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# Histone Deacetylases Regulate Multicellular Development in the Social Amoeba *Dictyostelium discoideum*

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Epigenetic modifications of histones regulate gene expression and lead to the establishment and maintenance of cellular phenotypes during development. Histone acetylation depends on a balance between the activities of histone acetyltransferases and histone deacetylases (HDACs) and influences transcriptional regulation. In this study, we analyse the roles of HDACs during growth and development of one of the cellular slime moulds, the social amoeba *Dictyostelium discoideum*. The inhibition of HDAC activity by trichostatin A results in histone hyperacetylation and a delay in cell aggregation and differentiation. Cyclic AMP oscillations are normal in starved amoebae treated with trichostatin A but the expression of a subset of cAMP-regulated genes is delayed. Bioinformatic analysis indicates that there are four genes encoding putative HDACs in *D. discoideum*. Using biochemical, genetic and developmental approaches, we demonstrate that one of these four genes, *hdaB*, is dispensable for growth and development under laboratory conditions. A knockout of the *hdaB* gene results in a social context-dependent phenotype: *hdaB*$^-$ cells develop normally but sporulate less efficiently than the wild type in chimeras. We infer that HDAC activity is important for regulating the timing of gene expression during the development of *D. discoideum* and for defining aspects of the phenotype that mediate social behaviour in genetically heterogeneous groups.



*Keywords:* histone acetylation; trichostatin A; cyclic AMP; cell adhesion; heterochrony

## Introduction

The hallmark of cell differentiation is the coordinated expression of genes dependent on extracellular cues such as diffusible signals and cell–cell contact. How cells perceive these stimuli and respond *via* the generation of internal messengers is well studied. However our understanding of how cells coordinate the expression of many genes in the course of responding is limited. It has been suggested that large regions of chromosomes become accessible to the transcription machinery following chromatin modification in response to extracellular factors.[1] Covalent epigenetic modifications of histones and DNA can change chromatin architecture and facilitate or prevent transcription of many genes in the same neighbourhood. Supporting this, genes that are expressed at the same time and in the same cell have been found to be physically closer to each other in the *Drosophila* genome than expected by chance.[2,3]

The reversible acetylation of histones at lysine residues is a covalent modification that promotes the formation of transcription-competent regions on a chromosome.[4] Lysine residues in histones that are acetylated are conserved across species, suggesting an ancestral conserved mode of regulation. Specific

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Abbreviations used: HDAC, histone deacetylase; HAT, histone acetyltransferase; HMM, hidden Markov model; TSA, trichostatin A; RT-PCR, reverse transcription-polymerase chain reaction; HDAH, histone deacetylase-like amidohydrolase; HDLP, histone deacetylase-like protein.

patterns of lysine acetylation are established and maintained by the activity of histone acetyl transferases (HATs) and histone deacetylases (HDACs).[5] HDAC-like proteins are found in all the major categories of organisms including prokaryotes, which lack nuclear histones.[6] Based on their domain sequence, HDACs can be classified into four classes[7].Classes I, II and IV use $Zn^{2+}$ in the deacetylation reaction. The evolutionarily unrelated class III HDACs use an $NAD^{+}$-dependent deacetylation mechanism and are called sirtuins. Structural and biochemical data on class I and II HDACs of bacterial and mammalian origin, combined with mutational analyses, have provided insights into the residues involved in catalysis and $Zn^{2+}$ binding.[8,9]

Much of our current understanding of the roles of HDACs in developmental processes is based on genetic or pharmacological interference with HDAC activity in cell lines and organisms.[10] The availability of a specific inhibitor, trichostatin A (TSA), makes it possible to study the role of HDACs in growth and development.[11] Such studies have shown that HDACs regulate macronuclear differentiation in *Tetrahymena*,[12] yeast apoptosis,[13] cell fate specification in *Arabidopsis*,[14] zebrafish liver development,[15] differentiation of stem cells *in vitro*[16] and mouse endoderm specification.[17] But, we lack a comprehensive picture of HDAC function in a cellular context and in relation to organismal phenotype. A large number of cell types and their interactions make it difficult to interpret the outcomes of experimental manipulations in higher metazoans. On the other hand, yeasts are experimentally tractable but do not exhibit features of multicellular differentiation. We therefore chose to study the social amoeba *Dictyostelium discoideum*, which exhibits a uni- to multicellular

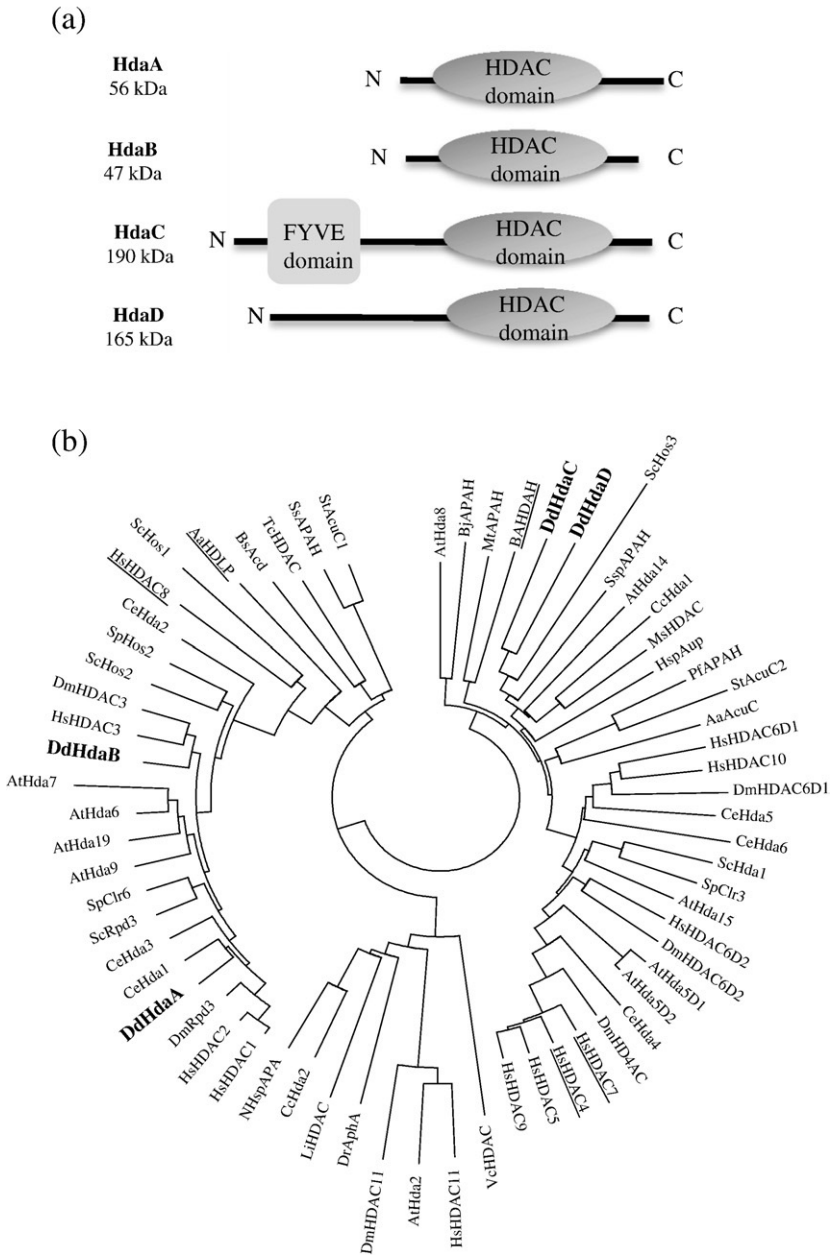


**Fig. 1.** Putative HDACs in *D. discoideum*. (a) Domain architecture of the four putative HDACs in *D. discoideum* with their name and predicted molecular mass. N and C indicate amino- and carboxy-termini of proteins. HDAC domain: histone deacetylase domain, FYVE domain: A domain found in *F*ab1, *Y*OTB/ZK632.12, *V*ac1, and *E*EA1. (b) A dendrogram of HDAC domain sequences (for details, see Supplementary Data Table 1) along with the four from *D. discoideum* DdHdaA, DdHdaB, DdHdaC and DdHdaD (indicated in bold). Proteins with known structures are underlined and have been used for the alignments shown in c and d. A multiple sequence alignment of DdHdaA and DdHdaB with class I homologues HDLP from *Aquiflex aeolicus* (AaHDLP) and HDAC8 from human (HsHDAC8) is shown in c and that of DdHdaC and DdHdaD with class II homologues HDAH from *Bordetella/Alcaligenes* (B/A HDAH), HDAC4 and HDAC7 from human (HsHDAC4, HsHDAC7) is shown in d. Identical and similar residues are marked with an asterisk and colon, respectively, below the alignment. Residues believed to be important for catalysis are highlighted in grey. The position corresponding to H976 of HsHDAC4 is marked with an arrowhead.

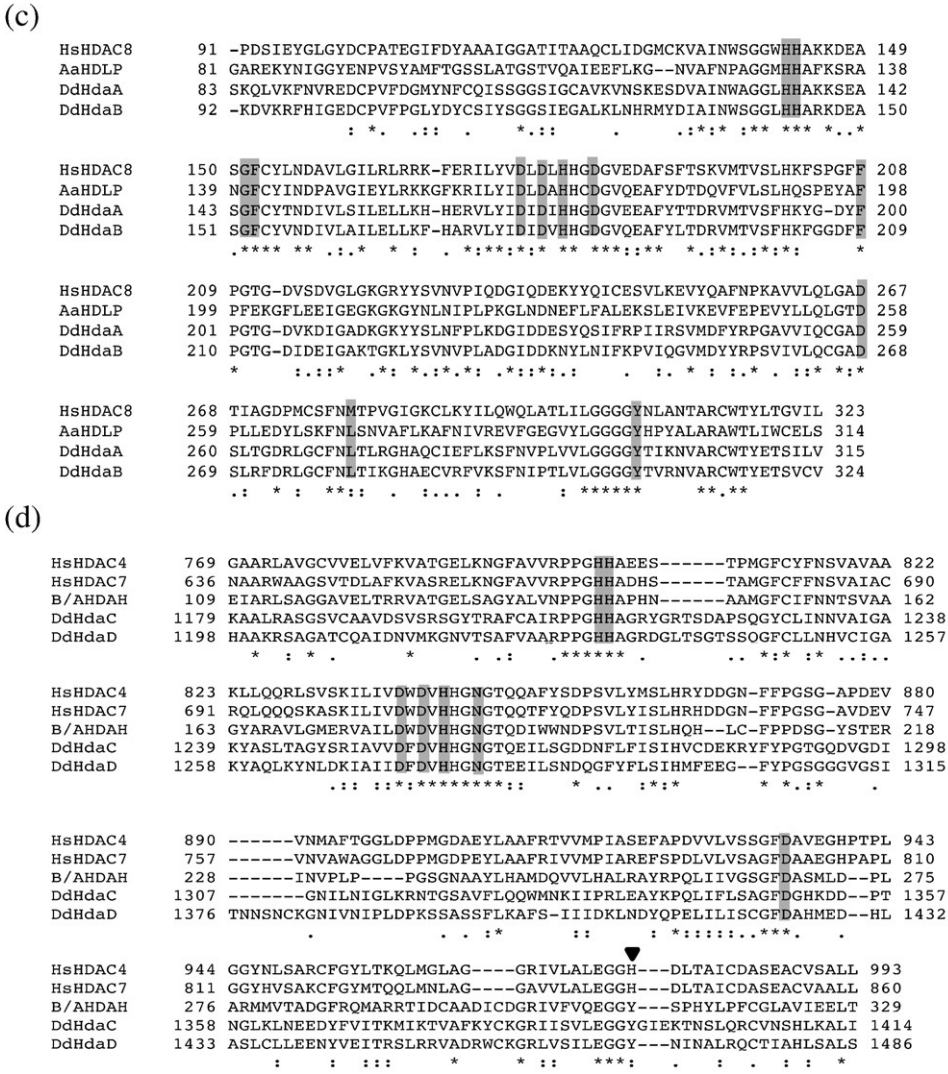


**Fig. 1** (*legend on previous page*)

transition, in order to address questions related to HDAC and development.

*D. discoideum* amoebae live in the soil and feed on bacteria. Following starvation, cells make and release cAMP every ~7 min and aggregate because of chemotaxis towards cAMP released by neighbouring cells.[18] Multicellular development follows and is accompanied by changes in gene expression and intercellular adhesion.[19] Each cell in the aggregate differentiates into one of two types. Prestalk cells die and contribute to an erect stalk, while prespore cells form dormant spores that lie atop the stalk. Up to a certain point, development is reversible: given appropriate conditions, prestalk and prespore cells can transdifferentiate from one to the other or dedifferentiate into the original stem cell-like state.[20,21]

Three features of *D. discoideum* make it an ideal system to study the epigenetic correlates of development: controlled and reversible entry into a developmental process accompanied by large-scale changes in gene transcription,[22] cell fate specification leading to just two cell types and a fully sequenced genome with ease of genetic manipulation.[23] *D. discoideum* histones exhibit diverse modification, such as lysine acetylation and methylation (B. Foeldesi and W.N., unpublished results) similar to that in other eukaryotes.[24] Deletion of one of the histone methyltransferases, *set1*, causes accelerated development and points to a role for chromatin modification in *D. discoideum* development.[25] Global gene transcription data point to a large number of genes regulated by extracellular factors, such as prestarvation factor and cAMP.[26] Many co-regulated genes are clustered in the genome.[23] For example, some genes expressed exclusively in prestalk cells are clustered in a region of chromosome 2;[22] and in the *set1* mutant, misregulated genes are clustered.[25] Thus, there could be a chromatin-level regulation of gene expression during *D. discoideum* development.

In what follows, we report on the roles of HDACs during multicellular development in *D. discoideum*. We identify four HDAC homologues in the genome.

Inhibition of HDAC activity by TSA influences the relative timing of developmental events; intriguingly, the absence of a functional *hdaB* gene gives rise to subtle consequences that come into play only in the context of intercellular competition within chimeras.

## Results

### Identification of putative HDAC homologues in *D. discoideum*

With the aim of identifying genes encoding histone-modifying enzymes, we searched the fully sequenced *D. discoideum* Ax4 genome using both PSI-BLAST and HMM from Pfam. The two approaches suggested that there are four non-sirtuin HDACs encoded by genes that we designate as *hdaA* (gene ID: DDB_G0268024, chromosome 1), *hdaB* (gene ID: DDB_G0270338, chromosome 1), *hdaC* (gene ID: DDB_G0280195, chromosome 3) and *hdaD* (gene ID: DDB_G0279267, chromosome 3). HdaA, HdaB and HdaD have no recognisable domain besides a single HDAC domain; HdaC has an additional FYVE-type zinc finger DNA-binding domain at the N-terminus (Fig. 1a).

We built a dendrogram using sequences of HDAC domains of previously categorised proteins along with those of the four putative *D. discoideum* homologues. The dendrogram grouped HdaA and HdaB along with the known class I HDACs, and HdaC and HdaD as class II HDACs close to yeast Hos3 (Fig. 1b). Both HdaC and HdaD have long N-terminal tails (Fig. 1a), characteristic of class II HDACs and known to regulate nuclear–cytosolic shuttling of the protein. Surprisingly, *D. discoideum* does not have an identifiable class IV HDAC similar to that found in bacteria, mammals and plants.

To see if residues required for the deacetylation reaction are present in the *D. discoideum* proteins, we aligned the domain sequences of class I HDACs HdaA and HdaB with the structurally characterised members of class I, *Aquifex* HDLP and human HDAC8 (underlined in Fig. 1b). The multiple sequence alignment confirmed that residues that co-ordinate the catalytic $Zn^{2+}$ in human HDAC8 (D178, H180 and D267) and HDLP are conserved in the both HdaA and HdaB (Fig.1c). Also, catalytic residues in HDAC8 (H142, H143, D176 and D183) and HDLP that form the Asp-His charge relay system for the deacetylation reaction are present in the two *D. discoideum* proteins (Fig. 1c).[27,28] Similarly, a sequence alignment of the class II members HdaC and HdaD with the well characterised human HDAC4, HDAC7 and *Bordetella/Alcaligenes* HDAH showed that the residues involved in catalysis and $Zn^{2+}$ binding are conserved (Fig. 1d).[8,9,29]

### Characterisation of HDACs

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA from various life stages of *D. discoideum* showed that the putative HDAC-encoding genes are expressed in amoebae (Fig. 2a). The class I HDACs, *hdaA* and *hdaB*, were expressed throughout growth and development, whereas the expression of the class II HDACs, *hdaC* and *hdaD*, was stage-dependent. *HdaC* was expressed after cells entered the multicellular phase upon starvation, and *hdaD* was down-regulated during terminal differentiation of cells into spores and stalk (Fig. 2a).

Having established that *D. discoideum* cells express HDAC-like genes, we sought to confirm the identification by measuring enzyme activity. Cytosolic and nuclear extracts of a mammalian cell line (HEK293) were used for comparison (Fig. 2b). HDAC activity of HEK293 cytosol was comparable to that of the nuclear extract; both were inhibited by TSA, a specific inhibitor of non-sirtuin HDACs.[11] In contrast, when normalised to total protein, *D. discoideum* extracts exhibited much lower activity than HEK293T extracts (Fig. 2b). Moreover, the cytosolic activity in *D. discoideum* was refractory to inhibition by TSA, while the nuclear activity was inhibited completely (Fig. 2b). We analysed the role of the nuclear HDAC activity during unicellular growth of *D. discoideum* by growing amoebae in the presence of different concentrations of TSA for 24 h (roughly two cell divisions). There was no alteration in the growth rate even at a high concentration of TSA (1 μM; Fig. 2c). Next, we assessed histone acetylation status in TSA-treated growing cells by immunoblotting with an anti-acetyl lysine-specific antibody (Fig. 2d). TSA caused an increase in acetylation of a band corresponding to histones as verified by an immunoblot with anti-histone H3 antiserum (lower panel in Fig. 2d). Since histones H2A, H2B, H3 and H4 do not separate during electrophoresis in a 12% (w/v) polyacrylamide gel as used in this experiment, we believe that the band in Fig. 2d (upper panel) represents the total histone pool and that TSA causes a hyperacetylation of all the histones. We have confirmed by mass spectrometry that *D. discoideum* histones are indeed acetylated (W.N., unpublished results).

### Effect of inhibiting HDAC activity on spatial and temporal features of development

We treated freshly starved cells with different concentrations of TSA for 12 h in phosphate buffer. As judged by immunoblotting, TSA induced hyperacetylation of a band corresponding to histones in a concentration-dependent manner (Fig. 2e). By immunoblotting histones isolated from control and TSA-treated cells, we confirmed that proteins with increased acetylation upon TSA treatment of starving cells were indeed histones (Fig. 2f). TSA at a concentration of 500 nM was sufficient to induce high levels of acetylation (Fig. 2e) and was used in further experiments.

Cells developing in the presence of 500 nM TSA showed a delay in development. Inhibitor-treated cells took about 30 h to form fruiting bodies as compared to 24 h taken by vehicle-treated control

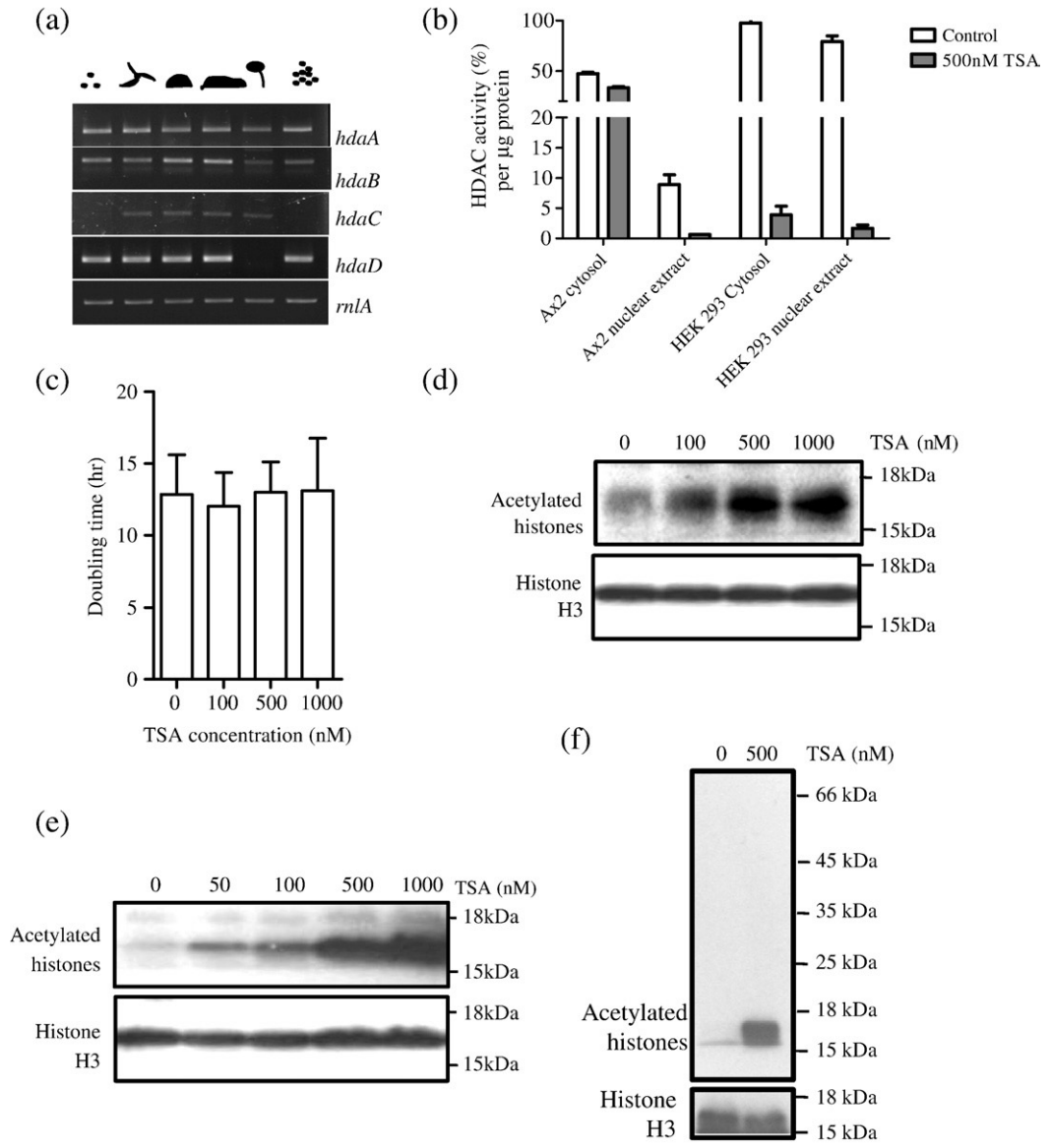


**Fig. 2.** Expression profiles of HDACs in *D. discoideum*. (a) Analysis of expression of the four putative HDAC genes during the *D. discoideum* life-cycle by RT-PCR. rnlA, a ribosomal gene, is the normalising control. Lanes from the left: exponentially growing cells, streaming cells (around 6–7 h post starvation), mounds (12 h post starvation), slugs (16 h), fruiting bodies (24 h), stationary phase cells (12 h post growth to a density of $2 \times 10^7$ cells/ml). (b) HDAC activity in crude extracts of cytosol and nuclei of vegetative amoebae of *D. discoideum* and human embryonic kidney cell line (HEK293T). The activity is normalised to protein amount and shown relative to the mean HEK293T cytosolic activity (mean + SD, $n = 2$). Note the change in the scale on the ordinate. (c) Effect of the indicated concentrations of TSA on the generation time of *D. discoideum* (mean + SD, $n = 2$). (d – f) Immunoblots with cell extracts after treatment with the indicated concentrations of TSA. (d) Vegetative cells incubated with TSA for 24 h. (e) Starving cells incubated with TSA for 12 h in suspension. (f) Purified histones from starving cells incubated with TSA for 12 h in suspension. Blots d – f were probed with anti-acetyl lysine-specific antibody (upper panel) or with anti-histone H3 antibody (lower panel) for normalisation. Molecular mass markers are indicated.

cells (Fig. 3a and b). Nonetheless, the final fruiting body morphology was comparable in the two cases (Fig. 3b). It was of interest to see if the delay caused by TSA was restricted to one or two stages, or was because of a generalised slowing of development. Control cells showed robust streaming by 8 h, formed mounds by 12 h, slugs by 16 h and culminated at 24 h (Fig. 3a and b). When cells were starved on buffered agar with 500 nM TSA, long continuous streams were observed only by 11–13 h, mounds by 18 h, slugs after 24 h and fruiting bodies after about 28 h (Fig. 3a and b). The delay was more significant in the initiation of aggregation by streaming of single cells and formation of mounds from streams, and imperceptible on development beyond the mound stage (Fig. 3a and b). To assess the effectiveness of TSA on histone acetylation during multicellular development on agar, we

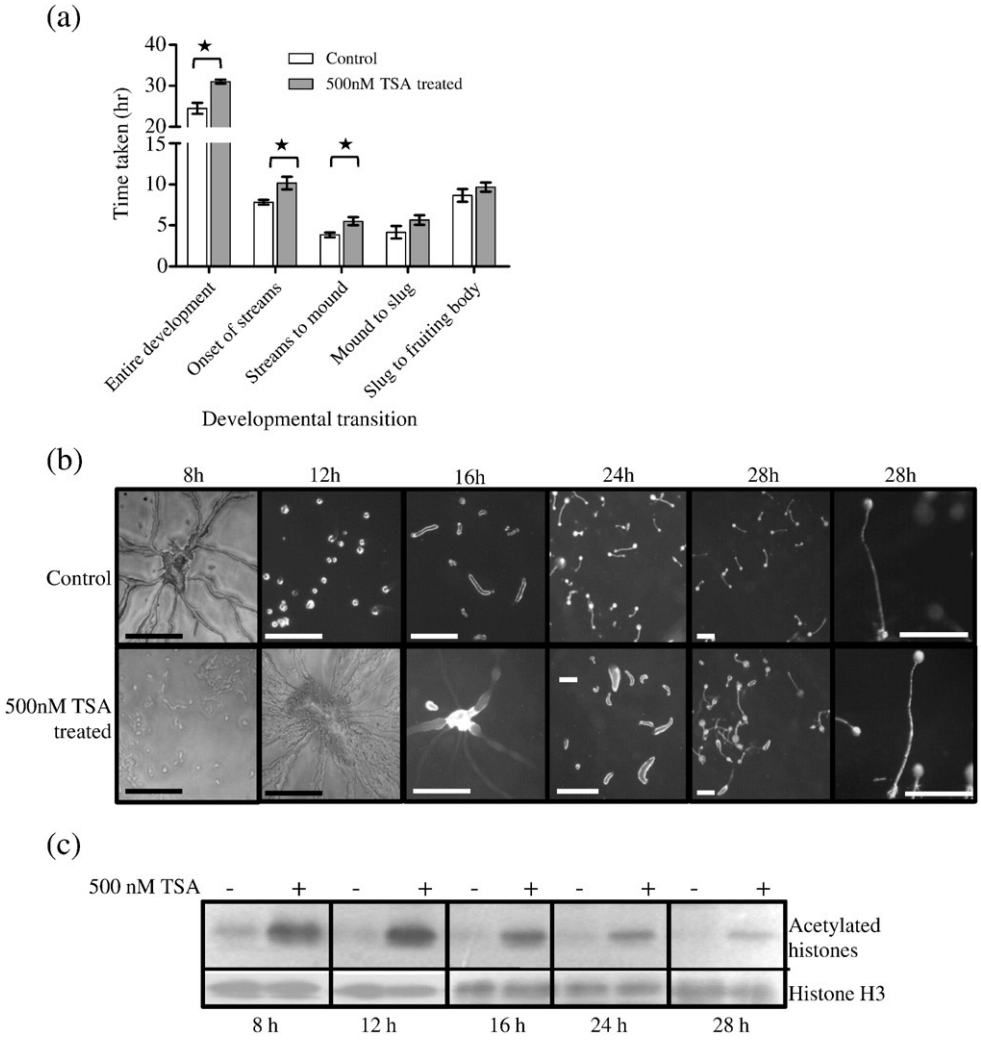


**Fig. 3.** Effect of TSA on the development of *D. discoideum*. (a) Time taken to form the fruiting body and for various developmental transitions in the presence or in the absence of TSA (mean + SD, $n=4$). Asterisks represent statistically significant differences ($p < 0.5$, Student's paired *t*-test). (b) Representative photographs of six independent experiments showing different stages of cells developing without TSA (control) or with 500 nM TSA at the indicated times following the onset of starvation. The scale bars in black represent 100 μm and the scale bars in white represent 250 μm. (c) Immunoblots of extracts from cells that were allowed to develop on agar in the presence or in the absence of 500 nM TSA for the indicated lengths of time. The blots were probed with anti-acetyl lysine-specific antibody (upper panel) or an anti-histone H3 antibody (lower panel) for normalisation.

immunoblotted extracts of cells developing in the absence or presence of 500 nM TSA for various lengths of time (Fig. 3c). TSA induced histone hyperacetylation at all stages monitored (Fig. 3c); as expected (see Figs. 3a and b), the extent of hyperacetylation decreased at the later stages of development.

Besides causing developmental delay, treatment with TSA affected development in many other ways, most obviously with respect to group traits. First, development became markedly asynchronous: at the later stages, many slugs remained on plates when other aggregates had formed fruiting bodies (data not shown). Second, the application of TSA led to a decrease in the number of fruiting bodies per unit area (Fig. 4a). Third, TSA caused a dramatic lowering in sporulation efficiency (Fig. 4a).

## Effect of inhibiting HDAC activity on gene expression

Given that so many aspects of development had been affected, we asked whether treatment with TSA could also affect cell differentiation as monitored by gene expression. We chose two genes as markers of differentiation for this purpose (Fig. 4b). The prestalk marker (*ecmA*) and the prespore marker (*cotC*) were expressed at 12 h (mound stage) in control cells, but only at 15 h upon treatment with TSA (Fig. 4b), as might be expected from the developmental delay shown in Fig. 3b and c. To see more directly if the two cell types suffered similar delays, we estimated their proportions after treating marked strains with 500 nM TSA. Green fluorescent protein (GFP) driven by cell-type specific

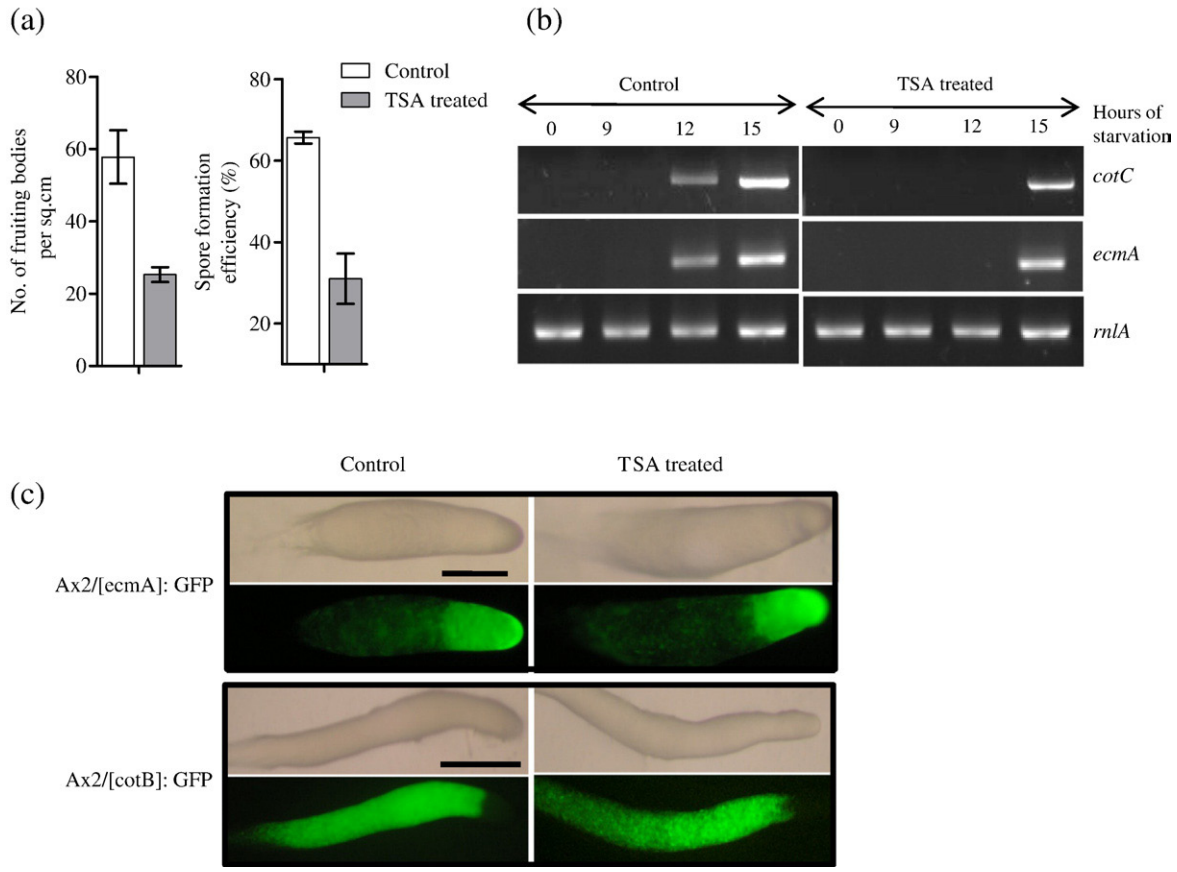


**Fig. 4.** Changes in developmental parameters upon TSA-induced histone hyperacetylation. (a) Effect of TSA on the number of fruiting bodies per unit area and on spore formation efficiency. Values indicate mean + SD ($n=3$). (b) Expression profile of two markers of spatial gene expression, *ecmA* and *cotC*, by RT-PCR in cells developing on agar in the absence or in the presence of 500 nM TSA for the indicated lengths of time. *rnlA* was used as a normalising control. (c) Photographs of spatial localisation of differentiation markers at the slug stage (representative of two independent experiments), indicating similar cell-type proportioning in control and TSA-treated cells. Transgenic cells expressing GFP under *ecmA* and *cotC* promoters were developed in the absence or in the presence of 500 nM TSA and photographed at their respective slug stage. The scale bar represents 250 μm. Slugs formed in the presence of TSA exhibited phototaxis comparable to controls (data not shown).

promoters localised to specific regions of the slug. Prestalk-promoter driven GFP (ecmA-GFP) localised to approximately the anterior one-fifth of the slug and GFP driven by a prespore promoter (cotB-GFP) showed fluorescence in the posterior four-fifths. The spatial distribution of the two markers was comparable in untreated and TSA-treated slugs (Fig. 4c).

The most significant contribution to the slowing of development seemed to occur at or before mound formation (12 h in controls; Fig. 3a). Therefore, we monitored the expression of genes whose activity is implicated in the transition of starving cells into multicellular mounds. Histone acetylation did not change appreciably in the untreated controls over 12 h; however TSA-treated cells showed a steady increase in histone acetylation, reaching a maximum at 12 h (Fig. 5a). Note that of the proteins of different sizes that are recognised by an acetyl lysine-specific antibody, only histone acetylation increased upon treatment with TSA (Fig. 5a). As judged by RT-PCR analysis, the expression of genes involved in cAMP signalling, namely adenylyl cyclase (*acaA*), a cell-surface receptor for cAMP (*carA*) and phosphodiesterase A (*pdsA*) was comparable in control and TSA-treated cells (Fig. 5b). Because the expression of genes involved in cAMP synthesis and degradation was not altered by treatment with TSA, we asked whether the periodic cAMP oscillations that accompany aggregation in *D. discoideum* also remained unaffected.[30] As expected, robust cAMP pulses of comparable intensity and periodicity were detected in both control and TSA-treated cells at the end of 5 h of starvation (Fig. 6a). However, TSA affects one cAMP-dependent cellular phenotype. Growing and freshly starved cellular slime mould cells are amoeboid. After some hours of starvation they respond to endogenously generated chemoattractant gradients, become polarised and appear elongated.[31] Control cells of *D. discoideum* that are starved for 7 h show this morphological change (Fig. 6b). TSA-treated cells remain amoeboid at 7 h, similar to freshly starved cells; they become elongated only by 10 h (Fig. 6b).

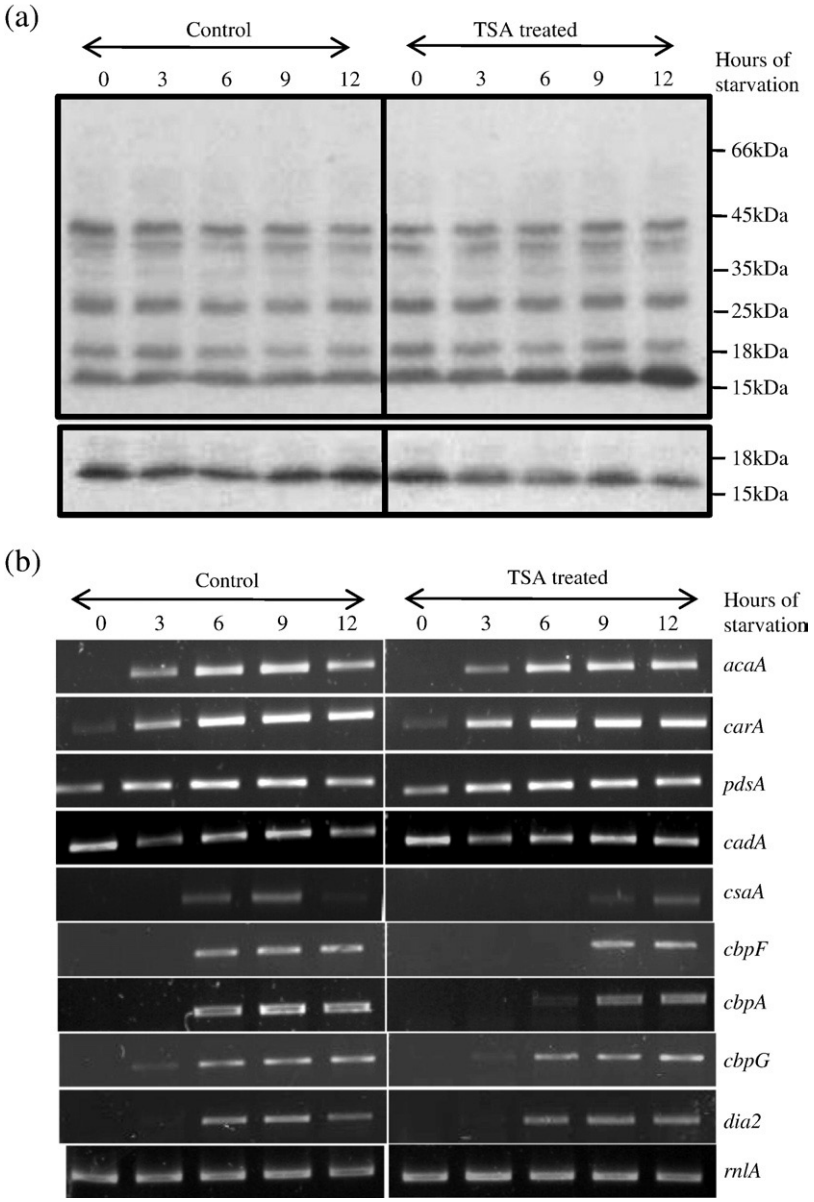


**Fig. 5.** Altered gene expression by TSA-induced histone hyperacetylation during early development. (a) An immunoblot of extracts of freshly starved cells treated with 500 nM TSA for the indicated lengths of time on agar. The blot was probed with anti-acetyl lysine-specific antibody (upper panel) or with an anti-histone H3 antibody (lower panel) for normalisation. Molecular mass is indicated at the right. (b) Expression profile of genes controlling early development in cells starved on agar in the absence or in the presence of 500 nM TSA. Analysis was done by RT-PCR using primers specific to various genes indicated on the right. *rnlA* acts as a normalising control.

Next, we studied the expression of two genes encoding cell-adhesion molecules, which are important for streaming and the formation of multicellular structures. Cadherin A (*cadA*), a gene that encodes an EDTA-sensitive adhesion molecule,[32] was expressed to similar extents in control and TSA-treated cells during the first 12 h of development (Fig. 5b). However, the expression of contact site A (*csaA*), a gene that encodes an EDTA-resistant adhesion molecule,[33] was affected by treatment with TSA. In control cells, the *csaA* transcript can be seen from 6 h to 9 h of starvation, but only a low level of the transcript can be detected after 9 h of starvation in the presence of TSA; the level rises slightly at 12 h (Fig. 5b). In accordance with this finding, a quantitative assay for cell adhesion shows that EDTA-sensitive adhesion is unchanged in the presence of TSA (Fig. 6c), whereas EDTA-resistant cell adhesion appears much later in TSA-treated cells than in controls (Fig. 6d).

Finally, we analysed a set of four genes that require the activation of cAMP-dependent protein kinase A for their transcription.[26] Of the four, two that encode calcium-binding proteins, *cbpF* and *cbpA*, showed a delayed expression upon treatment with TSA as compared to controls (Fig. 5b). Another calcium binding protein-encoding gene, *cbpG*, and a differentiation associated gene, *dia2*, showed a comparable timecourse of expression in control and TSA-treated cells.

## Generation and characterisation of strains lacking HDAC homologues in *D. discoideum*

Earlier observations suggested that the proper timing of aggregation initiation is dependent on normal HDAC activity. In an attempt to narrow down the cause we used bioinformatics and genetics to try and infer which HDAC homologues might be implicated. Hos3 in yeast is insensitive to TSA.[34] The

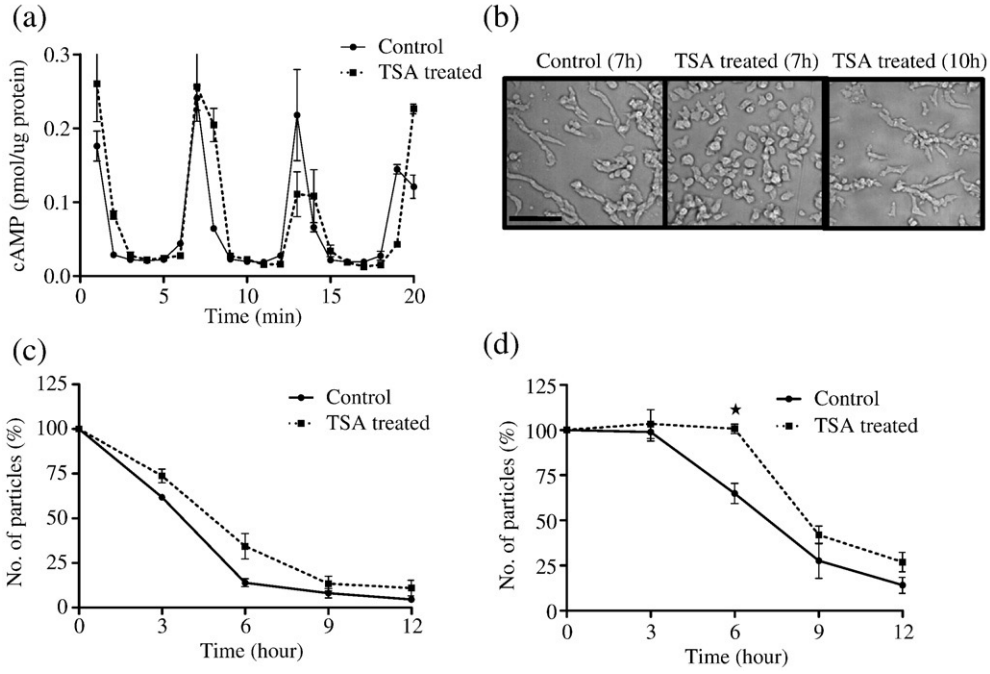


**Fig. 6.** Effect of TSA on factors controlling early development. (a) Quantification of total cAMP (intracellular + extracellular) levels in cell suspensions shaken for 5 h in phosphate buffer with or without 500 nM TSA. Sampling was done every minute for a period of 20 min after 5 h of starvation in suspension. The values are representative of two independent experiments and the profiles are superimposed. (b) Visualisation of starved cells incubated with or without TSA for 7 h on agar. The scale bar represents 50 μm. (c and d) Cell adhesion assay in the absence (c) or in the presence (d) of EDTA (for details, see Materials and Methods). The number of single particles (single cells or cell clumps) is plotted as a percentage of the number at the beginning of the experiment (mean + SD, $n=3$). An asterisk represents statistically significant differences ($p < 0.5$, Student's paired *t*-test).

close proximity of HdaC and HdaD to Hos3 in the sequence dendrogram (Fig. 1b) suggests that these two class II proteins may be resistant to TSA. That made us consider the class I HDACs, which are primarily involved in deacetylation of histones.[6] To estimate the relative contribution of the two class I genes in *D. discoideum* towards cytosolic and nuclear HDAC activity, we tried to knock them out individually and in combination. In spite of screening several thousand clones, the attempt to knock out *hdaA* was unsuccessful (Supplementary Data Fig. 1). In the case of hdaB, on the other hand, two independent knockout clones were obtained and confirmed by PCR (Fig. 7a and b), Southern blot analysis (Supplementary Data Fig. 2) and RT-PCR (Fig. 7c). The absence of a functional *hdaB* did not lead to misexpression of the other HDACs, as analysed by RT-PCR (Fig. 7c). The knockout strains were then analysed for HDAC activity in crude cytosolic and nuclear fractions. Cells deficient in HdaB had about 25% lower nuclear HDAC activity than the wild type (Fig. 7d), while the cytosolic activity of the two strains was comparable. HdaB may thus be localised to the nucleus or it may be present throughout the cell and require a nuclear factor to be catalytically active. When we visualised HdaB (tagged with GFP at its C-terminus) in live cells, fluorescence was restricted to the nucleus (GFP alone showed no specific subcellular localisation; Fig. 7e). The fact that the *hdaB*$^-$ strain has compromised nuclear HDAC activity and that HdaB may be nuclear-localised made us ask whether levels of acetylated histones in the knockout strain are higher than that in the wild type. However, an immunoblot analysis of samples from the wild type and *hdaB*$^-$ cells across all developmental stages failed to show any significant difference in general histone acetylation (Fig. 7f). We then studied various growth and development parameters in the knockout strain. The generation time of the knockout strain was comparable to that of the wild type (Supplementary Data Fig. 3). Also, starvation-induced multicellular development was similar in the wild type and the knockout strain with respect to developmental kinetics (Fig. 8a), cell type proportioning, aggregation territory size and germination efficiency (Supplementary Data Fig. 3). But when cells deficient in HdaB were mixed 1:1 with the wild type and allowed to form chimeric multicellular structures, the knockout cells formed significantly fewer spores than the wild type (Fig. 8b).

### Effect of TSA in the *hdaB*$^-$ and *set1*$^-$ backgrounds

Deletion of the histone methyltransferase *set1* leads to accelerated development,[25] whereas deletion of the histone deacetylase *hdaB* shows normal development (this work). To see if treatment with TSA causes a developmental delay in these two

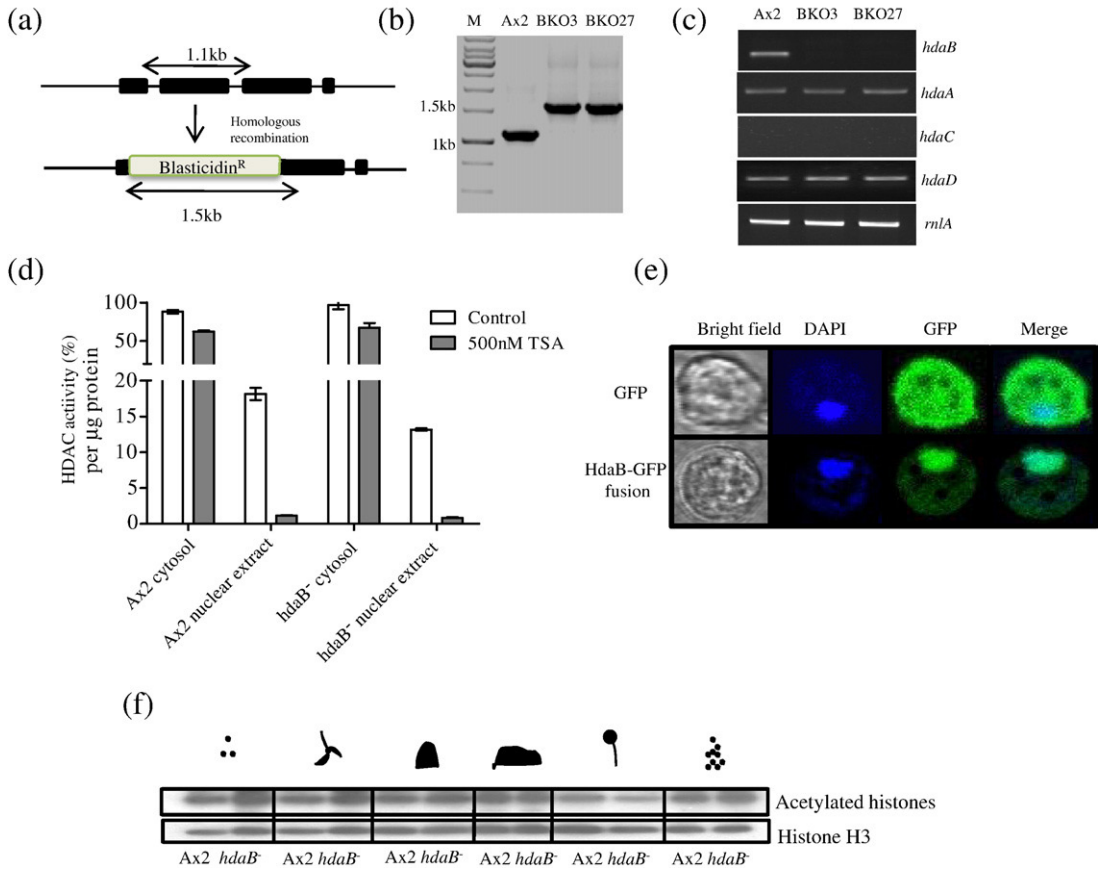


**Fig. 7.** Generation and characterisation of *hdaB* knock-out. (a) Scheme of the knock-out strategy. The upper line depicts a chromosomal copy of *hdaB* in wild type cells with dark boxes representing exons. The expected outcome of recombination of the blasticidin cassette (grey box) flanked by regions of homology is shown in the lower line. The primers used for screening would amplify 1.1 kb and 1.5 kb region from wild type and *hdaB*$^-$ cells, respectively, as shown. (b) PCR amplification with wild type or knockout genomic DNA (two independent knockout strains BKO3 and BKO27). (c) RT-PCR analysis with RNA extracted from Ax2 and the two knockout strains BKO3 and BKO27 using primers specific to various genes indicated at the right. *rnlA* acts as a normalising control. (d) HDAC activity of cytosol and nuclear extract of Ax2 and *hdaB*$^-$ normalised to protein amount and expressed as a percentage in comparison with wild type cytosolic activity. Values are mean + SD ($n=2$). HDAC activity of Ax2 and *hdaB*$^-$ nuclear extracts was significantly different from each other ($p < 0.5$, Student's paired *t*-test). (e) Images of cells expressing GFP alone (top) or HdaB-GFP fusion (bottom) taken by a confocal microscope. (f) An immunoblot of cell extracts of various developmental stages of Ax2 and *hdaB*$^-$ probed with anti-acetyl lysine-specific antibody (top panel) and anti-histone H3 antibody (lower panel). The developmental stages from the left are: exponentially growing cells, streaming cells (around 6-7 h post starvation), mounds (12 h post starvation), slugs (16 h post starvation), fruiting bodies (24 h post starvation), stationary phase cells (12 h post growth to a density of $2 \times 10^7$ cells/ml). Note that the protein loading is unequal across lanes, reflected in histone H3 blot (lower panel) and hence a higher intensity of the acetylated histone band does not indicate an increased histone acetylation.

histone modification mutants, we treated freshly starved cells of the two mutant genotypes with 500 nM TSA. The developmental delay and histone hyperacetylation in the two strains were comparable to what was seen in the wild type (Fig. 8c and d).

## Discussion

According to the current proteome-based phylogeny, multicellularity evolved independently in the social amoebae and the main metazoan lineage (e.g., nematodes, zebrafish and mouse).[35] Common features in the development of these diverse groups may point to evolutionary convergence *via* the independent deployment of ancestral "tool-kits".[35] In this study, we attempted to understand how a conserved process of protein acetylation participates in the ontogeny of *D. discoideum*.

### Genomic repertoire of protein acetylation-based signalling in *D. discoideum*

*D. discoideum* harbours many genes encoding proteins that control acetylation, e.g. HATs, HDACs and bromodomain-containing proteins that bind specifically to acetylated lysine in a protein.[36] *D. discoideum* homologues of several known HDAC substrates such as histones, Hsp90 and tubulin show conservation of lysine residues that are acetylated in

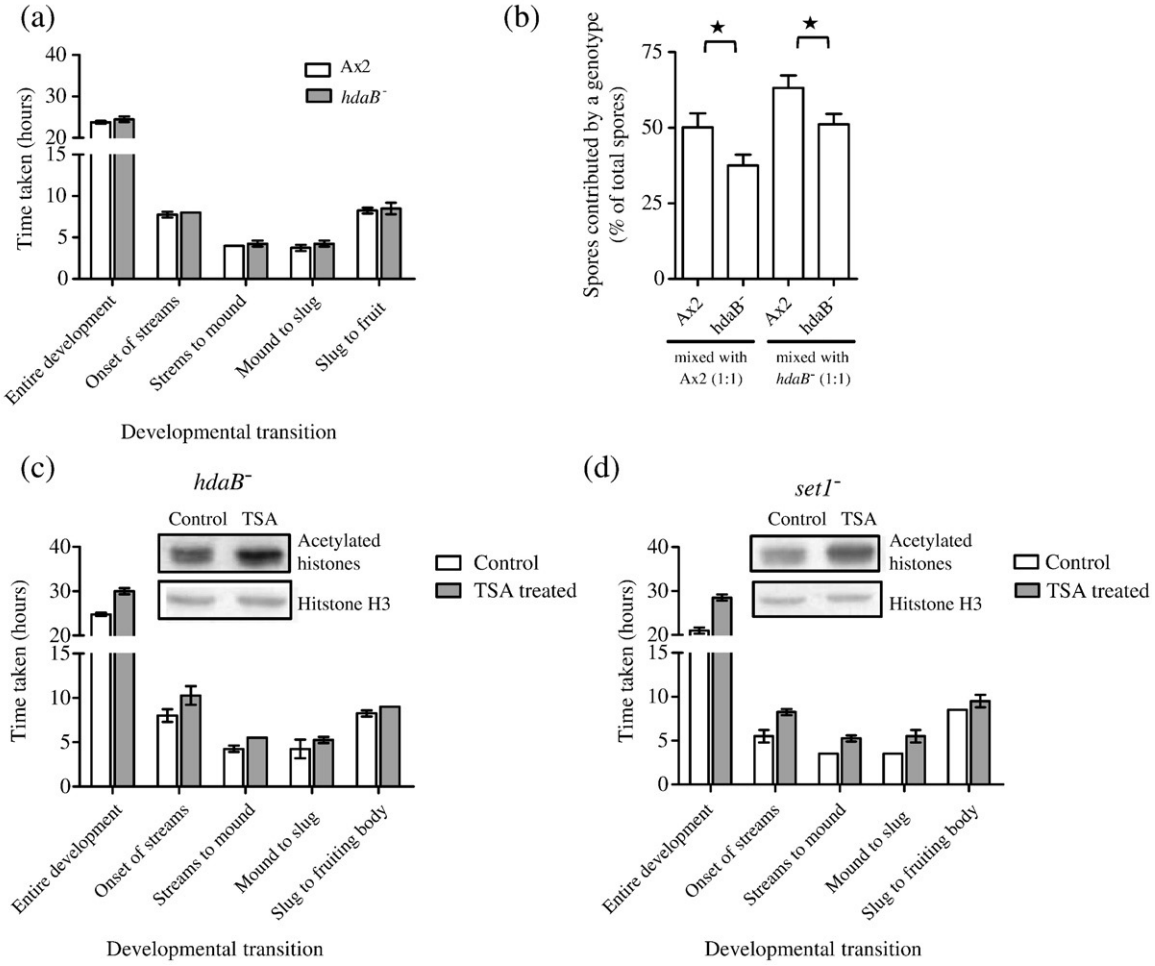


**Fig. 8.** Developmental parameters of histone modification mutants. (a) Time taken to form the fruiting body and for various developmental transitions of *hdaB*⁻ cells in comparison with Ax2 cells. Values are mean + SD ($n=5$). (b) Outcome of Ax2 and *hdaB*⁻ mixing experiment. Percentage of spores formed by a genotype mentioned immediately below *X*-axis when mixed 1:1 with the indicated genotypes. Values are mean + SD ($n=3$). Asterisks represent statistically significant differences ($p<0.5$, Student's paired *t*-test). (c and d) Effect of TSA on time taken to complete various developmental stages in *hdaB*⁻ (c) and *set1*⁻ (d). Values are mean + SD ($n=4$). Inset: respective immunoblots showing effects of TSA at the end of 12 h treatment of the indicated strains that had developed on agar.

other systems.[37,38] Hsp90 activity regulates *D. discoideum* development,[39] which means that modulation of Hsp90 activity by acetylation at a conserved lysine may influence development. This makes it plausible that *D. discoideum* utilises acetylation more generally as a post-translational modification in signal transduction.

## Possible roles of HDAC homologues in *D. discoideum*

The high level of sequence similarity of the two class I HDAC proteins with their well characterised counterparts suggests that HdaA and HdaB are TSA-sensitive, nuclear localised, and use histones as substrates.[6] Supporting this, we find a nuclear localisation of HdaB-GFP fusion protein (Fig. 7e) and a decrease in the TSA-sensitive nuclear HDAC activity upon *hdaB* deletion (Fig. 7d). This suggests that TSA inhibits both HdaA and HdaB and leads to histone hyperacetylation. We could not demonstrate this directly because neither protein could be expressed in a folded form in *Escherichia coli* (data not shown). HdaA could not be expressed as a GFP-fusion protein even in *D. discoideum*, possibly due to interference from a poly-asparagine tract at its C-terminus (data not shown).

Despite a decrease in HDAC activity in *hdaB*⁻, cells did not show major alterations in gross histone acetylation levels (Fig. 7f). The apparent contradiction can be rationalised in three ways. First, HdaB activity may be directed towards non-histone substrates in the nucleus. Second, there may be a functional overlap of the two class I HDACs under laboratory conditions (so that HdaA compensates for a lack of HdaB in regulation of histone acetylation). Third, there is only a minor difference in the levels of histone acetylation in the wild type and *hdaB*⁻, reflecting a 25–30% difference in HDAC activity (Fig. 7d); the effects on growth and development are insignificant (Supplementary Data Fig. 3). The technique of immunoblotting may not

resolve this difference in the steady-state levels of histone acetylation. A sensitive and quantitative technique will be required to resolve this issue. In *Aspergillus nidulans*, HDACs regulate clusters of genes involved in secondary metabolite diversity,[40] and in *D. discoideum* too, HdaB may be required for the regulation of genetic loci involved in restricted functions such as secondary metabolism.

Interestingly, *hdaB*$^-$ cells develop normally by themselves but are handicapped when examined in a social context. When *hdaB*$^-$ cells are mixed with wild type cells to form chimeras, they make significantly fewer spores than expected (Fig. 8b). These observations can be explained on the hypothesis that *hdaB*$^-$ and wild type cells differ with regard to traits that are not essential for viability but are important for determining the outcome of social interactions. Intercellular adhesion and long-range cell-to-cell signalling are examples. An alteration in such a trait may lead to no discernible consequence among a clone; the relative fitness of a cell will remain the same. However, the alteration could enhance phenotypic variation, and so re-order relative fitness, in a genetically heterogeneous group. Epigenetic regulation of development may serve to reduce phenotypic differences between the individuals that comprise a group (Bonner's "range variation"[41]). Thereby, they might ensure a reliable developmental outcome in diverse physical or biotic environments. This has been shown to be true of Hsp90 in *D. discoideum*[39]; it may be valid in the case of HDACs.

The class II members of *D. discoideum*, HdaC and HdaD, possess all the conserved residues required for catalysis in class II HDACs. The residue corresponding to H976 of human HDAC4 (marked with an arrowhead in Fig. 1d) is occupied by tyrosine in *D. discoideum* (Y1394 in HdaC and Y1469 in HdaD). The presence of tyrosine instead of histidine at this position allows a protein to use acetylated lysine as a substrate with greater efficiency.[42] HdaC and HdaD may use proteins acetylated at lysine as a substrate rather than small non-protein molecules carrying an acetyl modification. The catalytic domains of HdaC and HdaD have the greatest sequence similarity among those checked to that in yeast Hos3 (Fig. 1b). Hos3 belongs to a fungi-specific subgroup of class II HDACs that is TSA-insensitive[34,43]. We believe that HdaC and HdaD are localised in the cytosol and contribute to the TSA-insensitive cytosolic HDAC activity (Fig. 2b). Both proteins have long N-termini (Fig. 1a) that are believed to help in nucleo-cytosolic shuttling. Interestingly, there are many proteins in the range of 25 – 45 kDa that are recognised by anti-acetyl-lysine antibody on immunoblots and their acetylation status remains unchanged in the presence of TSA (Fig. 5a). These could well be substrates of the two class II proteins in the cytosol. Unlike *D. discoideum*, mammalian cells that have a high TSA-sensitive cytosolic HDAC activity (Fig. 2b) show an increased acetylation of several cytosolic proteins upon treatment with TSA.[44]

## Effects of TSA on histone acetylation, growth and development

The histone hyperacetylation caused by HDAC inhibition shows that cellular HATs are active in *D. discoideum*. The increased histone acetylation induced by TSA was more pronounced in starving (Fig. 3a) than in growing cells (Fig. 2d). This may reflect differences in the HAT activity in starving cells compared to vegetative cells. Alternatively, growing cells may have efficient mechanisms to get rid of xenobiotics like TSA. Another possibility is that TSA remains bound to growth medium components and is not readily available for targeting HDACs inside vegetative cells. Despite a moderate effect on histone acetylation in vegetative cells, TSA did not affect their growth (Fig. 2c). This finding is in contrast to an earlier report showing that sodium butyrate, an HDAC inhibitor, slows growth and causes histone hyperacetylation.[45] However, the latter results are difficult to interpret, as the high concentration of sodium butyrate used there (10 mM) may have affected growth by interfering with metabolism, and not *via* histone hyperacetylation. The finding that TSA does not change the growth rate in *D. discoideum* is in contrast to the observation that TSA inhibits growth of the closely related parasitic amoeba *Entamoeba histolytica*.[46]

Histone acetylation levels do not change appreciably during the course of normal development (Supplementary Data Fig. 4). Upon treatment with 500 nM TSA, however, starving cells exhibited a profound increase in histone acetylation and developmental delay (Fig. 3). The delay was in visible changes in morphology and in transcriptional changes that accompany differentiation (Fig. 4b), even though morphogenesis and differentiation are decoupled in *D. discoideum*.[47, 48] However, the delay in differentiation did not lead to improper proportioning of cell type (Fig. 4c). The marked decrease in spore-forming efficiency of TSA-treated cells was not because fewer cells took part in aggregation. Many cells may have been shed during slug migration or later, possibly due to a misregulation of cell adhesion molecules (see below).

## TSA-induced histone hyperacetylation and misregulation of genes

We have analysed the status of histone acetylation by using an anti-acetyl lysine antibody that does not differentiate between different sites of acetylation on different histones. Many studies have suggested that TSA-induced hyperacetylation occurs on all the sites of acetylation on the core histones and total acetylation of histones can serve as a good indicator of an increase in acetylation at individual sites.[49] Although a higher level of histone acetylation is believed to lead to increased transcription,[5] we found that a few genes were down-regulated upon TSA-induced hyperacetylation (Fig. 5b). This may be due to an increased level of a repressor whose

synthesis is transcriptionally activated by TSA. Alternatively, as has been suggested recently, histone acetylation may not be strictly linked to gene upregulation.[50] Several genes in cancer cell lines are specifically down-regulated despite TSA-induced histone hyperacetylation in the promoter region.[50] The mechanism underlying this phenomenon is not clear.

In *D. discoideum*, starvation and quorum sensing lead to an increase in transcription of genes responsible for cAMP pulses, namely *acaA*, *carA* and *pdsA*.[51] The fact that the regulation of these genes was unaltered upon treatment with TSA (Fig. 5b) suggests that cells were able to sense starvation appropriately. In fact TSA-treated cells exhibited robust oscillations in cAMP levels at 5 h post starvation, comparable to the controls (Fig. 6a; different profiles have been superimposed to emphasise the point). This is in contrast to the histone methyltransferase mutant *set1*$^-$, which shows precocious expression of *acaA* and early aggregation.[25] This indicates that histone methylation and acetylation influence *D. discoideum* development differently; *set1*$^-$ cells also show delayed development when starved in the presence of TSA (Fig. 8b), placing histone methylation and acetylation in parallel pathways that control early development.

### Regulation of a subset of cAMP-induced genes by HDAC activity

While cAMP oscillations are normal, cell polarisation and adhesion, both dependent on cAMP signalling,[31] are affected by TSA, as is the profile of developmental gene expression (Figs. 5 and 6). At least 15 genes are strongly induced by extracellular pulses of cAMP in a protein kinase A (PK-A)-dependent manner.[26] The three genes found to be misregulated upon treatment with TSA in this study—*csaA*, *cbpA* and *cbpF*—belong to this class. Further, both HdaA and HdaB have phosphorylation sites that are putative substrates of PK-A (our unpublished results). This suggests that, as in mammalian cells[6], TSA-sensitive HDACs sense PK-A activity and up-regulate the expression of genes in *D. discoideum*.

*cbpA* and *cbpF*, two genes that code for calcium-binding proteins, were misregulated upon treatment with TSA, suggesting an alteration in calcium signalling in TSA-treated cells. A deletion of this gene results in delayed development.[52] The TSA-induced developmental delay could be due to a delayed expression of *cbpA*. *cbpF* is in a cluster of four genes, all of which are regulated by cAMP.[26] We tested whether TSA misregulates the entire cluster of genes by analysing the expression profile of another gene, *cbpG*, located adjacent to *cbpF*. It remained unaffected (Fig. 5b), arguing that only a specific subset of cAMP and PK-A-regulated genes require HDAC activity for their transcription. A genome-wide expression profiling would be required to assess the extent of gene misregulation caused by TSA.

### Epigenetic regulation of early *D. discoideum* development

As seen, a decrease in HDAC activity causes a delayed expression of cAMP-regulated genes and, consequently, delayed differentiation and morphogenesis. Thus HDAC activity is crucial in controlling the most important developmental transition in *D. discoideum*, the initiation of aggregation. This transition can be reversed by supplying food to cells.[20,21] HDAC activity may serve as a way of keeping certain regions of chromatin poised for a quick transcriptional switch that can be readily modulated by extracellular cues.

There are two broad implications of our findings. The first pertains to heterochrony; namely, an alteration in the temporal profile of developmental events. Heterochrony has long been considered a possible route for the origin of evolutionary novelties and its importance has been emphasised in the specific context of the cellular slime moulds.[53] One means of achieving heterochrony would be to affect the timing of gene expression with respect to some genes but not others, which is what happens when HDAC activity is inhibited (Fig. 5b). Our study has shown that histone modification plays a significant role in the multicellular development of *D. discoideum* by influencing the temporal regulation of genes. This may point to a more general link between chromatin status and heterochrony. The second implication follows from the observation that there is no obvious effect of knocking out *hdaB* on growth or development. However, the efficiency of spore formation by *hdaB*$^-$ cells is depressed severely in *hdaB*$^-$–Ax2 chimeras (Fig. 8): a mutant with what appears to be a null phenotype turns out to be handicapped when forced to develop (and compete) with the wild type. This reinforces the point that the evolutionary consequences of a mutation depend on its effect on fitness over the entire set of relevant physical and biotic (here, social) environments.

## Materials and Methods

### Bioinformatics

Genes encoding putative HDACs in the *D. discoideum* genome were accessed online† and searched with BLAST using human, yeast and plant HDACs as a query. The pfam database of protein families groups non-sirtuin-type HDACs in a hidden Markov model (HMM, PF00850) built on 170 seed sequences.[54] The *D. discoideum* protein sequences were analysed using this HMM with a cut-off of 0.1 in the E-value. Multiple sequence alignments of catalytic domains of selected proteins were done using ClustalW2.[55] A dendrogram of the HDAC domains of various annotated proteins[7] from archea, eubacteria, plants, fungi and animals was built with the neighbour-joining method using MEGA 4.0 software. Branches with more than 50% support in a bootstrap test were retained.

† www.dictybase.org

### HDAC activity assay

The HDAC activity of HEK293 and *D. discoideum* cells was quantified using an HDAC assay kit (Cayman Chemical Co., USA; cat no. 10011563). Preparation of cell lysates and activity measurements were done according to the manufacturer's protocol with the following modifications in the assay conditions: the peptide substrate concentration was 32 μM, the reaction volume was 42 μl and the amount of cell lysate per reaction was 20 μg. Fluorescence was quantified with a 0.1 s time window in a Victor microplate reader (Perkin Elmer, USA).

### Cell culture and development

*D. discoideum* strains (Ax2, knockouts and transgenics) were grown as suspensions in HL5 at 22°C.[56] Cells were transformed by electroporation with the indicated DNA constructs.[57] Neomycin- and blasticidin-resistant transformants were grown in 15 μg $ml^{-1}$ G418 (Sigma, USA) and 5 μg $ml^{-1}$ blasticidin (MP Biomedicals, USA). To initiate multicellular development, exponentially growing amoebae ($2 \times 10^6 - 4 \times 10^6$ cells $ml^{-1}$) were washed with 16 mM potassium phosphate buffer (pH 6.4), deposited on buffered agar at a density of $5 \times 10^5$ cells $cm^{-2}$ and incubated in dark humid chambers at 22°C. To test the effect of different concentrations of TSA on growth or development, TSA (Sigma) was added to HL5 or phosphate buffer at the indicated concentrations from a 1 mM stock solution; equivalent amounts of DMSO were added as vehicle controls. For growth studies, cells were inoculated at $5 \times 10^5$ cells $ml^{-1}$ and followed for three days. For developmental studies, 500 nM TSA was incorporated in the phosphate-buffered agar. Spore formation efficiency was calculated as the number of spores formed divided by the number of cells plated for development (after accounting for a small post-starvation increase in cell number).

### Immunoblotting, histone extraction and gene expression analysis by RT-PCR

Cells from various stages were lysed with 1% (w/v) SDS followed immediately by heating to 90°C to inhibit proteases. Samples were stored at –20°C. Protein estimation was done using a micro-BCA kit from Pierce (USA): 20 μg of proteins was separated by electrophoresis in 12% (w/v) polyacrylamide gels and blotted onto a PVDF membrane. Note that individual histones are not resolved and appear as a single band (unpublished results). The blots were probed with either anti-acetyl lysine mouse monoclonal antibody (Cell Signalling Technology, USA; cat. no. 9681S) or anti-human histone H3 rabbit polyclonal antiserum (Active Motif, USA; cat. no. 39163). HRP-conjugated secondary antibodies were purchased from GE Healthcare (USA).

Histones were extracted from 12 h-starved cells by acid extraction of isolated nuclei (C Pears, personal communication). About $5 \times 10^7$ cells were washed with cold phosphate buffer and lysed with 0.6% (v/v) Triton X-100 at 4°C for 10 min in the presence of Protease Inhibitor Cocktail (Sigma, USA) and 10 mM sodium butyrate (HDAC inhibitor) in lysis buffer (50 mM Tris pH 8, 10 mM NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 0.5 M sorbitol). Nuclei were separated from cytosol by centrifuging at 10,000*g* for 5 min at 4°C and were washed twice with the lysis buffer containing protease and HDAC inhibitors. Acid extraction of nuclei was done by incubating isolated nuclei in 150 μl of 0.4 M HCl at 4°C for 1 h. The supernatant containing histones was neutralised with NaOH and used for immunoblotting.

RNA was isolated from various stages of development using TRI reagent (Sigma, USA) as per the manufacturer's protocol and subjected to oligo(dT)-primed first strand cDNA synthesis using MMLV reverse transcriptase (Promega, USA). PCR was performed on cDNA using Taq polymerase (Bangalore Genei, India) and gene-specific primers (the primer sequences are available in Supplementary Data Table 2). The PCR cycle numbers were standardised for each pair of primers such that the amplification was in a non-saturating range.

### Cell adhesion assay and cAMP measurements

Developing cells were harvested from buffered agar plates every 3 h after starvation and resuspended in phosphate buffer (containing 10 mM EDTA if needed) at a density of $10^7$ cells $ml^{-1}$. Cell clumps were vortex mixed and incubated for 30 min at 22°C for monitoring cell adhesion. At the end of incubation, single particles were counted in a haemocytometer.[58]

For measuring cellular cAMP levels, exponentially growing cells were washed, suspended in phosphate buffer at a density of $10^7$ cells $ml^{-1}$and shaken at 150 rpm. After starvation for 5 h under these conditions, 50 μl of suspension was removed and immediately added to 50 μl of 0.2 M HCl at 65°C to inactivate cellular phosphodiesterases. Sampling was carried out every minute for 20 min. Total cAMP (intra- and extra-cellular) was measured by a radioimmunoassay[59] and was normalised to cellular protein content as estimated by Bradford reagent (Sigma, USA).

### Generation of *hdaB* knockout and subcellular localisation of *hdaB*

Details of the primers used in this study are given in the Supplementary Data. The *hdaB* knockout construct was created by cloning two regions of homology flanking the blasticidin-resistance cassette in the pLPBLP plasmid.[60] The region corresponding to the *hdaB* gene between –700 and +47 (numbering with the start codon ATG at position +1) was cloned at the KpnI and HindIII sites of pLPBLP using primers hdaBKO1 and hdaBKO2, respectively. The region between +1061 and +1988 was cloned at the PstI and NotI sites of pLPBLP using primers hdaBKO3 and hdaBKO4, respectively. The cloned insert was released by KpnI and NotI, gel-purified and electroporated in *D. discoideum*. A successful recombination event removed part of the *hdaB* gene between +47 and +1061 (Fig. 7a), which comprises the region coding for the catalytic domain. Blasticidin-resistant clones were selected and confirmed to be knockouts by PCR and RT-PCR using *hdaB*-specific primers. The *hdaA* knockout construct was generated in a similar way (for details, see both Table 1 and Fig. 1 in the Supplementary Data ).

For subcellular localisation of HdaB, a full-length *hdaB* gene was amplified from cDNA made from vegetative cells using primers:

forward 5′ *GTCGAC*ACTGCAGAAATGGAATATAA-TACAATTTTA-AAC 3′
and reverse 5′ TTA*GGATCC*ACTAAAATCAT-TATGCTTTTTCTTTC 3′

with SalI and BamHI sites italicised. The PCR fragment was sub-cloned in pDNeo2a between SalI and BamHI

sites upstream of the GFP gene to make a fusion protein with GFP at the C-terminus. Ax2 cells were transformed with pDNeo2a-*hdaB*-GFP by electroporation.[57] Cells expressing GFP-fusion protein were visualised using a confocal microscope.

#### Ax2/*hdaB*⁻ mixing experiment

Freshly starved cells of each genotype were stained with CellTracker Blue (Molecular Probes, USA), mixed with unstained cells of the other genotype in a 1:1 ratio and allowed to develop together. Fruiting bodies were monitored and fluorescent spores were counted.

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## Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.06.067

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