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The ATAC acetyl transferase complex controls mitotic progression by targeting non-histone substrates

Meritxell Orpinell, Marjorie Fournier, Anne Riss, Zita Nagy, Arnaud R Krebs, Mattia Frontini[1] and Làszlò Tora*

Department of Functional Genomics, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS UMR 7104, INSERM U964, Université de Strasbourg, Illkirch Cedex, France

All DNA-related processes rely on the degree of chromatin compaction. The highest level of chromatin condensation accompanies transition to mitosis, central for cell cycle progression. Covalent modifications of histones, mainly deacetylation, have been implicated in this transition, which also involves transcriptional repression. Here, we show that the Gcn5-containing histone acetyl transferase complex, *A*da *T*wo *A* *c*ontaining (ATAC), controls mitotic progression through the regulation of the activity of non-histone targets. RNAi for the ATAC subunits Ada2a/Ada3 results in delayed M/G1 transition and pronounced cell division defects such as centrosome multiplication, defective spindle and midbody formation, generation of binucleated cells and hyperacetylation of histone H4K16 and α-tubulin. We show that ATAC localizes to the mitotic spindle and controls cell cycle progression through direct acetylation of Cyclin A/Cdk2. Our data describes a new pathway in which the ATAC complex controls Cyclin A/Cdk2 mitotic function: ATAC/Gcn5-mediated acetylation targets Cyclin A for degradation, which in turn regulates the SIRT2 deacetylase activity. Thus, we have uncovered an essential function for ATAC in regulating Cyclin A activity and consequent mitotic progression.
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Introduction

Eukaryotic cells must regulate accurately the packaging and unfolding of their chromatin throughout the cell cycle to ensure precise transcription and timely replication of their genetic material. The structural features of chromatin are controlled partially by post-translational modifications occurring on histones, among which acetylation has a major function (Kouzarides, 2007). Histone acetylation levels are defined by the co-ordinated but opposite action of histone acetyl transferase (HAT) and deacetylase (HDAC) enzymes, which regulate essential cellular processes, such as DNA replication, transcription and/or cell division. During mitotic chromosome condensation, HDAC activity is favoured on the histones resulting in a predominantly deacetylated state (Valls *et al*, 2005). HDAC enzymes also regulate mitotic progression by targeting non-histone substrates that drive chromosome separation (Dryden *et al*, 2003; Ishii *et al*, 2008). The high level of histone deacetylation during mitosis suggested that the activity of HAT complexes is downregulated during this process, and thus their potential contribution to cell division remained largely unexplored.

Gcn5, the founding member of a GNAT protein family, is a subunit of several transcriptional coactivator complexes (Brownell and Allis, 1996; Lee and Workman, 2007). In addition to the function of Gcn5 in transcription regulation, its potential involvement in cell cycle regulation has been recently described (Vernarecci *et al*, 2008; Paolinelli *et al*, 2009). In metazoans, at least two Gcn5 containing HAT complexes exist: *S*pt–*A*da–*Gcn5* *a*cetyltransferase (SAGA) and *A*da *T*wo *A* *c*ontaining (ATAC) (Lee and Workman, 2007; Nagy and Tora, 2007; Suganuma *et al*, 2008; Wang *et al*, 2008; Guelman *et al*, 2009; Nagy *et al*, 2010). These two complexes share a number of components, but differ in molecular size, subunit composition and substrate specificity (Martinez, 2002; Ciurciu *et al*, 2008; Suganuma *et al*, 2008; Nagy *et al*, 2010). Gcn5 and two adaptor proteins, Ada2b/Ada3 in SAGA or Ada2a/Ada3 in ATAC, form the catalytic core of the complexes, respectively (Suganuma *et al*, 2008; Wang *et al*, 2008; Gamper *et al*, 2009; Nagy *et al*, 2010). In addition, the mammalian ATAC complex harbours several *bona fide* subunits with distinct properties, such as a second putative HAT enzyme (Atac2), other subunits involved in transcription regulation (NC2β), nucleosome remodelling (Wdr5, Sgf29), cell growth (Yeats2) and potential DNA binding (Zzz3) (Wang *et al*, 2008; Guelman *et al*, 2009; Nagy *et al*, 2010). Recently, it has been shown that the presence of Gcn5-HAT, or its vertebrate paralogue, Pcaf, is mutually exclusive in mammalian ATAC complexes (Nagy *et al*, 2010).

Drosophila ATAC possesses different substrate specificity than dSAGA, as it mainly acetylates histone H4 (Ciurciu *et al*, 2006; Guelman *et al*, 2006; Suganuma *et al*, 2008). The H4-specific activity was suggested to result from the presence of the second HAT, Atac2, in the complex (Suganuma *et al*, 2008). However, when testing the HAT activity of different human ATAC preparations on free histones and nucleosomes, it acetylated histone H3 and H4, with histone H3 being the preferential target (Wang *et al*, 2008; Guelman *et al*, 2009; Nagy *et al*, 2010). As in human, both SAGA and ATAC complexes have same specificity towards histone H3 and

*Corresponding author. Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS UMR 7104, INSERM U964, Université de Strasbourg, 1 Rue Laurent Fries, BP 10142, Illkirch Cedex 67404, France.
Tel.: +33 388 65 34 44, Fax: +33 388 65 32 01;
E-mail: laszlo@igbmc.u-strasbg.fr
[1]Present address: Clinical Science Center, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK

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H4, acetylation of different non-histones targets could give functional specificity for each complex. However, at present the function of the metazoan ATAC complex is not clear, and the physiological targets of this complex await further analysis.

Here, we identify a function for the mammalian ATAC complex in orchestrating mitotic progression. We provide evidence that the specific depletion of the Ada core of ATAC leads to severe mitotic abnormalities including centrosome multiplication, defective midbody formation and completion of cytokinesis, appearance of binucleated cells, H4K16 and α-tubulin hyperacetylation, and impaired mitotic localization and activity of the SIRT2 deacetylase. We report that the presence of the ATAC complex is essential during mitosis to inhibit Cyclin A/Cdk2 activity by favouring Cyclin A degradation through acetylation. As the Cyclin A/Cdk2 kinase is essential for correct centrosome formation and inhibits SIRT2 function, our data positions the ATAC complex as an important regulator of mitosis, and thus uncovers an essential function for the ATAC acetyl transferase (AT) complex in cell division.

Results

Identification of the ATAC complex at the mitotic spindle

As Gcn5 has been implicated in cell cycle regulation (see Introduction), we aimed to investigate which of the two mammalian Gcn5-containing complexes, SAGA or ATAC, was involved in this function *in vivo*. Thus, we tested whether ablation of Spt20 (SAGA specific) and Ada2a (ATAC specific) by RNAi would affect normal cell division rates (Figure 1A–C). The indicated subunits were knocked down by either transfecting a mixture of four specific siRNAs against the respective mRNAs (Spt20 or Ada2a) into mouse NIH3T3 cells, or by transfecting different small hairpin (sh) DNA constructs targeting Spt20 or Ada2a into human HeLa or 293T cells. First, the efficiency of the depletion was verified and the specific effects were compared with a mixture of non-targeting siRNAs (Mock) (Supplementary Figure 1). As Spt20 or Ada2a knockdown (KD) was efficient and specific in the different cellular systems used, we tested whether the ablation of either SAGA or ATAC function would influence cell division. To this end, we scored the number of cells undergoing mitosis using time-lapse microscopy. The initial number of cells on the image field was considered the ‘Total’ cell number, and the cell cycle of each cell was followed for 30 h. For each cell, we determined whether it was (1) dividing properly or (2) displaying cell division defects (such as asymmetrical, delayed or failed division) and (3) multinucleation. Depletion of Ada2a led to reduced number of cells undergoing proper division (<50%), as these cells show several defects to complete mitosis. On the contrary, depletion of Spt20 had no significant effect (Figure 1A; Supplementary Figure 2). Consistent with this, depletion of Ada2a, but not that of Spt20, lead to increased mitotic abnormalities (delayed, asymmetric or incomplete cell divisions) and concomitantly, increased population of bi- or multinucleated cells (Figure 1B and C; Supplementary Figure 2). These data suggest that the ATAC complex is required for proper cell cycle progression in mammalian cells.

To define the cellular events in which ATAC is involved during cell cycle and to compare it with SAGA, we investigated the localization of these two complexes along different stages of the cell cycle in mouse fibroblasts using immunofluorescence labelling. Interestingly, ATAC specific subunits (Ada2a and Yeats2) and subunits of ATAC that are also present in SAGA, localized to the mitotic spindle (Gcn5 and Ada3) (Figure 1D, panels g–j; Supplementary Figure 3B). In contrast, SAGA-specific subunits, such as Spt20 or Usp22, were excluded from the chromatin and the mitotic spindle during mitosis (Figure 1D, panels k and l; Supplementary Figure 3A). These observations suggest that the whole ATAC complex localizes to the mitotic spindle. Note that in interphasic cells all the antibodies used gave a nuclear staining for the tested factors (Figure 1D, panels a–f).

To confirm that the observed specific localization corresponds to the ATAC complex, we compared the composition of ATAC in asynchronized and G2/M synchronized cells. Cells were either non-treated or synchronized with nocodazole and cell extracts prepared. From both cell extracts, ATAC complexes were immunopurified using three different antibodies against ATAC-specific subunits and the ATAC composition was then verified by western blot (WB) analysis (Figure 1E). The fact that no differences were detected between the compositions of the immunoprecipitated complexes prepared from non-synchronized (A) or mitotic cells (M) suggested that the ATAC complex does not dissociate during mitosis. This is in good agreement with the immunofluorescence experiments (Figure 1D; Supplementary Figure 3B). Consistent with these observations, ATAC subunits, such as Ada2a and Ada3 (hereafter Ada2a/3), co-localized during all mitotic stages (Figure 2A, panel g; Figure 2C, panels g–i). In addition, the KD of either Ada2a or Ada3 impaired the localization of Ada3 and Ada2a, respectively (Figure 2A, panels b–f). Finally, KD of Ada2a or Ada3 resulted in the dissociation of the Gcn5-HAT subunit from the complex (Figure 2B, lanes 4 and 8, for KD efficiency see Supplementary Figure 1A–C). Altogether, our data indicate that the ATAC complex requires its integrity to localize to the mitotic spindle.

To get a more precise description of the localization of ATAC subunits to the mitotic spindle, we compared the localization of Ada2a/3 to that of the microtubule network (exemplified by α-tubulin) and members of the chromosome passenger complex (CPC) (exemplified by Aurora B). These two markers were selected because of their important functions during cell division: the microtubule network, because it provides the pulling force for chromosome segregation (Dumont and Mitchison, 2009), and the CPC, because it is controlling several mitotic features ranging from chromosome-microtubule attachment to cytokinesis (Ruchaud *et al*, 2007). Interestingly, from early mitotic stages to anaphase, the ATAC subunits Ada2a and Ada3 strongly co-localized with the microtubule network, as exemplified by the α-tubulin staining (Figure 2C, panels p–r), but not with CPC components, such as Aurora B (Figure 2C, panels y, z, aa). These data suggest that during mitosis the ATAC complex is mainly associated with the microtubule network. The co-localization of Ada2a/3 with α-tubulin is restricted to mitosis, as during interphase, all ATAC subunits analysed were predominantly nuclear (Figure 1D, panels a–d), whereas the microtubule network is cytoplasmic.

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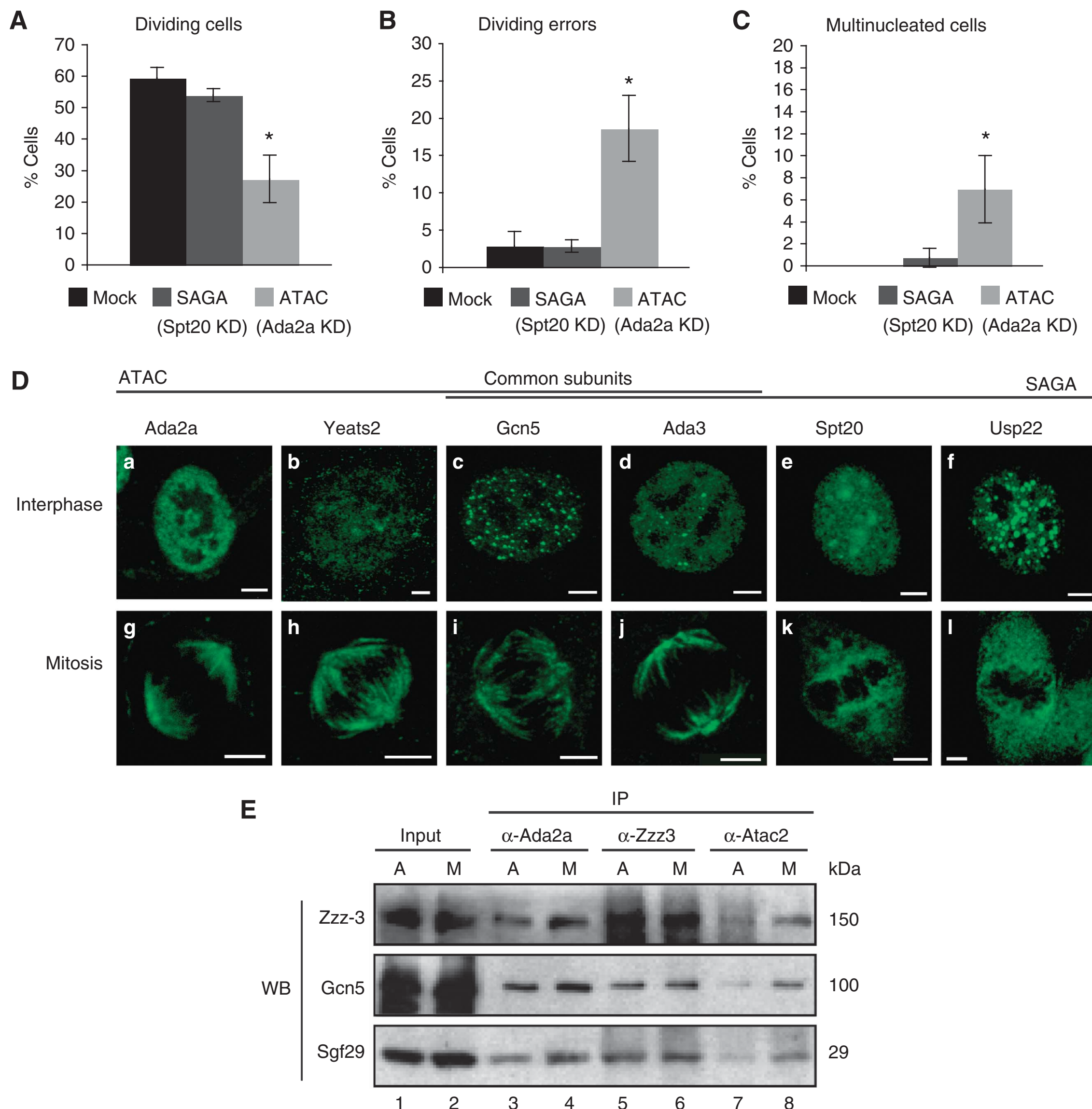

Figure 1 Identification of ATAC as a potential mitotic regulator. (**A–C**) Cell division analysis of NIH-3T3 cells transfected with control siRNA (Mock) or siRNA to achieve either SAGA (Spt20)- or ATAC (Ada2a)-specific subunit knockdowns (KDs). siRNA-transfected cells in prophase were randomly chosen and video imaged for 30 h. See Supplementary Figure 2 for representative images from the time-lapse analysis. The initial number of cells on the image field was considered the 'Total' cell number, and the life cycle of each cell was followed. For each cell, we determined whether it was (**A**) undergoing normal cell division, (**B**) displaying division errors (delayed, asymmetric or uncompleted cell divisions), or (**C**) multinucleation. The number of cells in (**A**), (**B**) or (**C**) is expressed as percentage of total cells analysed (>100 cells/condition, mean and s.d. from four independent experiments, $*P<0.05$). (**D**) Interphasic and mitotic localization of endogenous ATAC or SAGA subunits in mouse NIH-3T3 cells. Cells were stained with anti-ATAC (Ada2a, Yeats2)-, anti-ATAC/SAGA (Gcn5, Ada3)- or anti-SAGA (Usp22, Spt20)-specific antibodies, as indicated. Scale bars: 4 μm. (**E**) Composition of the ATAC complex in asynchronous (A) or mitotic (M) cell cultures. Cell extracts were prepared and immunoprecipitation (IP) of the endogenous ATAC complex was carried out using antibodies against the indicated subunits from the two cell populations. A total of 10% of the input extracts (Input) and the immune pellets were analysed by western blot (WB) using antibodies as indicated.

Depletion of the Ada core of ATAC leads to mitotic abnormalities

The specific positioning of ATAC subunits to the mitotic spindle prompted us to further investigate the function of ATAC at different mitotic stages. We explored how cells progressed through the cell cycle after G2/M arrest on Ada2a or Ada3 RNAi. Consistent with the above results, cells depleted for Ada2a or Ada3 showed a delayed M/G1 transition after nocodazole synchronization (Figure 3A). In agreement with this prolonged mitotic phase after Ada2a or Ada3 depletion, we also observed an increase in the mitotic index of these cells, which was measured on the basis of cells positive for mitotic-specific histone marks such as H3S10P and H4K20me1 (Figure 3B) (Wei *et al*, 1998; Oda *et al*, 2009). Moreover, visualization of centrosomes by γ-tubulin and chromatin by Hoechst staining in the Ada2a/3-depleted cells showed a drastic induction of centrosome multiplication, with a four-fold increase in cells containing more than two centrosomes (Figure 3C). Furthermore, imaging of α-tubulin or chromatin in these cells revealed that the ablation of Ada2a and Ada3 lead to the appearance of aberrant midbodies, which are thicker than those in the

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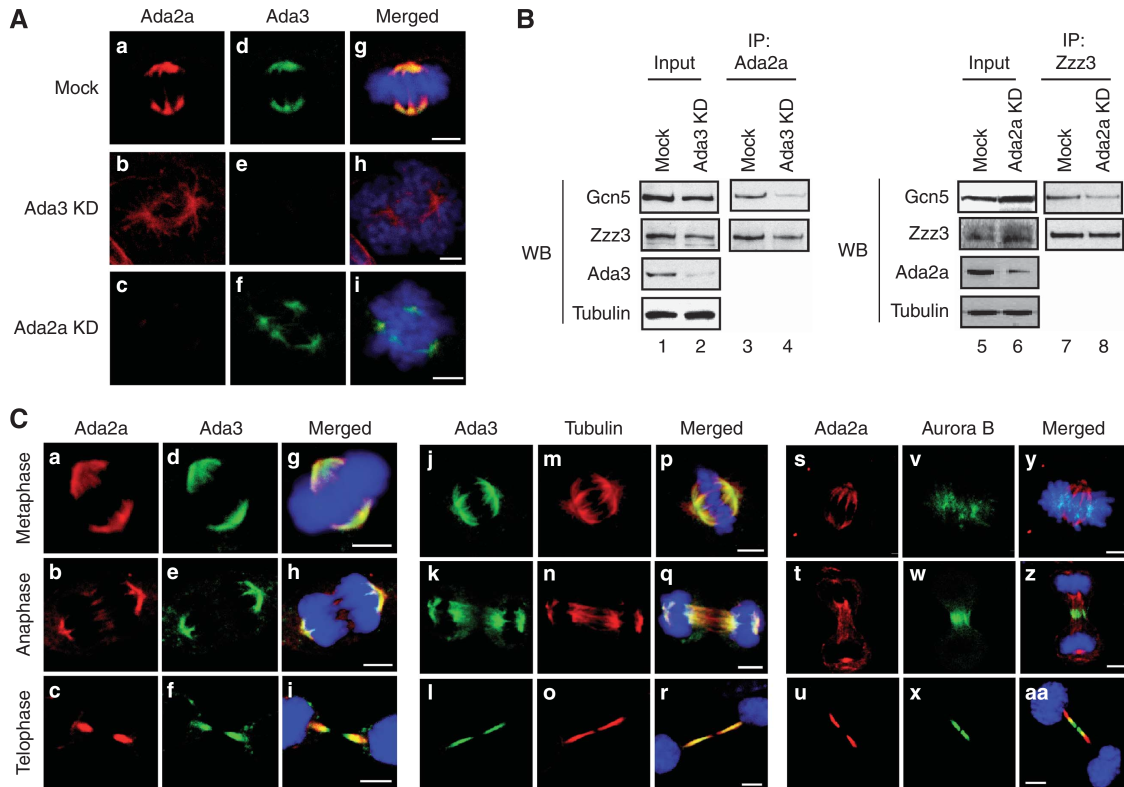

Figure 2 The ATAC complex stays associated during mitosis and colocalizes with the microtubule network. (**A**) Knock down (KD) of endogenous Ada2a (panels c, f, i) and Ada3 (panels b, e, h) protein expression after RNAi was verified by immunofluorescence and compared with cells transfected with a non-targeting control siRNA (Mock, panels a, d, g). The antibody used to label the cells is indicated at the top of the panels. Images are representative of $n>3$ independent experiments. Scale bars: 4 μm. (**B**) Composition of the ATAC complex on Ada3 and Ada2a knockdown. Cells were synchronized in G2/M phase with nocodazole and immunoprecipitation (IP) of the endogenous ATAC complex was carried out using an anti-Ada2a or anti-Zzz3 antibody, as marked. A total of 10% of the input extracts (Input) and the immune pellets were analysed by western blot (WB) using the antibodies indicated on the left of the panels. (**C**) The co-localization of Ada2a with Ada3 (panels a–i), Ada3 with α-tubulin (panels j–r) and Ada2a with AuroraB (panels s–aa) was tested by immunofluorescence in metaphase, anaphase and telophase cells (marked on the left). The merged images show the specific localization of the tested factors together with DNA (blue). Images are representative of at least three independent experiments. Scale bars: 4 μm.

control cells and twisted in shape, as well as to shortened distances between the dividing nuclei (Figure 3D). All these data show that cells lacking Ada2a or Ada3 are unable to proceed properly along cytokinesis and suggest that the mammalian ATAC complex is required for correct cell division, by affecting both initial and late stages of mitosis.

Depletion of the Ada core of ATAC leads to H4K16 and tubulin hyperacetylation

In metazoan organisms, the function of the ATAC complex has been associated with acetylation of histones (see Introduction), particularly H4K16. As the H4K16Ac mark is known to prevent chromatin compaction (Shogren-Knaak *et al*, 2006), the need of a functional ATAC complex during mitosis seems paradoxical with the notion that histone hypoacetylation is a prerequisite for mitotic chromosome formation (Kruhlak *et al*, 2001; Cimini *et al*, 2003; Valls *et al*, 2005) (Supplementary Figure 4). We thus examined whether global levels of acetylation on specific histone residues were affected in mitotic cells upon RNAi for Ada2a/3. Unexpectedly, knocking down the Ada2a or Ada3 components of the ATAC complex resulted in a specific increase of H4K16 acetylation (Figure 4A, panels d–l; Figure 4C), whereas H3K9, H3K14, H4K5 and H4K12 acetylation levels remained unchanged (Supplementary Figure 5). The hyperacetylation of H4K16 suggests a defective chromosome condensation following Ada2a/3 KD. As we identified ATAC subunits at the mitotic spindle together with α-tubulin, we also explored the state of α-tubulin acetylation under the same RNAi conditions. A similar hyperacetylation was observed on α-tubulin, whose mitotic function is also critically regulated by its acetylation (Zilberman *et al*, 2009 and refs therein). Thus, RNAi of either Ada2a or Ada3 induced hyperacetylation of both H4K16 and α-tubulin, which would consequently affect chromatin architecture and the tubulin network (Figure 4A and B, panels d–l; Figure 4C). Note, however, that the KD of the SAGA-specific subunit Spt20 did not result in hyperacetylation of α-tubulin (Supplementary Figure 6).

Earlier observations suggested that the Gcn5-HAT activity in the ATAC complex may be positively regulated by the Ada2a and Ada3 subunits that are directly interacting with Gcn5 (Balasubramanian *et al*, 2002; Gamper *et al*, 2009). Consistent with this, we observed that Gcn5 dissociates from the ATAC complex after Ada2a/3 KD (Figure 2B). Therefore, the increased acetylation of H4K16 and α-tubulin on Ada2a or Ada3 depletion may not be the result of an increased AT activity of ATAC. Rather, ATAC might affect other substrates

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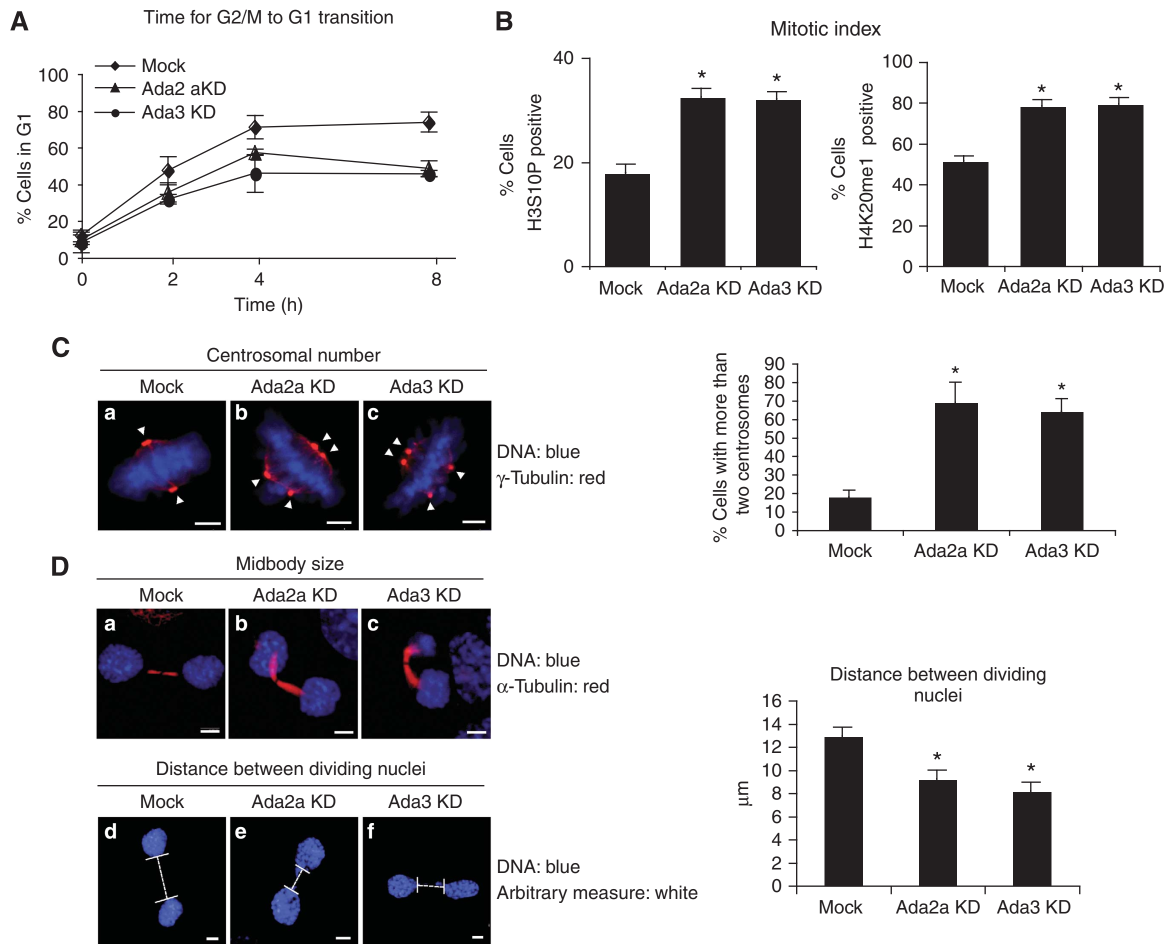

Figure 3 ATAC-depleted cells display delayed mitosis and defects in cytokinesis. NIH-3T3 cells were transfected with non-targeting control siRNA (Mock) or siRNAs against Ada2a or Ada3. After 72–96 h of transfection, cells were further analysed. (**A**) The different siRNA-treated cells were treated overnight with nocodazole to arrest them in G2/M and then released. After release, cells were harvested at different time points (as indicated) and the G1 population was monitored by FACS analysis. The results are expressed as percentage of total cells analysed (mean and s.d. from four independent experiments). (**B**) To analyse the mitotic index of siRNA-transfected cell populations, cells were stained with an anti-H3S10P and an anti-H4K20me1 antibody, and the DNA was visualized by Hoechst staining. The number of cells positive for the two marks were determined by immunofluorescence for each condition (>400 cells/condition, mean and s.d. from $n>4$ independent experiments, $^*P<0.05$). (**C**) To visualize centrosomes in siRNA-transfected mitotic cells, the cells were stained with an anti-γ-tubulin antibody and the DNA was visualized by Hoechst staining. Centrosome numbers were determined for each condition (>200 cells/condition, mean and s.d. from $n>4$ independent experiments, $^*P<0.05$). (**D**) To visualize the microtubule network in siRNA-transfected mitotic cells, cells were co-stained with an anti-α-tubulin antibody and Hoechst (DNA). A line (dashed-white line), placed on the basis of the position of the midbodies (α-tubulin staining), was used for measuring the distance between the daughter cells (>100 cells/condition, mean and s.d. from $n>3$ independent experiments, $^*P<0.05$).

that consequently lead to changes in the acetylation status of H4K16 and α-tubulin. Interestingly, the mammalian HDAC SIRT2 mediates both mitotic H4K16 and α-tubulin deacetylation (North *et al*, 2003; Vaquero *et al*, 2006, 2007), suggesting that it may be a potential target of the ATAC complex. SIRT2 belongs to the class III HDAC enzymes (sirtuins), which require nicotinamide (NAM) adenine dinucleotide [NAD(+)] for catalysis. SIRT2 resides in the cytoplasm during interphase, but at the onset of mitosis relocates to the nucleus, in which it deacetylates its substrates thereby ensuring mitotic progression (Vaquero *et al*, 2006, 2007). Thus, next we analysed the SIRT2 localization in cells in which ATAC subunits have been knocked down. In Ada2a/3-depleted cells, SIRT2 did not localize properly to the chromatin during mitosis (Figure 4D) that can explain the observed increase in the levels of acetylation of H4K16 and α-tubulin. To confirm our hypothesis, we tested whether overexpression of SIRT2 could rescue the deacetylation of H4K16 and α-tubulin in Ada2a/3-depleted cells. Indeed, overexpression of wild-type SIRT2 rescued both H4K16 and α-tubulin deacetylation in Ada2a/3-depleted cells (Figure 4E, compare lanes 1–3 with 4–6). In contrast, overexpressing the catalytically inactive SIRT2-H150Y mutant did not restore the H4K16 and α-tubulin deacetylation (Supplementary Figure 7) (Pandithage *et al*, 2008), further suggesting that the HDAC activity of SIRT2 is needed for this function. Consistent with this, inhibition of endogenous SIRT2 activity in cells with NAM (Bitterman *et al*, 2002) mimicked the effects of Ada2a/3 RNAi on H4K16 and α-tubulin acetylations (Figure 4E, compare lane 1 with lanes 2, 3 and 7, and see quantification on the right of the panel). Finally, *in vitro* SIRT2 tubulin deacetylase (TDAC) assays using overexpressed SIRT2 protein in Ada2a or Ada3 KD cell backgrounds further showed that the deacetylase activity of SIRT2 is impaired when the ATAC complex is lacking the Ada core (Supplementary Figure 8A). These observations together indicate that the

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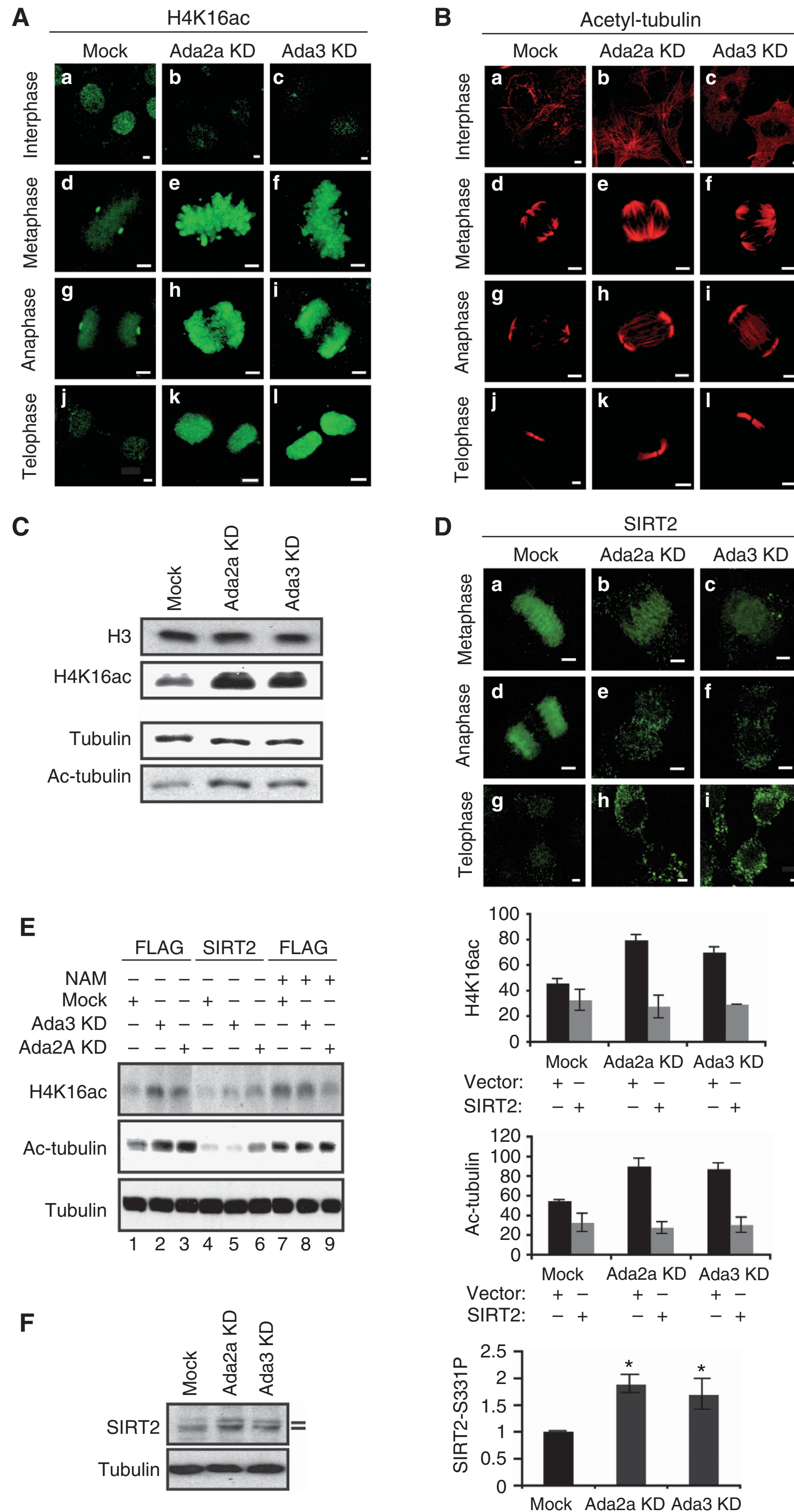
increased H4K16 and α-tubulin acetylation caused by KDs of Ada2a/3 is due to reduced/mislocalized SIRT2 activity and not because of increased activity of ATAC. To prove our hypothesis that SIRT2 is the major deacetylase involved in the observed phenotype, we aimed to exclude the potential contribution from the principal α-TDAC in the cells, HDAC6 (Hubbert *et al*, 2002; Zhang *et al*, 2003). To this end, we first tested the *in vitro* HDAC6 enzymatic activity in TDAC assays by using overexpressed HDAC6 protein in Ada2a or Ada3 KD cell backgrounds. This experiment showed that HDAC6 is fully active in the absence of the Ada core of the ATAC complex (Supplementary Figure 8B). Next, we



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inhibited the endogenous activity of HDAC6 with TSA (Minoru *et al*, 1995; Furumai *et al*, 2001) and compared the levels of α-tubulin acetylation to those observed on either NAM treatment or Ada2a/3 depletion. TSA treatments led to a more than 10-fold increase on α-tubulin acetylation levels, clearly larger than the 2.5-fold increase observed on either NAM treatment or Ada2a/3 depletion (Supplementary Figure 8C; Figure 4E, compare lanes 1–7). This result is consistent with the notion that HDAC6 is indeed the main α-TDAC acting in the cells. Furthermore, as earlier tested for SIRT2, we examined whether overexpression of HDAC6 could rescue the deacetylation of α-tubulin in Ada2a/3-depleted cells. Contrary to the SIRT2 overexpression experiment (Figure 4E), exogenous HDAC6 expression did not restore α-tubulin acetylation levels in Ada2a/3 backgrounds (compare Supplementary Figure 8D, lanes 2–6 with Figure 4E, lanes 2–6).

Overall, these results show that the hyperacetylation of H4K16 and α-tubulin observed on depletion of Ada2a or Ada3 is due to inefficient SIRT2 deacetylase activity. Furthermore, our data indicate that the ATAC complex modulates SIRT2 activity during mitosis, placing these two opposite enzymatic activities as parts of the same regulatory pathway.

ATAC regulates the phosphorylation state of SIRT2

SIRT2 activity can be regulated through post-translational modifications on several residues, among which S331 phosphorylation has been shown to partially block its HDAC activity (North and Verdin, 2007; Pandithage *et al*, 2008). Interestingly, after Ada2a/3 KD, a slower SIRT2 migrating form appeared on WB, suggesting that ATAC could regulate SIRT2 phosphorylation (Figure 4F, upper panel). The use of SIRT2-S331P-specific antibodies (Pandithage *et al*, 2008) confirmed that SIRT2 was phosphorylated on S331 in cells in which either Ada2a or Ada3 was depleted (Figure 4F, right panel). Expression of an SIRT2 mutant in which the S331 residue is mutated to alanine (S331A) was enough to prevent the hyperacetylation effects of Ada2a/3 KD on α-tubulin (Supplementary Figure 7). In contrast, overexpression of a SIRT2-S331E phospho-mimicking mutant phenocopied the effect of the Ada2a/3 KD, suggesting that Ada2a and Ada3 would indirectly regulate SIRT2 activity through changes in its phosphorylation status (Supplementary Figure 7).

ATAC acetylates Cyclin A

The inhibitory phosphorylation of SIRT2 on S331 is catalysed by the Cyclin A/Cdk2 complex (Pandithage *et al*, 2008). As ATAC had no detectable kinase activity on SIRT2 (data not shown), we next explored whether ATAC might indirectly affect SIRT2 phosphorylation, and thus its activity, by regulating Cyclin A/Cdk2 function through its acetylation. On the basis of the specific localization of Gcn5 to the spindle during mitosis (Figure 1D, panel i), we first checked whether this enzyme could acetylate Cyclin A/Cdk2. Using *in vitro* acetylation assays, we could detect that recombinant Gcn5 acetylates recombinant Cyclin A alone or in the context of the Cyclin A/Cdk2 complex (Figure 5A, lanes 11 and 12). However, as the ATAC complex contains other acetyltransferase activities such as Atac2 or Pcaf (see Introduction), we also tested these enzymes as potential Cyclin A acetyltransferases, along with Gcn5 and a catalytically inactive form of Gcn5 (rGCN5-ΔHAT) (Bu *et al*, 2007). As earlier described, the GCN5-ΔHAT mutant does not acetylate histone H3 and H4 peptides *in vitro* (Supplementary Figure 9A). We observed that Cyclin A is acetylated by Gcn5 and Pcaf, in agreement with a recent report (Mateo *et al*, 2009) (Figure 5B, lanes 8 and 9). On the contrary, neither the catalytically inactive Gcn5 (rGCN5-ΔHAT) nor Atac2 acetylated Cyclin A *in vitro* (Figure 5B, lanes 7 and 10), which further indicates that only the paralogue HATs, Gcn5 and Pcaf can acetylate Cyclin A. Next, we tested whether these enzymes could also mediate Cyclin A acetylation when incorporated in their respective endogenous complexes (ATAC or SAGA). To this end, we obtained highly pure ATAC or SAGA complexes from HeLa cells (Figure 5C) and used them to carry out *in vitro* acetylation assays as before. This experiment shows that only the ATAC complex mediates Cyclin A acetylation (Figure 5D, compare lane 10 with 11 and 12). Therefore, this result indicates that Cyclin A is a target for the acetyltransferase activity of ATAC and that the activity of the Cyclin A/Cdk2 kinase complex may be controlled by acetylation in the cells. To test whether Gcn5 can acetylate Cyclin A in a cellular context, cells were transfected with expression vectors for either Flag-Cyclin A together with Flag-Gcn5 wild type (Gcn5wt), or with a Flag-Gcn5 HAT-defective mutant (GCN5-ΔHAT). After transfection, an anti-Flag immunoprecipitation (IP) was carried out and the acetylation status of Cyclin A was analysed by using an antibody against Ac-Lys. We detected acetylated Cyclin A only in the wt Gcn5-expressing cells indicating that Cyclin A is a target of the Gcn5-HAT activity (Figure 5E).

Identification of Cyclin A residues targets for Gcn5 acetylation

In a recent study, lysines K54, K68, K95 and K112 of Cyclin A were identified as potential *in vitro* target sites for Pcaf

Figure 4 ATAC regulates the mitotic function of the SIRT2 HDAC. (**A–C**) Ada2a/3 depletion causes H4K16 and α-tubulin hyperacetylation. NIH-3T3 cells transfected with control (mock), Ada2a or Ada3 siRNA were visualized by immunofluorescence along mitosis using either (**A**) anti-H4K16ac or (**B**) anti-Acetyl-α-tubulin antibodies. (**C**) Western blot (WB) analysis of mitotic whole cell extracts from Ada2a/3 siRNA-transfected cells (indicated on the top) using the antibodies as indicated on the left. (**D**) Ada2a/3 KD disturbs the mitotic positioning of SIRT2. NIH-3T3 cells were transfected with the indicated siRNAs and mitotic cells were visualized using the anti-SIRT2 antibody in immunofluorescence. (**E**) SIRT2 overexpression restores H4K16 and α-tubulin deacetylation, whereas the inhibition of SIRT2 mimics the KD of either Ada2a or Ada3. The 293T cells were co-transfected with an empty vector (FLAG) or an expression vector for SIRT2-FLAG (SIRT2), and DNA constructs expressing shRNAs against Ada2a (Ada2a KD), Ada3 (Ada3 KD) or a scramble shRNA (Mock) as indicated on the top of the panel. Transfected cells were either untreated or treated O/N with 5 mM nicotinamide (NAM). After the indicated transfections and treatments, WCE was prepared from the cells and analysed by western blot (WB) with the indicated antibodies (left panel). Panels on the right: the results of the quantification of $n > 10$ independent experiments by densitometry ($*P < 0.05$). (**F**) Ada2a or Ada3 knockdown increases the phosphorylation of SIRT2 on S331. NIH-3T3 cells were treated with control (mock), Ada2a or Ada3 siRNAs for 48 h. Cell extracts were prepared after transfection and analysed by WB using an anti-SIRT2 antibody (left panel). Endogenous SIRT2 was immunoprecipitated from control and Ada2a or Ada3 KD 293T cells and analysed by an antibody that recognizes SIRT2 in general or specifically recognizing SIRT2 phosphorylated on S331P. WBs obtained with the phospho-specific antibody were quantified by densitometry. Indicated fold changes are mean from $n = 4$ independent experiments, $*P < 0.05$.

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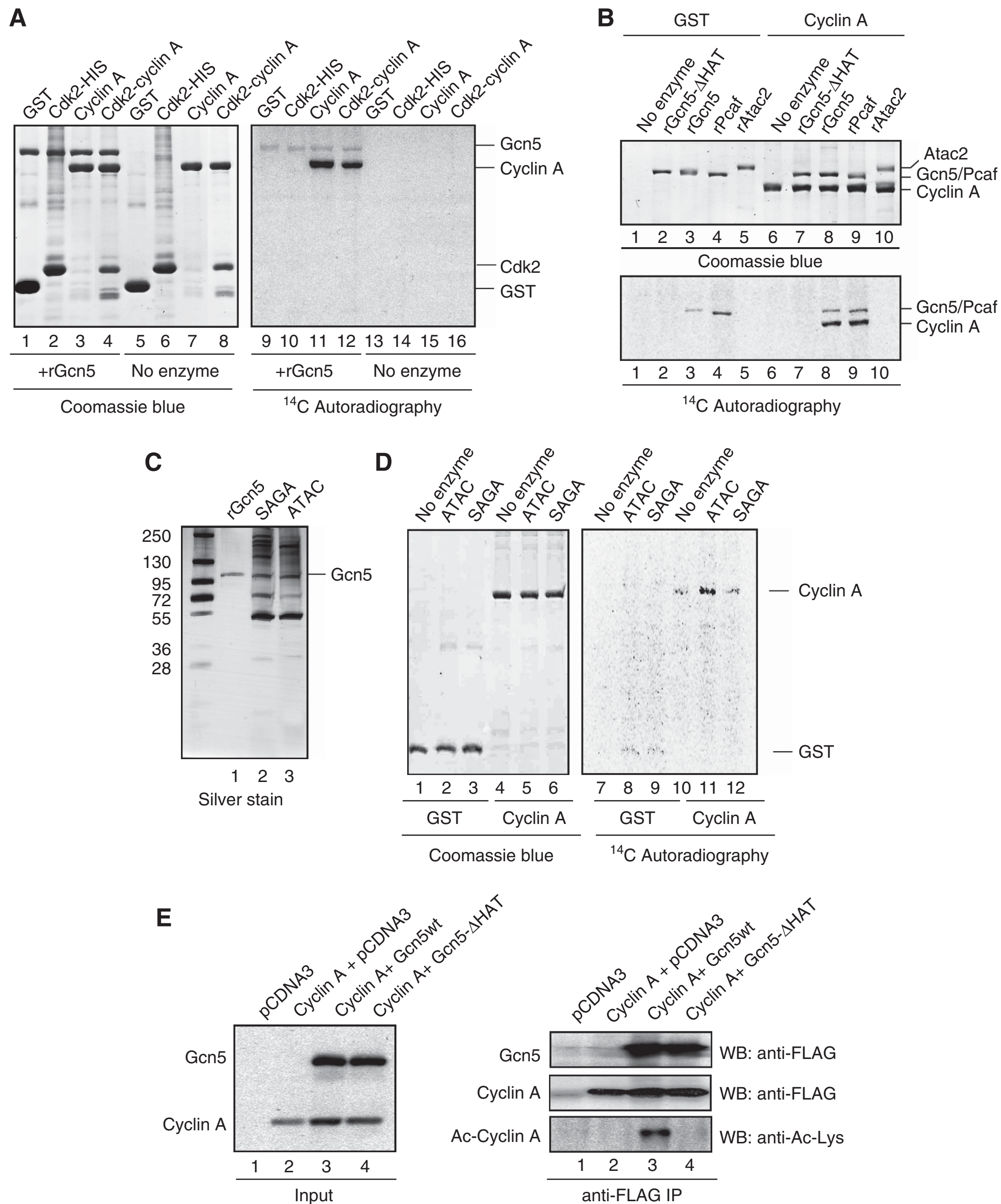

Figure 5 The ATAC complex acetylates Cyclin A/Cdk2 through Gcn5 or Pcaf. (**A**) Gcn5 acetylates the Cyclin A/Cdk2 complex *in vitro*. *In vitro* acetylation assays have been carried out using purified recombinant (r) Cdk2 and Cyclin A as substrates, and rGcn5 as enzyme. Reactions were separated by SDS–PAGE, visualized by coomassie blue staining (left panel) and labelled proteins were visualized by autoradiography (right panel). (**B**) Gcn5 and Pcaf, but not Gcn5-ΔHAT or Atac2, efficiently acetylate Cyclin A *in vitro*. *In vitro* acetylation assays have been carried out using purified recombinant (r) Cyclin A as substrate, and rGcn5, rGcn5-ΔHAT, rPcaf or rAtac2 as enzyme. Reactions were separated by SDS–PAGE, visualized by coomassie blue staining (upper panel) and labelled proteins were visualized by autoradiography (lower panel). (**C**, **D**) The ATAC complex, but not SAGA, acetylates Cyclin A *in vitro*. *In vitro* acetylation assays have been carried out using purified recombinant (r) Cyclin A as substrate, and the purified hATAC or hSAGA complexes as enzymes. (**C**) Shows a silver staining for the respective ATAC and SAGA purifications, with equivalent Gcn5 content. (**D**) Shows the acetylation experiment performed with the purified ATAC and SAGA complexes: reactions were separated by SDS–PAGE, visualized by coomassie blue staining (left panel) and labelled proteins were visualized by autoradiography (right panel). (**E**) Gcn5 acetylates Cyclin A *in vivo*. 293T cells were transfected with an empty vector (pCDNA3) (lane 1), or co-transfected with FLAG-Cyclin A and pCDNA3 (lane 2); FLAG-Cyclin A and FLAG-Gcn5 wild type (Gcn5wt) (lane 3), or Cyclin A and FLAG-Gcn5 mutated in the HAT domain (Gcn5-ΔHAT) (lane 4). WCEs from the transfected cells was prepared and analysed by western blot (WB) with the indicated antibodies (left panel). FLAG-tagged proteins were immunoprecipitated from WCEs, eluted with FLAG peptide and analysed with an antibody recognizing acetyl-lysines, Cyclin A and Gcn5 (as indicated).

acetylation (Mateo *et al*, 2009). These four lysine residues are located on the N-terminal domain of Cyclin A, in the so-called canonical degradation (D)-box (positions 46–63) or the extended D-box (65–82) (Tin Su, 2001 and references therein). Interestingly, these domains have been implicated in regulating the stability of the protein (Wolthuis *et al*, 2008) (Figure 6A). Moreover, it has been reported that the replacement of K54 and K68 of Cyclin A by arginines stabilizes

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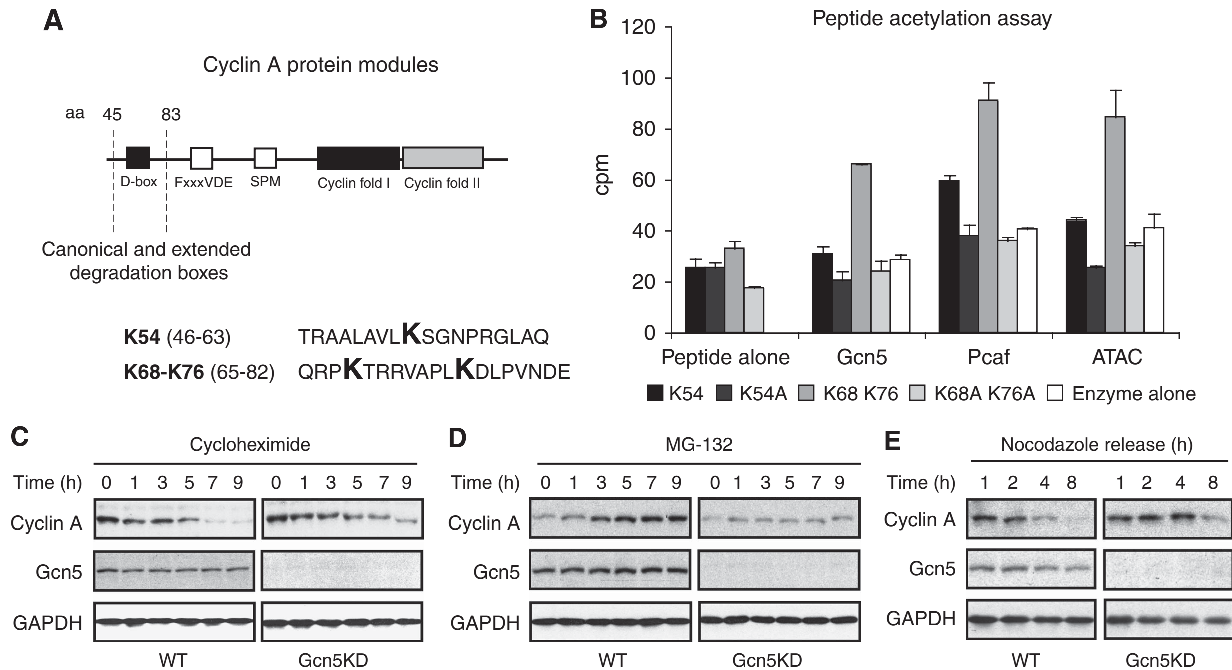

Figure 6 The ATAC complex regulates Cyclin A stability through acetylation. (**A**) Upper panel: schematic representation of the Cyclin A protein and its characteristic domains. The amino-acid (aa) positions of the canonical (D-box) and the extended degradation boxes are indicated. Lower panel: summary of the peptides synthetized to be used as substrate in the peptide acetylation assays shown in (**B**). (**B**) Peptide acetylation assays were performed using rGcn5, rPcaf or the ATAC complex as enzymes, and the peptides containing potential lysine target sites [wt (K) or mutated to Alanine (A)] as indicated. (**C**, **D**) Gcn5 acetylation promotes Cyclin A degradation *in vivo* through the proteasome. Inducible HeLa shRNA (Luc-KD or Gcn5-KD) cell lines maintained in the presence of doxicyclin were left untreated or exposed to cycloheximide (**C**) or MG-132 (**D**) for different time points (as indicated). Cells were collected, WCEs prepared and analysed by WB with the indicated antibodies. (**E**) Gcn5 acetylation promotes Cyclin A degradation during mitosis. HeLa shRNA (Luc-KD or Gcn5-KD) cell lines maintained with doxicyclin, blocked at the G2 phase of the cell cycle with Nocodazole for 18 h and then released from this block for several hours as indicated. Cells were collected, WCEs prepared and analysed by WB with the indicated antibodies.

Cyclin A in an ubiquitin-independent manner (Fung *et al*, 2005). As our mass spectrometric analysis suggested that Gcn5 may acetylate Cyclin A at positions K54 and K68 positions (data not shown) and as these lysines are located in the D-box of Cyclin A, we generated two peptides covering these positions, and their corresponding mutant versions in which the lysines (K) were substituted with alanines (A) (Figure 6A). Using the wild-type or mutated peptides in *in vitro* peptide acetylation assays, we observed that either the recombinant rGcn5 or rPcaf enzymes or the ATAC complex acetylated the Cyclin A peptide (from amino-acid 65–82) containing K68 and K76, which corresponds to the extended D-box (Geley *et al*, 2001) (Figure 6B). In contrast, the enzymatic activities were much less efficient on the Cyclin A peptide (from amino-acid 46–63) containing K54, present within the canonical D-box (Figure 6B). Note that the corresponding mutant peptides showed only background acetylation levels. Importantly, an earlier study showed that the single deletion of the canonical D-box (positions 45–58) does not affect Cyclin A protein turnover. However, the deletion of both the canonical and extended D-box (position 47–83) leads to stable Cyclin A protein levels (Geley *et al*, 2001). Our present results together with earlier observations suggest that acetylation of Cyclin A on the extended D-box may serve as a regulatory mechanism for Cyclin A protein stability along the cell cycle, and concomitantly, regulate Cyclin A mitotic function through its degradation.

Gcn5 regulates Cyclin A stability through acetylation

The function of Cyclin A/Cdk2 is restricted to early mitotic stages as Cdk2 is inactivated and Cyclin A degraded when cells enter in prometaphase (den Elzen and Pines, 2001). Thus, we tested whether the acetylation of Cyclin A by Gcn5 would affect the stability of Cyclin A in the cells. To this end, we generated an inducible HeLa cell line in which Gcn5 expression could be knocked down by the doxicyclin inducible expression of a specific shRNA (http://tronolab.epfl.ch/). As a control, we generated a cell line with an inducible shRNA against Luciferase (Luc-KD). After 48 h of induction by doxicyclin treatment, Luc-KD or Gcn5-KD cells were treated with cycloheximide to block *de novo* protein synthesis and the Cyclin A levels at different time points were measured by WB analysis (Figure 6C). In wild-type cells, degradation of Cyclin A was completed after 5–7 h of cycloheximide treatment. In contrast, in Gcn5-KD cells, this degradation was clearly delayed, as after 9 h of cycloheximide treatment Cyclin A was still detectable. This result is in good agreement with our hypothesis that Gcn5 activity could regulate Cyclin A protein stability. To further confirm this observation, we performed the same type of experiment, but on cells transfected with expression vectors for Gcn5wt or for Gcn5-ΔHAT. After transfection, cells were treated with cycloheximide and Cyclin A levels measured by WB analysis (Supplementary Figure 9B). In non-transfected cells, degradation of Cyclin A started at 6 h of cycloheximide treatment, whereas in Gcn5-expressing cells, this degradation started

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earlier (around 3 h of treatment), further confirming that Gcn5-mediated acetylation can trigger Cyclin A degradation. In contrast, in cells expressing the Gcn5-ΔHAT mutant, Cyclin A degradation started much later (around 9 h of treatment) further underlining the importance of the acetyltransferase activity of Gcn5 in Cyclin A degradation (Supplementary Figure 9B).

On the basis of these results and on the identification of the potential lysines as target of Gcn5 acetyltransferase activity within the extended D-box of Cyclin A, we next tested whether Gcn5-mediated Cyclin A degradation was proteasome dependent. For this aim, after 48 h of doxicyclin treatment, wild-type or Gcn5-KD cells were treated with MG-132 to block proteasome-mediated protein degradation, and Cyclin A levels were then measured by WB analysis at different time points of MG-132 treatment (Figure 6D). In wild-type cells, Cyclin A levels steadily increased over time, whereas in Gcn5-KD cells, Cyclin A levels remained unchanged. This result suggests that in the absence of Gcn5, Cyclin A is not targeted to proteasome degradation. This observation is in agreement with our working hypothesis that Gcn5-mediated acetylation on Cyclin A triggers Cyclin A degradation. Finally, we evaluated whether the mitotic degradation of Cyclin A was affected in the absence of Gcn5. Thus, we blocked both wt and Gcn5-KD cells at the G2 phase of the cell cycle by nocodazole treatment for 18 h, and then monitored Cyclin A protein levels by WB at different time points after releasing the block (Figure 6E). Consistent with our above results, we observed a delayed Cyclin A degradation on G2/M synchronization and release in Gcn5-KD cells, when compared with the wild-type cells (Figure 6E). These results together show that Gcn5 AT activity has a function in the regulation of the mitotic degradation of Cyclin A and thus the activity of the Cyclin A/cdk2 kinase. Our data show that Cyclin A is degraded in a Gcn5-dependent manner at the beginning of mitosis to ensure timely completion of cell division (Tin Su, 2001 and references therein).

Ada core of the ATAC complex regulates Cyclin A stability

Next, we tested whether depletion of the Ada2a/3 subunits of the ATAC complex could lead to changes in Cyclin A levels in the cells. To this end, we examined Cyclin A levels under Ada2a/3 KD conditions by immunofluorescence, either in late G2 or metaphase. In agreement with our above observations, in Ada2a/3-depleted cells, Cyclin A levels remained stable during late G2, suggesting that Cyclin A degradation at early mitotic stages did not occur (Figure 7A, compare panel a with c and e, or panel g with i and k). Next, we analysed endogenous Cyclin A levels by WB analysis in asynchronous, G1 or G2/M arrested cell populations. Asynchronous cultures showed increased Cyclin A levels after Ada2a/3 depletion (Figure 7B). After the release of control cells arrested in G1 or G2/M, Cyclin A levels steadily increased from G1 to G2 (Figure 7C, lanes 1–3), and were degraded on G2 to M transition (lanes 4 and 5 in Figure 7C). In contrast, Ada2a/3-depleted (KD) cells displayed higher and stable Cyclin A levels along G1–G2 (Figure 7C, lanes 1–3), and were unable to trigger Cyclin A degradation on mitosis (Figure 7C, lanes 4 and 5).

To test whether the increased Cyclin A protein levels on Ada2a/3 KD could be also due to transcriptional effects, we evaluated the impact of Ada2a KD on the transcription of the *Cyclin A* gene in cell populations synchronized at the G2 phase of the cell cycle (Figure 7D, left panels). After synchronization, control or Ada2a KD cells were collected and mRNA expression for *Cyclin A* or *Cyclin B* was measured. This experiment clearly showed that *Cyclin A* or *B* transcripts did not change on Ada2a KD (Figure 7D, right panel), further confirming our above results that the ATAC KD induced Cyclin A stabilization effects are taking place at the protein level. The lack of Cyclin A degradation on G2/M release in Gcn5-, Ada2a- and Ada3-depleted cells strongly suggests that ATAC-mediated acetylation contributes to Cyclin A degradation in wild-type conditions.

In summary, here we describe a new pathway in which the ATAC complex controls Cyclin A/Cdk2 and, indirectly, SIRT2 activity. The ATAC/Gcn5-mediated acetylation of Cyclin A targets it for degradation, which is indispensable for obtaining the non-phosphorylated form of SIRT2. This SIRT2 is consequently fully active and able to deacetylate its mitotic targets, H4K16 and tubulin.

Discussion

Although the function of HAT complexes in regulating chromatin structure and transcription activation is widely studied (Lee and Workman, 2007; Nagy and Tora, 2007), less is known about their non-histone substrates and the function they fulfil through acetylating other proteins than histones. In the present report, we describe that the Gcn5-containing ATAC complex localizes to the mitotic spindle in which it has an essential function in orchestrating the progression through mitosis. This is a specific feature of the ATAC complex that contrasts with the other Gcn5-containing complex, SAGA, showing a different localization and not being involved in mitosis. We show that cells display a number of mitotic abnormalities upon depletion of the Ada core of ATAC. Our observations suggest that in the absence of the Ada core, the Gcn5 AT, probably as a free protein or associated with a partial ATAC complex, acetylates inefficiently its mitotic substrates, and in turn globally impacts on mitotic progression.

Indeed, analysis of the phenotype arising on Ada2a/3 depletion reveals a number of defects in crucial stages of cell division. Altogether, Ada2a/3-depleted cells proceed with major difficulties through mitosis, by either dividing at very slow rates or asymmetrically. In the most severe cases, these cells fail to complete their division and generate multinucleated cells or cells that die soon after mitosis.

Depletion of Ada2a/3 critically affects both early and late mitotic states. Ada2a/3 KD leads to a four-fold increase in the number of cells possessing super-numeral centrosomes compared with control cells. Deregulated centrosome amplification has a major impact on cell division, as centrosome duplication is a prerequisite for proper bipolar spindle formation, correct chromosome segregation and symmetrical cell division (Heald *et al*, 1997). Importantly, centrosome duplication requires, among other activities, the function of the Cdk2 kinase in association with Cyclin A (Meraldi *et al*, 1999). In addition, Cyclin A/Cdk2 has been described to be essential to coordinate centrosomal and mitotic events (De Boer *et al*, 2008). Therefore, any modification of normal Cyclin A/Cdk2 activity would influence not only proper

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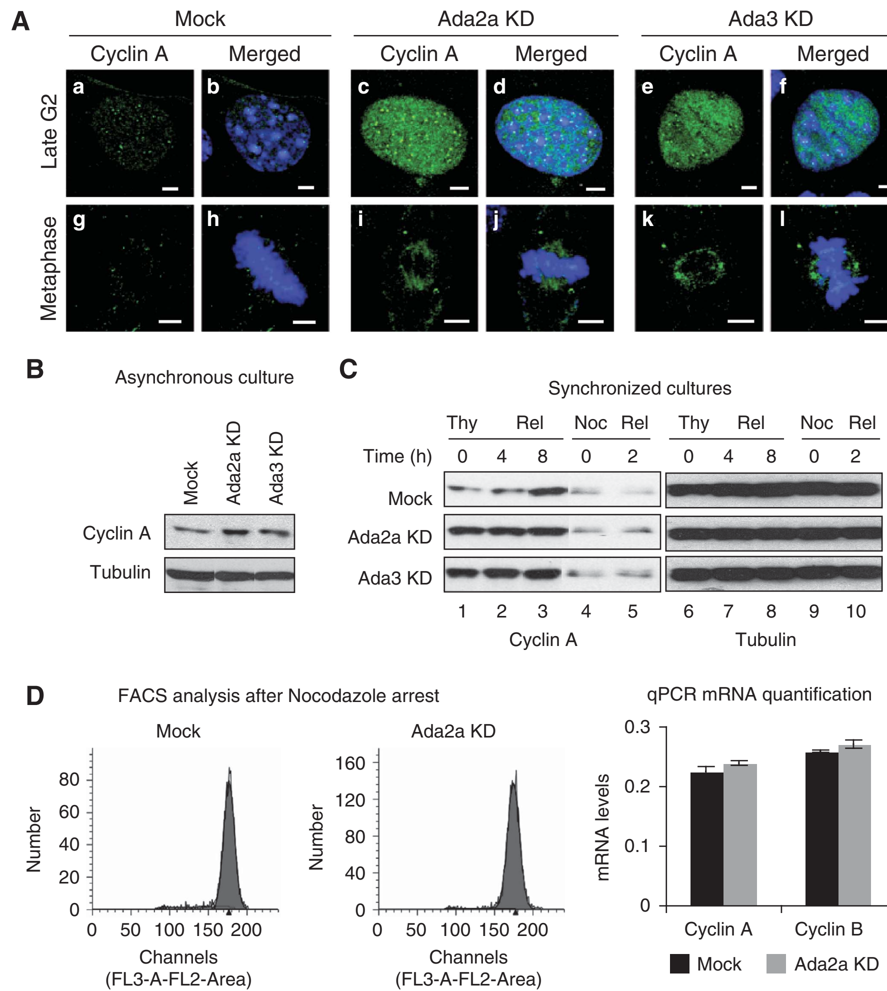

Figure 7 The Ada core of the ATAC complex regulates Cyclin A stability. (**A**) Mock or Ada2a/3 siRNA-transfected cells were visualized by immunofluorescence in late G2 and metaphase using an anti-Cyclin A antibody. Images are representative of three independent experiments. (**B**, **C**) WB analysis of Cyclin A levels in cell extracts prepared from wild-type or Ada2a and Ada3 KD cells. Asynchronous (**B**), G1 (thymidine: Thy) or G2/M (nocodazole: Noc) arrested cells (**C**) were released and Cyclin A protein level monitored using the indicated antibodies. (**D**) *Cyclin A* transcription is not affected after depletion of an ATAC subunit (Ada2a). Mock or Ada2a siRNA-transfected cells were blocked at the G2/M phase of the cell cycle by Nocodazole treatment for 18 h (as verified by the FACS analyses shown on the left). In the two left panels the x-axis represents the DNA content and the y-axis the cell numbers ($\times$ 1000). Cells were collected and their respective RNA extracted. This RNA was used for quantitative PCR analysis of the *Cyclin A* and *Cyclin B* transcripts for each condition (panel on the right). Mean and s.d. were calculated from three independent experiments.

centrosome duplication rates, but also centrosomal activities and mitotic events. Our results uncover the involvement of the ATAC complex, through Gcn5-mediated acetylation, in the regulation of Cyclin A stability and as a consequence, Cdk2 activity. Importantly, the depletion of Ada2a and Ada3 deregulates the enzymatic activity of ATAC, as the Ada core is essential to sustain Gcn5-HAT activity (Balasubramanian *et al*, 2002). Furthermore, we show that the depletion of the Ada2 or Ada3 subunits results in the disassembly of Gcn5 from the ATAC complex. Overall, the deregulated ATAC activity because of the Ada2a/3 KD would directly affect Cyclin A acetylation, stability and function, thus correlating with the observed centrosome abnormalities. Consistent with the aberrant centrosome multiplication, Ada2a/3-depleted cells manifest defects also in spindle and midbody formation, which in turn correlates with the observed difficulties to proceed through cytokinesis and complete cell division. Altogether, these abnormalities result in a time delay of mitosis and retarded entry into the G1 phase of the cell cycle.

In addition, Ada2a and Ada3 KD lead to changes in chromatin post-translational modifications, such an increase in H4K16 acetylation levels and a reduction of H3S10 phosphorylation levels (Ciurciu *et al*, 2008; Nagy *et al*, 2010 and data not shown). These two features oppose the normal mitotic scenario characterized by high H3S10P and low H4K16Ac levels required for normal chromatin condensation. Furthermore, we also show abnormal hyperacetylation of the microtubule network in ATAC-depleted cells. Microtubules act as pulling forces for chromosome segregation and their dynamics relies on hypoacetylated states, as α-tubulin acetylation marks static microtubule-based structures (Westermann and Weber, 2003; Hammond *et al*, 2008). Therefore, the observed hyperacetylation of α-tubulin would impair proper chromosome segregation and is consistent with the phenotype of binucleated cells that we describe. Thus, the absence of the Ada core of ATAC leads to inefficient chromosome compaction (because of H4K16 hyperacetylation) and increased microtubule stability (because of α-tubulin hyperacetylation), which together will impair mitotic progression.

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It seemed striking that the depletion of an AT complex could lead to the hyperacetylation of two proteins whose deacetylated state is a prerequisite for mitotic progression: α-tubulin and H4K16. We solved this apparent paradox by showing that the effects observed in the absence of the Ada core of ATAC derive from impaired SIRT2 activity on these substrates. Consistent with this, in the absence of Ada2a and Ada3, SIRT2 overexpression is enough to restore normal H4K16 and α-tubulin deacetylation. Moreover, we also show that the Cyclin A/Cdk2 complex constitutes the link between the activities of ATAC and SIRT2, and that ATAC-mediated acetylation of Cyclin A determines the fate of the Cyclin A/Cdk2 complex by priming Cyclin A for degradation. These observations are in good agreement with recent findings describing that Cyclin A degradation could be triggered by acetylation (Mateo *et al*, 2009). Furthermore, in this report, we show that both Gcn5 and the ATAC complex mediate lysine acetylation in the extended D-box of Cyclin A and thus regulate Cyclin A degradation along the cell cycle. Consequently, any dysfunction of the ATAC complex will have an impact on Cyclin A activity. At the onset of mitosis, Cyclin A has to be degraded to ensure faithful mitotic progression. Indeed, an earlier report shows that after deletion of its extended D-box, Cyclin A becomes non-degradable, which leads to mitotic delay, as cells show anaphase arrest and difficulties to complete cytokinesis (Geley *et al*, 2001). Our study shows a situation that mimics the phenotype arising after deletion of the Cyclin A extended D-box, as depletion of Ada2a or Ada3 also leads to Cyclin A stabilization and difficulties for mitotic completion. Overall, our data illustrate a pathway in which, during mitosis, ATAC inhibits Cyclin A/Cdk2 function by promoting Cyclin A degradation through Gcn5-mediated acetylation. This consequently renders SIRT2 non-phosphorylated and fully active, inducing deacetylation of H4K16 and α-tubulin, which allows chromatin compaction and segregation. In the absence of the Ada2a/3 core of ATAC, Gcn5 activity is not correctly targeted and thus, Cyclin A levels remain high, as Cyclin A fails to be efficiently degraded. This in turn would lead to amplified Cyclin A/Cdk2 kinase activity, which then would result in abnormal centrosome duplication and defective bipolar spindle formation, SIRT2 phophorylation and inactivation. All these effects together would then lead to defective chromatin compaction, microtubule dynamics, and thus, mitotic failure.

Altogether, our data highlight an essential implication of a mammalian AT complex in mitosis, by regulating the activity of crucial non-histone substrates. We have uncovered a novel function for a HAT complex (ATAC) that earlier has been mainly implicated in transcription regulation. Acetylation of non-histone substrates is now being generally accepted as a regulatory mechanism of protein activity. As we describe in this study for Cyclin A, protein acetylation seems to be a more general mechanism to control protein turnover and, therefore, the function of important cellular protein machines is controlled by regulating their cellular amounts (Caron *et al*, 2005; Sadoul *et al*, 2008). The involvement of the ATAC complex in targeting cell cycle kinase complex(es) reveals a novel regulatory function for this complex in controlling cell cycle progression. Moreover, our new results challenge the classical concept that HAT activities must be replaced by HDACs during mitosis, and indicate that ATs must remain active and act coordinately with HDACs to regulate cell division.

Materials and methods

Cell culture, reagents, treatments and transfections

Cell culture conditions, specific treatments and transfection reagents are detailed in the Supplementary data.

Generation of inducible HeLa shRNA cell lines

During a first infection, HeLa cells (ATCC) were transduced with a lentivirus encoding the tetraCycline repressor DNA-binding domain fused to a KRAB domain (pLV-tTRKRAB-red). Five days after infection, a cell population expressing similar levels of the dsRED marker was sorted using an FACS Diva. These cells were hereafter grown in DMEM supplemented with 10% Tet-free FBS. In a second round of infections, these cells were transduced with lentiviruses harbouring shRNA cloned in pLVTH either against Gcn5 (target sequences: CGTGCTGTCACCTCGAATGA) or GL2 luciferase as control (target sequences: CCTTACGCTGAGTACTTCGA) at a MOI of 20. All cell lines generated were checked for the presence of the EGFP marker after 2 days of doxicylin induction (1 μg/ml) by FACS. Lentivirus production and titre evaluation were carried out according to Professor D Trono's laboratory protocols (http://tronolab.epfl.ch/) pLV-tTRKRAB-red, pLVTH and the packaging systems were kindly provided by Professor D Trono and are described in Wiznerowicz and Trono (2003).

Antibodies

Antibodies used in this study are detailed in the Supplementary data.

Protein overexpression in baculoviruses

Expression and purification of human Cyclin A in complex with Cdk2 was performed as earlier described (Sarcevic *et al*, 1997). The baculovirus expressing rGcn5 was described in Demeny *et al* (2007). The rGcn5-ΔHAT construct is essentially the same containing two point mutations (E575A and D615A) generated by site-directed mutagenesis. The rATAC2 expressing baculovirus contains the amplified human ATAC2 cDNA obtained from the IMAGE clone IRAUp969G0838D. Primers used for the amplification were 5′-TCCTCGAGCTGTATTCGCATCAGCGCC-3′ and 5′-AAGAATTCGATG GATAGTAGCATCCACCTGAG-3′. The amplicon was inserted into the EcoRI XhoI sites of the HA tag containing pCDNA 3.1 vector and further cloned into pVL1393 (BD Biosciences) to generate recombinant viruses. The rPCAF was kindly provided by N Rochel.

Preparation of cell extracts and IP

Details concerning preparation of total or nuclear cell extracts from mammalian cells or insect cells, and immunoprecipitation are described in the Supplementary data.

HAT assays

Acetylation assays: proteins, purified either by GST pull down or by His-tag purification, were incubated in the presence of recombinant Gcn5, Gcn5-ΔHAT, Pcaf or Atac2 (purified from baculovirus-infected insect cells by anti-FLAG or anti-HA IP followed by elution with FLAG or HA peptides) or the human ATAC or SAGA complexes (purified from HeLa nuclear extracts) and ^{14}C-Acetyl-CoA. The reaction mixture (25 μl) containing 5X HAT buffer (250 mM Tris [pH 7.9], 50% glycerol, 0.5 mM EDTA, 250 mM KCl, 100 mM sodium butyrate, 5 mM and the protease inhibitor C-Complete (Roche) was incubated for 1 h at 30°C. The reaction was stopped by adding Laemmli buffer with 10 mM DTT and boiled for 5–10 min. Proteins from the reactions were separated on a 13% SDS–PAGE and analysed by coomassie brillant blue staining and then by radiography.

Peptide acetylation assays were performed as described in Nagy *et al* (2010).

TDAC assays were performed as described in North *et al* (2003).

Immunofluorescence

Details concerning indirect immunofluorescence, microscopy, and image analysis are described in the Supplementary data.

FACS analysis

Cells were trypsinized, washed with PBS, and fixed with ice-cold 70% ethanol O/N at 4°C. DNA was stained using a solution with 50 μg/ml propidium iodide (Sigma) and 1 mg/ml RNase A in PBS. The cells were analysed on FACScalibur (BD Biosciences) using CellQuest and ModFit data analysis software.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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