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Developmental timing in *Dictyostelium* is regulated by the Set1 histone methyltransferase

Jonathan R. Chubb [a,h,i,*], Gareth Bloomfield [b,c], Qikai Xu [d,e], Markus Kaller [f], Al Ivens [b,c], Jason Skelton [b,c], Bryan M. Turner [g], Wolfgang Nellen [f], Gad Shaulsky [d,e], Robert R. Kay [b,c], Wendy A. Bickmore [h], Robert H. Singer [a]

[a] *Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, The Bronx, NY 10461, USA*
[b] *MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*
[c] *The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK*
[d] *Graduate Program in Structural and Computational Biology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030, USA*
[e] *Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA*
[f] *Abt. Genetik, Universität Kassel, Heinrich-Plett-Str. 40, D-34132 Kassel, Germany*
[g] *Chromatin and Gene Expression Group, Anatomy Department, University of Birmingham Medical School, Birmingham B15 2TT, UK*
[h] *MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK*
[i] *Division of Cell and Developmental Biology, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK*

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Abstract

Histone-modifying enzymes have enormous potential as regulators of the large-scale changes in gene expression occurring during differentiation. It is unclear how different combinations of histone modification coordinate regimes of transcription during development. We show that different methylation states of lysine 4 of histone H3 (H3K4) mark distinct developmental phases of the simple eukaryote, *Dictyostelium.* We demonstrate that the enzyme responsible for all mono, di and tri-methylation of H3K4 is the *Dictyostelium* homolog of the Set1 histone methyltransferase. In the absence of Set1, cells display unusually rapid development, characterized by precocious aggregation of amoebae into multicellular aggregates. Early differentiation markers are abundantly expressed in growing *set1* cells, indicating the differentiation program is ectopically activated during growth. This phenotype is caused specifically by the loss of Set1 catalytic activity. *Set1* mutants induce premature differentiation in wild-type cells, indicating Set1 regulates production of an extra-cellular factor required for the correct perception of growth conditions. Microarray analysis of the *set1* mutants reveals genomic clustering of mis-expressed genes, suggesting a requirement for Set1 in the regulation of chromatin-mediated events at gene clusters.

Keywords: Chromatin; Dictyostelium; Set1; Lysine 4 methylation; Gene cluster; Clock

Introduction

Spatially and temporally coordinated gene expression requires structural reorganization of chromatin architecture, to permit or block access of regulatory proteins to DNA. To remodel chromatin architecture during development, eukaryotic cells use enzymes that covalently modify histones by phosphorylation, acetylation, methylation and ubiquitinylation. Another mechanism for changing chromatin involves the deposition of histone variants, which displace existing histones, and the modifications they carry. In addition, there are ATP-hydrolyzing chromatin-remodeling enzymes capable of sliding or displacing nucleosomes.

A key question is how chromatin modifications operate to regulate developmental gene expression. A chromatin-modifying enzyme could act at individual genes that are dispersed in

* Corresponding author. Division of Cell and Developmental Biology, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK.
E-mail address: j.chubb@dundee.ac.uk (J.R. Chubb).

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the genome, or might act locally, for instance at co-regulated gene clusters. Indeed, large proportions of the genomes of eukaryotes consist of clusters of co-expressed genes (Boutanaev et al., 2002; Lercher et al., 2002; Spellman and Rubin, 2002). Some modifications spread locally along chromatin, in part due to the self-interaction of chromatin proteins and the potential for some modifying enzymes to interact with their cognate modification while marking nearby nucleosomes (Danzer and Wallrath, 2004). Other modifications are more punctate in their genomic distribution (Bernstein et al., 2005), so their effects could be distributed throughout the genome, rather than involving specialized chromatin events at gene clusters. One view is that a chromatin modification or combination of modifications marks a battery of genes specific to a particular program of cellular differentiation (Kurdistani et al., 2004). This reflects the histone code hypothesis (Strahl and Allis, 2000), which takes into account the large number of post-translational modifications of histones and the combinatorial potential of these modifications to specify different programmes of gene expression.

To address the requirement for different chromatin modifications in the regulation of developmental gene expression ideally requires a genetically tractable developmental model. Genetic studies in higher eukaryotes have proven difficult to interpret because of the complexity of these developmental systems. In contrast, analysis in yeast is hampered by a lack of developmental options. We therefore decided to address the role of histone modification in development using the simple eukaryote *Dictyostelium*. It has a simple and well-defined developmental programme, and a small and fully sequenced genome (Eichinger et al., 2005) that encodes chromatin proteins and modifying enzymes conserved with those in higher eukaryotes. In addition, the organism is highly amenable, with powerful haploid molecular genetics (De Lozanne and Spudich, 1987). Individual *Dictyostelium* cells enter their program of differentiation upon nutrient depletion. Upon starvation, the cells aggregate, using cAMP as a chemoattractant, into a multicellular mound. After a series of morphogenetic steps, cells in the aggregate adopt predominantly only two developmental fates. The final developed structure consists of a spore head, containing approximately 80% of the cells, held over the substrate by a thin stalk structure, containing the remaining 20% of cells.

In this study, we have analyzed the developmental role of the histone methyltransferase (HMTase) required for methylation of the lysine 4 residue of histone H3 (H3K4). Methylation of H3K4 is associated with active chromatin in a wide range of eukaryotes. Lysine methylation of histones can occur in three states: mono-, di- and tri-methyl (Strahl et al., 1999). Dimethylation of H3K4 (H3K4me2) is associated with euchromatic regions, but not silent heterochromatic states (Noma et al., 2001), while trimethyl H3K4 (H3K4me3) is present exclusively at active genes (Santos-Rosa et al., 2002) and is a mark laid down during transcriptional elongation by RNA polymerase II (Ng et al., 2003). The chromatin states marked by monomethyl H3K4 (H3K4me1) are not known. In the yeast *S. cerevisiae*, the Set1 HMTase is required for di- and tri-methylation of H3K4 (Santos-Rosa et al., 2002) and is recruited to coding regions by the transcription elongation complex (Hampsey and Reinberg, 2003). Set1 yeast mutants have defects in ribosomal and telomeric silencing, and DNA repair (Briggs et al., 2001; Bryk et al., 2002; Kanoh et al., 2003; Krogan et al., 2002). A *Drosophila* H3K4 HMTase, TRR, is required for morphogenetic furrow progression during embryogenesis and retinal development (Sedkov et al., 2003), although the absence of TRR does not remove all meH3K4, since *Drosophila* has two additional H3K4 HMTases, Ash1 and TRX.

Here, we show that different levels of methylation of H3K4 mark different developmental phases of *Dictyostelium*. We have identified the *Dictyostelium* homologue of Set1, and show that s*et1* mutant cells completely lack mono, di and tri-methylation of H3K4. Loss of Set1 gives rise to cells displaying unusually rapid development, characterized by precocious aggregation of cells into multicellular aggregates. Early starvation markers are abundantly expressed during growth, indicating the mutants inappropriately enter the differentiation program, even in the presence of nutrients. We demonstrate a strong non-autonomous component to the developmental timing phenotype, indicating Set1 regulates production of an extra-cellular factor required for the correct perception of growth conditions. Microarray analysis of the *set1* mutants indicates many of the mis-expressed genes are tightly grouped in the genome, suggesting Set1 may act to regulate chromatin-mediated events at gene clusters.

Materials and methods

Disruption of the set1 locus

Dictyostelium AX2 cells and their derivatives were grown, developed and transformed as described (Chubb et al., 2000). To disrupt the *set1* locus, we inserted a blasticidin resistance cassette into the *Swa*I site of *Dictyostelium* genomic clone JC1b237cO3, which spans the 3′ end of the *set1* gene. The *Swa*I site is upstream of the SET domain, so insertion renders the Set1 protein catalytically inactive. *Bgl*II linkers were inserted into the *Swa*I site, and a *Bam*HI fragment containing the blasticidin resistance cassette was inserted into the linkered site. A targeting fragment was released by digestion of *Bam*HI and *Kpn*I sites in the polylinker. To assemble a Set1 genomic clone, we inserted the *Sac*I/*Cla*I fragment from Dict_IV_V660d03 into Dict_IV_V649d06 and the *Sty*I/*Eco*RI fragment from Dict_IV_V71c10 into Dict_IV_V590g11. A *Kpn*I/*Spe*I fragment from the first product and a *Spe*I/*Eco*RI fragment from the second, were cloned together into *Eco*RI/*Kpn*I-digested pBluescriptII. Catalytic dead N1425Q and C1474A mutations were generated by PCR, then spliced into the full genomic clone using *Cla*I/*Acc*I digestions. For expression of these clones in cells, we place an *Eco*RI linker into the *Nhe*I site just upstream of the Set1 ATG, then digested out the full length *set1* clones, using *Eco*RI, into the pDEXH 82 vector, 3′ of the GFP coding sequence, thus generating GFP-Set1 fusions.

Analysis of cellular DNA, RNA and protein

Southern, Northern and Western blotting was carried out as described (Chubb et al., 2000). For confirming disruption of the *set1* locus, we BclI-digested genomic DNA from transformants and probed Southerns with a *Bam*HI/*Kpn*I fragment from JC1b237cO3. For Northerns, the following probes were used: cAR1 and ACA (from cDNA clones), rasG and V18 (PCR products) and discoidin Ia (from clone SLB855). RNA FISH experiments were carried out as described (Femino et al., 1998) with cells plated on acid-washed coverslips fixed in 4% paraformaldehyde prior to lysis with 0.5% triton.

For Western blotting, we used rabbit antisera against histone H3 and their modifications. We used a commercial C-terminal histone H3 antibody (ab1791, Abcam) at 1 in 2000 dilution. Antisera to mono-, di- or tri-methylated H3K4 were raised in rabbits by immunization with linear synthetic peptides conjugated to ovalbumin, using the procedures described by White et al. (1999). The peptide sequence used for immunizations corresponded to the ten N-terminal residues of H3 and incorporated mono-, di- or tri-methyl lysine at position 4 (A.R.T.meK.Q.T.A.R.K.S.C). The C-terminal cysteine was added to facilitate coupling to affinity gels. Specificity was tested by inhibition ELISA (White et al., 1999). By this criterion, each of the antisera used here was specific for only one of the three possible H3K4 methylation states (Breiling et al., 2004). The H3K4 antisera were used at dilution of 1 in 1000. GFP was detected with the JL-8 monoclonal antibody (Sigma). We used HRP-conjugated anti secondaries (Jackson) for detection.

Immunofluorescence on H3 modifications was carried out on cells fixed in 4% paraformaldehyde (K4) or 3.7% formaldehyde (K9) on acid-washed coverslips. Lysis was carried out using 0.2% Triton X100 and antibodies were used at 1 in 500. For K9 di-methylation, we used a rabbit IgG directed against the H3K9me2 epitope (Upstate). A Cy3-conjugated anti-rabbit secondary was used for detection. ChIP assays were carried out using anti-H3K9me2 sera from Abcam.

ChIP assays

10^8 Cells were resuspended in 50 ml KK2 buffer (20 mM potassium phosphate, pH 6.2), formaldehyde was added to 1.2% and the mixture was shaken for 15 min. Fixation was quenched with 360 mM glycine for 5 min. Cells were pelleted, washed in 10 ml RLB lysis buffer (0.32 M sucrose, 10 mM Tris 7.5, 5 mM $MgCl_2$, 1% triton) then resuspended in a small volume of RLB. 0.5 ml GPA buffer (10 mM Tris 7.5, 10 mM EDTA) was added, followed by 0.5 ml GPB buffer (10 mM Tris 7.5, 0.7% SDS). The suspension was sonicated 6 times with 10 s pulses at 10 μ on a 550 Sonic Dismembrator sonicator (Fisher). The chromatin-containing supernatant was recovered after a 5-min full speed spin at 4°C in microcentrifuge. A 50-μl aliquot was retained (input). 50 μl proteinA-sepharose beads were added to the extract, which was incubated on a wheel for 30′ at 4°C. The beads were pelleted, the supernatant divided between samples. Aliquots were topped up with IP buffer (0.5% triton in TBS, 1 mM EDTA, protease inhibitors) to 1 ml. 2 μl antibody and 50 μl beads were added to each sample and the tubes were rotated overnight at 4°C. The chromatin-bound beads were recovered by centrifugation, washed twice with 1 ml IP buffer then 4 times with 1 ml W buffer (10 mM Tris pH 8, 500 mM LiCl, 1% deoxycholate, 1% NP40, 1 mM EDTA). Finally, the beads were resuspended in 150 μl E buffer (50 mM Tris 7.5, 1% SDS, 1 mM EDTA), vortexed then incubated at 37°C for 15 min. The beads were re-extracted with E buffer, extracts were pooled and treated overnight at 65°C with 200 μg/ml proteinaseK. Digestion was duplicated with the input fraction. Free DNA was isolated on PCR purification columns (Qiagen). Quantification of the amount of a DNA sequence associated with a H3K4 modification was carried out using Real Time PCR with a SYBR Green reagent mix on a 7900HT cycler (ABI). Results were normalized with respect to histone H3 controls or the input fraction. The developmental changes in ChIP values were similar irrespective of the standard used.

Cell signaling assays

To assess the responsiveness of *set1* mutant cells to folate, axenically growing cells were inoculated at a density of 5×10^5 cells/ml in HL5 (Sussman and Sussman, 1967). supplemented with 1 mM folate. To assess the requirement for PKA signaling in the development of *set1* mutants, we developed cells at a density of 2×10^7 cells/ml in KK2 suspension supplemented with 50 μM H89 (Calbiochem), an inhibitor of PKA signaling (Zeng et al., 2001). For this experiment, we used cells grown in bacterial suspension, to give a relatively undifferentiated starting population. To quantify the ability of *set1* mutants to condition media at different cell densities, we cultured AX2 and *set1* null cells at a variety of different densities. The cultures were inoculated with 1.25×10^4 cells/ml of an AX2 clone expressing the firefly luciferase gene under the control of the discoidin 1γ promotor (a gift from Harry MacWilliams). Cells were withdrawn and assayed for luciferase activity using the Dual-Glo Luciferase Assay System (Promega) on a FLUOstar Optima luminometer (BMG labtechnologies).

Genome-wide expression profiling

Total RNA was extracted from two separate vegetative samples each of two independent *set1* mutant clones and the parental strain AX2 using RNeasy columns (Qiagen). 25 μg of each sample was primed with anchored oligo(dT) and separately labelled with Cy3 and Cy5 using Superscript III reverse transcriptase (Invitrogen). Each set of mutant labelled cDNA was then paired with the parental cDNA labelled with the complementary fluorophore, and the mixture hybridized to a DNA microarray. The microarrays comprised 7346 PCR products designed to be specific to predicted *Dictyostelium discoideum* genes, plus controls, printed in duplicate on Codelink slides (Amersham); the design and construction of the arrays will be described in detail elsewhere, and detailed protocols are available at http://www.sanger.ac.uk/PostGenomics/Pathogen-Arrays/protocols/Dicty. Images were acquired and quantified using a Genepix 4000B scanner and its associated software (Axon Instruments).

Data were read into the R environment and normalization and further analysis carried out using the Bioconductor package LIMMA (Smyth, 2004; Gentleman et al., 2004). Briefly, background fluorescence was subtracted using the method of Kooperberg et al. (2002) then the resulting intensities were adjusted using print-tip loess normalization. A simple linear model was fitted to estimate the log-ratios of each gene, comparing mutant with parent across all slides, and an empirical Bayes method was used to assess whether genes were differentially expressed. After ranking, genes with a P value less than 0.05 were provisionally accepted as having altered expression in the mutant cell lines. P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg (1995).

Analysis of genomic clustering of mis-expressed genes

Grouping of affected genes within the genome was assessed separately using the CLAC method of Wang et al. (2005). Cluster trees were constructed along entire chromosomes without data smoothing (window size of one) for all four Set1 versus Ax2 slides, in comparison with three reference slides on which RNA from the parental strain labelled with both dyes had been hybridized. Clusters were reproduced across all mutant/parental slides and had an FDR score far less than 0.01 and thus accepted as significant.

We also investigated the distribution of significantly mis-expressed genes on chromosome 2. A 100-gene window was moved along chromosome 2 (1963 unique chromosome 2 genes on the array) to find regions on the chromosome enriched with mis-expressed genes (Cohen et al., 2000) in the *set1* mutant. The probability of finding at least the number of target genes in a given window was calculated using the cumulative hyper-geometric distribution (Rice, 1995). At around 6.3 Mb, we found that 6 of the 32 target genes were in the 100-gene window ($P < 0.005$). To account for the multiple-testing problem, we performed a Monte Carlo simulation and verified the statistical significance of the clustering ($P < 0.01$). To get rid of the effect of possible recent gene duplication, we performed a BLASTN (Altschul et al., 1990) search on all genes on chromosome 2 (against themselves) and identified 69 families from 186 highly similar genes (E value < 1e–30). We then repeated the simulation by randomly picking one gene as the representative of each family.

Results

Chromatin proteins in Dictyostelium

The *Dictyostelium* sequence databases reveal a large complement of chromatin proteins and histone modifiers known in higher eukaryotes. Linker histones (Hauser et al., 1995), histone deacetylases and acetyl transferases, ATP-dependent chromatin-remodeling enzymes, RNAi proteins (Martens et al., 2002), chromodomain proteins, SET domain proteins and variant histones can all be found. The *Dictyostelium* genome encodes a homolog of the histone H3 lysine 9 (H3K9) methyltransferase su(var)3–9 (DDB0190352), a protein absent

in the yeast *S. cerevisiae.* Homologs of all core nucleosomal histones were identified. The *Dictyostelium* H3 family comprises three genes with 85% identity to human H3 (DDB0191157 and DDB0216291 — two adjacent genes) and two divergent variants. The three H3 genes similar to human H3 are identical in their extreme N-termini to the human H3 (N-ARTKQTARKSTG-). The H3 residues known in higher eukaryotes to be post-translationally modified (lysine, arginine and serine/threonine) are highly conserved. Substitutions in their N-terminal and histone fold domains indicate all three are H3.3-type histones.

The developmental regulation of H3K4 methylation in Dictyostelium

We chose to study the modification of an amino acid in the conserved N-terminus of histone H3, methylation of H3K4. H3K4 can exist in three methylation states: mono-, di- and trimethyl. In other models, meH3K4 is considered to be a mark of active chromatin.

If histone modification regulates batteries of genes associated with developmental transitions, we should to see differences in the modification states of genomes at different developmental states. We addressed this hypothesis by looking at the total genomic levels of the different methylation states of H3K4 through the entire program of *Dictyostelium* development, from growing cells to the mature fruiting body (Figs. 1A, B). The levels of H3, relative to total cellular protein, increase as differentiation proceeds towards the final culminant. This may reflect the loss of cytoplasmic contents that occurs during spore formation, or increased DNA packaging during dormancy. The levels of H3K4me3 diminish considerably during the process of differentiation. In contrast, the level of H3K4me1 becomes significantly enhanced during differentiation. We see a slight

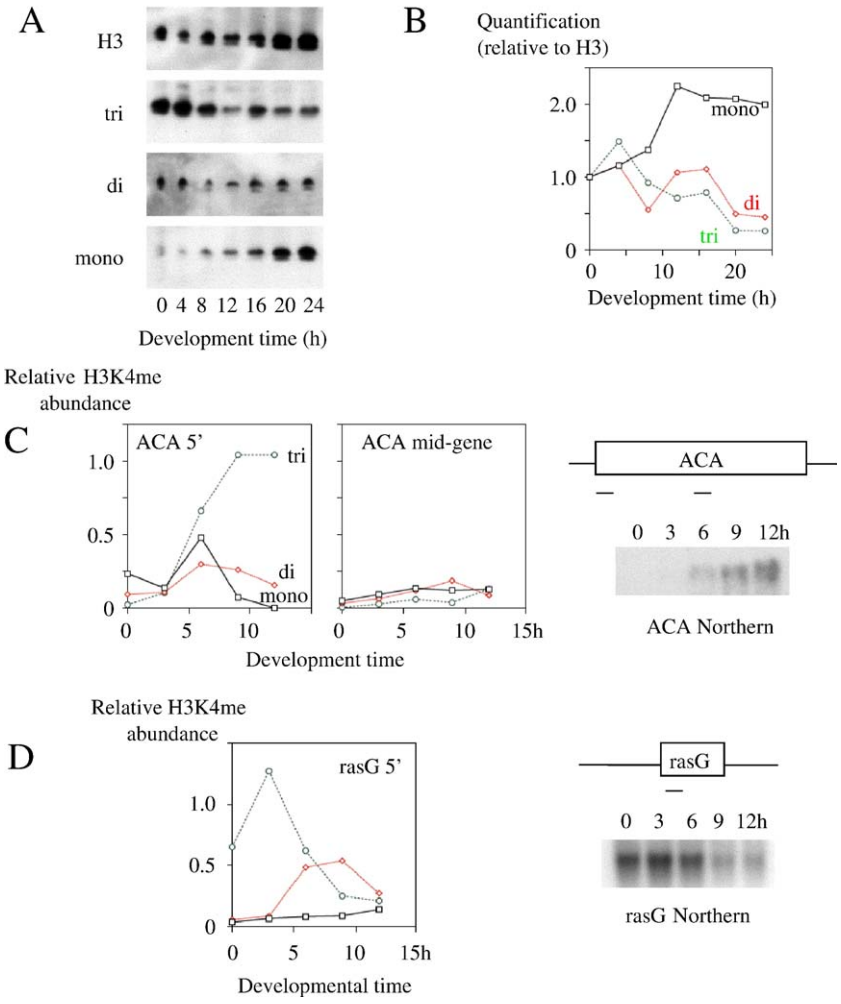

Fig. 1. Different H3K4 methylation states mark distinct phases of differentiation. (A) Western blots of different methylation states of H3K4, of extracts taken throughout the development of wild-type *Dictyostelium*. Equal amounts of protein were loaded. (B) Quantification of the intensity of the chemiluminescent signals from the blots in A. (C) H3K4me3 at the 5′ end of the ACA gene correlates with transcriptional activation (green). (D) H3K4me3 at the *rasG* gene is lost as the gene is inactivated. Levels of the lower modification states increase as trimethylation disappears (H3K4me2 in red, H3K4me1 in black). (C and D) Amount of DNA immunoprecipitated by antibodies against meH3K4 was assessed by quantitative PCR using primers amplifying the regions marked by black bars. Gene expression is shown by Northern blots of RNA from the same cells.

but reproducible dip in H3K4me2 around the time of aggregation (8 h) and the level of this mark again dips during the final stages of spore formation (20–24 h).

In budding yeast and vertebrates, H3K4me3 is enriched at the 5′ ends of active genes (Ng et al., 2003; Bernstein et al., 2005). To validate the *Dictyostelium* for the study of H3K4 methylation, we analyzed H3K4Me at genes whose expression changes during *Dictyostelium* development, using chromatin immunoprecipitation (ChIP) assays and quantitative PCR. We see a strong correlation between H3K4 trimethylation and gene activation, in a manner similar to other eukaryotic models. The adenylate cyclase gene (*ACA*) is induced a few hours after the onset of differentiation (Pupillo et al., 1992), which parallels levels of H3K4me3 at the 5′ end of the gene (Fig. 1C). The level of enrichment of H3K4me3 2 kb downstream of the transcription start site is very low (Fig. 1C). We also see a correlation between gene activity and H3K4me3 for genes that are inactivated during early differentiation. The expression of the growth stage GTPase, *rasG* (Robbins et al., 1992), declines a few hours after the onset of differentiation, which correlates with a significant drop in the level of H3K4me3 at the *rasG* locus (Fig. 1D).

The levels of H3K4me1 and H3K4me2 rise during the inactivation of *rasG* that occurs after the onset of differentiation (Fig. 1D). The level of dimethylation at this locus peaks coinciding with the loss of H3K4me3. This enrichment of dimethyl H3K4 declines as the rise in the level of H3K4me1 continues. We see a similar trend for the growth gene *V18* (data not shown, Singleton et al., 1989).

Identification of the H3K4 HMTase in Dictyostelium

The Set1 methyltransferase is required for di- and trimethylation of H3K4 in the yeast *S. cerevisiae* (Santos-Rosa et al., 2002). We searched *Dictyostelium* sequence databases with the Set1 sequence and identified a gene predicted to encode a 1486 amino acid protein with a C-terminus showing high homology to the catalytic (SET) domain of Set1 (DDB0188336, Fig. 2). Alignment of its SET domain with other SET proteins indicates it is member of the Set1 family and is distinct from other SET proteins (Fig. 2B). We named this gene *Dictyostelium set1*. The closest homolog to the *Dictyostelium* Set1 catalytic domain is the human Set1/Ash2 HMTase (KIAA0339, Fig. 3) a protein known to methylate H3K4 (Wysocka et al., 2003). Unlike some Set1 proteins, there is no RNA recognition motif (RRM). The *Dictyostelium* genome appears to encode at least 25 additional SET proteins.

To investigate the role of Set1 in chromatin regulation in *Dictyostelium*, we mutated the *set1* locus by homologous recombination. A blasticidin resistance cassette (Sutoh, 1993) was integrated into the *set1* coding sequence upstream of the SET domain, (Fig. 3A) confirmed by Southern analysis (Fig. 3B). We obtained a high frequency (95%) of recombinants carrying the disrupted allele, indicating Set1 is dispensible for

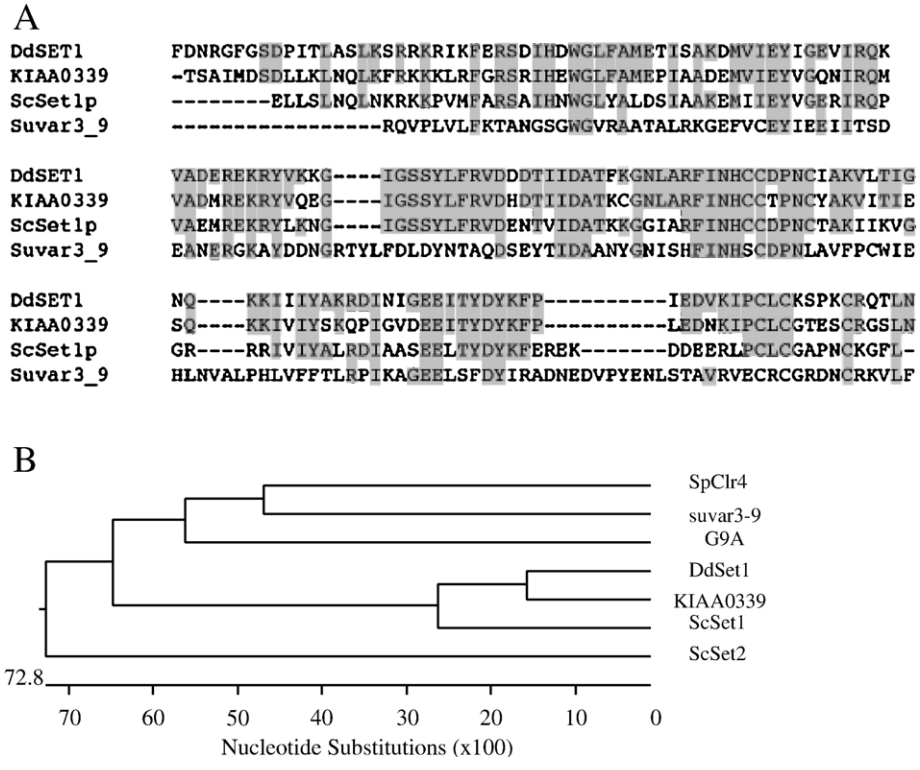

Fig. 2. A Set1 homolog in *Dictyostelium.* (A) Alignment of the C-terminal SET domain of *Dictyostelium* Set1 with other SET proteins. The sequence is more closely related to Set1 family members, than to the H3-K9 methyltransferase su(var)3–9. Identities are highlighted. (B) Phylogenetic tree comparing the SET domain of *Dictyostelium* Set1 with other SET domains. Alignments and tree building carried out using ClustalW.

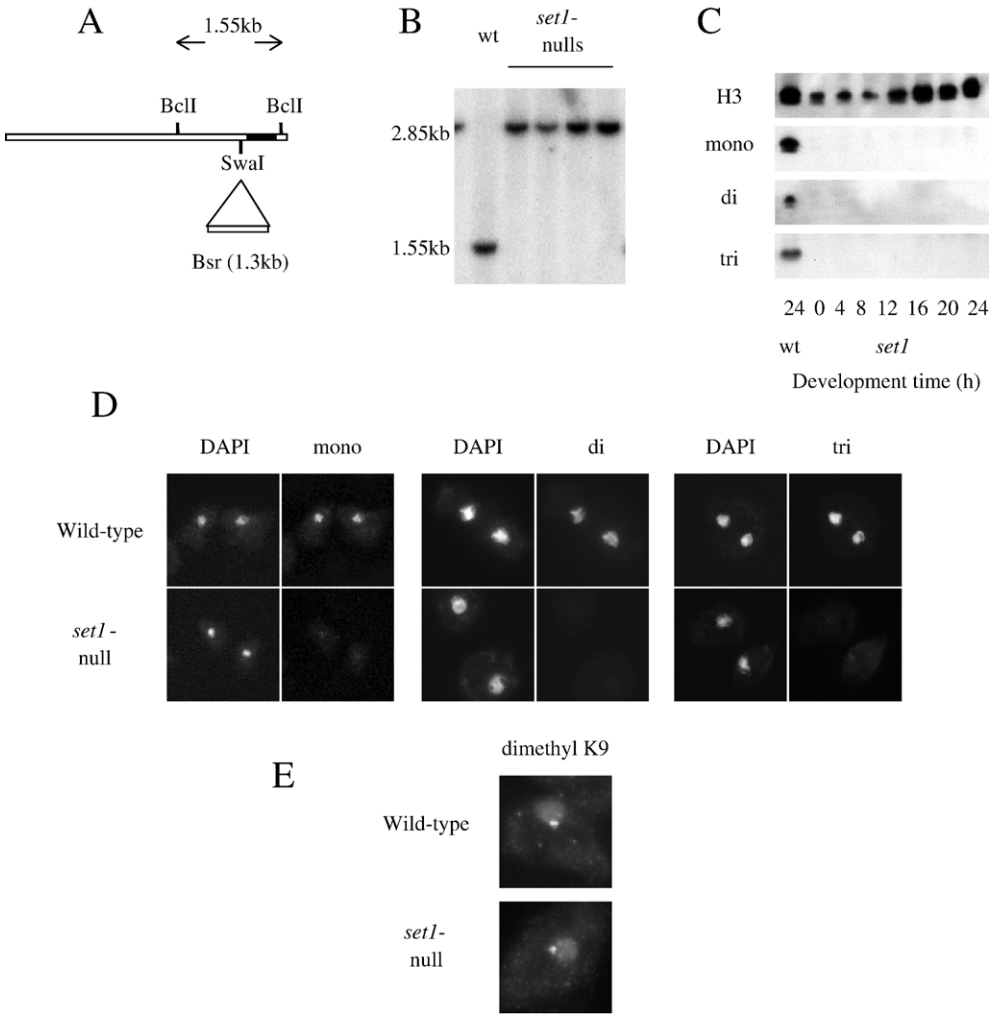

Fig. 3. Targeted disruption of the Set1 gene results in the loss of all methylation at H3K4. (A) Scheme for disruption of the *set1* locus. A blasticidin resistance cassette was inserted into a genomic clone spanning the Set1 C-terminus, upstream of the SET domain. (B) Southern analysis of clones derived from transformation of wild-type *Dictyostelium* cells with the gene disruption construct in A. Disruptants are revealed by a shift of the wild-type band by 1.35 kb, equivalent to the size of the blasticidin cassette. (C) H3K4 methylation is lost in *set1* mutants. Western blots, probed with antibodies against different H3K4 methylation states are shown, with extracts from *set1* cells throughout development. (D) Immunofluorescence of growing wild-type cells with antibodies against the different H3K4 methylation marks reveals a diffuse nuclear-wide staining pattern lost in *set1* cells. (E) Immunofluorescence with a H3K9me2 antibody indicates this modification is not overtly affected by loss of Set1.

cell viability. Western blots show that mono-, di- and trimethylation of H3K4 are absent throughout the life cycle of the mutant (Fig. 3C), confirmed by immunofluorescence (Fig. 3D). In the wild-type cells, all three meH3K4 marks distribute throughout the nucleus. In the mutant cells, all three modifications are absent. We can also demonstrate the loss of the H3K4 methylation by ChIP (data not shown). At the gross nuclear level, the distribution of dimethyl H3K9 is unchanged in the *set1* cells (Fig. 3E). In both wild-type and mutant cells, H3K9me2 staining is localized to a single spot adjacent to the nuclear envelope.

Precocious development of set1 mutants

The growth rates of wild-type and *set1* cells are not significantly different during short term culture, although when wild-type and *set1* mutant cells were co-cultured for a week, wild-type cells were observed to slowly take over the culture (as monitored by immunofluorescence), suggesting the *set1* mutants have a mild growth disadvantage. At the gross morphological level, *Dictyostelium* development is normal in the absence of Set1. When developed on non-nutrient agar, *set1* mutants form normal-sized fruiting structures with a wild-type morphology (Supplementary Fig. 1).

The *set1* mutants are accelerated in their early development (Fig. 4A). After nutrient removal, wild-type cells enter a program of differentiation and approximately 6 h later, chemotax to form a multicellular mound. *Set1* cells aggregate approximately 2 h more rapidly than wild type. This result was seen for *set1* clones derived from independent transformations (Fig. 4A).

Rapid development phenotypes are observed in two classes of *Dictyostelium* mutant. Some mutants activate the developmental gene expression program early or rapidly, and others

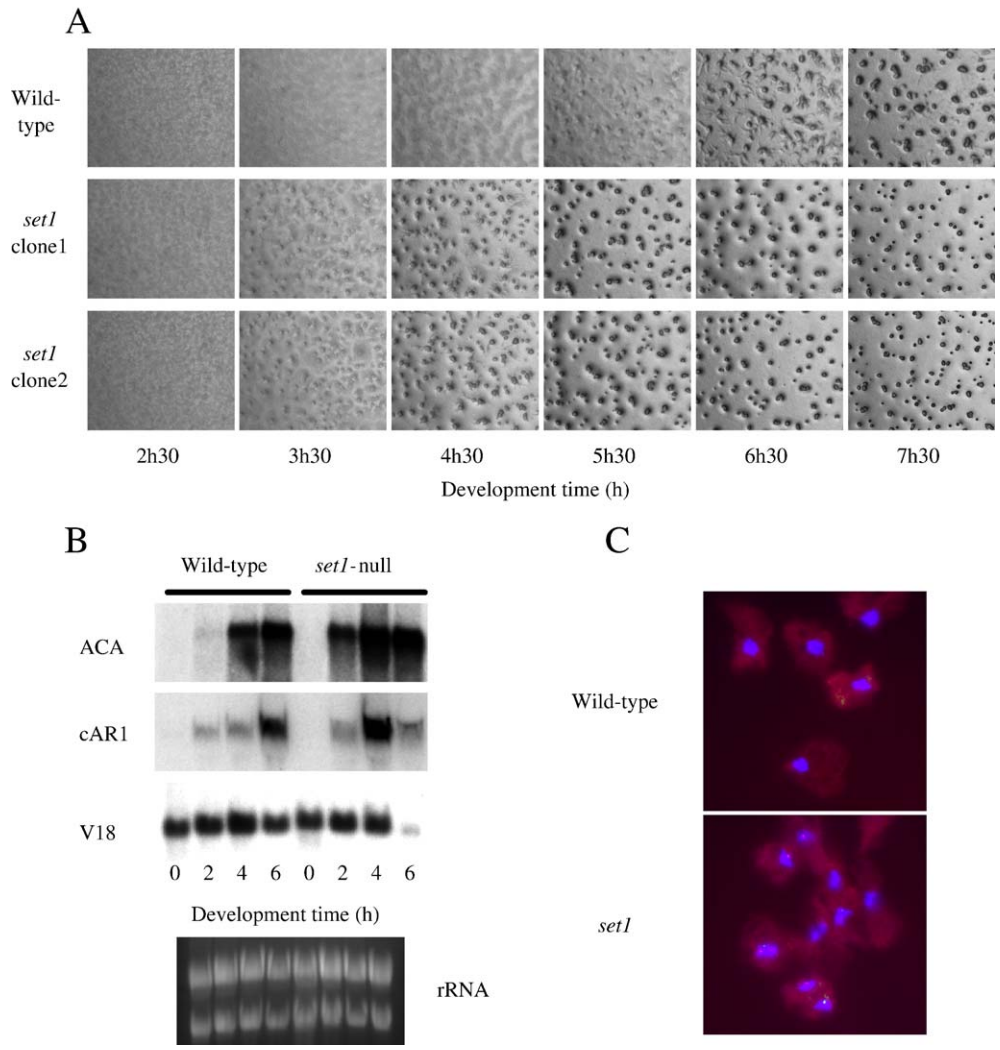

Fig. 4. Precocious development of *set1* mutants. (A) The timing of early development is accelerated in *Dictyostelium* cells lacking Set1. Cells were removed from growth media and developed on non-nutrient agar plates. Two independent *set1* clones aggregate 2 h more rapidly than wild-type cells. (B) Accelerated induction of aggregation-stage gene expression in *set1* cells. Shown are Northern blots of wild-type and *set1* RNA with probes used against the early developmental genes cAR1 and ACA and the growth gene V18. (C) RNA FISH on 4 h developed wild-type and *set1* cells reveals an abundance of prominent ACA (red) and cAR1 (green) transcription sites in the mutant cells.

have aberrant chemotactic signaling (Luo et al., 2003). We studied the RNA expression of two genes normally induced a few hours after nutrient removal, cAR1, which encodes an aggregation stage cAMP receptor (Sun and Devreotes, 1991) and ACA, which encodes the cAMP synthesis enzyme, adenylate cyclase (Pupillo et al., 1992). Both genes are induced precociously in the *set1* mutant (Fig. 4B). ACA shows a strong induction by 2 h of development in the mutant, to a level comparable to that shown at 4 h in the wild-type strain. The expression of cAR1 RNA in the *set1* mutant peaks early. We can also detect enhanced expression of ACA and cAR1 in *set1* mutants at the single cell level, using RNA FISH on 4 h developed cells (Fig. 4C). In addition, the expression of the growth phase gene, V18 (Singleton et al., 1989), declines early in the *set1* mutants (Fig. 4B). These data indicate the timing phenotype is caused by early or rapid activation of the differentiation program.

Ectopic activation of the differentiation program during growth

Set1 mutants could develop precociously because they proceed rapidly through the differentiation program after nutrient removal. Alternatively, they might be already partially differentiated during growth. To distinguish between these possibilities, we studied the expression of the early developmental marker discoidin I (Devine et al., 1982). This gene is normally induced immediately after nutrient depletion, and also in growth media at high culture densities (Clarke et al., 1987). We assessed discoidin I RNA levels in wild-type and *set1* cells at a range of cell densities (Fig. 5A). In wild-type cells, discoidin I becomes significantly induced at a density of 2×10^6 cells/ml. In *set1* cells, discoidin I is strongly induced even at very low culture densities (5×10^5 cells/ml). Indeed, the level of discoidin RNA at this density far exceeds the expression level seen in the wild-type cells at a 10-fold higher density (Fig. 5A).

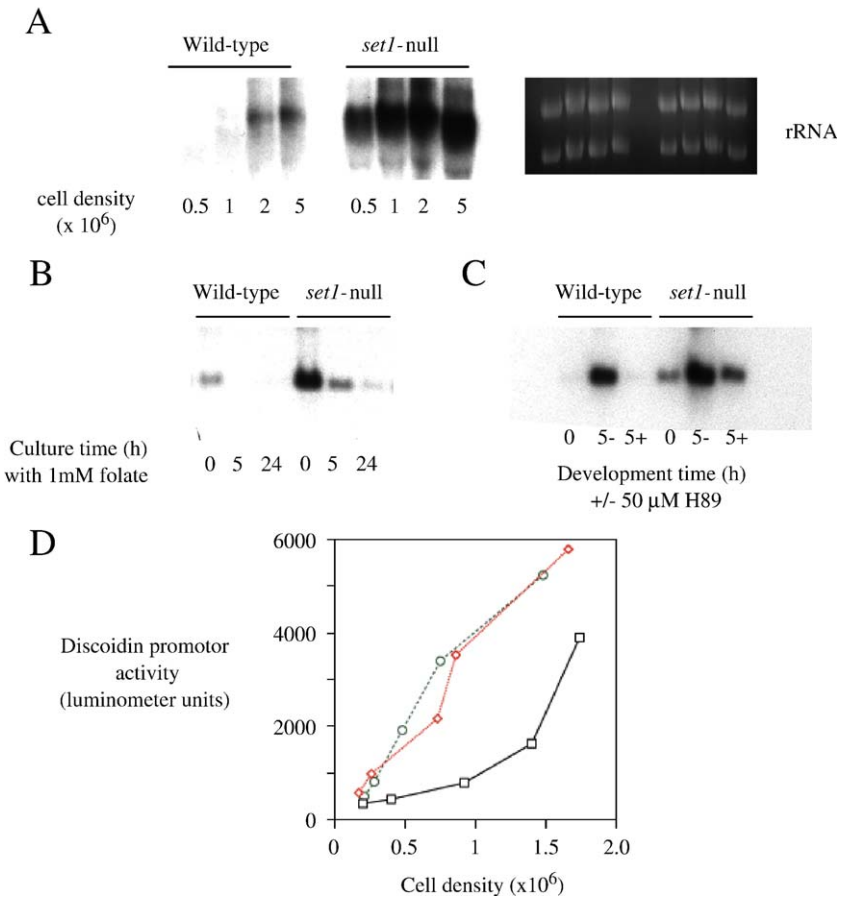

Fig. 5. *set1* mutants differentiate inappropriately during growth. (A) The early developmental marker discoidin Ia is strongly induced at low cell densities in growing *set1* cells. A Northern blot is shown, of RNA from wild-type and *set1* cells grown at different culture densities. (B) *set1* cells retain responsiveness to folate. The discoidin Ia Northern is of RNA extracted from wild-type and *set1* cells after inoculation of cultures with 1 mM folate. (C) The early development of *set1* cells retains its dependence upon PKA signaling. The discoidin Ia Northern is of RNA extracted from wild-type and *set1* cells developed for 5 h with or without 50 µM of the PKA inhibitor H89. (D) *set1* cells induce an inappropriate starvation response in wild-type cells. Wild-type and two independent *set1* cell lines were co-cultured with a starvation reporter cell line transformed with a luciferase gene under the control of a discoidin promotor. The level of luciferase expression, and therefore the degree to which the reporter cells are differentiated, is shown at different culture densities. Both *set1* clones (red and green lines) induce reporter cell differentiation more strongly than wild-type cells (black) at all cell densities.

In our developmental timing experiments, cells were taken out of media at a density of 1×10^6 cells/ml. We conclude that *set1* cells enter the differentiation program even under conditions of nutrient abundance and low cell density. Inappropriate activation of developmental gene expression occurs during growth.

There are a several signals, in addition to nutrient depletion, which trigger differentiation. We addressed whether these signals were perturbed in *set1* cells. The bacterial metabolite, folate, represses differentiation of *Dictyostelium* (Blusch and Nellen, 1994). We inoculated cultures of wild-type and *set1* cells with 1 mM folate, and monitored discoidin I expression. Although *set1* cells have a higher starting level of discoidin I than wild-types, after 24 h of folate treatment the level of this marker is negligible (Fig. 5B) indicating the mutants are responsive to folate. Early *Dictyostelium* development requires signaling through protein kinase A (PKA) dependent pathways (Firtel and Chapman, 1990). We assessed the requirement of *set1* cells for PKA signaling during early development (Fig. 5C). Both wild-type and *set1* cells show a strong increase in discoidin I induction after 5 h of differentiation. If PKA signaling is inhibited using the inhibitor H89, discoidin induction is impaired in both wild-type and *set1* cells. We conclude that *set1* cells still require PKA for differentiation.

Another differentiation trigger in *Dictyostelium* is high cell density. One pathway for sensing cell density is the prestarvation pathway (Clarke et al., 1988). Growing cells continually secrete a prestarvation factor (PSF) and when a critical level of PSF is present (at the critical population density) the cells differentiate (Rathi et al., 1991). One possibility is that *set1* cells secrete an excess PSF or fail to secrete a PSF antagonist. We therefore assessed the ability of *set1* mutants to induce the starvation response in wild-type cells. Cultures of *set1* and wild-type cells were seeded with reporter wild-type cells expressing luciferase under the control of a discoidin promotor (Fig. 5D). The differentiation of the reporter cells was monitored as an increase in luciferase production. When wild-type cells are cultured with the reporter cells, a surge in discoidin promotor activity occurs at a density of 1.5×10^6 cells/ml. When reporter cells were cultured with two different *set1* clones, the discoidin promotor activity was considerably enhanced over wild type levels at all cell

densities. Therefore, there is a strong non-autonomous component to the developmental timing phenotype of *set1* cells. The *set1* cells might secrete an excess of a starvation-promoting factor, such as PSF. Alternatively, Set1 could be required to express an inhibitor of the starvation response.

Genome organization of Set1-regulated genes

To evaluate the role of Set1 in orchestrating changes in gene expression during the growth to development transition, we compared the genome-wide transcript differences between growth phase wild-type and *set1* cells using DNA microarrays. A surprisingly small number of genes are significantly mis-expressed in the mutant. 44 genes are overexpressed and 28 genes underexpressed ($P < 0.05$. These data can be accessed at http://www.sanger.ac.uk/Users/alicat/chubb.html). Of these 72 genes, only 19 (26%) are part of the normal early developmental response, in that they are normally altered in wild-type cells after 2 h of starvation. A comparison of growing wild-type cells with 2 h starved wild-type cells reveals 605 genes significantly different in their expression profiles ($P < 0.05$). Therefore, although the *set1* mutants are precociously differentiated with respect to certain genes and cellular responses, the initial developmental response is neither complete nor concerted. A survey of the classes of gene mis-expressed in the *set1* mutants and a comparison with other studies using the same DNA arrays, reveals no obvious bias in the functional classes of the mis-expressed proteins. To validate the microarray data, the expression changes for 5 mis-expressed genes were assessed by Northern blots of independently derived RNA. The alterations detected by microarray were confirmed for all 5 genes (data not shown).

The genes mis-expressed in the *set1* mutants are non-randomly distributed in the genome. To assess possible clusters of differentially expressed genes along chromosomes we used the CLAC (Cluster Along Chromosomes) method implemented in the CGH-Miner software of Wang et al. (2005). The method uses agglomerative clustering to group together adjacent genes on the basis of their log-ratios and assesses how long strings of up and down-regulated genes are. The arrays tested were compared against control arrays in which no significant clustering should be seen to estimate the false discovery rate (FDR) among the positive clusters (Benjamini and Hochberg, 1995). We identified 12 gene clusters reproduced in all arrays tested that passed our FDR cutoff of 0.01 (Fig. 6, Supplementary Fig. 2). Of the 12 clusters, 6 were predominantly composed of genes with significant sequence homology to their neighbors (Fig. 6, green bars). The other 6 clusters are comprised of non-homologous genes (Fig. 6, red bars), so their clustering does not reflect co-expression of gene family members or cross hybridization.

We took an alternative approach to assessing the clustering of mis-expressed genes, using only genes identified as significantly altered ($P = 0.05$ or less). We concentrated our analysis on chromosome 2, which encodes nearly one half of the mis-expressed genes (32/72), and provides a large pool for

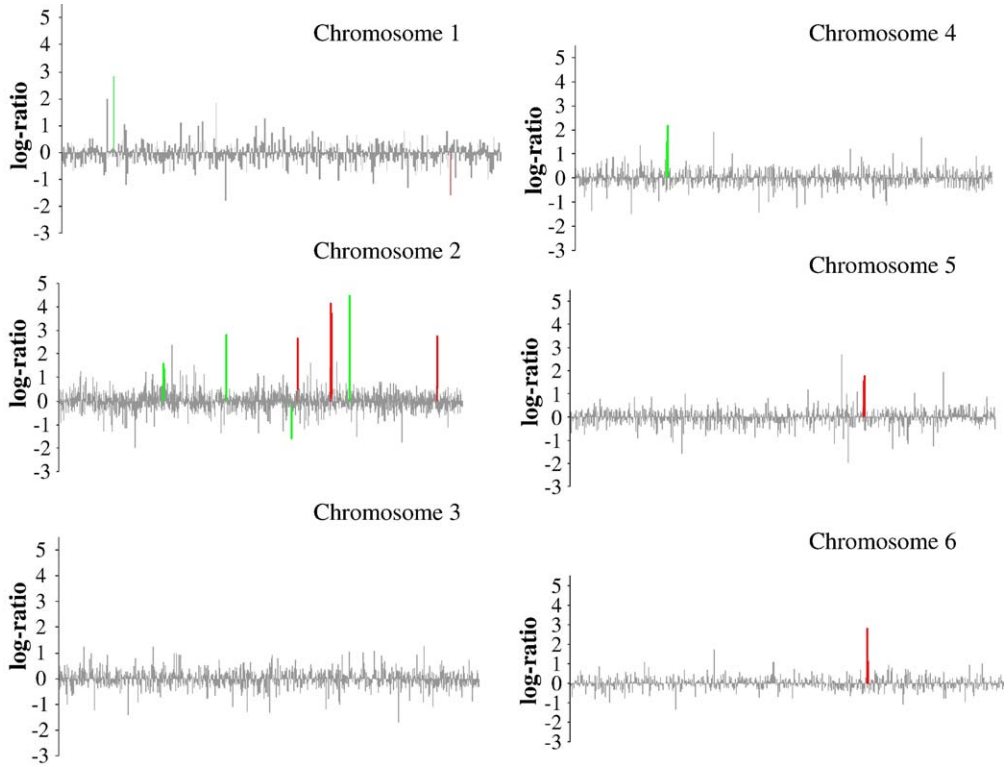

Fig. 6. Genomic clustering of Set1-regulated genes. DNA microarrays were used to identify expression changes in the Set1 mutant. The figure shows plots of log-ratio of expression against chromosomal location for the four arrays used in the analysis. We used the CLAC (Cluster Along Chromosomes) method to assess the significance of the strings of up- and down-regulated genes. Strings passing the cut-off FDR (False Discovery Rate) of 0.01 were accepted as significant. Of 12 such strings identified, all of which were reproduced in all arrays, six (in green) contain predominately closely-related sequences. The remaining 6 are highlighted in red.

rigorous quantitative analysis. We moved a window of 100 genes along the chromosome to identify portions enriched with differentially expressed genes. A region at 6.3 Mb was filtered out and inspection of that region revealed 4 genes within 30 kb ($P < 7 \times 10^{-6}$) and a total of 6 genes falling in the 100-gene window ($P < 0.005$). These four genes also show the most significant perturbations of gene expression in the *set1* mutants and this clustering was also detected by CLAC (Supplementary Fig. 2, cluster 8). We then restricted our analysis to dissimilar genes. We performed a BLASTN search (Altschul et al., 1990) on all chromosome 2 genes against themselves and identified 69 gene families (*E* value < 1e−30). By randomly picking one gene to representative of each family, we repeated the simulation and found the clustering at 6.3 Mb was still significant ($P < 0.02$).

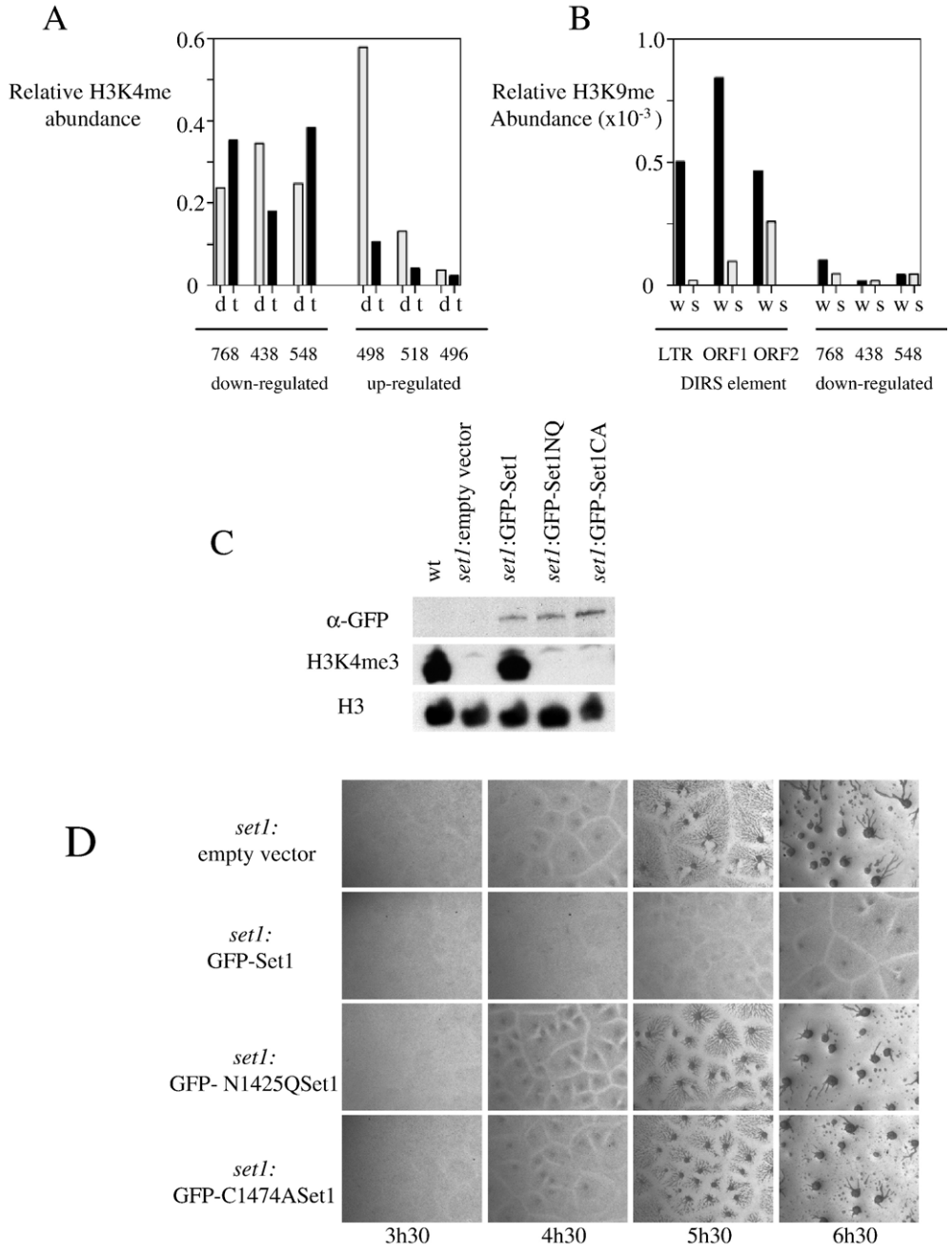

Fig. 7. Set1 catalytic activity and the *set1* mutant phenotype. (A) Methyl H3K4 detected on genes mis-expressed in the absence of Set1. ChIP studies on wild-type cells were carried out using antibodies against H3K4me2 and H3K4me3 for the following genes — DDB0169498, DDB0203518, DDB0169496, DDB0217768 (all from the 6.3 Mb cluster) and also DDB0188438 and DDB0185548. Results were normalized to H3 antibody controls. The background levels obtained from normalized ChIP values from *set1* mutant cells were subtracted from these data. (B) No increase in H3K9Me2 on genes repressed in *set1* cells. ChIP experiments were carried out to detect changes in H3K9Me2 levels at genes repressed in *set1* cells. These were compared to the changes in H3K9Me2 levels observed at the LTR and at two points in the reading frame of the DIRS transposon (w = wild-type, s = set1 mutant). (C) Western blots showing rescue of H3-K4 methylation by expression of GFP-Set1, but not catalytically dead N1425Q and C1474A GFP-Set1 proteins (D) Rescue of the *setI* mutant phenotype by a wild-type GFP-Set1 clone, but not catalytically dead GFP-Set1 proteins. An empty vector *set1* control was used so all cells experience the same selective growth conditions (10 μg/ml G418).

We also found 3 pairs of genes within 3 kb and 4 pairs within 7 kb. To test whether these pairs could arise randomly, we ran a simulation that randomly picked 32 genes from the chromosome, and assessed how often pairs fall within 3 kb and 7 kb. We found significant clustering of our data relative to random pairs using both a 3 kb and 7 kb window ($P < 0.003$ and $P < 0.009$, respectively).

H3K4 methylation of Set1-regulated genes

To assess whether the genes mis-expressed in the *set1* mutant cells are normally methylated by the Set1 enzyme, we carried out ChIP experiments to assess the levels of H3K4me2 and H3K4me3 at these loci in growing wild-type cells (Fig. 7A). We studied six loci, three over-expressed homologous genes at the 6.3-Mb cluster (DDB0169498, DDB0203518 and DDB0169496), one under-expressed gene adjacent to this cluster (DDB0217768) and two other repressed genes (DDB0188438 and DDB0185548). The data were normalized by subtraction of the background levels of immunoprecipitated chromatin from *set1* cells. All the down-regulated genes show high levels of both H3K4me2 and H3K4me3. The up-regulated gene DDB0169498 is strongly dimethylated. The other up-regulated genes show more modest dimethylation, but the levels we detect are consistently above background.

A possible reason for the silencing of genes in the *set1* mutants could be spreading of redundant silencing factors in the absence of H3K4me. To address this possibility, we carried out ChIP studies using antisera against H3K9me2 (Fig. 7B). The levels of H3K9me2 at these repressed genes are at background levels (no antibody controls) in both wild-type and *set1* mutant cells, so there is no evidence that deposition of H3K9me2 is responsible for the silencing. We observe robust levels of H3K9me2 at the DIRS transposon, both in the LTR and at two sites in the open reading frame. H3K9me2 is reduced, notably in the LTR, in the *set1* cells, indicating that the loss of H3K4Me causes dissipation of silencing factors from some locations. These observations on H3K9me2 distribution were reproduced with different H3K9me2 antisera.

To address whether the precocious development phenotype is caused by the lack of Set1 catalytic activity in the *set1* mutant cells, we transformed *set1* cells with *set1* genes engineered to express mutations in the SET domain of the protein. We used two substitutions which render the protein catalytically inactive (N1425Q and C1474A; Santos-Rosa et al., 2003). Expression of wild-type GFP-Set1 reverts the loss of meH3K4 in the *set1* mutants, whereas transformation of the same sequence encoding the N1425Q or C1474A mutations does not recover meH3K4 (Fig. 7C). The wild-type clone retards the precocious development of the *set1* mutant (Fig. 7D) while the two mutant clones fail to rescue the phenotype.

Discussion

We have studied the function of Set1, a histone methyltransferase homolog in *Dictyostelium*. *Set1* mutant cells lack all methylation of the lysine 4 residue of histone H3. At the genome-wide level, the different methylation states mark different developmental phases of the organism. Depletion of Set1 gives rise to cells displaying unusually rapid development, characterized by precocious aggregration of amoebae into multicellular aggregates after nutrient removal. This phenotype is caused specifically by the loss of Set1 catalytic activity. Early developmental markers are abundantly expressed in growing *set1* cells, revealing that the mutants differentiate ectopically during growth. Co-culture of wild-type cells with *set1* mutants induces the starvation response in the wild-type cells, indicating Set1 regulates production of an extra-cellular signal required for the correct perception of growth conditions. Microarray analysis of the *set1* mutants indicates significant genomic clustering of mis-expressed genes.

Histone modification and development

A number of reports have linked histone modification to the timing of developmental transitions. Rhythmic histone H3 acetylation of circadian clock genes has been reported in the mouse, with acetylation levels mimicking corresponding RNA levels (Etchegaray et al., 2003), and interfering with histone deacetylation with the drug trichostatinA impairs light-induced gene expression (Naruse et al., 2004). Vernalisation, the stimulation of flowering by long periods of cold, has been correlated to deposition of dimethyl H3K9 and H3K27 at the promotor of the *FLC* gene (Bastow et al., 2004). Sustained down-regulation of the *FLC* is required for the normal timing of flowering, and these repressive modifications are required for maintenance of a silent *FLC* gene. In this study, we correlate a genome-wide loss of H3K4 methylation with unusually rapid development. The role of meH3K4 in gene regulation is unclear. Although H3K4me3 appears to be a mark of recent RNA polymerase II activity (Ng et al., 2003), the functional consequences of this mark are ambiguous. A possible role of H3K4me3 is the maintenance of a transcription-competent chromatin state, but other models include H3K4me3 acting as a signal for the recruitment of RNA processing factors (Ng et al., 2003).

In this study, we have shown H3K4 methylation is distributed throughout the nucleus, implying modification of much of the genome. Although possible that the effects of loss of meH3K4 act at a single dominant locus, we must also consider a concerted transcriptional change, distributed through the genome, or more locally, at gene clusters. This is pertinent when considering the phenotype of the *set1* mutants, which show accelerated, but normally organized development, consistent with the concerted mis-expression of a battery of early differentiation genes. Concerted genome-wide transcriptional regulation by histone modifiers has been suggested in a recent study on the yeast *S. cerevisiae*. A combination of ChIP and microarrays revealed that different states of H3 and H4 hyper- and hypoacetylation define groups of biologically related genes (Kurdistani et al., 2004). Similar to microarray studies on the yeast s*et1* mutant, we see only a small percentage of the genome is mis-expressed in the *Dictyostelium set1* cells. This implies redundancy in the histone code, with meH3K4, an abundant mark, required for normal expression of small proportion of genes. In addition,

only 26% of these mis-expressed genes are part of the normal early developmental response, and the normal early developmental response involves altering the expression profile of at least 8-fold more genes than are mis-expressed in the *set1* mutants. The *set1* phenotype therefore appears not to be concerted at the level of the whole genome. However, it is possible that loss of *set1* could have a concerted effect on a subset of genes involved in the growth to development switch, and that these genes, when mis-expressed, have a penetrant effect.

One possibility we addressed was whether any of the mis-expressed genes are tightly linked on chromosomes. Several well-documented examples of gene clusters encoding proteins with similar biological functions exist, notably the Hox, globin and myosin heavy chain clusters. In addition, significant proportions of the genomes of metazoa are organized with respect to shared expression profiles (Boutanaev et al., 2002; Lercher et al., 2002; Spellman and Rubin, 2002). Genes that are clustered in the genome have a tendency to be co-expressed. This is true even of clusters comprising non-homologous sequences. The *Drosophila* genome contains at least 200 groups of adjacent and similarly expressed genes (Spellman and Rubin, 2002) and these groups account for over 20% of genes. A recent study on FLC indicates this gene is also part of a cluster which is coordinately modified, to give common expression profiles to FLC and its neighbors (Finnegan et al., 2004). The corepressor CoREST acts during extraneuronal restriction to mediate the silencing of a chromosomal interval enriched for neuronal specific genes (Lunyak et al., 2002).

We detected clustering of mis-expressed genes in *set1* mutant cells. These clusters were not solely comprised of duplicated genes, perhaps reflecting the co-regulation of non-homologous proteins involved in similar physiological processes. One hypothesis is that these clusters represent functional chromatin domains, which permit co-regulation of expression by concerted changes in local chromatin topology. The topology of these clusters is particularly sensitive to loss of meH3K4, hence mis-expression in the *set1* mutant background. A pertinent question here is how H3K4me3, a modification restricted to a specific portion of a gene, could exert effects on neighbouring loci. Indirect models are possible, with a mis-expressed protein being responsible for activation of gene clusters via shared regulatory elements, although the cluster we detect at 6.3 Mb on chromosome 2 also includes down-regulated genes, in addition to three up-regulated homologous genes. Another possibility is that H3K4me3 allows docking of factors capable of exerting more long-range effects on transcription. Alternatively, the lower methylation states of H3K4 may have the more dominant influence on clustered expression. The 6.3 Mb cluster is only weakly trimethylated, but more robustly dimethylated. In both *Drosophila* and yeast, H3K4me2 appears to be more uniformly distributed through the genome, so could potentially have more long-range effects than H3K4me3 (Noma et al., 2001; Santos-Rosa et al., 2002; Schubeler et al., 2004). In mouse and human cells, the distribution of H3K4me2 appears generally more punctate, although broad regions of meH3K4 overlay the Hox clusters (Bernstein et al., 2005).

Developmental regulation of H3K4 methylation

Genome wide levels of the different H3K4 methylation states are enriched at different stages during *Dictyostelium* development. Levels of H3K4me3 drop as cells enter the multicellular phase of the lifecycle, while H3K4me1 increases. A drop in total H3K4me2 occurs at the end of development. H3K4me3 and H3K4me2 are modifications associated with transcriptional activation and euchromatin, respectively, so the reductions in these marks may reflect silencing and compaction of the genome occurring as the cells approach the dormant spore state. Alternatively, the loss of these higher methylation states may reflect slowing of the cell cycle rather than differentiation, as observed for meH3K4 in resting lymphocytes (Baxter et al., 2004). The association of H3K4me1 with the transcriptionally dormant spore state is interesting, and may reflect a primed ground state.

H3K4me3 is a mark of active chromatin, yet we see an ectopic induction of the *ACA* gene in *set1* mutants, where H3K4me3 is absent. This apparent inconsistency can be resolved. Firstly, we observe precocious starvation properties in the *set1* mutants prior to nutrient removal, 2 h before ACA induction. A primary cause of the rapid development phenotype occurs during growth, so the effect of loss of H3K4me3 on ACA expression will be overridden by this dominant early response. Secondly, the microarray experiments in this work and those performed on yeast *Set1* mutants indicate that a number of genes appear to be repressed (directly or indirectly) by Set1. Loss of Set1 results in the loss of all three H3K4 methylation states, so up-regulation of genes in the absence of Set1 could also be a consequence of the loss H3K4me1 or H3K4me2, or differing background modification contexts.

When H3K4me3 diminishes, as genes are inactivated, we observe a transient rise in the level of H3K4me2 and a more sustained increase in the level of H3K4me1. Our observations are consistent with the stepwise removal of methyl groups from H3K4 as transcription ceases. This could involve lysine demethylation involving a FAD-dependent monoamine oxidase (Shi et al., 2004), as *Dictyostelium* has genes for these enzymes, one being a few kilobases from the *set1* locus. Alternatively, histone replacement might be used as the mechanism for removing methylated lysines (Ahmad and Henikoff, 2002; Janicki et al., 2004). At the whole genome level, we also observe an increase in H3K4me1 as H3K4me3 diminishes during differentiation. The *Dictyostelium* H3 proteins all appear to be H3.3 variants, so a mechanism of demethylation via histone replacement, followed by constitutive activity of Set1 methylating newly incorporated histones is certainly feasible. It will be interesting to address these possibilities in *Dictyostelium* cells mutated for H3 variants and nuclear monoamine oxidases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.054.

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