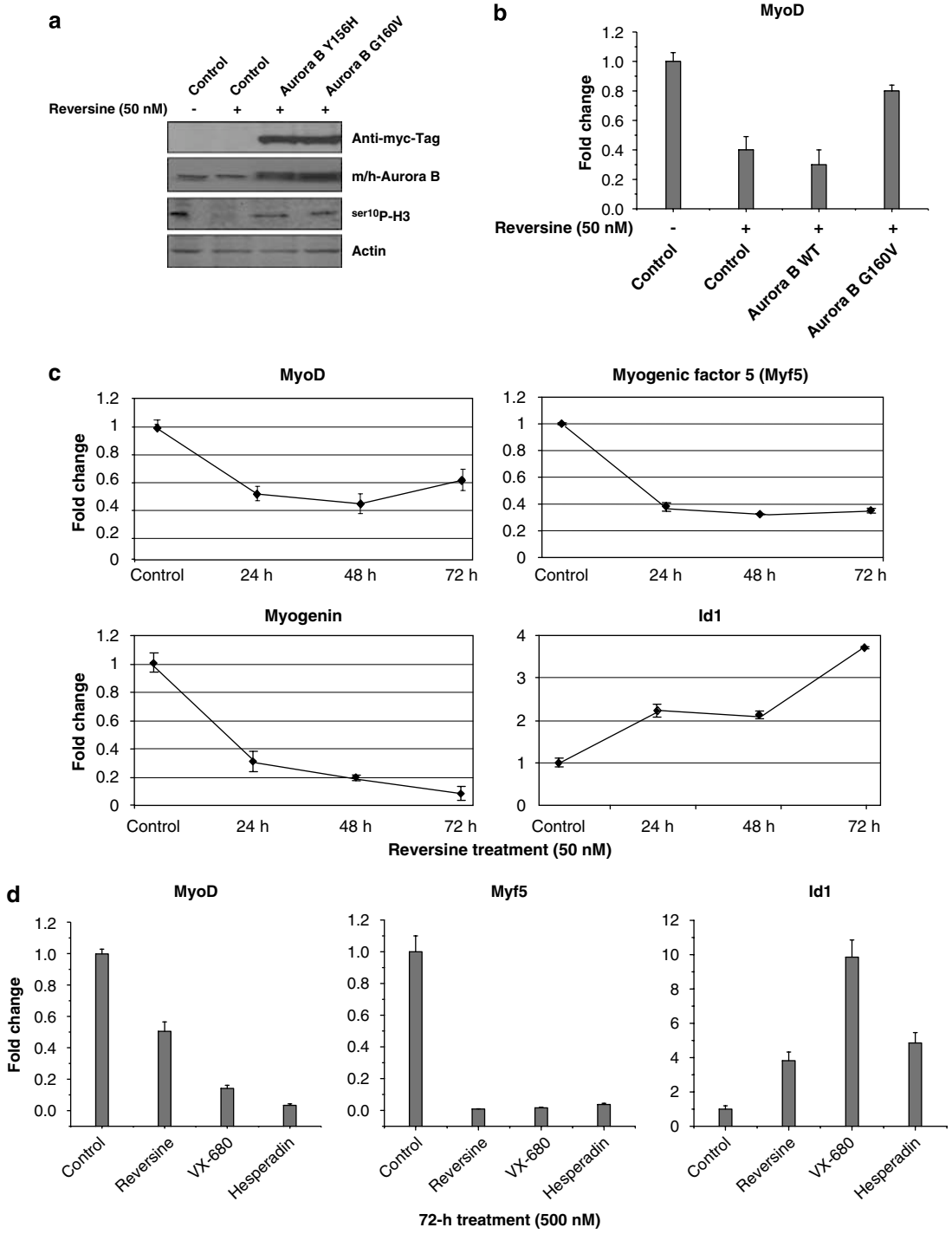

Figure 6 Reversine inhibits histone H3 phosphorylation on Ser10 and induces the downregulation of MRFs expression . (**a**) C2C12 trasfected with Aurora B mutants were treated with 50 nM of reversine for 48 h. After the treatment, the Ser10-H3 phosphorylation status was analyzed by western blot. Anti-Myc and anti-Aurora antibodies were used as transfection control, whereas actin was used as loaded control. (**b**) MyoD mRNA levels were evaluated in untreated cells, cells treated with reversine and in cells previously transfected with Aurora B G160V, Aurora B wild type (WT), and then treated with reversine for 72 h. (**c**) C2C12 cells were treated with 50 nM of reversine for 24, 48 and 72 h. MRFs and Id1 expressions were analyzed using the real-time PCR with Taqman (MyoD) and SYBR green technologies (Myogenin, Myf5 and Id1). MyoD mRNA levels were normalized to 18S mRNA levels. Myf5, myogenin and Id1 mRNA levels were normalized using Gapdh. The untreated C2C12 cells were used as standard to calculate the fold enrichment. (**d**) C2C12 cells were treated with 500 nM of reversine, VX-680 and hesperadine for 72 h. MRFs and Id1 expressions were analyzed using the real-time PCR with Taqman (MyoD) and SYBR green technologies (Myogenin, Myf5 and Id1)

MyoD, Myogenin and Id1 promoters. In essence, at the level of the repressed genes (MyoD and Myogenin), we observed the expected inhibition of phospho-Ser10 but no other reversine dependent change, neither in the overall H3 acetylation, nor methylation (Lys4 and Lys9 were analyzed; Figure 7). Interestingly, but not surprisingly, the dual

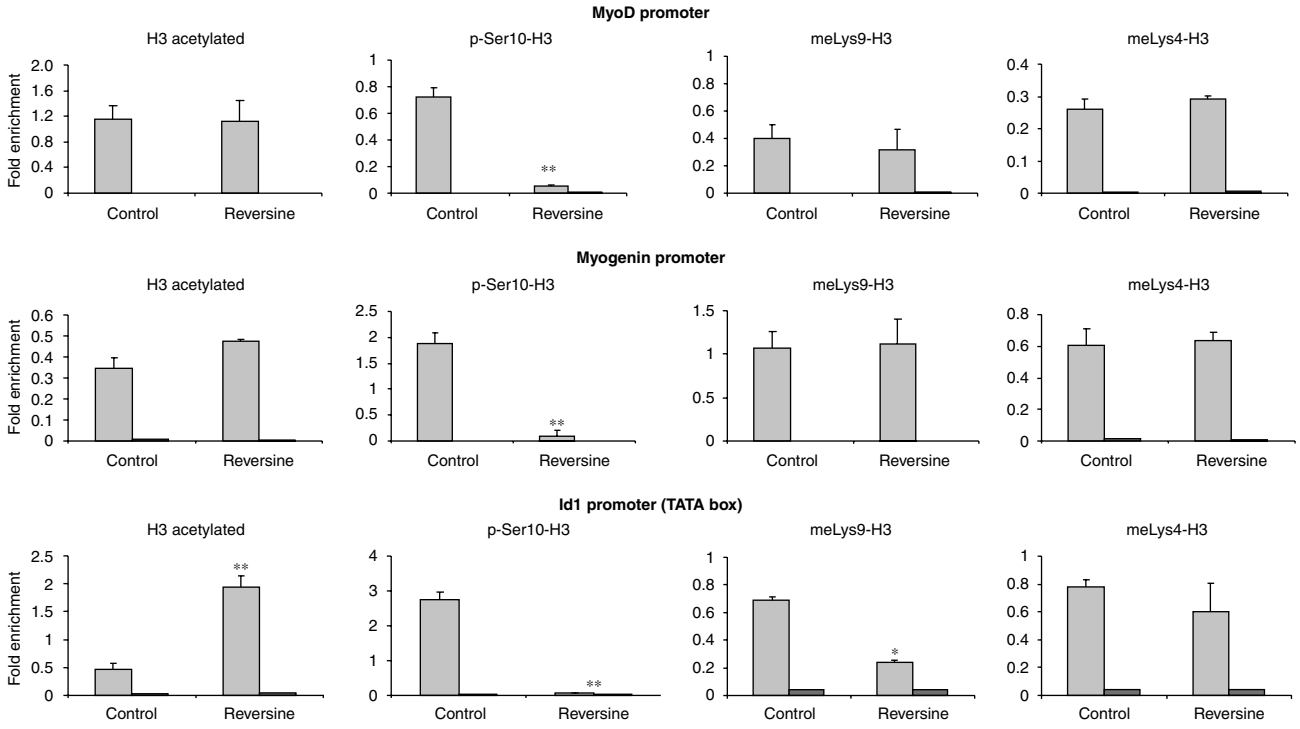

Figure 7 Aurora B is involved in affecting chromatin status. Chromatin immunoprecipitation analysis was performed on C2C12 cells treated with reversine 50 nM. Polyclonal antibodies against H3 acetylated at lysine 9 and 14, against phospho-Ser10-H3, against dymethil Lys 9-H3 and against dymethil Lys 4-H3 were used to precipitate the protein–chromatin complex. The DNA was purified, and specific regions of mouse MyoD, Myogenin and Id1 promoters were analyzed using the real-time PCR (see Materials and Methods). Rabbit IgG was used as control of the assay specificity (purple barr). Asterisks indicate the values of fold enrichment statistically different with ($*P<0.05$) and ($**P<0.01$)

modification of acetyl Lys14 and phospho-Ser10 that represent a marker of gene induction[29] was drastically reduced in the genes repressed by reversine (Figure 8). This result is in agreement with the findings of Mal *et al.*,[30] who reported that the presence of phospho-Ser10 at the level of myogenin promoter is a marker of active chromatin. Therefore, it is likely that the reversine causes a decrease in the expression of the myogenin and MyoD genes by inhibiting the phospho-Ser10-H3. In contrast to the repressed genes, the pattern of histone modification at the promoter level of the induced gene Id1 was drastically changed following the treatment with reversine. We not only observed the expected inhibition of phospho-Ser10 but also a significant increase of overall H3 acetylation as well as a decrease of the Lys9 methylation.

In summary, it appears that the common feature after treatment with reversine is the drastic reduction of phospho-Ser10-H3 on all the promoters analyzed (Figure 7), as expected for a potent Aurora B kinase inhibitor. Surprisingly, inhibition of phospho-Ser10-H3 leads to opposite effects in Id1 gene (induction) and in MyoD and myogenin (repression). Recently, Sabbattini *et al.*[17] reported the results that are in line with our observations. These authors described a new role of Aurora B kinase in the repression of genes in postmitotic differentiated cells. Specifically, they reported that phospho-Ser10 could be a marker of gene repression. Here, We show that the Id1 gene is induced as a consequence of Aurora B inhibition, and the chromatin at the level of its promoter is drastically remodeled with markers typical of active genes (increase in acetylation, decrease of Lys9 methylation). It is likely that Aurora B associates with different protein complexes to mark active and repressed genes. In the future, it will be important to elucidate the molecular bases for these gene-specific effects.

Materials and Methods

Cell culture, treatment and transfection. C2C12 myoblasts (ATCC Manassas, VA) were cultured in growth medium consisting of DMEM supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen Corp., Carlsbad, CA) and 10% (v/v) FBS (HyClone, South Logan, UT). C2C12 cells were treated with reversine, VX-680, hesperadin, MLN-8054 at a concentration range from 20 nM to 5 μM, whereas control cells were incubated with 0.01% DMSO. Treatment was maintained for 48 and/or 72 h without the growth medium changes. C2C12 cells were transiently transfected with Lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. The cells were screened for the presence of ectopic gene expression after 48 h by western blot.

Kinase assays. Recombinant human Aurora $B^{45–344}$ and human $INCENP^{835–903}$ were expressed and purified as described previously.[23] Kinase assays were carried out in 30 μl reaction mix containing 50 μM ATP, 1 mM DTT, 1 mM Na_3VO_4, 5 μCi [γ-^{32}P]-ATP, 5 μg of histone H3 as substrate, 1 μl DMSO or drugs dissolved in DMSO, and 5 nM of WT or G160V mutant Aurora B/INCENP. Reaction mixes were incubated for 1 h at 30°C, quenched with SDS-loading buffer and resolved on 14% SDS-polyacrylamide gel (SDS-PAGE). Incorporation of ^{32}P was visualized by autoradiography.

RNA preparation and quantitative analysis. Total RNA was prepared from C2C12 cells by using Qiagen's RNeasy® midi kit (Qiagen Inc., Valencia, CA). *MyoD* expression was assessed after the treatments as indicated using TaqMan™ chemistry with the ABI 7700 Prism real-time PCR (RT-PCR) instrument (Applied

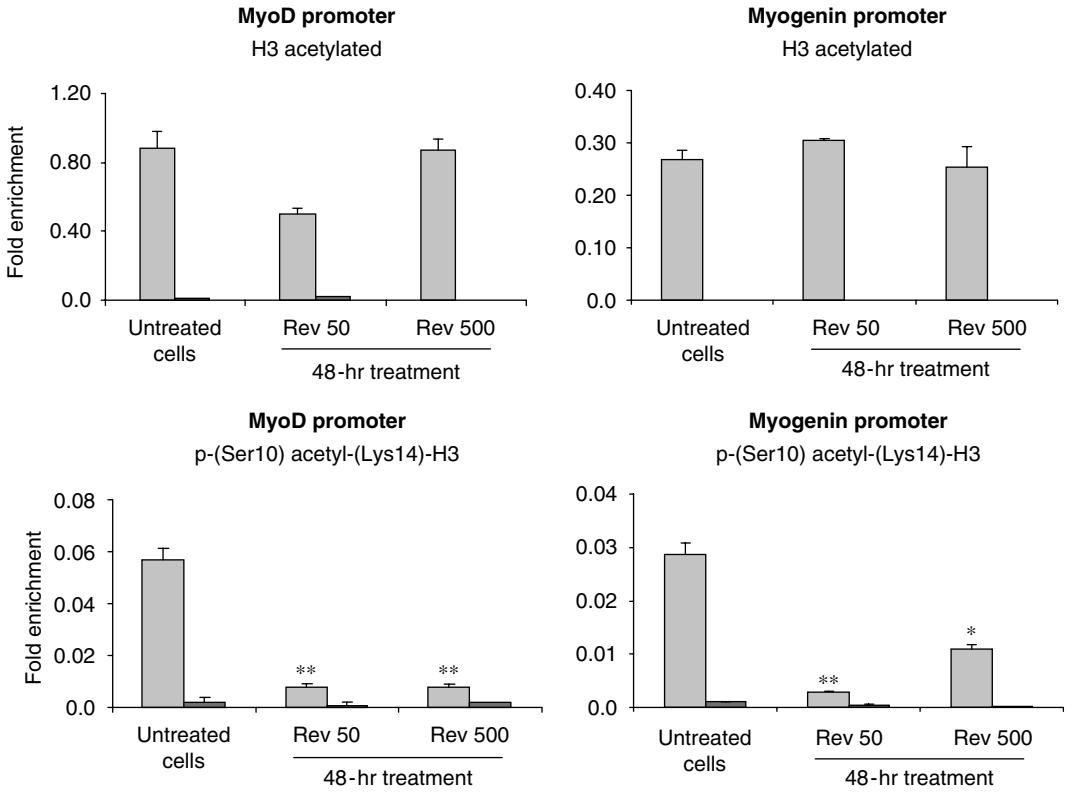

Figure 8 Reversine decreases drastically Ser10-H3 phoshorylation at levels of MRFs genes. Chromatin immunoprecipitation analsysis was performed on C2C12 cells treated with reversine 50 and 500 nM as indicated. Polyclonal antibodies against H3 acetylated at lysine 9 and 14 and against anti-phospho-Ser10-acetyl (K14)-histone H3 were used to precipitate the chromatin—protein complex. The DNA was purified and specific regions of mouse MyoD and myogenin promoter were analyzed using the real-time PCR (see Materials and Methods). Rabbit IgG was used as control of the assay specificity (purple barr). Values obtained for the fold enrichment were statistically different with ($*P<0.05$) and ($**P<0.01$)

Biosystems, Foster City, CA). The forward and reverse primer (200 nM) sequences for mouse *MyoD* were 5′-TTCTTCACCACACCTCTGACA-3′ and 5′-GCCGT GAGAGTCGTCTTAACTT-3,′respectively, and the probe sequence (100 nM) was 5′-ACAGCCGGTGTGCATTCCAA-3′. Data analysis was performed using 18S as internal standard. The forward and reverse primer sequences for mouse 18S were 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3,′ respectively, and the probe sequence was 5′- TGCTGGCACCAGACTTGCCCTC-3′.

Mouse *Myf5* and *Myogenin* were analyzed as reported previously,[31] whereas *Id1* was analyzed as reported by Gersbach *et al*.[32] The expression was measured using SYBR green chemistry with the ABI 7700 Prism RT-PCR instrument (Applied Biosystems). The forward and reverse primer sequences of mouse *Gapdh* used as internal standard were 5′-GGAGATTGTTGCCATCAACGACC-3′ and 5′-GGTCAT-GAGCCCTTCCACAATGC-3,′ respectively. Osteopontin, collagen type 1, ap2 and LPL expressions were analyzed with SYBR green method as reported previously.[18]

Dedifferentiation assay. C2C12 cells were cultured in the presence of reversine, VX-680, hesperadin, MLN-8054 as described. After 72 h of treatment, compounds were removed and cells were cultured in (ADM or in ODM. Adipogenic-inducing medium contained DMEM 10% FBS, 4.5 g/l glucose, 1 μM dexamethasone, 0.5 mM isobutylmethylxantine and 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), whereas osteogenic-inducing medium contained DMEM-10% FBS, 50 μg/ml ascorbic acid-2-phosphate, 0.1 μM dexamethasone and 10 mM β-glicerophosphate (Sigma). For hystochemical analysis, after 7 days of culture in ADM or ODM, cells were fixed with 4% paraformaldehyde for 15 min and washed twice with PBS. Cells were then incubated for 2 h with Oil Red O reagent (Sigma) to evaluate the presence of adipocytes and with mixture of 0.1 mg/ml of naphthol AS-MX phosphate (Sigma), 0.5% N,N-dimethylformamide, 2 mM $MgCl_2$ and 0.6 mg/ml of fast blue BB salt (Sigma) in 0.1 M Tris-HCl, pH 8.5, to evaluate the presence of osteoblasts. Cells were examined by phase-contrast microscopy with DFC320 microscope (Leica Microsystems, Wetzlar, Germany). For quantitative analysis, after being cultured for 7 days in ADM, cells were incubated with AdipoRed reagent for 10 min. ALP activity was measured with a fluorescent substrate system (AttoPhos AP; Promega, Madison, WI). After 7 days in ODM, cells were incubated with AttoPhos Substrate (Promega, Madison, WI) for 15 min. Fluorescence was measured as suggested by the manufacturer using Fusion Universal Microplate Analyzer and Envision Microplate Analyzer (Perkin Elmer).

Western blot analysis. Cells were lysed in 50 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 1% Nonidet P-40, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM Na_3VO_4. Equal amounts of extracted proteins (50 μg) were loaded and separated on SDS-PAGE (Invitrogen), and then transferred onto a nitrocellulose membrane (Schleicher & Schuell Biosciences, Sandford, ME). Immunodetection was performed using the following primary antibodies: anti-Myc-Tag and anti- ^{ser10}P-H3 (Millipore, Billerica, MA), antimouse Aurora B (Becton Dickinson, San Jose, CA) and antimouse/human Aurora B (Abcam Inc., Cambridge, MA).

Cell cycle analysis. C2C12 cells were incubated with reversine as indicated for 48 h, then the growth medium contained reversine was changed and the cells were cultured with a medium suitable for the development of osteoblstasts (ODM) for 96 h. Cells were fixed in 70% ethanol over night. After double washing with PBS, cells were labeled with cell–cycle-staining reagent PBS/0.In all, 1% Triton X-100, 200 μg/ml DNAse-free RNAse, 25 μg/ml propidium iodide (Invitrogen) and incubated at room temperature, in the dark, for 30 min. DNA content was analyzed using FACS Canto II (Becton Dickinson).

Immunofluorescence. In all, 5×10^4 cells were cultured on poly-lysine (Sigma)-coated coverslips and incubated in the presence of nocodazol 500 nM and

reversine as indicated for 4 h. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature. After two washes with PBS, cells were stained with DAPI 0.5 μg/ml for 1 h. The samples were analyzed with Confocal Microscopy with LSM 510 META microscope (Zeiss, Thornwood, NY).

Chromatin immunoprecipitation. Cells (10^6 for each antibody) were used to cross-link chromatin using the protocol from Upstate Biotechnology (Milton Keynes, UK) with minor modifications. Briefly, cells were incubated in 1% formaldehyde for 10 min at room temperature. Glycine (0.125 M) was added to stop cross-linking. Cross-linked chromatin was sonicated three times for 10 s at 80% amplitude (Vibra-Cell sonicator; Sonics and Materials, Meryin/Satigny, Switzerland). Polyclonal antibodies raised against H3 acetylated at lysine 9 and 14 (αH3-Ac) and against antiphospho-S10-acetyl (K14) histone H3, both from Millipore, whereas antihistone H3 dymethil K4, antihistone H3 dymethil K9 and anti-phospho-Ser10-H3 were purchased from Abcam. DNA was purified by phenol–chloroform extraction, and specific regions of the indicated promoters were amplified by RT-PCR using SYBR green method (Applied Biosystem). The primers used for mouse MyoD promoter were 5′-CGCCCCCAGCCTCCCTTTCCA-3′ and 5′-TGTCAGAGGTG TGGTGAAGAAA-3′; mouse myogenin promoter was analyzed as reported previously.[30] The primers used to analyze the TATA box of mouse Id1 promoter were 5′-CTTATAAAAGACTGGCTCCAGC-3′ and 5′-GGAGGCTGAGAACA GAAACAGAGTGTG-3′ To calculate the fold enrichment, a previously published method was used.[33]

Statistical analysis. Statistical analysis was performed by the analysis of variance (ANOVA) test using Origin 6.1 software for Windows (Microcal Software Inc., Northempton, MA).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)