Nuclear organisation and epigenetic regulation of gene expression in *Dictyostelium discoideum*

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Zusammenfassung:

Für die Überexpression markierter Proteine in *Dictyostelium discoideum* wurde eine Serie von Konstrukten kloniert und getestet. Diese Vektoren ermöglichen die Fusion von N- oder C-terminalen Tags (GFP, RFP, 3xFLAG, 3xHA, 6xMYC und TAP). Sie beinhalten eine optimierte Polylinker-Sequenz und haben keine zusätzlichen Aminosäuren zwischen dem Tag und dem Gen von Interesse. Außerdem sind verschiedenen Selektionsmarker (Blasticidin und Geniticin) verfügbar. Darüber hinaus gibt es extrachromosomale Versionen der Vektoren, die in bestimmter Kopienzahl vorliegen und somit ein kontrollierbares Expressionslevel der Zielproteine in *D. discoideum* erlauben. Diese Vektoren können im Hinblick auf Komplementation, Co- und Supertransformation flexibel eingesetzt werden. Schließlich haben die Vektoren standardisierte Restriktionsschnittstellen, die für die Klonierung genutzt werden können. Das Gen von Interesse kann in Abhängigkeit von den experimentellen Anforderungen leicht in verschiedene Vektoren eingebracht werden.

Die Organisation und Dynamik des *D. discoideum* Zellkerns wurde während des Zell-Zyklus untersucht. Es ist bekannt, dass die Zentromer spezifische Variante des Histons H3 (CenH3) als Ansatzpunkt des Kinetochors am Zentromer dient und ermöglicht so die korrekte Verteilung der Chromosomen während der Mitose und der Meiose. Eine Reihe endogene *Dictyostelium* Proteinen, die eine Histon H3-Domäne besitzen, wurden als GFP-markierte Fusionsproteine exprimiert. Es stellte sich heraus, dass eines dieser Proteinee Verhalten verantwortlich ist.

In *D. discoideum* konnte ein Homolog von DET1 nachgewiesen werden. DET1 Homologe sind nur von multizellulären Eukaryoten bekannt, wo sie eine Rolle in der Entwicklungsregulation spielen. Wie auch in anderen Spezien ist das DET1-Homolog in *Dictyostelium* im Zellkern lokalisiert. In ChIP Experimenten zeigte sich, dass DET1 an Promotoren bindet, die in der Entwicklung reguliert werden. Im Gegensatz zu anderen Organismen ist der DET1-Knockout nicht lethal, wobei die Lebensfähigkeit der KO Zellen sehr beeinträchtigt ist. Der Verlust von DET1 führt zu Störungen in der Entwicklung von *D. discoideum*, wobei vergrößerte Aggregatzonen gebildet werden. CenH3-Funktion hat. Genau wie CenH3-Proteine in anderen Organismen besitzt das *Dictyostelium*-Homolog eine erweiterte N-terminale Domäne, die keine Ähnlichkeit zu anderen Proteinen zeigt. Die A-Helix 1 und der Loop 1 der N-terminale Domäne werden für die Bindung von CenH3 am Zentromer benötigt. Im Gegensatz zu bekannten und putativen CenH3-Homologen ist die Loop 1-Region bei dem *D. discoideum* Homolog verkürzt.

Die Lokalisation von Histonen mit bestimmten Modifikationen sowie der modifizierenden Enzyme wurden untersucht. Mit Fluoreszenz-in-situ-Hybridisierung (FISH) und CenH3 Chromatin

Immunopräzipitation (ChIP) konnte gezeigt werden, dass alle sechs telozentrischen Zentromere DIRS-1 und die meisten DDT-A und Skipper Transposons beinhalten. In der Interphase sind die Zentromere an dem Zentrosom gebunden, wodurch sich ein einzelner CenH3 perizentromerischer Cluster ergibt. Dieses Cluster umfasst die putative H3K9 Methyltransferase SuvA, H3K9me3 und HP1 (Heterochromatin Protein 1). Abgesehen vom zentromeren Cluster und einer Anzahl von kleinen Bereichen an der Peripherie des Nukleus gegenüber der Zentromere ist der Nukleus frei von Transposons und Histonmodifikationen, die auf Heterochromatin schließen lassen. Einige der eben angesprochenen Bereiche stimmen mit den distalen Telomeren überein, was die Vermutung nahe legt, dass die Organisation der Chromosomen ein Rabl-ähnliches Verhalten zeigt. Im Gegensatz zu Metazoaen konnte gezeigt werden, dass die Beladung der *Dictyostelium* Zentromere mit CenH3 in der späten G2-Phase statt findet.

Die Transformation von *Dictyostelium* mit Vektoren, die eine G418 Resistenz Kassette tragen, führt typischerweise zu einer Integration der Vektoren in das Genom von *D. discoideum*. Dabei integiert der Vektor in einem oder wenigen repetitiven Clustern von ungefähr ein hundert Kopien. Im Gegensatz dazu integiert ein Plasmid mit einer Blasticidin Resistenz Kassette nur einmal oder in wenigen Kopien in das Genom von *D. discoideum*. Das Verhalten der Transgene im Zellkern wurde mit Hilfe von FISH untersucht und es wurde festgestellt, dass sie mit einer niedrigen Kopienzahl zufällig über den ganzen Nukleus verteilt sind. Im Gegensatz dazu integieren Transgene mit einer Kopienzahl von mehr als 10 in Clustern im oder nahe dem Zentromer in Interphase Zellen. Während der Mitose zeigen die Transgene ein zentromer-ähnliches Verhalten. In ChIP Experimenten zeigte sich, dass die Transgene sowohl den Euchromatin Zeiger H3K4me3 wie auch den Heterochromatin Zeiger H3k9me2 und die zentromerische Histonvariante H3v1 besitzen. Repetitive Cluster und das zentromer-ähnliche Verhalten konnten weder bei extrachromosomalen Transgenen noch bei Stämmen festgestellt werden, bei denen die Transgene in das extrachromosomale rDNA Palindrom integriert sind. Das legt die Vermutung nahe, dass die repetitive Natur der Transgene für das zentromer-ähnlich

Summery

A series of vectors for the over-expression of tagged proteins in *Dictyostelium* were designed, constructed and tested. These vectors allow the addition of an N- or C-terminal tag (GFP, RFP, 3xFLAG, 3xHA, 6xMYC and TAP) with an optimized polylinker sequence and no additional amino acid residues at the N or C terminus. Different selectable markers (Blasticidin and gentamicin) are available as well as an extra chromosomal version; these allow copy number and thus expression level to be controlled, as well as allowing for more options with regard to complementation, co- and super-transformation. Finally, the vectors share standardized cloning sites, allowing a gene of interest to be easily transfered between the different versions of the vectors as experimental requirements evolve.

The organisation and dynamics of the *Dictyostelium* nucleus during the cell cycle was investigated. The centromeric histone H3 (CenH3) variant serves to target the kinetochore to the centromeres and thus ensures correct chromosome segregation during mitosis and meiosis. A number of *Dictyostelium* histone H3-domain containing proteins as GFP-tagged fusions were expressed and it was found that one of them functions as CenH3 in this species. Like CenH3 from some other species, *Dictyostelium* CenH3 has an extended N-terminal domain with no similarity to any other known proteins. The targeting domain, comprising α-helix 2 and loop 1 of the histone fold is required for targeting CenH3 to centromeres. Compared to the targeting domain of other known and putative CenH3 species, *Dictyostelium* CenH3 has a shorter loop 1 region.

The localisation of a variety of histone modifications and histone modifying enzymes was examined. Using fluorescence *in situ* hybridisation (FISH) and CenH3 chromatin-immunoprecipitation (ChIP) it was shown that the six telocentric centromeres contain all of the DIRS-1 and most of the DDT-A and *skipper* transposons. During interphase the centromeres remain attached to the centrosome resulting in a single CenH3 cluster which also contains the putative histone H3K9 methyltransferase SuvA, H3K9me3 and HP1 (heterochromatin protein 1). Except for the centromere cluster and a number of small foci at the nuclear periphery opposite the centromeres, the rest of the nucleus is largely devoid of transposons and heterochromatin associated histone modifications. At least some of the small foci correspond to the distal telomeres, suggesting that the chromosomes are organised in a Rabl-like manner. It was found that in contrast to metazoans, loading of CenH3 onto *Dictyostelium* centromeres occurs in late G2 phase.

Transformation of *Dictyostelium* with vectors carrying the G418 resistance cassette typically results in the vector integrating into the genome in one or a few tandem arrays of approximately a hundred copies. In contrast, plasmids containing a Blasticidin resistance cassette integrate as single or a

few copies. The behaviour of transgenes in the nucleus was examined by FISH, and it was found that low copy transgenes show apparently random distribution within the nucleus, while transgenes with more than approximately 10 copies cluster at or immediately adjacent to the centromeres in interphase cells regardless of the actual integration site along the chromosome. During mitosis the transgenes show centromere-like behaviour, and ChIP experiments show that transgenes contain the heterochromatin marker H3K9me2 and the centromeric histone variant H3v1. This clustering, and centromere-like behaviour was not observed on extrachromosomal transgenes, nor on a line where the transgene had integrated into the extrachromosomal rDNA palindrome. This suggests that it is the repetitive nature of the transgenes that causes the centromere-like behaviour.

A *Dictyostelium* homolog of DET1, a protein largely restricted to multicellular eukaryotes where it has a role in developmental regulation was identified. As in other species *Dictyostelium* DET1 is nuclear localised. In ChIP experiments DET1 was found to bind the promoters of a number of developmentally regulated loci. In contrast to other species where it is an essential protein, loss of DET1 is not lethal in *Dictyostelium,* although viability is greatly reduced. Loss of DET1 results in delayed and abnormal development with enlarged aggregation territories. Mutant slugs displayed apparent cell type patterning with a bias towards pre-stalk cell types.

Contributions

All experiments described in this thesis are my own work except for the cloning of the suvA gene and the creation of the pDneo2a-SuvA-6xMYC and pDneo2a-SuvA-RFP expression constructs (Table 3.2) which was preformed by Vladimir Maximov, Department of Genetics, Kassel University.

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Annotations

Histone modifications are annotated according to (Turner, 2005). The order is: histone type – modified residue – type of modification – level of modification. For example, H3K4me3 denotes the core histone H3 tri-methylated at lysine 4. Abbreviations for different modifications used in this thesis are as follows: ac – acetylation, me – methylation, ph – phosphorylation, ub – ubiquitination. The level of modification is only specified where more than one modification state can occur at a particular residue (usually methylation) and we want to refer to a specific level of modification. For example, for H3K9ac no additional number is needed as lysines can only be mono-acetylated. H3K9me2 would indicate histone H3 di-methylated at lysine 9, while H3K9me indicates H3 with any level of methylation at lysine 9 (mono, di or tri).

In many cases the context (flanking amino acid sequence) of a modified amino acid is well conserved across different species. As *Dictyostelium* core histones are slightly larger than the core histones from commonly studied organisms such as yeast and animals, this often results in the orthologous modified residue (and its "context") being situated at a slightly different position on *Dictyostelium* histones. If the context of a modified residue is found to differ from that in other species by a few residues, the amino acid is numbered according to the general convention and not according to its actual position on the *Dictyostelium* histone. For example, the H3K36me modification is conserved in *Dictyostelium*, but due to the slightly larger size of *Dictyostelium* H3 gene, the actual modified residue is K39. To maintain consistency with the published literature this modification will be referred to as H3K36me and <u>not</u> H3K39me.

Abbreviations

5meC:	DNA methylated at the 5 position of cytosine
BSA:	bovine serum albumin
CaCl2:	calcium chloride
CCD:	charge-coupled device
ChIP:	chromatin immunoprecipitation
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI:	4’,6-diamidino-2-phenylindole
DDW:	double distilled water
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
EGTA:	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
FISH	fluorescence *in situ* hybridisation
GFP:	green fluorescent protein
HA:	Influenza hemagglutinin epitope tag
HEPES:	N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)
HCl:	hydrochloric acid
IF:	immunofluorescence
kb:	kilo base
kDa:	kilo Dalton
MES:	2-(N-morpholino)ethanesulfonic acid
min:	minute/minutes
NaCl:	sodium chloride
NP40:	Igepal CA-630 detergent
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PFA:	paraformaldehyde
PIPES:	piperazine-N,N’-bis(ethanesulfonic acid)
PMSF:	phenylmethylsulphonyl fluoride
PVDF:	polyvinylidene fluoride
REaL:	restriction enzyme digest and Ligation
RFP:	red fluorescent protein
SDS:	sodium dodecylsulphate
SDS-PAGE:	sodium dodecylsulphate polyacrylamide gel electrophoresis
SSC:	saline-sodium citrate
SSR:	sequence-specific recombination
TAP:	tandem affinity purification
TEV:	tobacco etch virus
Tris-HCL:	tris (hydroxymethyl) aminomethane hydrochloride

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1. Introduction

1.1 Dictyostelium

The model organism *Dictyostelium discodeum* is a member of the Amoebozoa, an ancient family of soil and leaf-litter inhabiting protists that diverged from the opisthokont linage shortly after the plant-animal split (Baldauf and Doolittle, 1997; Eichinger et al., 2005). *Dictyostelium* has a number of features, which makes it an attractive model to study the epigenetic control of gene regulation, development and nuclear organisation. Although the Amoebozoa diverged from the animal linage prior to fungi, the relatively low rate of evolution of the Amoebozoa, together with the extensive gene loss associated with the adoption of a parasitic or saprophytic lifestyles among the fungi (Iyer et al., 2008) means they have to share some gene families with animals that are not found in fungi. Examples include SH2 domains, EGF domains and the B-box zinc-binding domain (Eichinger et al., 2005).

When food is plentiful *Dictyostelium* are uni-cellular and commonly used lab strains can be cultured in a defined medium. Under these conditions most of the molecular techniques available in yeast can also be applied to *Dictyostelium*. However, when the food supply is exhausted or removed, starvation triggers the onset of the developmental program (Bonner, 1970). A few cells in the population start emitting cAMP pulses, which act as a chemoattractant for other cells. The other cells chemotax towards the cAMP source, thus aggregating into mounds of approximately 100,000 cells. The cells in the mound enter a developmental pathway, differentiating into a number of different cell types to form a multicellular slug. After a period of migration (towards light and away from heat) the slug develops into a fruiting body supported by a stalk (Strmecki et al., 2005; Bonner, 1959; Bonner, 1970; Fig. 1.1). While multicellularity in *Dictyostelium* has evolved independently from that in plants and animals, the role of Retinoblastoma (Rb), histone acetylation and histone methylation in development appear to be conserved (MacWilliams et al., 2006; Sawarkar et al., 2009; Chubb et al., 2006). The ease of molecular manipulation, combined with a relatively simple developmental program has made *Dictyostelium* an attractive model for studying developmental processes (Strmecki et al., 2005; Williams, 2006). It provides a useful intermediate step between fission yeast where the mating-type switch can be studied and more complicated “obligate” multicellular organisms such as *C. elegans* and *Drosophila*. An interesting feature of *Dictyostelium* differentiation and development is that it must reset without passing through the meiotic cycle.

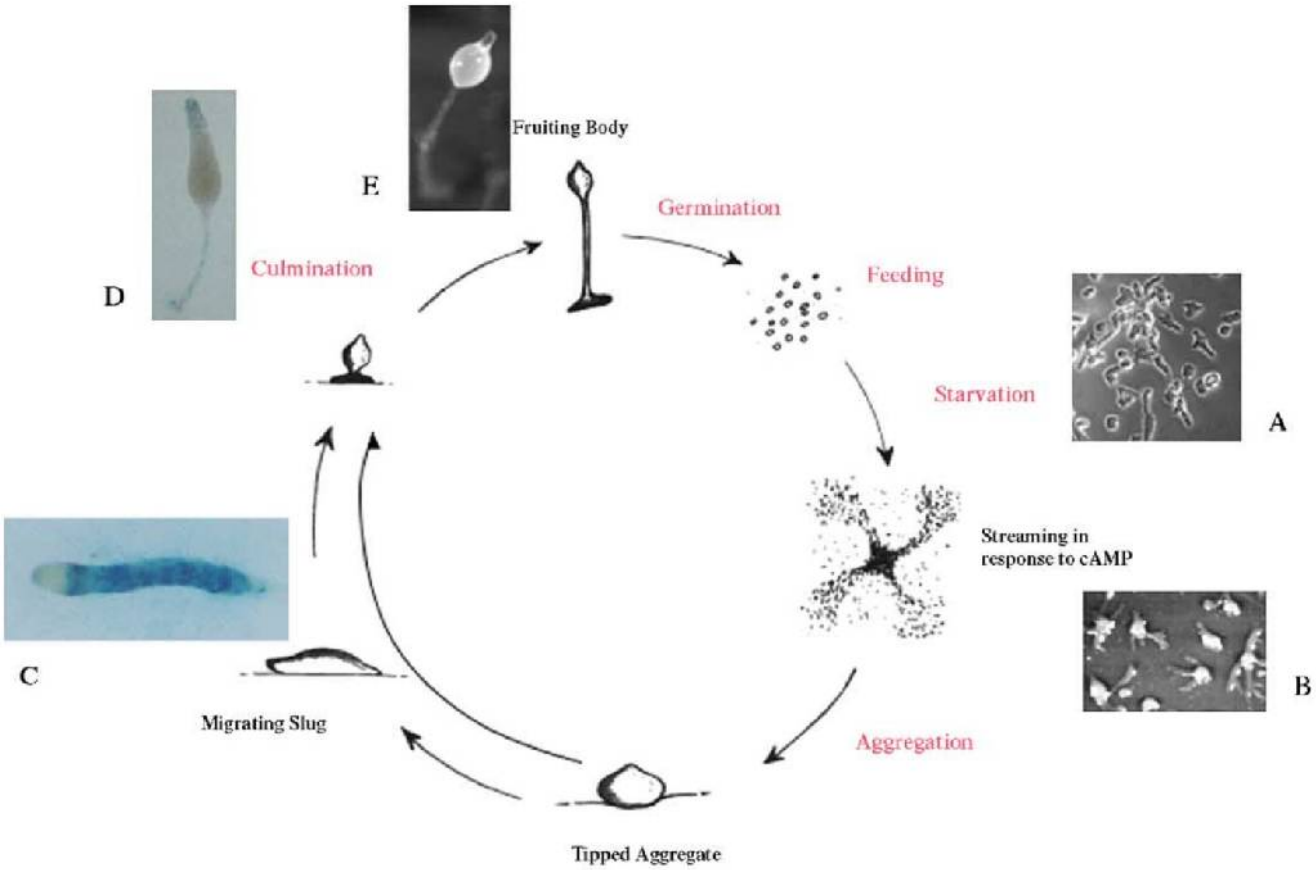


Fig. 1.1 ***Dictyostelium*** **Life Cycle** (from Strmecki et al., 2005). In the presence of a food source *Dictyostelium* amoeba are unicellular. On starvation some cells start to emit cAMP and the other cells chemotax towards them (**A**). The Streaming cells (**B**) form mounds of up to $1x10^5$ cells. The mound of aggregated cells forms a tip and then develops into a slug consisting of pre-stalk cells at the anterior, and pre-spore cells at the posterior (stained blue in panel **C**). The slug migrates towards an area that will favour spore dispersal and then culminates to form a fruiting body on a stalk (**D**). The spores after dispersal will germinate when food is again available.

1.1.1 Genome Features

Dictyostelium features a compact 34 Mbp haploid genome that encodes approximately 12 500 genes. This is significantly more than unicellular yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (approximately 6,000 and 5,000 genes respectively), and is closer to that of metazoans such as *Drosophila* (13,500 genes). 50% of the genome is arranged into 6 chromosomes, 30% consists of mitochondrial DNA and the remaining 20% makes up the extrachromosomal 88 kB palindromes which are present at about 100 copies per cell and contain the genes encoding the ribosomal RNA. The genome is extremely compact (62% protein coding) resulting in few and short introns. Non-coding regions between genes are short, typically around 50-500 bp, while repetitive elements comprise only 10% of the chromosomal DNA (Eichinger et al., 2005). Another notable feature of the genome is a strong AT bias (78%) and a pronounced AT difference between coding and non-coding regions.

1.2 Regulation of gene expression

The DNA sequence or genome contains the genetic information that serves as a “blueprint” which encodes the proteins and non-coding RNAs that along with a multitude of other enzymatic products which comprise an organism. In eukaryotes, this DNA is wrapped around proteins known as histones to form a DNA-protein complex known as chromatin. In addition to the genome, it is apparent that an additional level of information is present in eukaryotes. This information is at least partly contained within the chromatin but is independent of the DNA sequence, and is thus referred to as “epigenetic” information.

1.2.1 Chromatin

Eukaryotic chromosomes are packaged into arrays of nucleosomes which are arranged on the DNA like “beads on a string”. Each nucleosome consists of an octameric complex of histone proteins around which the DNA duplex is wrapped two and a half times (approximately 146 base pairs; Luger, 2003). These nucleosome arrays are arranged into higher order structures such as the proposed 30 nm fiber, although there is still debate regarding the nature of these higher order structures (Luger, 2006). Histone octamers consist of a core tetrameter comprised of two histone H3 and two histone H4 proteins. Two histone H2A/H2B dimers are bound to this tetrameter to form the complete histone octamer (Fig. 1.2, Luger, 2006).

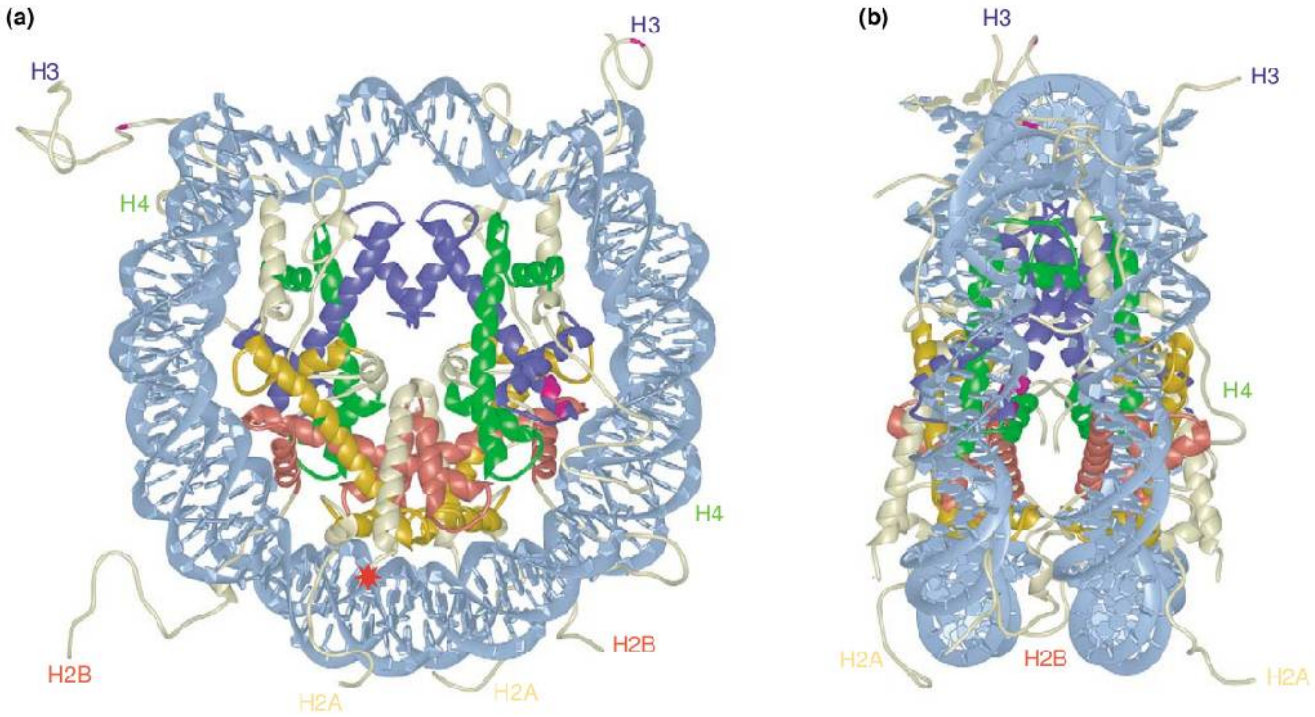


Fig. 1.2 **Nucleosome Structure** (from Luger, 2003). Front view (**a**) and side view (**b**) of nucleosome core particle (protein database 1KX5). Histones H2A, H2B, H3 and H4 core domains are shaded yellow, red, blue and green respectively. DNA double helix is coloured light blue.

The four core histones are among the most highly conserved proteins in eukaryotes. In addition to the core histones, variants of histone H3 (such as the centromeric histone H3 variant (CenH3) and the active chromatin H3.3 variants and histone H2A (e.g. macro-H2A , H2AX and H2AZ) are present in many, if not most eukaryotes. These variants replace the equivalent core histone in certain specific regions of the genome and serve to modify the physical properties of these loci and/or “mark” them in some manner (Hake and Allis, 2006; Luger, 2003). Linker histones such as histone H1, bind the naked linker DNA sequence in-between adjacent nucleosomes in a sub-stoichiometric manner and influence chromatin compaction.

In many eukaryotes histone genes are typically present in multiple identical, or near identical copies. In agreement with this a search of the *Dictyostelium* genome reveals two copies of histone H4: H4a and H4b which encode identical polypeptides, three near identical copies of H2B (H2Bv1-3), three histone H3 genes (H3a-c). two histone H2A genes, one with homology to the H2AX variant and the other with homology to the H2AZ variant. A single linker histone H1 is present. In addition, the genome contains 2 proteins with weak homology to histone H3 and 3 with weak homology to histone H2A (Table 1.1, www.dictybase.org).

Histone Family	Name	Gene ID	Predicted Size*	Comments
H1/H5	H1	DDB_G0285319	19 kDa**	Parish and Schmidlin, 1985; EST support
H2A	H2AX	DDB_G0279667	17 kDa	Hudson et. al., 2005; EST support
	H2AZ	DDB_G0286747	16 kDa	2 predicted introns, EST support
	H2Av1	DDB_G0289187	29 kDa	2 predicted introns
	H2Av2	DDB_G0289193	31 kDa	1 predicted intron
	H2Av3	DDB_G0289197	15 kDa	
H2B	H2Bv1	DDB_G0293758	25 kDa	EST support
	H2Bv2	DDB_G0276273	54 kDa	EST support
	H2Bv3	DDB_G0286509	17 kDa	EST support
H3	H3a	DDB_G0267402	16 kDa	EST support
	H3b	DDB_G0270838	16 kDa	1 predicted intron, EST support
	H3c	DDB_G0271092	16 kDa	1 predicted intron
	H3v1	DDB_G0291185	68 kDa	
	H3v2	DDB_G0277979	10 kDa	1 predicted intron
H4	H4a	DDB_G0277183	12 kDa	EST support
	H4b	DDB_G0276863	12 kDa	EST support

Table 1.1 **Putative *Dictyostelium* Histone Genes.** *Predicted size of protein products of histone genes, due to the lysine rich composition of these proteins, it is not uncommon for them to run at higher than the predicted level when analysed SDS-PAGE. ** for example, histone H1 shows an electrophoretic mobility corresponding to a molecular weight of 26 kDa (Parish and Schmidlin, 1985).

1.2.2 Epigenetics

Epigenetics is the transmission of a phenotype or gene expression patterns in a mitotically and/or meiotically inheritable manner from generation to generation or from mother to daughter cell independent of DNA sequence. This allows gene expression profiles to be “remembered” after mitosis or another biological event. The maintenance of cell-type specific expression profile in differentiated cells and the transmission of these expression profiles to their daughter cells rely on epigenetic mechanisms. This allows specific regions of the genome to be specified as active or inactive and influences their localisation within the nucleus and how tightly packed, or condensed they are. Some genes, often those involved in the regulation of embryogenesis and development are expressed in a parent-of-origin specific manner (i.e. only from the maternal or paternal allele) a process known as imprinting. These monoallelic gene expression profiles are specified during gametogenesis and transmitted by epigenetic mechanisms. Epigenetic processes are also involved in specifying and maintaining the function of genomic features such as telomeres, which protect the ends of chromosomes and centromeres, which are regions of the chromosomes that interact with the cell’s cytoskeleton and ensure correct segregation of the chromosomes during cell division.

1.2.3 Mechanisms

Epigenetic mechanisms usually function by marking the specific gene or locus whose expression is to be modified without changing the DNA sequence. This can be achieved by marking the DNA itself (methylation of cytosine at position 5) or by post-translation modification of the histone proteins around which the DNA is wrapped (Goldberg et al., 2007).

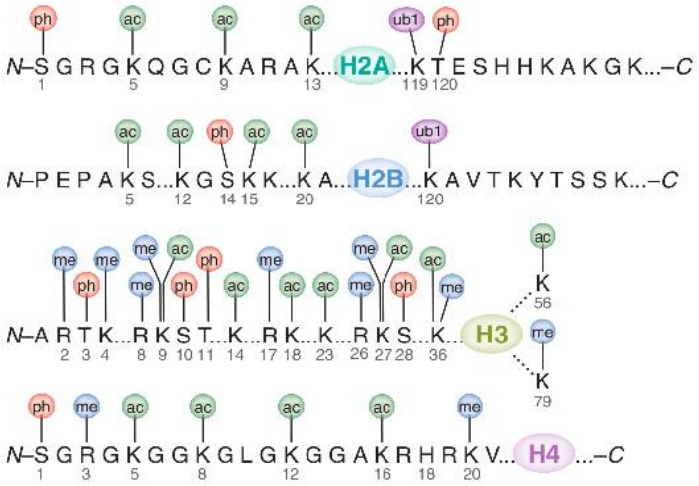


Fig. 1.3 **Post-Translational Modifications of Histones** (from Bhaumik et al., 2007).
Schematic representation of post-translational modifications that occur on mammalian histone tails: ac, acetylation; me, methylation; ph, phosphorylation; ub1, monoubiquitination.

These modifications include acetylation, formylation, methylation, ADP-ribosylation, ubiquitination, sumoylation, biotinylaltion (Fig. 1.3, Table 1.2). Alternatively, the expression of genes can be modulated by small RNAs, a class of short, 21-24 nucleotide RNAs with perfect or almost perfect complementarity to the gene of interest (or an adjacent region). Small RNAs can silence genes by inhibiting their translation, or, by recruiting DNA and/or histone modifying enzymes to the target locus, or by destruction of the mRNA transcripts.

DNA Methylation

In many, but not all eukaryotes DNA can be modified by the addition of a methyl group at the 5 position of cytosine (5meC). This modification is both mitotically and meiotically inheritable. In most organisms, the bulk of DNA methylation occurs on transposons and other invasive elements and serves to silence them. In addition, some silent genes are also methylated in their coding and/or regulatory regions. 5meC is catalysed by the Dnmt1 and Dnmt3 family of DNA methyltransferases. In animals this occurs mostly in the CG dinucleotide context while in plants it can occur in the CG, CNG and CNN contexts. In some cases small RNAs can trigger the methylation of homologous DNA sequences, although the exact mechanism is not yet clear (Wassenegger, 2005; Jeltsch, 2008). A third type of DNA methyltransferase, the Dnmt2 family, is widely distributed within eukaryotes and is highly conserved. Until recently, Dnmt2 was believed not to methylate DNA. Although it does appear to be able to methylate DNA, at least in *Drosophila* (Schaefer et al., 2008; Phalke et al., 2009), *Entamoeba histolytica (*Fisher et al., 2004) and *Dictyostelium* (Katoh et al., 2006; Kuhlmann et al., 2005), the major target of Dnmt2 appears to be the $tRNA^{Asp}$ (Goll et al., 2006). Nevertheless, in a recent publication Phalke et al. have shown that methylation of histone H4 at lysine 20 is reduced when *dnmt2* is knocked out in *Drosophila*.

Histone Acetylation

Acetylation of lysine residues in histone tails is one of the most abundant post-translational histone modifications. The N-terminal tails of all four histones contain lysine residues that are extensively acetylated. These residues (and their acetylation) appear to be highly conserved in eukaryotes, being present in plants, fungi and metazoans, as well as the apicomplexan *Plasmodium falciparum* and other basal eukaryotes. The acetylation of histone tails is catalysed by a family of enzymes known as histone acetyltransferases (HATs), examples of which include the GNAT (Gcn5) family and MYST family (ESA1; Turner, 2000). In contrast to most other histone modifications, acetylation is rather transient and is rapidly turned over (typical half lives are in the range of minutes; Waterborg and Matthews, 1984). The removal of acetylation marks is carried out by the histone deacetylase family of enzymes (HDACs) examples of which include Rpd3 and Sir2 from yeast. Histone acetylation is usually associated with actively transcribed genes, and some HATs are associated with the transcription activator complex, e.g. Gcn5 and p300 (Bhaumik et al., 2007).

The exact mechanisms by which histone acetylation regulates gene expression is unclear. One proposal is that acetylation acts to neutralise the positive charge of the histone tails and thus serves to dissociate the tails from the DNA and render the chromatin more labile (Ausio and Van Holde, 1986). Alternatively, histone acetylation may function by recruiting bromodomain containing proteins to bind directly to acetylated histones (Taverna et al., 2007). Bromodomains are found in many HAT members such as Gcn5/PCAF, CBP/p300 and TAF1, a component involved in initiation of transcription machinery assembly. While histone acetylation is strongly associated with actively transcribed genes, there are exceptions. For example, H3K56ac is associated with maintenance of genome stability and histone H4K12ac is associated with transcriptional silencing in *S. cerevisiae* and *Drosophila* (Bhaumik et al., 2007).

Histone Methylation

Many of the lysine residues within the tails of the core histones (and a few within the core domain) can be mono-, di- or tri-methylated. Likewise, arginine residues can be mono- or di-methylated. Unlike histone acetylation, histone methylation is a fairly stable modification and was considered to be irreversible until a few years ago. This modification is catalysed by the histone methyltransferase (HMT) family of proteins. Lysine histone methyltransferases usually contain a SET catalytic domain (Suppressor of variegation, Enhancer of zeste and Trithorax). Many of the HMTs were originally identified in suppressor screens in *Drosophila* where they are involved in the regulation of variegated gene expression (silencing of loci due to epigenetic phenomena). Methylated lysines can be selectively recognised and bound by proteins containing a “Royal family” domain (e.g. chromodomain, tudor domain, MBT domain) or a PHD finger (Plant homeodomain), a type of zinc finger. Unlike bromodomains, which recognise acetyl-lysine largely independent of sequence context, methyl-lysine binding domains are specific for particular lysines and their levels of methylation (i.e. mono, di- or tri-methylation). For example chromodomains (such as found in the heterochromatin protein HP1) are selective for H3K9me2,3 and H3K27me2,3. Recently proteins that can remove methyl groups from histones have been described. Amine oxidases such as LSD1 can remove mono- or di-methyl groups. Hydroxylases of the JmjC family are capable of removing mono-, di- or tri-methyl groups (Brosch et al., 2008). In some cases it has been shown that other histone modifications in the proximity affect the level of lysine methylation levels (H2BK123ub and H3R2me on H3K4me, H3S10ph on H3K9me (Kouzarides, 2007; Fig. 1.4).

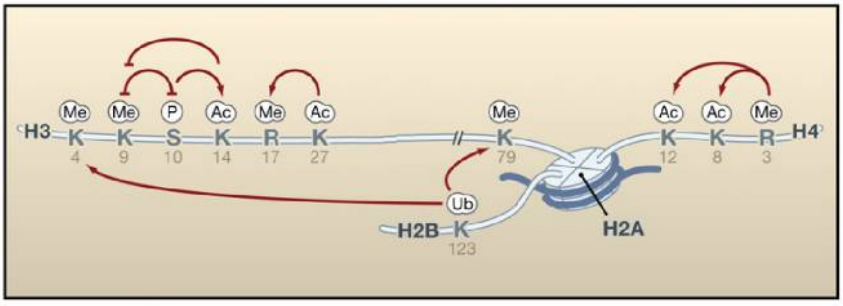


Fig. 1.4 **Cross-talk Between Histones Modifications** (from Kouzarides, 2007).
Positive effect of one histone modification on another is indicated with an arrow, negative effects with a dish-line. Ac: acetylation, Me: methylation, P: phosphorylation, Ub: mono-ubiquitination.

Arginine Methylation

Histone H3 can be mono- or di-methylated on arginine residues 2, 8, 17, 26 and histone H4 at arginine 3. Arginine methylation is catalysed by the CARM1/PRMT Arginine histone methyltransferases. With the exception of histone H3R8me, methyl-arginine of histones is associated with actively transcribed genes (Bhaumik et al., 2007). The target arginine residues are largely conserved in *Dictyostelium*, and the genome encodes several PRMT homologs (Kaller et al., 2006a).

H3K4me

Methylation of histone H3 at lysine 4 is a highly conserved modification present at the promoters of actively transcribed genes where it appears to have a role in transcription initiation. This modification is catalysed by HMT Set1 that is part of a multiprotein complex known as COMPASS (in yeast, the mammalian homologue of Set1 is called MLL, Bhaumik et al., 2007). The *Dictyostelium* Set1 protein is a homolog of yeast SET1 and H3K4me is completely abolished in *set1*$^{-}$ *Dictyostelium* cells (Chubb et al., 2006). Loss of H3K4me in *Dictyostelium* results in accelerated development at the onset of starvation as well as aberrant gene expression patterns during development (Chubb et al., 2006).

H3K9me

Methylation of histone H3 at lysine 9 is a highly conserved histone modification generally associated with heterochromatin and is found in almost all eukaryotes with the notable exception of the yeast *S. cerevisiae* (Krauss, 2008). The enzyme responsible for this modification is highly conserved and known as Su(var)3-9 in *Drosophila*, Set39h1, Set39h2, G9a and ESET in humans and Clr4 in the yeast *S. pombe*. A BLAST search of the *Dictyostelium* genome revealed a single Su(var)3-9 homologue (SuvA; DDB_G0269554, Kaller et al., 2006a; M. Essid, Diploma thesis, Kassel University, 2004). Attempts to knock-out the *suvA* gene in *Dictyostelium* have so far not been successful suggesting it is an essential gene in *Dictyostelium* (M. Essid, Diploma thesis, Kassel University, 2004). H3K9me2 has previously been detected (Kaller et al., 2006b) and more recently (this thesis) H3K9me3 has also been detected in *Dictyostelium*. H3K9me is responsible for recruiting a number of chromatin binding proteins to heterochromatin such as HP1 which has two homologs in *Dictyostelium*, HcpA and HcpB.

H3K27me

Histone H3 can be methylated at lysine 27 in many organisms including metazoans, plants and *Giardia* and is catalysed by Ezh2, a component of the Polycomb 2 (PCR2) complex (Garcia et al., 2007; Fuchs et al., 2006). H3K27me is associated with developmentally regulated gene silencing. The *Dictyostelium* genome does not appear to contain any homologs of the Ezh2 or other

components of the PCR2 complex, and while the lysine residue at position 27 itself is conserved in *Dictyostelium*, the flanking residues are not.

H3K36me

The histone methyltransferase SET2, which associates with elongating RNA pol II and is responsible for H3K36 methylation has been found in all eukaryotes examined (Bhaumik et al., 2007). H3K36me is distributed throughout the coding region of active genes where it may have a role in transcriptional elongation. In *Dictyostelium* a putative homolog of SET2 has previously been annotated (DDB_G189799; Kaller et al., 2006a). The residues surrounding this lysine in *Dictyostelium* are well conserved with respect to other eukaryotes.

H3K79me

H3K79me is catalysed by the methyltransferase DOT1 (defective of telomere silencing 1). Unlike all other histone methyltransferases, DOT1 does not contain a SET domain. While most SET domain proteins display methyltransferase activity on a peptide consisting of their target site and the 3-4 flanking residues on either side, DOT1 can only methylate histone H3K79 in the context of a nucleosome, suggesting that a different mode of recognition is used (Taverna et al., 2007). Histone H3K79me is necessary for proper telomeric silencing (Bhaumik et al., 2007) and has also been implicated in stem cell differentiation (Barry et al., 2009). The lysine residue at position 79 is conserved in *Dictyostelium* histone H3 and a gene encoding a DOT1 homolog (DDB_G0271626) is present in the genome (Kaller et al., 2006a).

H4K20me

Methylation of lysine 20 is a repressive mark found in plants, metazoans and protists (Cui et al., 2008) but not in fungi. Histone H4K20me is associated with heterochromatin and can be selectively recognised by proteins containing a double tudor domain such as 53BP1. While the genome of *Dictyostelium* contains a number of putative histone methyltransferase in addition to the already identified SET1, SET2, SuvA and DOT1 homologs, none of them has homology to the Suv(4-20) /SET8 histone methyltransferases responsible for methylation of lysine 20. In agreement with this the N-terminal tail of *Dictyostelium* H4 around lysine 20 is poorly conserved (Fig. 3.5), suggesting that this modification is not present in *Dictyostelium*.

Histone Phosphorylation

Histones can be phosphorylated at serine and threonine residues. Histone H3S10 and H3S28 are phosphorylated by the highly conserved Aurora kinases during cell division. Phosphorylation of H3S10 serves to disrupt binding of the heterochromatin protein HP1 to H3K9me (Johansen and Johansen, 2006; Fuchs et al., 2006). H3S10ph has also been associated with gene activation

(Ivaldi et al., 2007). In response to DNA double strand breaks, H2AXS139 adjacent to the damage site becomes rapidly phosphorylated by ATM kinase or DNA dependent protein kinase (DNA-PK) and this serves to recruit the DNA damage machinery (Redon et al., 2002). A DNA-PK homolog is present in *Dictyostelium* and has been shown to be required for efficient DNA damage induced H2AX phosphorylation (Hudson et al., 2005). An Aurora kinase homolog is present in *Dictyostelium* (Li et al., 2008) and the histone H3S10 residue is conserved, suggesting that this residue can be phosphorylated in *Dictyostelium*.

Histone Ubiquitination

Ubiquitin is a small, highly conserved protein that is conjugated via its C-terminal lysine residue to other ubiquitin moieties forming poly-ubiquitin chains. These poly-ubiquitin chains can be conjugated to a lysine residue on a target protein which targets that protein for destruction by the 26S proteosome. It is however worth noting that ubiquitin was first identified as a conjugate on histones. Histones are usually mono-ubiquitinated which in contrast to poly-ubiquitination, does not target them for proteolytic degradation, instead mono-ubiquitination on histones appears to serve as an epigenetic mark (Weake and Workman, 2008).

Histone H2B can be mono-ubiquitinated at lysine 123 on its C-terminal tail, this modification is catalysed by the Bre1/Rad6 ubiquitin-ligase complex. The modification is occurs at active genes where ubiquitinated H2B must be present for Set1 to catalyse H3K4me2 and me3, but not H3K4me1. This is one of the best examples of cross-talk between different histone modifications (Kouzarides, 2007). *Dictyostelium* has homologues of both Rad6 (DDB_G0275787) and Bre1 (DDB_G0274241). The C-terminus of H2B also has a conserved lysine residue that could be the target of these enzymes.

Histone H2A can be mono-ubiquitinated at lysine 119. This modification is catalysed by the polycomb repressive complex 1 (PCR1), and in female metazoans H2A on the inactive X-chromosomes is heavily ubiquitinated (Redon et al., 2002). *Dictyostelium*, like yeast and plants, lacks the PCR1 complex, and an antibody against H2AK119ub gives no signal in any of these species (de Napoles et al., 2004). Since the target lysine and the residues flanking it are at least partially conserved in these species, the post-translational modification of this residue may nevertheless still occur.

Interestingly, it has been shown recently that in response to DNA damage H2A can be ubiquitinated by a second complex containing the WD40 domain containing protein DDB1 (damaged-DNA binding protein 1) and the ubiquitin ligase adaptor Cullin4 in response to H2AXS139 phosphorylation (Li et al., 2006; Kapetanaki et al., 2006). DDB1 and Cullin4 homologs are highly conserved and found in all eukaryotes, including *Dictyostelium* where they are named RepE (DDB_G0286013, Alexander et al., 1996) and CulD (DDB_G0292794) respectively.

Histone Sumoylation

All four histones can be posttranslationally modified by SUMO (small ubiquitin-like modifier) which, like ubiquitin can be conjugated to lysine residues. Reported sites of SUMO conjugation include lysine 126, on H2A, lysines 6,7,16 and 17 on histone H2B and at lysines 5, 8, 12, 16 and 20 on histone H4 (Bhaumik et al., 2007). The modification is catalysed by the enzyme UBC9 and is associated with transcriptional repression. It appears to function by antagonising histone acetylation (Iñiguez-Lluhí, 2006). SUMO proteins are present in *Dictyostelium* (Sobko et al., 2002), but it is not possible to identify a UBC9 homolog in the *Dictyostelium* genome based on sequence alone, as UBC9 has high homology with ubiquitin E3 ligases.

Histone Citrullination

Citrullination has been has been detected at histone H3R17 and H4R3. This modification is catalysed by the enzyme peptidylarginine deiminase 4 (PAD4) which converts the methylated arginine to citrullinated arginine (Wang et al., 2004). PAD4 associates with HDAC1 and functions as a transcriptional repressor (Denis et al., 2009). Peptidylarginine deiminase encoding genes appear to be absent from the *Dictyostelium* genome, suggesting this modification may not be present in this species.

Histone ADP-Ribosylation

At sites of DNA damage the histone variant macroH2A1.1 can be (poly)ADP-ribosylated by the enzyme PARP1 on its macro domain (Timinszky et al., 2009). The recruitment of macroH2A1.1 results in a transient compaction of the chromatin structure. Although a few genes in the *Dictyostelium* genome encode macro domains, none are present on histones, suggesting that this mechanism is not conserved in this organism.

Modification	Enzyme responsible	*Dictyostelium* homolog
H3K4me	Set1 (Sc), SET7/SET9 (Hs), MLL (Hs), Smyd3 (Hs)	Set1 (DDB_G0289257)
H3K9me	SUV39H1 & SUV39H2 (Mm, Hs), G9a (Mm, Hs), Eu-HMTase1 (Hs), ESET & SETDB1 (Mm, Hs), Clr4 (Sp), Dim5 (Nc), Kryptonite (At), Ash1 (Dm)	SuvA (DDB_G269554)
H3K27me	E(z) (Dm), EZH2 (Hs, Mm)	-
H3K36me	SETD2/HYPB (Hs), NSD1 (Hs), Set2 (Sc)	DDB_G0648132
H3K79me	DOT1 (Sc), DOT1L (Hs)	DotA (DDB_G0271626)
H4K20me	Pr-SET7/SET8 (Hs, Dm), SUV4-20 (Hs), SET9 (Sp)	-
H3R2me	CARM1 (Mm, Hs)	prmt1 (DDB_G0235399), DDB_G0276237
H3R26me	CARM1 (Mm, Hs)	
H4R3me	PRMT1 (Hs), RMT1 (Sc)	
H3K9ac	Gcn5 (Sc), Src1 (Mm)	Gcn5 (DDB_G0283459), DDB_G0274269, DDB_G0275159,
H3K14ac	Gcn5 (Tt, Sc), Src1 (Mm), TAF1 (Dm, Hs), CBP & p300 (Hs), Sas3 (Sc),MOZ & MORF (Hs), PCAF & hGcn5 (Hs)	
H3K18ac	Gcn5 (Sc), CBP & p300 (Hs)	
H3K23ac	Gcn5 (Sc), CBP (Hs), Sas3 (Sc)	
H3K36ac	Gcn5 (Sc)	
H3K56ac	Rtt109 (Sc)	
H4K5ac	Esa1 (Sc), Hat1 (Tt, Dm, Hs), p300 (Hs), Tip60 (Mm), HBO1 (Hs)	
H4K8ac	p300 (Hs), Esa1 (Sc), Tip60 (Mm), p300 (Hs), HBO1 (Hs)	
H4K12ac	Hat1 (Sc), Esa1 (Sc), Tip60 (Mm) and CBP & p300 (Hs), HBO1 (Hs)	
H4K16ac	Mof (Dm), hMof (Hs), Sas2 (Sc), Tip60 (Mm), Esa1 (Sc)	
H2AK5ac	Tip60 (Hs, Dm)	
H2BK11ac	Gcn5 (Sc)	
H2AZK14ac	Esa1 (Sc), Gcn5 (Sc)	
H3S10ph	Snf1 (Sc), Jil-1 (Dm), Rsk2 (Mm, Hs), Msk1 (Mm), Ip11(Sc), Aurora B (Ce, Dm, Hs),	AurK (DDB_G0279343)
H3S28ph	Aurora B (Mm, Hs)	
H4S1ph	Sps1 (Sc), CKII (Sc)	?
H2BS14ph*	Mst1 (Hs), Ste20 (Sc)	?
H2AXS139ph**	Tel1 & Mec1 (Sc), ATM & ATR & DNAPK (Hs)	DNAPKcs (DDB_G0281167)
H2AK119ub	Ring1B (Dm, Mm, Hs)	-
H2BK120ub***	Rad6 (Sc), Bre1 (Sc), HR6A (Hs), HR6B (Hs)	DDB_G0275787, DDB_G0274241

Table 1.2 **Histone Modifying Enzymes.** Table of histone modifications together with the enzymes responsible for catalysing these modifications and possible *Dictyostelium* homologs. Hs: *Homo sapiens,* At: *Arabidopsis thaliana*, Sc: *Saccharomyces cerevisae*, Xl: *Xenopus laevis*, Ce: *Caenorhabditis elegans*, Dt: *Drosophila teissieri*, Dm: *Drosophila melenogaster*, Mm: *Mus musculus,* Sp: *Schizosaccharomyces pombe.* *H2BS10ph in yeast, **H2AS129ph in yeast, *** H2BK123ub in yeast.

1.3 Chromatin Associated Proteins

1.3.1 Linker Histone H1

In addition to the core histones, most eukaryotes have one or more variants of the linker histone H1 or the linker histone H5. Histone H1 variants are present in chromatin in a sub-stoichiometric ratio and bind the free DNA in between nucleosomes. This is thought to stabilise chromatin and favour the formation of heterochromatin. Histone H1 variants have been shown to have a role in developmental regulated gene repression (Gilbert et al., 2003). *Dictyostelium* has a single linker histone H1 (DDB_G0285319) which has been characterised at the biochemical level (Parish et al., 1985; Res, 1985; Blumberg et al., 1991).

1.3.2 Heterochromatin Protein 1

Heterochromatin protein 1 (HP1) was first described as a protein binding to heterochromatin in *Drosophila.* It consists of an N-terminal chromodomain, which recognises methylated lysines separated by a hinge region which has nucleic acid binding activity and a C-terminal chromoshadow domain which mediates homodimerisation (Fanti and Pimpinelli, 2008). HP1 binds to H3K9me and H3K27me *in vitro,* although it has rather low affinity and other factors appear to be necessary for *in vivo* targeting (Eskeland et al., 2007). HP1 is recruited to constitutive heterochromatin where it helps to maintain gene silencing and is required for centromere formation (Fischer et al., 2009; Folco et al., 2008). HP1 is also necessary for telomere maintenance and some isoforms such as HP1γ in mammals and LHP1 in *Arabidopsis* are involved in the formation of facultative heterochromatin and developmental gene regulation (Mylne et al., 2006). *Dictyostelium* has three genes encoding HP1 homologs HcpA (DDB_G0283023), HcpB (DDB_G0282987) and HcpC (DDB_G0270198). HcpA and HcpB have been characterised and at least one is required for cell viability in *Dictyostelium*, while HcpC is expressed at a very low level, if at all (Kaller et al., 2006b).

1.3.3 Polycomb Proteins

A series of developmental mutants identified in *Drosophila* were found to encode components of the two multiprotein complexes, PCR1 (the polycomb repressive complex 1) and PCR2. In animals the polycomb complexes act as a cellular “memory” that serve to control body patterning by regulating the expression patterns of the HOX transcription factors in a segment specific manner. They also have roles in the regulation of epigenetic cellular memory, pluripotency and stem cell self renewal (Bantignies and Cavalli, 2006). Some examples include X-inactivation in mammals and vernalisation in plants. The core PCR2 complex consists of the SET domain containing protein Enhancer of zeste (E(z)), Extra sex combs (Esc), Suppressor of zest 12 (Su(z)12) and the 55 kDa

nucleosome remodelling factor Nurf55. PCR2 catalyses the repressive H3K27me3. PCR1 consists of the chromodomain containing Polycomb (Pc) protein, dRing, Posterior sex combs (Psc) and Polyhomeotic (Ph). The chromodomain of Pc binds H3K27me3, while dRing catalyses the monoubiquitination of histone H2A at lysine 119 (de Napoles et al., 2004). PCR1 and PCR2 are present in most animals, while plants and nematodes only have the PCR2 complex. The lack of PCR1 and the modification it catalyses (H2AK119ub) may be at least partly responsible for the greater de-differentiation potential of plants compared to animals. *Dictyostelium* appears to lack both the PCR1 and PCR2 complexes, which is consistent with previous results suggesting both H3K27me and H2AK119ub are absent from this species (de Napoles et al., 2004).

1.4 The role of RNAi in gene expression and nuclear organisation

Over recent years it has become apparent that most of the eukaryotic genome is transcribed to some extent, regardless of whether it is protein coding or not. Consistent with this RNA pol II can be detected at many loci in the genome, including genes thought to be silent (Bühler and Moazed, 2007). In general it appears that many loci need to be transcribed to some extent in order to be silenced. Plants also have additional RNA polymerases, RNA pol IV and RNA pol V, that transcribe mainly silent loci (Matzke et al., 2009). In plants, fungi, amoebozoa, and some animals such as *C. elegans,* RNA pol II derived transcripts serve as a substrate for the RNA dependent RNA polymerase (RdRP) which uses it as a template to generate a second strand, resulting in double stranded RNA (dsRNA; Fig. 1.5). In turn, Dicer cleaves the dsRNA into small 21-24 nucleotide RNAs known as small interfering RNA (siRNA; Verdel et al., 2009). These siRNAs can be bound by an Argonaut protein which uses it to target a complementary nucleic acid sequence by base pairing. The Argonaut protein can slice the target nucleic acid sequence, which, in case of an mRNA (messenger RNA) results in RNA decay and is known as PTGS (post-transcriptional gene silencing).

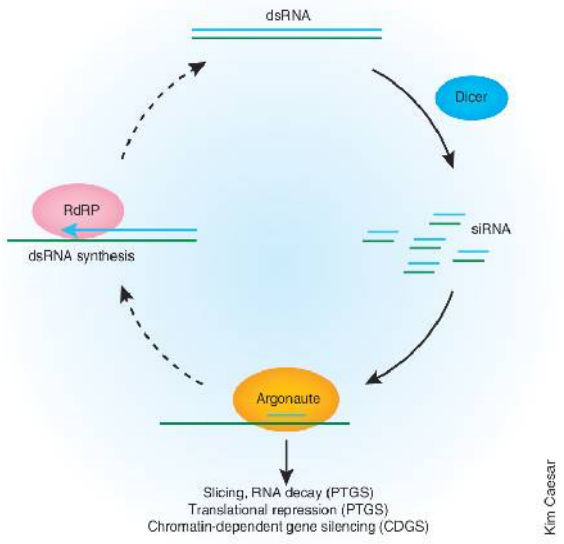


Fig. 1.5 **RNA Mediated Silencing Pathways** (from Bühler and Moazed, 2007). Schematic representation of the core components of the RNA silencing pathways.

Translation of the mRNA into a polypeptide can be inhibited by incomplete base pairing of a small RNA (miRNA) usually to untranslated regions of an mRNA (translational gene silencing). At least in fission yeast and plants, the siRNA can target the chromosomal locus from which it was originally transcribed or another locus with sequence homology. In fission yeast siRNAs generated from the pericentromeric repeats are bound by Ago1 which forms part of a complex known as RITS (RNA-induced transcriptional silencing). RITS is guided by the bound siRNA and via its chromodomain containing Chp1 protein to the histone H3K9me decorated centromeric repeats where it recruits an RdRP containing complex which converts the nascent pericentromeric transcript to dsRNA (Zofall and Grewal, 2006). The dual requirement of RNAi and histone H3K9me provides specificity for the RNAi machinery. Clr4, the H3K9 HMT homolog of *S. pombe* is part of the Rik1 complex which also contains Cullin4, Rik1 (a homolog of DDB1 (Damaged DNA binding protein 1)). The Rik1 complex is necessary for histone H3K9me in the pericentromeric region (Hong et al., 2005). The RNAi pathway must be functional for targeting of the Rik1 complex to the pericentromeric regions (Kapetanaki et al., 2006) although the mechanism by which this happens is not clear. In plants siRNAs can induce DNA methylation at homologous sequences (Wassenegger et al., 1994; Matzke et al., 2007; Teixeira et al., 2009).

1.5 Nuclear Organisation

Eukaryotic nuclei display extensive organisation with individual chromosomes occupying their own discreet territory within the nucleus (Cremer et al., 2006; Lieberman-Aiden et al., 2009). The localisation of a locus within the 3D space of a chromosome territory is also correlated with its expression status. Inactive loci tend to be present in the interior of a chromosome territory, while active loci are often localised at the inter-chromosome boundaries. Recruitment of genes to the nuclear periphery or to nuclear pores has been associated with changes in gene expression level (Finlan et al., 2008; Wiblin et al., 2005). Using FISH and fluorescently tagged proteins it has been shown that many genes are repositioned within a chromosome territory in response to changes in gene expression levels. Developmentally regulated genes that are differentially expressed in different cell types, such as the immunoglobulin loci, not only are decorated with different chromatin modifications in these different cell types but are localised to different positions within the nucleus (Lanctôt et al., 2007). Some differences in the position of entire chromosomes within nuclear space have been observed between different cell types (Sexton et al., 2007).

1.5.1 Constitutive heterochromatin

Heterochromatin was originally described cytologically as regions of the nucleus that remain dense or compacted throughout the cell cycle (Heitz, 1928). These regions, which are now referred to as constitutive heterochromatin typically contain few protein coding genes. Instead they contain most of the transposons and other repetitive elements found within the an organisms genome.

Constitutive heterochromatin is visible as bright regions when nuclei are stained with DAPI, and remain optically dense in acid giemsa staining. This suggests that the chromatin in these regions is more compacted and in a closed conformation. Constitutive heterochromatin often has low levels of histone modifications associated with active genes such as acetylation and methylation of lysines 4 and 36 on histone H3. Histone H3K9 methylation is present at high levels at these loci (although in some species it is also present in other regions of the nucleus). The chromodomain containing protein HP1 (or certain isoforms of it) are enriched within heterochromatin. In many organisms the chromosomal regions responsible for chromosome segregation, the centromeres, are located within or adjacent to constitutive heterochromatin (in which case it is also known as pericentromeric heterochromatin). Heterochromatinisation appears to repress expression of genes and transposons, although even heterochromatin seems to be transcribed to some extent by RNA polymerases. Constitutive Heterochromatin is both mitotically and meiotically inheritable. Stable meiotic inheritance is mediated at least in part by DNA cytosine methylation patterns, which are transmitted to the progeny (Bártová et al., 2008).

1.5.2 Centromeres

Centromeres are essential regions of eukaryotic chromosomes that link them to the cytoskeleton. A protein complex, the kinetochore, is formed on the centromeres (Gieni et al., 2008) and constitutes the attachment site for the microtubule spindle, which grows out of the centrosome (microtubule organizing centre = MTOC).

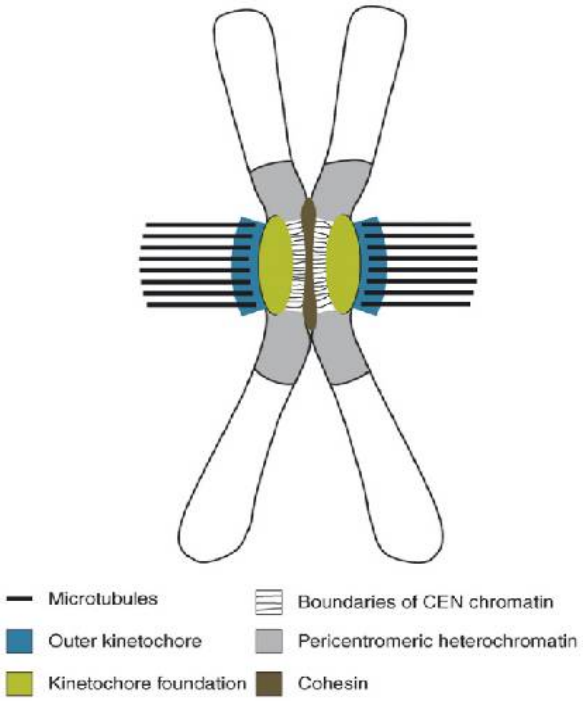


Fig. 1.6 **Centromere Structure** (Dawe and Henikoff, 2006). Schematic representation of the proposed organisation of eukaryote centromeres.

Centrosomes are in turn anchored via further microtubules to the rest of the cytoskeleton and provide the physical force that separates the sister chromatids and localises them to the daughter cells during mitosis and meiosis (Fig. 1.6). The proteins comprising the kinetochore complex are highly conserved and a single evolutionary origin for centromeres early in eukaryotic evolution has been proposed (Tyler-Smith and Floridia, 2000). Centromere size is extremely variable, ranging from the 125 bp point centromeres in *S. cerevisiae* to the holocentric centromeres of *C. elegans* which span the entire length of the chromosome. Most eukaryotes have regional centromeres consisting of arrays of satellite repeats and transposons or retrotransposons typically spanning 100kB to 100Mb (Ekwall, 2007; Fig. 1.7).

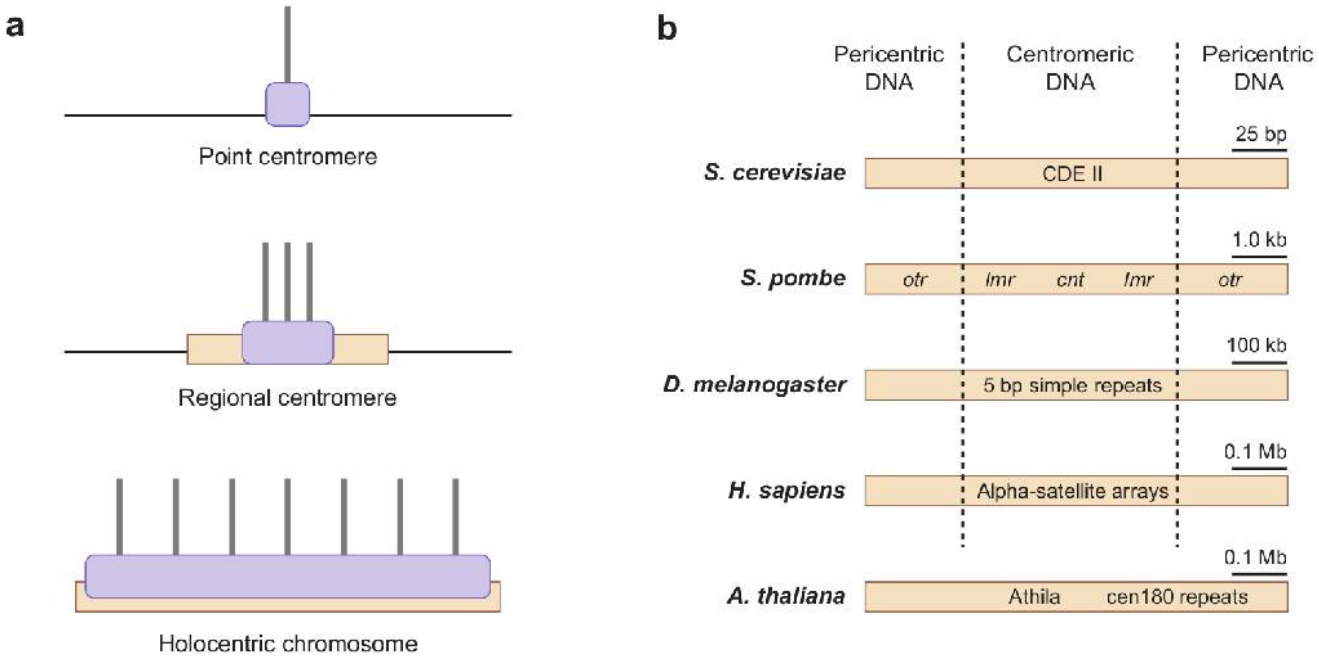


Fig. 1.7 **Centromere Organisation in Different Species** (from Ekwall, 2007). **a:** Different types of centromeres, Grey lines: kinetochore microtubules, purple box: kinetochore, tan box: centromere DNA. **b:** Centromeric DNA organisation in different species.

It has been suggested that modern centromeres evolved from an ancestral holocentric centromere (Dernburg, 2001), while others have proposed that regional centromeres evolved from telomeres which also typically contain repetitive sequences and transposons (Villasante et al., 2007). The existence of conventional regional centromeres in the basal eukaryote *Giardia lamblia* is consistent with this latter view (Dawson et al., 2007). In contrast to the highly conserved kinetochore proteins, the DNA sequence composing the centromeres is highly variable (Cooper and Henikoff, 2004; Malik and Henikoff, 2001), and in some species is not even conserved between the different chromosomes (Sanyal et al., 2004). It has been hypothesised that the rapid evolution of centromeres is a major driver of speciation as sequence divergence leads to incompatibility in meiosis (Henikoff et al., 2001). With the exception of point centromeres such as those of *S.*

cerevisiae, DNA sequence alone appears insufficient to specify centromere function. Instead centromere formation usually requires the presence of pericentromeric heterochromatin, which flanks a core centromere region (Fig. 1.7a). In the fission yeast *S. pombe*, pericentromeric heterochromatin formation is RNAi dependent, where small RNAs serve to recruit repressive chromatin modifications such as H3K9me2. The RNAi pathway also appears to be necessary for pericentromeric heterochromatin maintenance in mammals and *Drosophila* (Ekwall, 2007). In some nucleosomes within the core centromere histone H3 is replaced by a centromere specific variant know as CSE in fungi, Cid in *Drosophila*, CENP-A in metazoans and Htr12 or CenH3 in plants. This centromeric H3 variant appears to be essential and interacts directly with components of the kinetochore complex. Unlike the highly conserved kinetochore proteins, CenH3 is poorly conserved between different species. CenH3 appears to co-evolve with the rapidly evolving centromere DNA sequences, which are also not conserved between species. CenH3 differs from conventional histone H3 by a divergent and longer loop1 and also a divergent alpha-helix 2 (Sullivan et al., 1994; Black and Bassett, 2008). Many CenH3 variants, such as Cid from *Drosophila* have a long N-terminal domain, however, this appears to be dispensable for proper targeting of CenH3 (Vermaak et al., 2002). In contrast to the surrounding pericentromeric heterochromatin, the core centromere has properties that resemble those of euchromatin such as H3K4 methylation and low levels of H3K9 methylation (Gieni et al., 2008).

The sequence identity of the centromeres has not been confirmed in *Dictyostelium*, but is thought to be defined by a large cluster of retrotransposons, of which DIRS-1 is the most prominent component in a telocentric position of each chromosome. The centromeres have been defined by cytological methods as a single locus that is intensely stained for H3K9me2, GFP-HcpA and GFP-HcpB during interphase (Kaller et al., 2006b).

1.5.3 Facultative Heterochromatin

Facultative Heterochromatin contains loci that are silent in a particular cell type, stage of the cell cycle, or point in time of development. Facultative heterochromatin is generally devoid of “active” modification marks such as histone acetylation and methylation of histone H3 at lysines 4 and 36. In higher eukaryotes it is associated with high levels of H3K9me, H3K27me and H4K20me. In some cases, isoforms of HP1, such as HP1γ in mammalian cells and LHP1 in *Arabidopsis* may be associated with these loci (Trojer and Reinberg, 2007). The repressive polycomb complexes PCR2 and PCR1 are involved in specifying facultative heterochromatin. Facultative heterochromatin is mitotically but not meiotically inheritable and unlike constitutive heterochromatin, is not normally associated with DNA methylation. Unlike constitutive heterochromatin, facultative heterochromatin does not form distinct structures at the resolution of light microscopy, but rather appears scattered throughout the nucleus (Bártová et al., 2008).

1.5.4 Euchromatin

Euchromatin usually consists of regions containing transcribed genes. Euchromatin is decondensed and characterised by high levels of histone acetylation and methylation of histone H3 on lysine H3K4 and K36 and low levels of repressive modifications (although H3K9me has been reported on some active genes). Complexes such as SAGA, COMPASS and the activating polycomb complex trithorax (TRX) are associated with euchromatin (Bártová et al., 2008).

1.5.5 Ribosomal DNA

The RNA components of the ribosome are transcribed from the ribosomal DNA genes which normally occur as tandem arrays present at a few hundred copies per genome. The 5S ribosomal RNA gene is transcribed by RNA polymerase III while RNA polymerase I transcribes the 45S ribosomal RNA precursor, which is further processed to yield the 18S, 5.8S and 28S ribosomal RNAs. (17S, 5.8S and 26S in *Dictyostelium*). The ribosomal RNA genes are generally the most highly transcribed genes in the eukaryotic nucleus and are localised to bodies known as the nucleoli (McKeown and Shaw, 2009). In most eukaryotes the ribosomal DNA genes are clustered in arrays at one or a few chromosomal loci but in some cases such as the ciliate *Tetrahymena,* and the Ameobazoa *Dictyostelium* and *Physarum* the ribosomal DNA genes are not on the chromosomes, but rather are present as tandem repeats on extrachromosomal palindromes (Cockburn et al., 1978). The nucleus of *Dictyostelium* contains approximately 100 copies of the 88 kb rDNA extrachromosomal palindrome. A master copy of the rDNA palindrome appears to be present on chromosome 4, but the exact mechanism by which the extrachromosomal palindromes are replicated and maintained free of sequences heterogeneity is not known (Sucgang et al., 2003). The ends of the palindromes consist of AG_7 repeats similar to those of normal eukaryote telomeres.

1.5.6 Telomeres

Telomeres are specialised regions that occur at the ends of eukaryotic chromosomes. They serve to protect the ends of the chromosomes from degradation and also serve to avoid triggering a DNA damage response, which usually occurs when free ends of DNA are present in the nucleus (Weaver, 1998; Gasser, 2000). Like centromeres, telomeres often contain transposons and repetitive elements. In most species the very ends of the telomeres consists of 10's to 100's of a simple DNA repeat (TTAGGG in vertebrates) that is added to the ends by the telomerase RNA – protein complex to avoid the loss of chromosome ends during replication. In the case of *Drosophila,* telomerase does not seem to be present and transposon activity appears to be sufficient to compensate for replication mediated loss of chromosome ends. Loss of telomeric repeats has been implicated in cell ageing (Blackburn et al., 2006; Armanios et al., 2009). *Dictyostelium* has an unusual telomere organisation with each end of the chromosomes containing

a fragment of the extrachromosomal rDNA palindrome, including the distal repeats which serves to protect the ends of the rDNA palindrome (Eichinger et al., 2005; Glöckner and Heidel, 2009).

1.5.7 Tranposons and Repetitive Elements.

A considerable part of most eukaryotic genomes consist of repetitive elements. These can be simple repeated sequences, which consist of tandemly repeated short nucleotide sequences. While these simple repeated sequences are generally confined to non-coding genomic regions in most organisms, 34% of *Dictyostelium* genes contain simple sequence repeats that result in homo-polymer stretches (typically of glutamine or aspartate; Eichinger et al., 2005). Genomes also contain complex repeat elements, which are larger (often over three kb) and usually contain coding sequences. As these elements are or were originally mobile and can move to other loci in the genome, they are referred to as transposable elements (TEs). They seem to provide no obvious function to the host cell and thus they are often considered to be “selfish DNA” (Flavell, 1995). TEs are the major constituent of the repetitive DNA within the constitutive heterochromatin in many eukaryotes. They are characterised by the presence of direct or inverted repeat sequences at their ends, the presence of truncated or partial copies in the genome, and regions with the potential to form secondary structures. Some TEs are excised from the genome and reinserted into another locus, in which case they are known as DNA transposons. In contrast, retroelements are transcribed into an RNA intermediate, which can then be reverse-transcribed into DNA by a reverse-transcriptase. These copies can then be inserted into a new genomic locus. Approximately 10% of the *Dictyostelium* genome is comprised of repetitive elements (Eichinger et al., 2005). The LTR (long terminal repeat) containing DIRS-1 (*Dictyostelium* inverted repeat sequence 1) family (Cappello et al., 1984; Cappello, et al., 1985a) is the most abundant with approximately 240 complete and incomplete copies composing approximately 3% of the *Dictyostelium* genome. DIRS-1 is 4.8 kB long and has an unusual replication method. Instead of an integrase enzyme found in most retroelements, it encodes a tyrosine recombinase which can perform recombination on double stranded DNA sequences that contain sequence similarity. DIRS-1 contains non identical inverted repeat sequences, which apparently allow it to replicate via a circular DNA intermediate (Cappello, et al.,1985b). RNA from DIRS-1 is detectable at low levels on northern blots and is present at much higher levels in the rrpC- mutant (Martens et al., 2002; Kuhlmann et al., 2005). Approximately 2/3 of all endogenous siRNAs are derived from DIRS-1 (Hinas et al., 2007). The *skipper* retrotransposon is a 7 kB LTR transposon of the Ty3/*gypsy* family present at about 50-60 copies, comprising almost 1% of the genome (Leng et al., 1998). *skipper* transcripts are readily detected on northern blots and transcripts increase during *Dictyostelium* development (Martens et al., 2002). The *Dictyostelium* genome also contains 5-20 copies of the 5 kB DGLT-A (*Dictyostelium* Gypsy-like transposon A) retrotransposon and ten copies of DGLT-P, a non-functional pseudo-transposon (Glöckner et al., 2001; Table 1.3).

Subgroup	Class	Transposon	Accession no.	Consensus length (bp)	LTR type	LTR length (bp)	TSD (bp)	Genome content (% nt)	Fragment no. N_r	Fragment no. ML (N)	Fragment no. FI
LTR transposons	DIRS-1	DIRS-1*	M11339	4826	IR	320	0	3.260	235	302	1.3
	gypsy	skipper*	AF049230	6994	DR	390	5	0.997	50	82	1.7
		skipper_LTR*		390				0.011	10	n.d.	n.d.
		DGLT-A	AF298204	5054	DR	268	4/5	0.067	5	7	1.5
		H3R*	X59570	268				0.013	15	n.d.	n.d.
		DGLT-P	AF298205	(6017)	n.d.	n.d.	n.d.	0.047	10	17	1.5
Non-LTR transposons	TRE3	TRE3-A*	AF134169	5243	—	—	n.d.	0.960	60	82	1.3
		TRE3-B*	AF134170	5292	—	—	n.d.	0.770	50	68	1.3
		TRE3-C*	AF134171	4751	—	—	n.d.	0.450	30	36	1.1
		TRE3-D	AF135841	(2816)	—	—	n.d.	0.051	5	16	2.7
	TRE5	TRE5-A*	X57034	~6200	—	—	n.d.	1.220	70	92	1.3
		TRE5-B	AF298209	~5700	—	—	n.d.	0.200	15	36	2.1
		TRE5-C	AF298210	(890)	—	—	n.d.	0.012	5	12	3.0
DNA transposons	Tdd-4	Tdd-4*	U57081	3843	IR	146	5	0.425	40	55	1.4
		Tdd-5	AF298206	4031	IR	183	5	0.076	5	20	3.3
	DDT	DDT-A	AF298201	5169	IR	48	2	0.309	20	65	3.3
		DDT-B	AF298202	5471	IR	38	2	0.314	20	68	3.6
		DDT-S	AF298203	758	IR	27	2	0.295	130	175	1.3
Unclassified	thug	thug-S	AF298207	2192	IR	18	4	0.058	10	19	2.1
		thug-T	AF298208	1132	IR	8	4	0.038	10	18	1.6
		Total						9.573	750	1195	

DR, direct repeat; IR, inverted repeat; Nr, rounded copy number as estimated from genome content; ML(N), maximum likelihood estimate of fragment number; FI, fragmentation index; TSD, size of target site duplication.

Table 1.3: ***Dictyostelium* Repetitive Elements** (From Glöckner et al., 2001)

In addition, the *Dictyostelium* genome contains Non-LTR retrotransposons that integrate in a position and orientation specific manner upstream (TRE5) or downstream (TRE3) of tRNA genes (Winckler et al., 2005). Three closely related subfamilies of TRE5 (TRE5-A, TRE5-B and TRE5-C) are present in the *Dictyostelium* genome at approximately 90 copies in total. TRE3 transposons are related to the TRE5 family and contain 4 members at approximately 150 copies (Fig. 1.8). Unlike retrotransposons, DNA transposons are not amplified during the process of transposition, but are rather excised and transposed to another location in the genome. The related DNA transposons Tdd-4 and Tdd-5 are present at approximately 40 and 5 copies respectively. Another class of DNA transposons, the DDT (*Dictyostelium* DNA transposon) contain inverted terminal repeats. Three members of this family appear to be present; 20 copies of DDT-A, 20 copies of DDT-B and 130 copies of DDT-S (Winckler et al., 2005; Fig. 1.9).

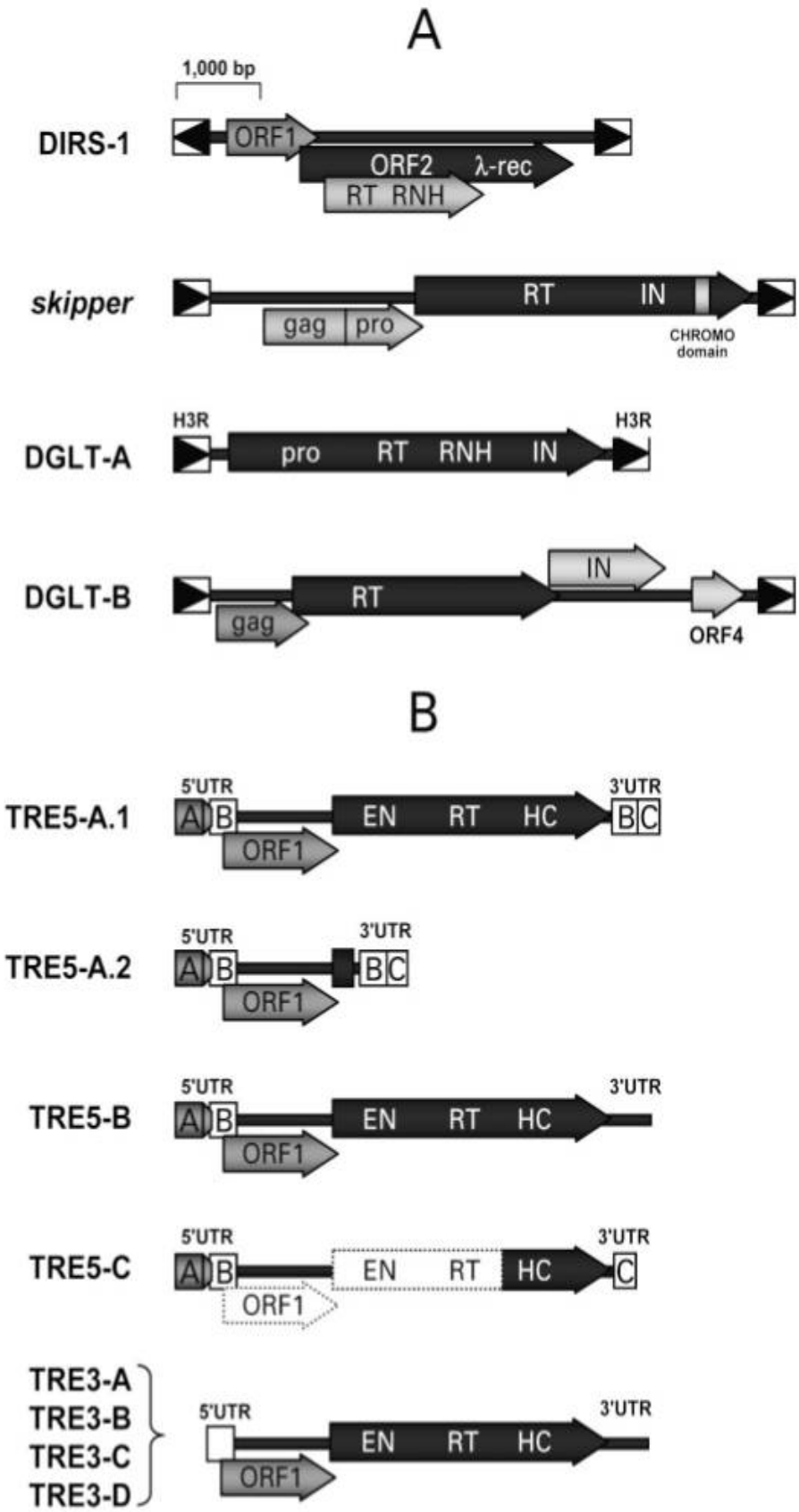


Fig. 1.8 ***Dictyostelium*** **Retrotransposons** (from Winckler et al., 2005) **A**: LTR transposons. **B**: Non-LTR transposons. RNH, RNase H domain; λ-rec, domain with homology to bacteriophage λ recombinase; CHROMO, chromatin organizer domain; EN, endonuclease domain; RT, reverse transcriptase domain; HC, domain containing a zinc finger-like histidine/cysteine-rich motif.

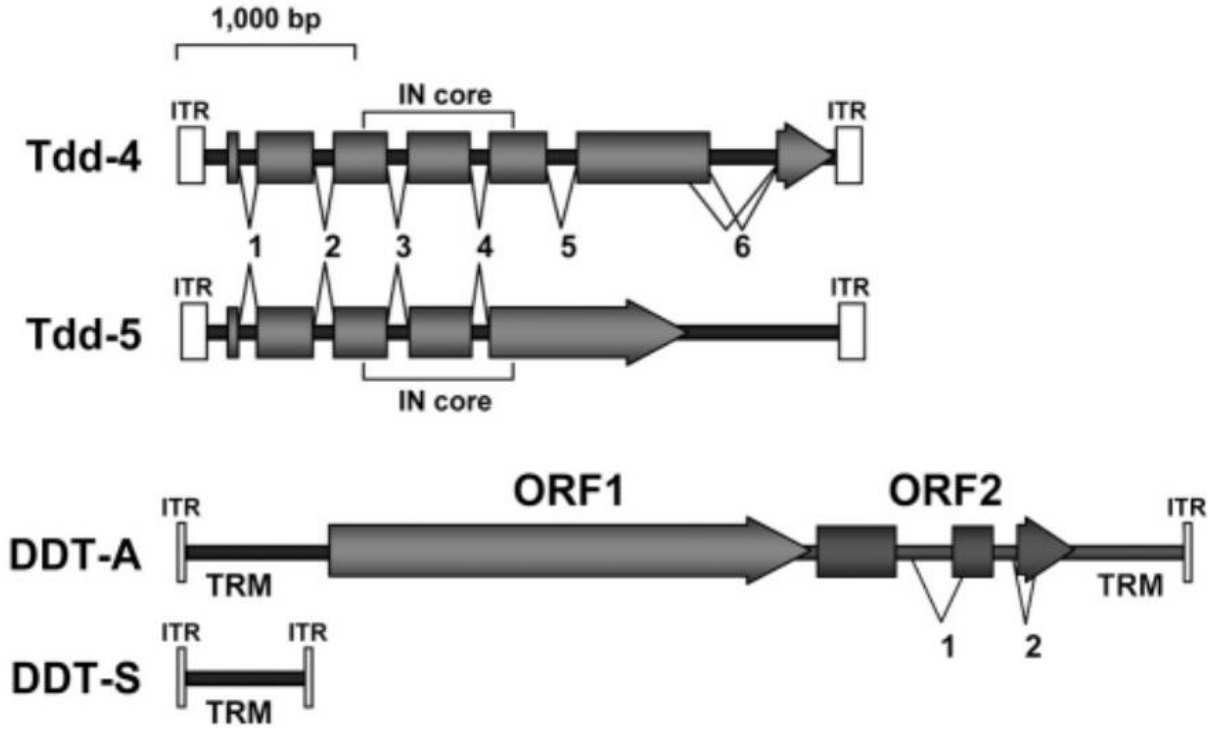


Fig. 1.9 ***Dictyostelium*** **DNA Transposons** (from Winckler et al., 2005). The Genes encoded by these transposons contain introns (indicated by numbers). ITR: inverted terminal repeat, IN core: retroviral integrase core domain, TRM: tandemly repeated motif.

Previously it has been proposed that DIRS-1, the most abundant retroelement in *Dictyostelium* may constitute the centromeres as it appears to be present in 6 linkage groups, one on each chromosome (Loomis et al., 1995). Using FISH it has been shown that 6 foci are visible at the edge of the nucleus (Eichinger et al., 2005). Is has previously shown that DIRS-1 DNA is methylated (Kuhlmann et al., 2005) and that DIRS-1 sequences associate with H3K9me2 (Kaller et al., 2007).

1.4 Differentiation and Multicellularity

Many unicellular organisms are capable of differentiating into different cell types. Examples include the mating type switch in yeast and spore/cyst formation in various eukaryotes (Bonner, 2003; Rokas, 2008). Differentiation is usually accompanied by exit from the cell cycle in G1, a process which is highly conserved in different eukaryotic lineages and in which the retinoblastoma (Rb) protein and E2F transcription factors play a crucial role (Ferreira et al., 2001; Poznic, 2009). Multicellularity appears to have evolved independently on a number of occasions during evolution in different lineages including plants, animals, fungi and amoebozoa (Rokas, 2008). Multicellularity allows larger organisms which can confer advantages in terms of protection from predation, food

consumption, or spore dispersal, the latter of which appears to be the case in *Dictyostelium*. Compared to unicellular organisms, multicellular organisms are normally more complex, with cells differentiating into different cell types and organs. Plants and animals have many (up to several hundred) distinct cell types while multicellular fungi and *Dictyostelium* only have seven to nine (Rokas, 2008; Gaudet et al., 2008).

1.4.1 Regulation of Development and Multicellularity

Multicellularity and cellular differentiation necessitates the evolution of a molecular machinery to control differentiation and pattern formation. Compared to their unicellular relatives, multicellular organisms often have expanded families of proteins involved in signalling such as cadherins, intergrins and receptor tyrosine kinases. Also in different lineages it is apparent that certain transcription factor families have been greatly expanded relative to unicellular organisms, for example MADS-box and homeodomain containing genes in plants. Similarly, in metazoans the homeodomain (HOX) proteins, immunoglobulin domain, helix-loop-helix and C2H2 zinc finger transcription factors are greatly expanded and responsible for pattern formation and regulation of differentiation/development (Rokas, 2008).

In *Dictyostelium* cAMP regulates aggregation and induces differentiation of pre-spore cells. Pre-spore cells in turn produce a chlorinated polyketide DIF-1 that induces pre-stalk cell differentiation (Brookman et al., 1987). A number of small polypeptides including conditioned medium factor (CMF, Gomer et al., 1991), pre-starvation factor (PSF, Rathi et al., 1991) and spore differentiation factors (SDFs, Anjard et al., 1997) are also involved in regulating differentiation and pattern formation. These factors are frequently detected by sensor-coupled histidine kinases which initiate signalling cascades (Schaap, 2007). While the entire downstream cascade from DIF-1 is not known, DIF-1 induces the nuclear accumulation of the bZIP transcription factors DimA and DimB which bind the promoter of the prestalk *ecmA* gene (Zhukovskaya et al., 2006) and regulate its expression. DIF-1 also modulates the activity of the GATA family transcription factor GtaC (Keller and Thompson, 2008). In addition, Myb, MADS-box, Cbl and STAT transcription factors are involved in the regulation of *Dictyostelium* development (Langenick et al., 2008; Yamada et al., 2008).

1.4.2 Epigenetic Regulation of Development

Differentiation into distinct cell types requires differential gene expression. Studies have shown that undifferentiated embryonic stem cells (ES cells) have relativity decondensed chromatin with high levels of histone acetylation and histone H3K4me3 but low levels of histone H3K9me3, histone H3K27me3 and histone H4K20me. As cells differentiate, the number of genes being expressed drops, and thus the global levels of H3K9me3 increases and histone acetylation is reduced. Protein complexes such as the polycomb proteins are recruited to silence genes and are

responsible for the accumulation of H3K9me, H3K27me and H4K20me. The TRX (trithorax) complex helps to maintain the expressed genes in an active state by counteracting the repressive polycomb complexes (Vasanthi and Mishra, 2008; Bantignies et al., 2006).

1.4.3 Differentiation and Development in *Dictyostelium*

When their food supply is exhausted *Dictyostelium* cells exit their unicellular proliferative phase and displaying facultative multicellularity. The unicellular amoeba aggregate to form a mound in which cells then differentiate to form pre-spore cells or pre-stalk cells. The mound then forms a slug with the pre-stalk cells at the anterior fifth and pre-spore cells and anterior-like cells (ALC) in the posterior four fifths. The slugs migrate towards light and away from heat and the pre-stalk cells differentiate further into different subtypes (pstO, pstA, pstB, pstAB and tip organiser region, (Fig. 1.10). Microarray analysis has shown that roughly 1/3 of the genes examined show more than a two-fold change in expression during early development (Iranfar et al., 2003). This is similar to the changes of gene expression observed during developmental transitions in plants and animals. Unlike the germline in plants and animals, the *Dictyostelium* spores return to the undifferentiated state without passing through meiosis, instead the undifferentiated state may be reset during mitosis.

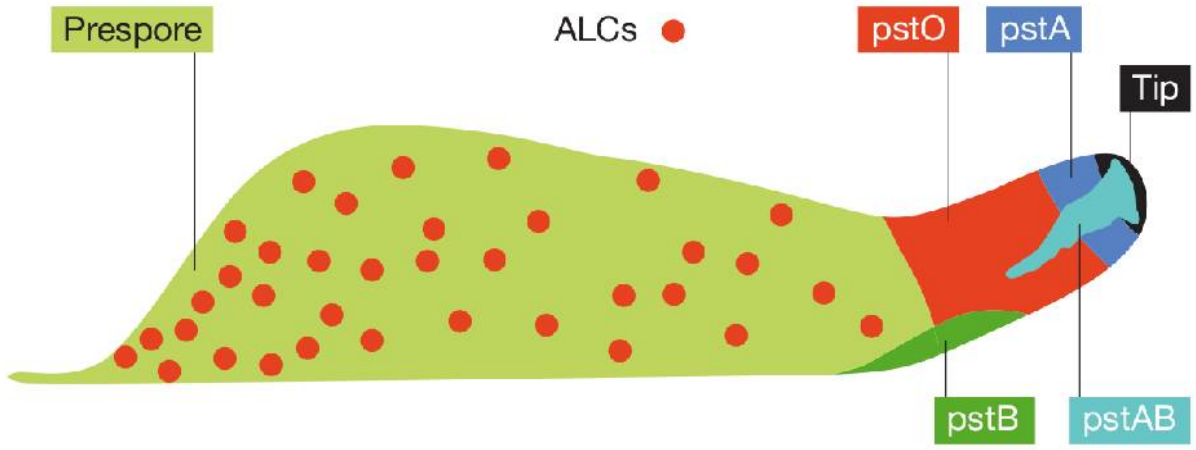


Fig. 1.10 **Cell Types Present in *Dictyostelium* Slugs** (from Williams, 2006). ALCs: anterior like cells, pstO: pre-stalk zone O, pstA: pre-stalk zone A, pstB: pre-stalk zone B, pstAB: pres-talk zone AB.

In contrast to higher eukaryotes, *Dictyostelium* cells only undergo one mitotic cycle at the onset of development, and another during the slug stage when they are differentiated (Muramoto and Chubb, 2008). Consistent with this the *Dictyostelium* genome seems to lack a the machinery thought to be required for epigenetic maintenance of gene expression through mitosis such as the polycomb PCR1, PCR2 and TRX complexes as well as those which are responsible for catalysing

or recognising histone modifications such as H3K27me, H4K20me and H2AK121ub (www.dictybase.org). Interestingly this is in contrast to many “primitive” protozoan parasites such as the trypanasomatids and apicomplexa where histone modifications and modifying/recognition proteins typical of higher eukaryotes are present (Iyer et al., 2008; Gissot et al., 2009). Protozoan parasites avoid host immune responses by cellular differentiation and antigenic variation and the presence of these epigenetic components is presumed to allow them to maintain the associated gene expression patterns in a stable manner (Sullivan et al., 2006).

DNA methylation is associated with the maintenance of epigenetic changes in gene expression in both plants and animals and is present in the multicellular yeast *Neurospora crassa* and *Dictyostelium* (Kuhlmann et al., 2005). However DNA methylation in *Dictyostelium* appears restricted to transposons, and loss of the sole DNA methyltransferase, Dnmt2 does not result in any obvious defect in development (Kuhlmann et al., 2005). This would suggest that in *Dictyostelium* the main role of DNA methylation is in silencing transposons and other parasitic elements rather than in gene regulation. Some epigenetic regulators have been shown to affect *Dictyostelium* development. Loss of the cell cycle regulator Rb causes DIF-1 hypersensitivity and cells to be biased towards the pre-stalk pathway (MacWilliams et al., 2006). The loss of the H3K4 methyltransferase results in accelerated development (Chubb et al., 2006). Treatment with HDAC inhibitors leads to histone hyperacetylation and results in a developmental delay (Sawarkar et al., 2009).

1.4.4 A Role for Ubiquitination in the Regulation of *Dictyostelium* Development?

The Cullins are a small family of evolutionary conserved proteins that are components of ubiquitin ligase complexes. Together with substrate adaptors they catalyse the conjugation of ubiquitin chains to their targets, resulting in their degradation by the 26S proteosome. Cullins have been shown to have critical roles in transducing hormone signals, developmental regulation and environmental signalling and cell-cycle regulation (Hotton and Callis, 2008; Sumara et al., 2008). In agreement with this, the loss of either *culA* or *culB* in *Dictyostelium* results in developmental defects (Mohanty et al., 2001; Wang and Kuspa, 2002). Cullin4, the homolog of *Dictyostelium* CulD has been implicated in the ubiquitination of various chromatin components including the transcription factor c-Jun, the cell-cycle regulator E2F and the DNA replication licensing factor CTD1 (Jackson and Xiong, 2009; Zhang et al., 2008; Wertz et al., 2004). It is also part of the DNA damage repair complex containing DDB1 (RepE in *Dictyostelium*) which can catalyse the mono-ubiquitination of the histone H2A (Kapetanaki et al., 2006; Dai and Wang, 2006). In fission yeast Cul4 also forms part of a complex with the DDB1 homolog Rik1, which acts to target the H3K9 methyltransferase Clr4 to pericentromeric heterochromatin (Hong et al., 2005) although this complex does not appear to be conserved in other eukaryotes.

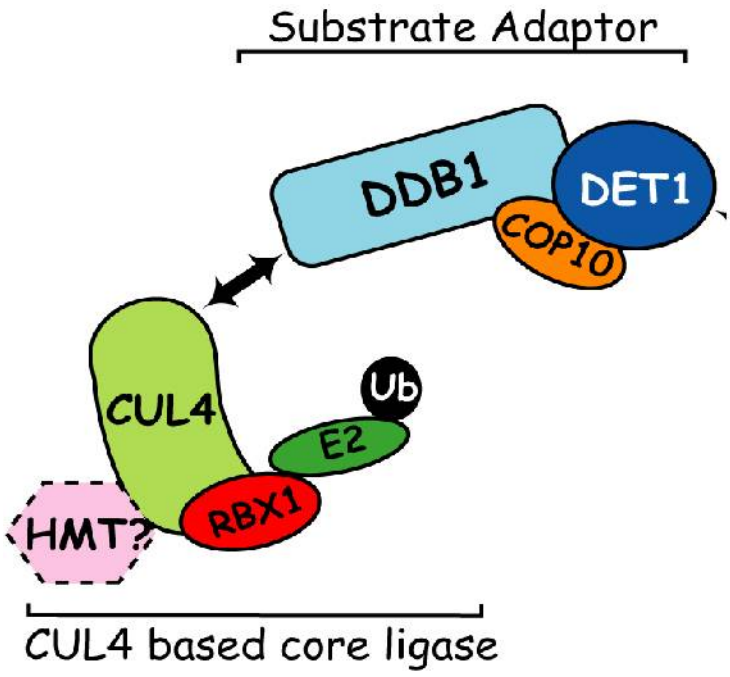


Fig. 1.11 **Cul4/DET1 Complex.** Schematic of the DET1 complex. DET1, DDB1 and COP10 form the DET1 core complex, which acts as the substrate adaptor for a CUL4-based ubiquitin ligase, which contains CUL4, the adaptor protein RBX1, and a ubiquitin E2 ligase. The presence of a histone methyltransferase in the complex has only been demonstrated in *S.pombe* and may not be conserved in other organisms.

In plants and animals DDB1 and Cul4 occur together in a complex with the DET1 (De-etiolated 1) protein (Schroeder et al., 2002; Wertz et al., 2004; Fig. 1.11). DET1 was identified in mutant screens for developmental mutants in *Drosophila* (Tomkiel et al., 1995), and light-signalling mutants in A*rabidopsis* (Chory and Peto, 1990) and tomato (Mustilli et al., 1999). DET1 binds to chromatin via the N-terminal tail of histone H2B and acts as a transcriptional repressor at its target genes (Berloco et al., 2001; Benvenuto et al., 2002; M. Dubin, Masters thesis, The Open University, 2006). While DDB1 and Cul4 are highly conserved and appear to be ubiquitous in eukaryotes, DET1 has a much more limited distribution, and occurs almost exclusively in multicellular plants and animals. Interestingly a homolog of DET1 appears to be present in *Dictyostelium*, but not in its unicellular relative *Entamoeba histolytica*. Given its role in developmental regulation in higher eukaryotes (to which it is almost entirely restricted) and its presence in *Dictyostelium*, it would seem that DET1 is a promising candidate for an epigenetic regulator of development in *Dictyostelium*. Interestingly in plants DET1 regulates development in response to environmental stimuli (in this case their primary energy source, light) while *Dictyostelium* development is also regulated in response to environmental factors, namely their food source.

1.5 Aims of this Thesis

As part of a wider study into nuclear organisation we will attempt to identify which histone modifications are present in *Dictyostelium* using commercially available antisera against different modifications. The distribution of these modifications in the nucleus will be examined using cytological methods (immunofluorescence) and also the distribution at different genomic loci determined by using chromatin immunoprecipitation (ChIP). This will allow for the correlation between gene expression and histone modifications in this organism. The genes encoding some of the proteins responsible for catalysing, recognising and removing post-translational modifications will be clone and the proteins expressed as epitope tagged fusions to determine their role in epigenetic regulation in *Dictyostelium*. Knowledge of histone modifying enzymes, histone modifications, and proteins that "read" these modifications in *Dictyostelium* will allow a comparative analysis with other unicellular and multicellular species. This may provide insight into which histone modifications (if any) are important for developmental regulation. The presence of histone modifications on developmentally regulated genes will be examined by CHIP on *Dictyostelium* cells at different stages of the developmental cycle.

In order to gain a better understanding of the centromeres and nuclear organisation in general in *Dictyostelium* we will examine the localisation of DIRS-1 and other retroelements during the cell cycle using fluorescence *in situ* hybridisation (FISH). The localisation of other complex loci such as telomeres and the extrachromosomal rDNA palindrome will also be examined. We will further investigate the organisation of transgenes to possibly find answers to the question why they are frequently expressed at unexpectedly low levels. While the *Dictyostelium* genome does not encode any proteins with high homology to CenH3 a number of histone H3 like genes are present in the genome. These will be cloned and expressed as epitope tagged fusions to see if any of them are orthologs of CenH3.

The aforementioned experiments (as well as other ongoing experiments in the lab) will require a number of proteins to be expressed as epitope, affinity, or fluorescent protein tagged fusions at the N- or C-terminus. Other requirements include being able to express the protein at different levels and with different resistance cassettes to allow super-transformation of existing transgenic lines. As it is difficult to predict in advance which particular combination will be required for a particular protein, it was desirable to have some kind of system where the protein of interest, can be easily and quickly introduced into the desired expression vector as soon as the experimental need is identified. A series of expression vectors that meet these requirements will be designed and constructed.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals, Solvents and Reagents

GE Healthcare (Little Chalfont, UK): Protein-A sepharose beads

Sigma-Aldrich (St. Louis, MO): bovine serum albumin (BSA), DAPI (4'6-diamidino-2-phenylindole), NP40 detergent, paraformaldehyde, sodium-deoxycholate, Tween20 detergent, Triton-X 100 detergent.

Roche (Basel, Switzerland): Complete EDTA-free protease inhibitor tablets,

Carl Roth (Karlsruhe, Germany): ampicillin (sodium salt), N,N,N,N- tetramethylethylenediamine (TEMED), Acrylamide, Agrose,

All other standard reagents were obtained from the following suppliers: Sigma-Aldrich (St. Louis, MO), JT Barker (Phillipsburg, NJ), Carl Roth (Karlsruhe, Germany), Promega (Madison, WI) or Boehringer Mannheim (Amsterdam, Netherlands).

2.1.2 Enzymes

Taq DNA polymerase:	Dept. Genetics, University of Kassel
Pfu DNA polymerase:	Dept. Genetics, University of Kassel
Proteinase-K:	Carl Roth; Karlsruhe, Germany
RNase-A:	Carl Roth; Karlsruhe, Germany
Dnase-I:	Fermentas; Burlington, Canada
T4 DNA polymerase:	Fermentas; Burlington, Canada
T4 DNA Ligase:	Fermentas; Burlington, Canada
DNA polymerase I:	Fermentas; Burlington, Canada
Klenow Fragement:	Fermentas; Burlington, Canada
Calf alkaline phosphatase:	Fermentas; Burlington, Canada
Shrimp alkaline phosphatase:	USB Corporation; Cleveland, OH
Type II restriction endonulceases:	Fermentas; Burlington, Canada

2.1.3 Antibodies

Table 2.1 **Antibodies**. List of antibodies used in this study. Unless otherwise indicated all antibodies raised in mouse or rat are monoclonal and those raised in rabbit goat are polyclonal. The dilution at which were antibody used in different types of experiments is indicated. Suppliers were as follows:

DCB:	Dept. Cell Biology, University of Kassel; Kassel, Germany
S.Cruz:	Santa Cruz Biotechnology; Santa Cruz, CA
S. Aldr.:	Sigma-Aldrich; St. Louis, MO
DG:	Dept. Genetics, University of Kassel; Kassel, Germany
Dianova:	Dianova; Hamburg, Germant
Millipore*:	Millipore; Billerica, MA
Abcam:	Abcam; Cambridge, UK
Epitom.:	Epitomics; Burlingame CA

*Some antibodies listed as been from Millipore were purchased from Upstate, prior to its takeover by Millipore.

Antibody	Source	Dilutions used		
		Western Blot	Immunofluorescence	ChIP (per $2x10^{-7}$ cells)
mouse anti-myc(9E10) hybridoma supernatant	DCB	1:5	1:2	50 µl
mouse anti-gfp hybridoma supernatant	DCB	1:5	1:2	50 µl
mouse anti-DdCP224 hybridoma supernatant	DCB		1:25	
rat anti-tubulin (Y1/2) hybridoma supernatant	DCB		1:5	
rabbit anti-HA (sc805)	S. Cruz	1:5000	1:500	2 µl
mouse anti Flag (mAb2)	S. Aldr.	1:500	1:100	5 µl
rabbit anti-EB4	DG			
rabbit anti-H3K9me2 (#07-441)	Millipore	1:2000	1:200	2 µl
mouse anti-H3K9me2 (ab1220)	Abcam		1:50	
rabbit anti-H3K9me3 (#07-523)	Millipore	1:5000	1:1000	2 µl
rabbit anti-H3K4me2 (monoclonal, #1347-1)	Epitom.	1:10 000	1:1000	
rabbit anti-H3K4me3 (ab8580)	Abcam	1:5000	1:1000	2 µl
rabbit anti-H3K4me3 (#05-745)	Millipore		1:500	
rabbit anti-H3K36me1 (#07-548)	Millipore		1:400	
rabbit anti-H3K36me2 (#07-274)	Millipore		1:400	
rabbit anti-H4K20me2 (ab9052)	Abcam		1:200	
rabbit anti-H4K20me3 (ab9053)	Abcam		1:200	
rabbit anti-H4ac (#06-866)	Millipore		1:1000	
rabbit anti-H3ac (#06-599)	Millipore		1:1000	
rabbit anti-H3K27me3 (#07-449)	Millipore		1:200	
rabbit anti-H3K27ac (#07-360)	Millipore		1:400	
rabbit anti-H3K14ac (#07-353)	Millipore		1:400	
rabbit anti-H3K4ac (#07-339)	Millipore		1:400	
rabbit anti-H3K9ac (#07-352)	Millipore		1:400	
rabbit anti-H2AK9ac (#07-289)	Millipore		1:400	
rabbit anti-H2AK5ac (#07-290)	Millipore		1:400	
rabbit anti-H2Bac (#07-373)	Millipore		1:1000	
rabbit anti-H2BK5ac (#07-386)	Millipore		1:100	
rabbit anti-H2BK12ac (#07-343)	Millipore		1:200	
rabbit anti-H4K5ac (#07-327)	Millipore		1:100	
rabbit anti-H4K8ac (monoclonal, EP1002Y)	Epitom.		1:100	
rabbit anti-H4K16ac (#07-329)	Millipore		1:500	
mouse anti-H3S10ph (#07-360)	Millipore		1:100	
rabbit anti-H2AXS139ph (#07-164)	Millipore		1:400	
goat Cy3-conjugated anti-rabbit	Dianova		1:1000	
goat Cy3-conjugated anti-mouse	Dianova		1:1000	
goat Alexa488-conjugated goat anti-mouse	Millipore		1:500	
goat alkaline phosphatase-conjugated anti-mouse	Dianova	1:10 000		
goat alkaline phosphatase-conjugated anti-mouse	Dianova	1:10 000		

2.1.4 Oligonucliotides

Single strand oligonucleotide primers for DNA fragment synthesis or quantitative PCR were obtained from Invitrogen (Carlsbad, CA) or obtained from existing lab stocks.

Name	Sequence		Description
MJD1	GATTGAAATTTTCAAA**CCATGG**GTGG	F	Oligos to remove the start of the *actin*6 gene from pDneo2
MJD2	CATCTTCTCC**CTGCAG**TTTATATTATATTTATTTATTTG	R	
MJD3	**CTGCAGG**TCAAATG**GGATCC**ATGAGTAAAGGAGAAGAACTTTTC	F	For amplification of GFP (S56T) gene (from Harry McWilliams)
MJD13	**CTCGAG**TTAT**GTCGAC**CCTTTGTATAGTTCATCCATGCC	R	
MJD5	**CTGCAG**AAATGTCCACAACAACAACATCTAC	F	Amplification of EriA for N-terminal cloning site from gDNA
MJD6	**GGATCC**TTTTACTGATTTCATTGTTGAAACTAAATC	R	
MJD7	**GTCGAC**AATGTCCACAACAACAACATCTAC	F	For amplification of EriA for C-terminal cloning site from gDNA
MJD8	**CTCGAG**TTATTTTACTGATTTCATTGTTGAAACTAAATC	R	
MJD9	CAGGTCGACGGTAT**GGATCC**AAAGCTATGGAGC	F	For amplification of 6xMYC gene from pEn3
MJD14	GTACCGGATCCC**GTCGAC**CCGGAATTCAGGTCC	R	
MJD11	A**GGATCC**GGTGGATATCCATACGATGTTCCAGATTATGCAGGAGGATACCCTTATGATGTACCTGACTAC	F	Overlaping primers for PCR amplification of 3xHA gene (no additional template needed)
MJD12	G**GTCGAC**CCACCTGCATAATCTGGAACATCGTATGGATAACCACCTGCGTAGTCAGGTACATCATAAGGG	R	
MJD15	A**GGATCC**GGTGGAGATTATAAAGATCATGACGGTGATTATAAGGACCACGATATAGAC	F	Overlaping primers for PCR amplification of 3xFLAG gene (no additional template needed)
MJD16	A**GTCGAC**CCACCTTTATCATCGTCATCTTTATAGTCTATATCGTGGTCCTTATAATC	R	
MJD59	A**CTGCAG**AAATGGCAGGCCTTGCGCAACACG	F	For amplification of nTAP
MJD18	**GTCGAC**CC**GGATCC**CTTATCGTCATCATCAAGTGCCC	R	
MJD19	**GGATCC**GG**GTCGAC**AATGGAAAAGAGAAGATGGAAAAAG	F	For amplification of cTAP
MJD20	**CTCGAG**TTAGGTTGACTTCCCCGCGG	R	
MJD25	**CTGCAG**AAATGATCCCAAAAAAACAAAAAGGATTC	F	Amplification of AgnB for N-terminal cloning site from gDNA
MJD26	**GGATCC**AAGGAAGAATAATTTATCAGATAATTGAGG	R	
MJD29	**GTCGAC**AATGATGGATCCATCCACTATATC	F	Amplification of N-terminal fragment of DET1 for N-terminal cloning site from gDNA
MJD32	CATGTAAAAGCTTTTTCTCGTGAG	R	
MJD31	CTCACGAGAAAAAGCTTTTACATG	F	Amplification of C-terminal fragment of DeDET1 for N-terminal cloning site from gDNA
MJD30	**CTCGAG**TTATGATACTGTATTATTATTATTGTTGTTGTTATTA	R	
MJD36	GCAAATAAAGGATTCATTGGGGTG		Screening primer for DET1 knocklout (together with #386)
MJD61	CATTATCGCGAGCCCATTTA		qPCR primer (together with #1261) for G418 cassette
MJD62	GCTAAAA**CTGCAG**AAAATGGGAAAAAGAGATAA	F	For amplification of HcpB for N-terminal cloning site from gDNA
MJD54	A**GGATCC**ACTTGGCTGACCACTATAACC	R	
MJD55	CGATCCAACTGCTGTAGAAATATCATCATC	F	For removal of internal *Pst* I site from HcpB by PCR mutagenisis
MJD5	GATGATGATATTTCTACAGCAGTTGGATC	R	
MJD63	**GTCGAC**AATGTCAAATAAATCTCATTTATCTGAAAAGGTTC	F	For amplification of DrnB for C-

MJD64	CTCGAGGTTAATCAGTTTCAGAGAATTTTAAATCATC	R	terminal cloning site from gDNA
MJD72	TGAAACACACACACTCACTCACTT	F	qPCR primers for *cotB* promoter
MJD73	TTGTGTATGAATGTGTTGAACTGG	R	
MJD74	TGCAAAGAGTTGTTGTGGTGA	F	qPCR primers for *cotB* coding sequence
MJD75	TTGACCAGTTGCACTTCCAG	R	
MJD79	CGGAAGAAGAAGAAGAAGAAACACATGTAGTATCAGAAGAAGAAAAAGAAGAGG	F	For removal of *Pst* I site from within the DPN1 extra-chromosomal origin of replication by PCR mutagenisis
MJD80	CCTCTTCTTTTTCTTCTTCTGATACTACATGTGTTTCTTCTTCTTCTTCTTCCG	R	
MJD81	A**GGATCC**GGTGGAATGGCATCATCAGAAGATGTTATTAAAG	F	For amplification of mRFPmars
MJD82	T**GTCGAC**CCACCTGCACCTGTTGAATGTCTACC	R	
MJD83	GGAAGAAGAAAGCCCCATTC	F	qPCR primers for DIRS-1 ORF2
MJD84	CAGAGAAGCCATAGCGGAAC	R	
MJD85	A**GTCGAC**AATGACAAGTGTTAATAATAATATGACAAG	F	For amplification of H3v2 for C-terminal cloning site from gDNA
MJD86	A**CTCGAG**TTAATAACGTGGCAAATAAAATGGTTTGATG	R	
MJD87	A**GTCGAC**AATGGCTAACAAACCCAAACCCTC	F	For amplification of H3v1 for C-terminal cloning site from gDNA
MJD88	A**CTCGAG**TTAAAAAAGAAAATGTCTAGCCCTTTTCC	R	
MJD89	A**GTCGAC**AATGGCCCGTACAAAACAAACCG	F	For amplification of H3c for C-terminal cloning site from gDNA
MJD90	A**CTCGAG**TTATGATCTTTCACCTCTGATACGTC	R	
MJD101	CCCCCATTCGCTATTTTATC	F	qPCR primers for EcmAO distal promoter (EcmO)
MJD102	AAAATTGTGGGGTGTAACTGTTT	R	
MJD103	CCAAAATTATACACCACCACCA	F	qPCR primers for EcmAO proximal promoter (EcmA)
MJD104	AAGACCGCAAAACCATTACG	R	
MJD105	ACCTACCGATTGTCAATAGCA	F	qPCR primers for Chr1L/Chr2R subtelomere
MJD106	CAAATTGCTCATTTGGGAAAA	R	
MJD111	GGGTTCGATTCTCGGTTTATT	F	qPCR primers for non-coding region of rDNA palindrome
MJD112	CCATGAGGTAACAGTGGATGC	R	
MJD113	TTCTAATTCCGCCTCACCTTT	F	qPCR primers for 26S rDNA
MJD114	GGTTTGAACCTTGGGCTATTC	R	
MJD115	CACTGGAGGCATACATGAACA	F	qPCR primers for *rasG* promoter
MJD116	TGTTGTTTTGTGTGTGTGGAAA	R	
MJD117	TGGTTGAAAGTGGGTTGGAT	F	qPCR primers for *acA* promoter
MJD118	TTTTTGTGTGTGTTGGAATTATTTA	R	
MJD119	A**CTGCAG**AAATGGTATTCGTTAAAGGTCAAAAGAAAG	F	Amplification of H2Bv3 for N-terminal cloning site from gDNA
MJD120	A**GGATCC**GTTTTTGCTTTCAGTTGGATTGTAC	R	
MJD121	AACATAT**GTCGAC**AATGGTATTCGTTAAAGGTCAAAAGAAAG	F	Amplification of H2Bv3 for C-terminal cloning site from gDNA
MJD122	A**CTCGAG**TTAGTTTTTGCTTTCAGTTGGATTGTAC	R	
MJD123	A**GTCGAC**AATGCAAATCTTTGTCAAAACATTAACTGG	F	Amplification of ubiquitin for C-terminal cloning site from gDNA
MJD124	A**CTCGAG**TTAACCACCTCTTAATCTGAGGAC	R	
MJD125	GCACACTTTCACCCACTTTG	F	qPCR primers for *psvA* promoter
MJD126	GGGTGAGTGGGTTGGTGTAT	R	
MJD127	AGATTGTGGTGGCACAGGAT	F	qPCR primers for *psvA* Exon2

MJD128	CAAGACAACCCAAAACACCA	R	
MJD129	TCTCCACCAAGACCAGACAA	F	qPCR primers for *psvA* Exon3/ anti-sense promoter
MJD130	TCTCCACCAAGACCAGACAA	R	
MJD140	A**GTCGAC**AATGTCAGAAACCAAACCAGCCTC	F	Amplification of H2AX for C-terminal cloning site from gDNA
MJD141	T**CTCGAG**TTAATAGATTTGAGATGAACCTTCAGCTG	R	
MJD142	A**GTCGAC**AATGAGTTCAGCACAATCAAGAGGTG	F	Amplification of H4a for C-terminal cloning site from gDNA
MJD143	T**CTCGAG**TTATGAGTTGAAACCGTATAAAGTTCTACC	R	

Table 2.2 **Oligonucleotides**. Oligonucleotide primers used in this work, restriction sites introduced for cloning purposes are indicated in bold.

2.1.5 Standard Vectors

DNA fragments amplified by PCR with *Pfu* or *Taq* polymerase were ligated into the vector pJET1.1, pJET1.2 (Fermentas; Burlington, Canada) or pGEM-T Easy (Promega; Madison, WI) using T4 DNA ligase. *Dictyostelium* expression vectors were derived from the vector pDneo2 (Witke et al., 1987).

2.1.6 Strains and Growth

The *E. coli* strain DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lac*ZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK^- mK^+), λ–) was used for cloning and plasmid isolation. *E. coli* cells were cultured at 37°C in Luria-Bertani (LB) broth (1% (w/v) casein peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) with the antibiotic selection (50 µg ml^{-1} ampicillin) where appropriate. Cell selection was carried out using LB-agar plates (LB broth + 1.5% (w/v) agar) supplemented with 50 µg ml^{-1} ampicillin.

The *Dictyostelium discodeum* strain Ax2-214 (axeA2, axeB2, axeC2; Watts and Ashworth, 1970) was cultured in petri dishes or shaking culture at 20°C in HL5 medium (Formedium; Hunstaton, UK) supplemented with 100 µg ml^{-1} ampicillin, 100 µg ml^{-1} amphotericin-B and the appropriate selective agent (10 µg ml^{-1} Geneticin and/or 10 µg ml^{-1} Blasticidin). Alternatively *Dictyostelium* were grown on Bacterial lawns of *Klebsiella aerogenes* on SM-agar plates.

2.2 Methods

2.2.1 Cloning

Unless otherwise mentioned all methods used were according to Sambrook et al., 1989, or where a commercial kit was used, according to the protocol supplied by the manufacturer. DNA fragments were amplified by PCR using *Pfu* polymerase from existing constructs or *Dictyostelium* genomic DNA. Larger fragments amplified with a mixture of *Pfu/Taq* (1:30) polymerases. Amplified fragments were ligated into the vector pJET (or when *Pfu/Taq* had been used into pGEM-T Easy) using T4 DNA ligase. The cloned DNA fragments were sequenced to confirm the absence of mutations. Correct clones were excised from the cloning vector using restriction sites introduced during the PCR amplification and ligated into the desired vector digested at the complementary restriction sites.

2.2.2 Construction of Vectors

The 3' end of the *actin6* promoter was amplified from the vector pDneo2 (Witke et al., 1987) using the primers MJD1 and MJD2. The amplified promoter fragment was cloned into the plasmid pJET. The fragment was excised from the vector using the restriction enzymes *Nco* I and *Pst* I and ligated into pDneo2 digested with the same two enzymes. This replaces the original *actin6* promoter that contains the first 8 codons of the *actin6* gene with a with a truncated version that lacks an initiation codon (AUG), this vector was designated pDneo2a.

CS1-GFP-CS2 was amplified from the plasmid GFP-S65T (Harry McWilliams) using the primers MJD3 and MJD13. This clone also contains the neutral H80R mutation found in the original published GFP expression construct (Chalfie et al., 1994) and which is also present in many of its derivatives. The amplified fragment was cloned into pJET and sequenced. CS1-GFP-CS2 was excised using the restriction enzymes *Pst* I and *Xho* I and ligated into pDneo2a which had been digested with the same two enzymes thus creating the vector pDneo2a-GFP.

The 3xHA and 3xFLAG tags were created by PCR amplification of two overlapping (partially complimentary) primers, MJD11/MJD12 and MJD15/MJD16 respectively. The 6xMYC tag was amplified using the primers MJD9 and MJD14 from the plasmid pE3n (Dubin et al., 2008) as a template. RFP was amplified using the primers MJD81/MJD82 from the plasmid pDbsrH-mRFPmars (Fischer et al., 2004). The *Pfu* polymerase amplified products were ligated into the vector pJET and sequenced to checked for any amplification errors. The Tags were then excised from pJET using the restriction enzymes *Bam* HI and *Sal* I and were ligated into pDneo2a-GFP which had been digested with the same two enzymes. This results in GFP been replaced with the new tag, resulting in the constructs pDneo2a-3xHA, pDneo2a-3xFLAG, pDneo2a-6xMYC and pDneo2a-RFP.

2.2.3 Tandam Affinity Purification Vectors

The primers MJD59/MJD18 were used to amplify nTAP using the plasmid pDV-NTAP (Meima et al., 2007) as a template. The PCR product was cloned into the vector pJET and sequenced to verify its identity. nTAP was excised from pJET using *Pst* I and *Sal* I and ligated into pDneo2a-GFP which had been digested with the same two enzymes to create the vector pDneo2a-nTAP. Alternatively nTAP was excised from pJET using *Pst* I and *Bam* HI and ligated into pDneo2a-GFP, pDneo2a-3xHA or pDneo2a-3xHA which had been digested with the same two restriction enzymes resulting in the vectors pDneo2a-nTAP-GFP, pDneo2a-nTAP-3xHA and pDneo2a-nTAP-6xMYC respectively.

cTAP was amplified from the plasmid pDV-CTAP (Meima et al., 2007) using the primers MJD19/MJD20 and ligated into the vector pJET. After sequencing to verify its identity, cTAP was excised from pJET using the *Bam* HI and *Xho* I restriction enzymes and ligated into pDneo2a-GFP which had been digested with the same two enzymes to create pDneo2a-cTAP. cTAP was also excised using *Sal* I and *Xho* I and ligated into pDneo2a-GFP, pDneo2a-3xHA or pDneo2a-3xHA which had been digested with the same two restriction enzymes resulting in the vectors pDneo2a-GFP-cTAP, pDneo2a-3xHA-cTAP and pDneo2a-6xMYC-cTAP respectively.

2.2.4 Blasticidin Resistant Vectors

The Blasticidin resistance cassette was excised from the vector pGEM-BSR (#987) using the restriction enzyme *Xba* I and ligated into the vector pDneo2a-nTAP which had been digested with *Xba* I and alkaline phosphatase treated, resulting in the vector pDbsr2a-nTAP. Other variants were obtained by excising the desired tag was excised from the appropriate pDneo2a vector using *Pst* I and *Xho* I and ligated into pDbsr2a-nTAP which had been digested with the same two enzymes resulting in the following vectors pDbsr2a-cTAP, pDbsr2a-6xMYC-TAP, pDbsr2a-TAP-6xMYC,

2.2.5 Extrachromsomal Vectors

The *Dictyostelium* extrachromosomal origin of replication Dpn1 (Noegel et al., 1985) was excised from the vector pTX-GFP (Levi et al., 2000) using the restriction enzymes *Pvu* I and *Bss* HII followed by T4 polymerase treatment to generate blunt ends. The Dpn1 fragment was ligated into pDbsr2a-GFP which had been digested with *Sma* I and alkaline phosphatase treated, generating the vector pDbsrXa-GFP. The primers MJD79 and MJD80 were used to remove a *Pst* I site from within the Dpn1 origin of replication by PCR mutagenesis resulting in the vector pDbsrXP-GFP. Other variants were obtained by excising the desired tag was excised from the appropriate pDneo2a vector using *Pst* I and *Xho* I and ligated into pDbsrXP-GFP which had been digested with the same two enzymes resulting in the following vectors pDbsrXP-6xMYC, pDbsrXP-3xHA, pDbsrXP-3xFLAG, pDbsrXP-RFP.

2.2.6 Cloning of Genes of Interest

Genes for which a C-terminal tag is desired are cloned into CS1, the oligos used from their amplification are designed so that the 5' end of the gene has a *Pst* I site followed by two alanines, then the initiation codon (the two alanines from an “A -run” which is necessary for efficient mRNA translation in *Dictyostelium* (Vervoort et al., 2000). At the 3' end of the gene the stop codon is replaced by a *Bam* HI site with no additional nucleotides added.

Genes for which a N-terminal tag is desired are cloned into CS2, the oligos used from their amplification are designed so that the 5' end of the gene has a *Sal* I site followed by a single alanines to maintain the correct reading frame. At the 3' end of the gene an *Xho* I site is placed after the stop codon.

2.3 Dictyostelium Transformation and Culture

2.3.1 Transformation

Dictyostelium cells grown axenically in shaking culture were transformed by electroshock transformation as previously described (Howard et al., 1988). Cells were grown in HL5 medium a further 12-16 hours and then cultured in HL5 supplemented with the selective agent (10 μg ml^{-1} Geneticin and/or 10 μg ml^{-1} Blasticidin). Every 72 hours the selective medium was aspirated and replaced with fresh selective medium.

2.3.2 Subcloning

Once resistant colonies were obtained, approximately 100-200 amoeba were spread on SM-agar plates with *Klebsiella aerogenes.* After 72 hours individual clones were picked from these plates and placed in liquid culture. For each transformation 4-6 individual clones were assayed for expression of the transgene by western blot and/or fluorescence microscopy.

2.3.3 Knockout of Dictyostelium *det1*

The N-terminal domain of *Dictyostelim* DET1 (DET1n) was amplified with the primers MJD29 and MJD32 and ligate into pGEM-T-Easy as described in section 2.2.1. pGEM-T-DET1n was double digested with *Pst* I/*Sma* I, blunted with T4 polymerase, then re-ligated with T4 ligase in order to remove the *Nhe* I site within the vector backbone. The resulting vector (pGEM-T-DET1n (-*Nhe* I) was then digested with *Nhe* I (which is present within the DET1 gene), blunted with T4 polymerase and alkaline phosphatase treated. A *Sma* I digested fragment from the vector pLPBLP (ref) containing a Blasticidin cassette flanked by LoxP sites was ligate in resulting in the vector pGEM-T-det1-LPBLP. The vector was linearise by digesting with *Eco*R I and used to transform Ax2 cells. Blasticidin resistant cells were subcloned. Putative disrupted clones were screened with the

primers #386/ MJD36. The disruption of the *det1* gene was confirmed by Southern bloting as described in Sambrook et al., 1989.

2.3.4 Diploids

To create diploid *Dictyostelium* equal amounts of two lines, one with Blasticidin resistance, one with Geneticin resistance were grown together in HL5 without selection in a shaking culture for 24-48 hours, diluting where appropriate to ensure that cell density did not exceed $2x10^{-6}$ cells ml^{-1}. $1x10^{-8}$ cells were allowed to settle for 30 minutes in a 10 cm Petri dish. The medium was aspirated and replaced with HL5 supplemented with 10 µg ml^{-1} Geneticin and 10 µg ml^{-1} Blasticidin. Every 72 hours the selective medium was aspirated and replaced with fresh selective medium. Putative diploid lines were examined by allowing cells to settle on glass slides for 10min. The cells were then fixed in 3:1 methanol:acetic acid at -20ºC for 60 min then air dried. A drop of mounting media (250 ng $µl^{-1}$ DAPI in 90% (v/v) glycerol, 20 mM Tris-HCL, 1 µg ml^{-1} DABCO, pH 8.3) was placed on the slide and overlaid with a coverslip. Cells were examined by fluorescence microscopy to ensure that up to 12 or 13 chromosomes were visible in metaphase cells.

2.4 Western Blotting

$1x10^{-7}$ cells were placed in a 1.5 ml Eppendorf tube, centrifuged (500 *g*, 2 min, 4ºC) and the supernatent was aspirated. The cell pellet was resuspended in 100 µl 1x Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 0.0005% (w/v) bromophenol blue). The Eppendorf tubes were place on a heat block at 100ºC for 5 min, centrifuged (13 000 *g*, 2 min, room temp). 10 µl of the SDS soluble supernatant was run on an SDS-page gel. Proteins were transferred to PVDF membrane (45 µM pore size) by semi-dry transfer (2 mA cm^{-2} for 90 min at 4ºC). Homogeneous loading and transfer of proteins was verified by staining the membrane with Ponceau-S dye (0.1% (w/v) in 5% acetic acid) for 2 min at room temperature. Excess dye was removed by washing 2x 1 min in 1% acetic acid and an image of the membrane was acquired. After washing in PBS to remove the Ponceau-S dye, the membrane was blocked (5% (w/v) BSA in PBS) for 60 min at room temperature. The membrane was incubated with the primary antibody in 3% (w/v) BSA in PBS overnight at 4ºC. After washing 3 x 5min with PBST (PBS + 0.05% (v/v) Tween20 detergent) the membrane was incubated with the appropriate secondary antibody conjugated to alkaline phosphatase (Dianova; Hamburg, Germany) at a dilution of 1:10 000 in 5% (w/v) non-fat skim milk powder in PBS) for 45 min at room temperature. After washing 3 x 5 min with PBST the membrane was incubated in development buffer (0,2 mg/ml BICP, 100mM Tris-HCl, 100mM NaCl, 5mM MgCl2, 0.05% Tween20, pH 9.5) until blue staining was apparent, at which point the filters were washed twice with water and photographed.

2.5 Tap-tag Purification

Cells expressing the TAP tagged protein of interest were grown in a 2 liter shaking culture to a density of $2x10^{-6}$ cells ml^{-1}. All procedures after this point were carried out at 4ºC unless otherwise indicated. The cells were centrifuged and resuspended in 40 ml of ice cold IP150S buffer (10 mM Tris-HCL, 150 mM NaCl, 250 mM sucrose, 0.1 mM PMSF, 1mM β-mercaptoethanol, pH 8.0). Cells were lysed by passing twice through a 5 µM filter. The lysate was centrifuged 2 min at 16 000 *g* and the supernatant was transferred to a new falcon tube. 250 µl of IgG-agrose was added to the falcon which was then incubated for 2 hours at 4ºC with gentle rotation. The supernatant -agarose slurry was loaded onto a polyprep column (Bio-rad; Hercules, CA) and the unbound fraction eluted off. The column was washed with 30 mL IP150 buffer (IP150S without the sucrose) then with 10 ml of TEV cleavage buffer (10 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 0.1 % (v/v) NP40, 1 mM DTT, pH 8.0). The resin was transferred to a 1.5 ml eppendorf tube containing 500 µl TEV cleavage buffer and 100 units of AccTEV protease (Sigma-Aldrich; St. Louis, MO). The tube was rotated for 2 hours at 16ºC. The beads were pelleted by centrifuging the tube 21 *g* for 1 min, the supernatant was transferred to 15 ml falcon and the beads were washed twice with 500 uL TEV cleavage buffer. The supernatant from the wash steps was combined with the first supernatant in the falcon tube to give a final volume of 1.5 ml. 3 ml of calmodulin binding buffer (10mM Tris-HCL, 150 mM NaCl, 0.1 %(v/v) NP40, 1 mM Mg-acetate, 1 mM imidazol, 2mM $CaCl_2$, 10 mM β-mercaptoethanol, pH8.0) and 30 µl of 100 mM $CaCl_2$ and 250 µl of calmodulin beads were added to the tube which was then rotated for 2 hours at 4ºC. The beads were transferred to a polyprep column and washed with 10 ml calmodulin binding buffer. Elution buffer (camodulin binding buffer supplemented with 2 µM EGTA) was added to the column and 500 µl fractions were eluted off the column. Elution fractions were run on an SDS-PAGE gel and analysed by western blotting.

2.6 Identification of Homologs, Alignments

Dictyostelium homologs of characterised genes were identified in the *Dictyostelim* genome (Eichinger et al., 2005) using BLAST search (Karlin and Altschul, 1993), a reciprocal BLAST search against the NCBI databank (non-redundant protein sequences) was preformed to confirm identity. Orthologs of H3v1 were identified using a PSI-BLAST search (Altschul et al., 1997) against the NCBI databank (non-redundant protein sequences). Sequence alignments were preformed using Clustal W (v1.83; Thompson et al., 1994). Phylogenetic trees were infered using MrBayes (v3.1.2; Huelsenbeck and Ronquist, 2001; Huelsenbeck et al., 2001). A mixed amino-acid model was run for 1000000 iterations using default settings, Similar results were obtained using Clustal X (Thompson et al., 1997). Phylogenetic trees were rendered using Dendroscope (v1.4; Huson et al., 2007).

2.7 Immunofluorescence

Cells were grown overnight on coverslips to approximately 80% confluence and fixed in 4% (w/v) paraformaldehyde (PFA) dissolved in 20 mM phosphate buffer (pH 6.7) for 10 min at 22ºC. The PFA solution was removed and cells were permeabilised for 5 min in 0.2% (v/v) triton-X 100 in 20 mM phosphate buffer (pH 6.7). Alternatively cells were fixed in methanol for 10 min at 20ºC. Cells were blocked with 2% (w/v) BSA in PBS pH 7.6 for 30 min at room temperature. The coverslips were incubated overnight at 4ºC with the primary antibody was diluted in 1% (w/v) BSA in PBS pH 7.6. The coverslips were washed 3x 5min in PBS then incubated with the secondary antibody diluted in 1% (w/v) BSA in PBS pH 7.6 for one hour at room temperature. The coverslips were washed 3x 5min in PBS then mounted on a slide with a drop of mounting media (250 ng μl^{-1} DAPI in 90% (v/v) glycerol, 20 mM Tris-HCL, 1 µg ml^{-1} DABCO, pH 8.3). Cells were examined by fluorescence microscopy.

2.8 Fluorescent Labelling of DNA Probes by Nick Translation

Labelling reactions were set up as follows on ice:

template DNA	1.5 µg
dNTP mix (250 µM dATP 250 µM dCTP 250 µM dGTP 50 µM dTTP)	5 µl
1 mm Cy3-dUTP or FITC-dUTP	4 µl
NT buffer (0.5 M Tris HCl, 50 mm MgCl2 0.05% BSA, pH 7.5)	5 µl
0.1 M β-mercaptoethanol	5 µl
DNase I (1: 250)	2.6 µl
DNA polymerase I	1 µl
DDW	to 50 µl

Reactions were incubated for 120 min at 15°C in a water bath. The reaction was quenched by adding 1 µl 0,5M EDTA, pH 8,0 and incubating for 10 min at 65°C. The probe was then ethanol precipitated and resuspended in 50 µl of DDW.

2.9 Fluorescence In Situ Hybridisation (FISH)

The fluorescence *in situ* hybridisation protocol was adapted from Moerman and Klein, 1998; and Sucgang et al., 2003. Cells were allowed settle on slides for 10min then fixed in 3:1 methanol:acetic acid at -20ºC for 60 min then air dried. Slides were placed at 60ºC for 30 min on a hot plate then RNase treated (200 µg ml^{-1} in 2xSSC for 40 min at 37ºC). Slides were washed 2x 5 min in 2xSSC, 1x5 min in PBS then post-fixed with 4% PFA in PBS for 10 min at room temperature. Slides were washed 2x 5 min in PBS and then dehydrated using an ethanol series of 70% for 3 min, 90% for 3 min and 99,7% for 3 min then air dried. Approximately 30 ng of fluorescent labelled probe was resuspended in 12 µl hybridisation solution (50% formamide, 10% dextran sulfate, 2xSSC) and denatured for 5 min at 95°C then cooled on ice. The hybridisation mix was placed on slide, overlaid with a coverslip, and sealed with rubber cement. The slide was placed on a 80ºC heat block to denature the genomic DNA for 2 min and then incubated in a moist chamber at 37ºC overnight to hybridise the probe. Slides were wash twice in 2xSSC for 5 min at

42ºC and then three times 5 min in 2xSSC, 50% formamide at 42ºC then dehydrated by ethanol series of 70% for 3 min, 90% for 3 min and 99,7% for 3 min then air dried. A drop of mounting media (250 ng/uL DAPI in 90% (v/v) glycerol, 20 mM Tris-HCL, 1 mg/ml DABCO, pH 8.3) was placed on the slide and overlaid with a coverslip.

2.10 IF-FISH

Cells were immunostained as described above. After immunostaining cells were fixed with 4% (w/v) PFA, 4% (w/v) sucrose in 20 mM phosphate buffer (pH 6.7) for 10 min at room temperature. Slides were washed 1x 5min with PBS then 2x5 min in 2xSSC and then processed according to the FISH protocol starting from the RNase treatment step.

2.11 Microscopy

Images were acquired on a Leica DMIRB inverted microscope equiped with a DC350 camera and IM50 Acquisition software (Leica Microsystems; Wetzlar, Germany). Live cell imaging was preformed with a Zeiss Axiovert 200 M CellObserver HS system (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) equipped with a Sutter DG-4 light source (Sutter instruments, Novato, CA, USA), an ASI piezo stage (Applied Scientific Instruments, Eugene, OR, USA), a Zeiss PlanApo 63x/1.2 NA glycerol immersion lens, a Zeiss Axiocam MRm Rev. 3 CCD camera, and Axiovision 4.6.3 software. Images were quantified and prepared for presentation using ImageJ v1.42n-v1.43b (http://rsbweb.nih.gov/ij/).

2.12 Chromatin Immunoprecipitation

All steps were preformed on ice unless otherwise stated. $1x10^{-8}$ cells were fixed in 1% (w/v) PFA in phosphate buffer at 22ºC. After 10 min glycine was added to a final concentration of 200 mM and the cells were incubated a further 5 min. Cells were washed with ice-cold phosphate buffer then with 1% Triton-X100 in phosphate buffer. Cells were resuspended in 500 µl of nuclear lysis buffer (50 mM HEPES, 10 mM EDTA, 1% Triton-X100, 1% SDS 0.1 mM PMSF, complete protease inhibitors (Roche) ph 8.0) in an eppendorf tube. Samples were sonicated with a UP 200S Sonicatior (Dr. Hielscher GmbH, Stansdorf, Germany) in an ice-water bath (5x10 sec, 25% output, 45% duty cycle) with a 50 sec pause between bursts. 900 µl ChIP dilution buffer (16.7 mM HEPES, 1% Triton-X100, 1.2 mM EDTA, 167 mM NaCl, 0.1 mM PMSF, complete protease inhibitors (Roche) pH 8.0) was added per 100 µL of sonciated lysate. Samples were centrifuged for 2 min at 16 000 *g* at 4ºC and the supernatant was transfered to a new tube. The supernatant was pre-cleared with protein-A sepharose for 60 min @ 4ºC then centrifuged and transfered to a new tube. Supernatant was divided into 1 ml fractions (approximately $2x10^{-7}$ cells) and 2 µg of the appropriate antibody was added. Samples were rotated overnight at 4ºC. 30 µl protein-A-sepharose was added and the samples rotated a further 60 min @ 4ºC. Beads were washed twice with with low salt wash

buffer (150 mM NaCl , 0.1% SDS , 1% Triton X-100, 2 mM EDTA, 20 mM HEPES, pH 8), once with high salt wash buffer (500 mM NaCl , 0.1% SDS , 1% Triton X-100, 2 mM EDTA, 20 mM HEPES, pH 8), twice with LiCl wash buffer (250 mM LiCl , 1% NP40 , 1% sodium deoxycholate, 1 mM EDTA, 10 mM HEPES, pH 8) and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). After the final wash 250 µl of elution buffer (1% (w/v) SDS, 100 mM NaHCO3) was added to the beads and the resultant slurry was incubated at 65ºC for 15 min with gentle agitation. After a brief centrifugation to pellet the beads, the supernatant was transferred to a new tube. Another 250 µl of elution buffer was added to the beads and the extraction was repeated. The two elutions were then combined. 20 µl of 5M NaCl was added to the elutant, which was then incubated overnight at 65ºC to reverse the crosslinking. In the morning 20 µl of protease K buffer (250 mM EDTA, 1 M Tris-HCl (pH 6.5)) and 2 µl of proteinase-K (10 mg ml^{-1}) were added and the solution incubated at 45°C for 3 hours. After phenol extraction the DNA was ethanol precipitated and resuspended in 50 µl DDW containing 10 ng ml^{-1} RNase-A.

2.13 Analysis ChIP Samples

Enrichment of loci of interest in the immunoprecipitated chromatin was detected by semi-quantitative PCR. Oligonuclotide primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cg). Standard settings, except for setting minimum and maximum primer GC content at 40% and 60% respectively, and setting amplified fragment length to 150-200 base-pairs were used. 1.5 µl of sample DNA from each chromatin immunoprecipitation was used in each 20 µl PCR reaction of 20 µl, (1 unit of *Taq* DNA polymerase, 1.5 mM MgC2,1 ρM of each oligonucloitde primer, 1 nM dNTP's). PCR was performed using a programmable thermocycler using the following scheme : 94°C for 90 seconds once, followed by cycles of 94°C for 30 sec, 60°C for 1 min, for X times, and then once at 60°C for 3 min. The PCR product was then analysed on an Ethidium bromide stained 1% agarose/TBE gel. The number of PCR cycles used was determined empirically as the number that did not give any signal in the no antibody control but gave a robust signal from total genomic DNA equal to 0.1% of that used in each chromatin immunoprecipitation. For each locus the PCR assay was performed in triplicate and each chromatin-immunoprecipitation was performed at least twice. Alternatively samples were analysed using a Real time PCR machine from Eppendorf (Hamburg, Germany) or Bio-rad (Hercules, CA) using Eva-Green nucleic acid dye (Jena Bioscience; Jena, Germany) according to the manufactures instructions.

2.14 Developmental Assay

Ax2 or strains of interest were grown in shaking culture to a density $1x10^{-6}$ cells ml^{-1}. $1x10^{-7}$ cells were centrifuged for 2 min, 200 *g* @ 4ºC. Cells were washed twice in phosphate buffer then

adjusted to a cell density of $4x10^{-6}$ cells ml^{-1}. 50 µl of each strain was spoted in triplicate on phosphate agar plates, so that each spot has area of approximately 1 cm^2. Plates were left open in a sterile hood for 30 min to allow excess buffer to evaporate. Plates were examined at regular intervals to examine development.

2.15 GFP Reporter Constructs

Ax2 or the mutant of interest were transformed with [ecmAO]:GFP or [cotB]:GFP reporter constructs (a gift from from Rick Fritel) and subcloned as described in section 2.3.2. Cells were grown in shaking culture to a density of $1x10^{-6}$ cells ml^{-1}. $1x10^{-7}$ cells were centrifuged 2 min 200 *g* @ 4ºC. Cells were washed 3 times in ice cold water and then resuspended in a minimal amount (approximately 100 µl) of water and deposited in 3-4 small drops on freshly prepared 1% phosphate-agar plates. The Plates were placed in a light-proof humid chamber to develop for 18-20 hours at 20ºC in the dark. Slugs were examined using a fluorescence microscope taking care to minimise the exposure of the plates to light beforehand.

2.16 LacZ Reporter Constructs

Ax2 or the mutant of interest were transformed with [ecmB]:LacZ, [EcmA]:LacZ, [EcmO]:LacZ or [EcmAO]:LacZ reporter constructs (from Dictybase) as described in section 2.3.2 except that selection was carried out using 50 µg ml^{-1} Geneticin. Strains of interest were grown in shaking culture to a density $1x10^{-6}$ cells ml^{-1}. $2x10^{-7}$ cells were centrifuged 2 min 200 *g* @ 4ºC. Cells were washed twice in phosphate buffer then resuspended in 800 µl of phosphate buffer. The cell suspension was distributed evenly of white, 47 mm diameter Nitrocelulose filters (47 mm diameter) placed on phosphate buffer soaked filter paper in a humid chamber. At the first time point the filter was sliced into 4 using a sharp scalpel, one piece was removed for fixation, while the others were allowed to continue development for the desired time. The filter quarter removed was briefly placed on a piece of tissue paper to absorb excess liquid and then placed in a petri dish. 800 µl of 3.7 % (w/v) PFA freshly dissolved in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) was gently pipetted onto the filter and allowed to sit for 10 min. The PFA solution was gently removed with a pipet and the filter briefly placed on a piece of tissue paper to absorb excess liquid. The filter was returned to the peitri dish and 800 µl of 0.1 % (v/v) NP40 dissolved in Z-buffer, gently pipetted onto the filter and allowed to sit for 20 min. The fixation solution was gently removed and the filters washed twice for 5 min with 800 µl of Z-buffer. The Z-buffer was removed and 800 µl of fixation solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.4 mg ml^{-1} X-Gal in Z-buffer) was added. When blue staining was apparent, the reaction was quenched by washing the filters twice with 800 µl of Z-buffer.

2.17 Anti-EB4 staining of developing Dictyostelium.

Staining of developing *Dictyostelium* with the anti-EB4 antibody was carried out as describe for LacZ staining, up until the washing steps with Z-buffer. After washing twice with Z-buffer, the cells were incubated with 5% (w/v) non-fat milk powder in PBS for 30 min at room temperature. The blocking solution was removed and the filters were incubate with anti-EB4 antiserum (1:1000, v/v) in 3% (w/v) non-fat milk powder in PBS overnight at 4 C. Filters were gently washed with 3 x 800 µl PBS then incubate with Alkaline-phosphatase conjugated Goat-anti rabbit (1:10 000, v/v) in 3% (w/v) non-fat milk powder in PBS for 60 min at room temperature. Filters were gently washed with 3 x 800 µl PBS, then incubated with development buffer (0,2 mg ml^{-1} BICP, 100mM Tris-HCl, 100mM NaCl, 5mM MgCl2, 0.05% Tween20, pH 9.5) until blue staining was apparent, at which point the filters were washed twice with PBS.

3. Results

3.1 Cloning vectors for Dictyostelium

As part of a wider project examining nuclear organisation in *Dictyostelium* it was necessary to express a number of proteins of interest with N- or C-terminal epitope or fluorescent protein tags at different levels of expression and with different selectable markers. As it is difficult to predict in advance which particular combination of tag and selectable marker will be required for a given experiment, it was desirable to have a system where the gene of interest can be easily transferred between the different expression vectors.

3.1.1 Considerations and background for *Dictyostelium*

Transformation of *Dictyostelium*, either by calcium-phosphate precipitation (Nellen et al., 1984) or by electroporation (Howard et al., 1988) is a well established and relatively simple process. However, there are some limitations including a limited choice of selectable markers and for many of the available vectors with an *actin* promoter, the first few amino acids of the *actin* gene are upstream of the multiple cloning site. This can cause problems as in some cases (approximately 15-20%, Hammarstrom et al., 2002) adding a tag or additional amino acids to the N-terminus can interfere with protein function. As an experimental project evolves, the need to express a protein with a different tag or with a different selectable marker often becomes apparent. When using the currently available traditional Restriction enzyme digest and Ligation (REaL) based cloning methods, this typically necessitates the design of new primers, re-amplification of the gene by PCR, cloning and resequencing of the gene of interest, a time-consuming and expensive process. In recent years a number of high throughput cloning systems have become available based on sequence-specific recombination (SSR), such as the Gateway® system commercialised by Invitrogen, the Creator® system from Clonetech, or the Stargate® system from IBA. These systems allow the gene of interest once cloned to be rapidly introduced into a series of different destination vectors and have proven to be particularly useful for high throughput projects (Matsuyama and Yoshida, 2009). In order to facilitate ongoing experimental work a cloning system was developed that met the following requirements:

1: no extra residues at the N- or C-terminus that can affect protein folding/function

2: optimal poly-linker design

3: variety of available tags

4: ease of transfer of gene of interest between vectors

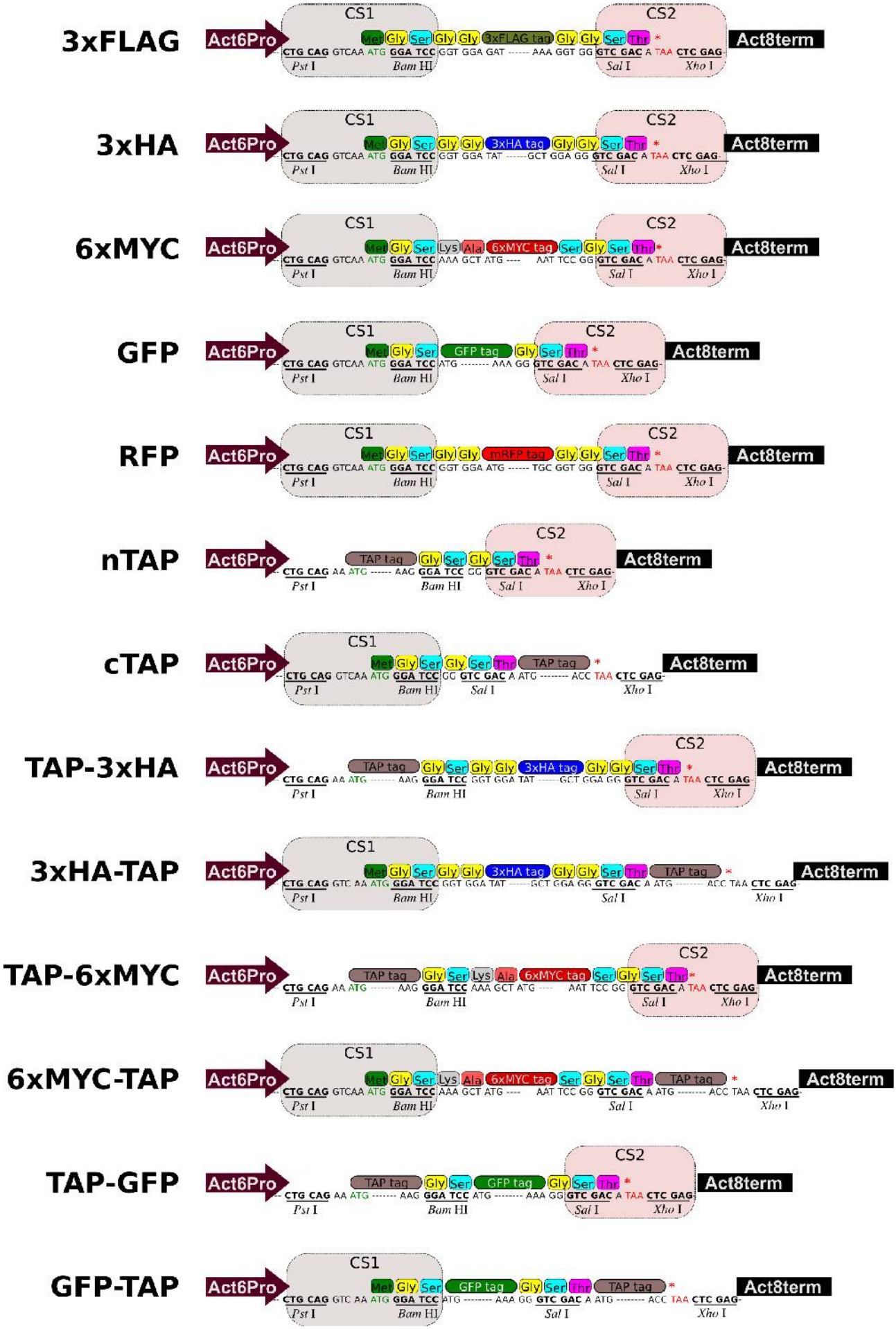
5: series of expression cassette allowing choice of expression level and selectable marker

3.1.2 Choice of Tags

Green-Fluorescent Protein (GFP) and Red-Fluorescent Protein (RFP) fusions with the protein of interest allows visualisation of protein localisation in living cells by fluorescence microscopy. While brighter variants of GFP with mammalian optimised codon-usage such as EGFP (Enhanced GFP) from Clonetech are now available and widely used, the S56T variant of the original *Aequorea victoria* gene (Heim et al., 1995) was chosen. Its high AT content is closer to that of *Dictyostelium* and the additional mutation present in EGFP (F64L) serves only to improve protein folding at 37 °C but is not beneficial at 20-22 °C (Patterson et al., 1997), the growth temperature of *Dictyostelium*. For RFP the variant mRFPmars (Fischer et al., 2004) which has been optimised for expression in *Dictyostelium* was used. For antibody based experiments such as immunolocalisation, immunoprecipitation, co-immunoprecipitation and chromatin immunoprecipitation the 6xMYC tag, the 3xHA tag and 3xFLAG epitope tags were selected. For affinity purification the TAP (tandem affinity purification) tag was chosen.

3.1.3 Design of Vectors

While a SSR based cloning system such as Gateway® would satisfy many of the requirements, it has a number of disadvantages, notably the recombination sequences result in long, and often highly charged polylinkers between the protein and tag. In some cases this can adversely affect protein expression levels (Colwill et al., 2006). Gateway® vectors have been available for *Dictyostelium* for a number of years (Thomason et al., 2006), but have not been widely used in this organism. We have chosen to develop a REaL system based on standardised restriction sites that should combine many of the benefits of both traditional REaL cloning and SSR method. The pDneo2 vector (Witke et al., 1987) was modified by removing the start of the *actin* gene. The desired tag flanked by an N-terminal and C-terminal cloning sites was then introduced (Fig. 3.1). The gene of interest can be cloned into the N-terminal cloning site 1 (CS1) using the *Pst* I and *Bam* HI restriction enzymes. The C-terminal cloning site 2 (CS2) contains a stop codon which is replaced with the gene of interest when an N-terminal tag is desired using the *Sal* I and *Xho* I sites (Fig. 3.1). Different tags can be introduced in place of the existing tag by cloning them into the *Bam* HI-*Sal* I site.


3xFLAG
Act6Pro
CS1
Met Gly Ser Gly Gly 3xFLAG tag Gly Gly Ser Thr *
CTG CAG GTCAA ATG GGA TCC GGT GGA GAT ------ AAA GGT GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
3xHA
Act6Pro
CS1
Met Gly Ser Gly Gly 3xHA tag Gly Gly Ser Thr *
CTG CAG GTCAA ATG GGA TCC GGT GGA TAT ------ GCT GGA GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
6xMYC
Act6Pro
CS1
Met Gly Ser Lys Ala 6xMYC tag Ser Gly Ser Thr *
CTG CAG GTCAA ATG GGA TCC AAA GCT ATG ---- AAT TCC GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
GFP
Act6Pro
CS1
Met Gly Ser GFP tag Gly Ser Thr *
CTG CAG GTCAA ATG GGA TCC ATG -------- AAA GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
RFP
Act6Pro
CS1
Met Gly Ser Gly Gly mRFP tag Gly Gly Ser Thr *
CTG CAG GTCAA ATG GGA TCC GGT GGA ATG ------ TGC GGT GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
nTAP
Act6Pro
TAP tag Gly Ser Gly Ser Thr *
CTG CAG AA ATG ------ AAG GGA TCC GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
cTAP
Act6Pro
CS1
Met Gly Ser Gly Ser Thr TAP tag *
CTG CAG GTCAA ATG GGA TCC GG GTC GAC A ATG -------- ACC TAA CTC GAG
Pst I
Bam HI
Sal I
Xho I
Act8term
TAP-3xHA
Act6Pro
TAP tag Gly Ser Gly Gly 3xHA tag Gly Gly Ser Thr *
CTG CAG AA ATG ------ AAG GGA TCC GGT GGA TAT ------ GCT GGA GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
3xHA-TAP
Act6Pro
CS1
Met Gly Ser Gly Gly 3xHA tag Gly Gly Ser Thr TAP tag *
CTG CAG GTC AA ATG GGA TCC GGT GGA TAT ------ GCT GGA GG GTC GAC A ATG -------- ACC TAA CTC GAG
Pst I
Bam HI
Sal I
Xho I
Act8term
TAP-6xMYC
Act6Pro
TAP tag Gly Ser Lys Ala 6xMYC tag Ser Gly Ser Thr *
CTG CAG AA ATG ------ AAG GGA TCC AAA GCT ATG ---- AAT TCC GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
6xMYC-TAP
Act6Pro
CS1
Met Gly Ser Lys Ala 6xMYC tag Ser Gly Ser Thr TAP tag *
CTG CAG GTCAA ATG GGA TCC AAA GCT ATG ---- AAT TCC GG GTC GAC A ATG -------- ACC TAA CTC GAG
Pst I
Bam HI
Sal I
Xho I
Act8term
TAP-GFP
Act6Pro
TAP tag Gly Ser GFP tag Gly Ser Thr *
CTG CAG AA ATG ------ AAG GGA TCC ATG -------- AAA GG GTC GAC A TAA CTC GAG
Pst I
Bam HI
CS2
Sal I
Xho I
Act8term
GFP-TAP
Act6Pro
CS1
Met Gly Ser GFP tag Gly Ser Thr TAP tag *
CTG CAG GTC AA ATG GGA TCC ATG -------- AAA GG GTC GAC A ATG -------- ACC TAA CTC GAG
Pst I
Bam HI
Sal I
Xho I
Act8term

The most commonly used resistance cassette for over-expression in *Dictyostelium* is the Geneticin (G418) resistance cassette. This typically results in about 100 copies of the transgene integrating into the genome mostly in tandem arrays (Nellen and Firtel, 1985). While the expression level of a transgene is determined by a number of factors, of which copy number is only one, these vectors normally result in a level of expression that allows many transgenes be readily detectable as GFP or epitope tagged fusions. The Blasticidin resistance cassette on the other hand, typically integrates at 1-5 copies, thus typically resulting in a lower level of expression of the transgene. A series of vectors with the Blasticidin resistance cassette instead were made for use when a lower level of transgene expression was required.

In some cases it is also desirable to be able to express transgenes at higher levels from vectors encoding Blasticidin resistance. For example when co-expressing the transgene together with another transgene on a Geneticin resistant vector it may be advantageous to express both proteins at a similar level. To achieve this, the origin of replication from a naturally occurring *Dictyostelium* extrachromosomal plasmid, Ddp1 (Noegel et al., 1985) was inserted into the Blasticidin resistant vector. This allows the transgene to being maintained on a stable extrachromosomal plasmid at approximately 100 copies per cell. This results in an expression level similar to, or slightly higher than that of G418 resistance based vectors. This is useful for co-expression of two proteins at roughly equal levels (Fig. 3.2). The vectors created are listed in table 3.1.

Fig. 3.1 **Schematic of the cloning sites and tags in the vectors constructed.** The gene of interest can be cloned into either cloning site 1 (CS1) using the *Pst* I and *Bam* HI restriction sites (underlined) or cloning site 2 (CS2) using the *Sal* I and *Xho* I restriction sites (underlined). The amino-acid composition of the short linkers added between the tag and the protein and their reading frame is shown for all the constructs. Initiation and termination codons are shaded green and red respectively. The *actin*6 promoter and *actin*8 terminator are labelled Act6Pro and Act8term respectively. The following tags were used: 3 copies of the FLAG epitope tag (3xFLAG), 3 copies of the HA epitope tag (3xHA), 6 copies of the MYC epitope tag (6xMYC), green florescent protein (GFP), monomeric red fluorescent protein (RFP), tandem affinity purification tag (TAP tag).

Vector	resistance	replication type	cloning site(s)
pDneo2a	G418(R)	integrating	both
pDneo2a-3xHA	G418(R)	integrating	both
pDneo2a-6xMYC	G418(R)	integrating	both
pDneo2a-GFP	G418(R)	integrating	both
pDneo2a-mRFP	G418(R)	integrating	both
pDneo2a-3xFLAG	G418(R)	integrating	both
pDneo2a-cTAP	G418(R)	integrating	CS1
pDneo2a-3xHA-TAP	G418(R)	integrating	CS1
pDneo2a-6xMYC-TAP	G418(R)	integrating	CS1
pDneo2a-GFP-TAP	G418(R)	integrating	CS1
pDneo2a-nTAP	G418(R)	integrating	CS2
pDneo2a-TAP-3xHA	G418(R)	integrating	CS2
pDneo2a-TAP-6xMYC	G418(R)	integrating	CS2
pDneo2a-TAP-GFP	G418(R)	integrating	CS2
pDbsr2a-nTAP	BS(R)	integrating	CS2
pDbsr2a-cTAP	BS(R)	integrating	CS1
pDbsr2a-TAP-6xMYC	BS(R)	integrating	CS2
pDbsr2a-6xMYC-TAP	BS(R)	integrating	CS1
pDbsrXP-3xHA	BS(R)	extrachromosomal	both
pDbsrXP-6xMYC	BS(R)	extrachromosomal	both
pDbsrXP-3xFLAG	BS(R)	extrachromosomal	both
pDbsrXP-GFP	BS(R)	extrachromosomal	both
pDbsrXP-mRFP	BS(R)	extrachromosomal	both

Table 3.1 **Expression vectors for *Dictyostelium*.**

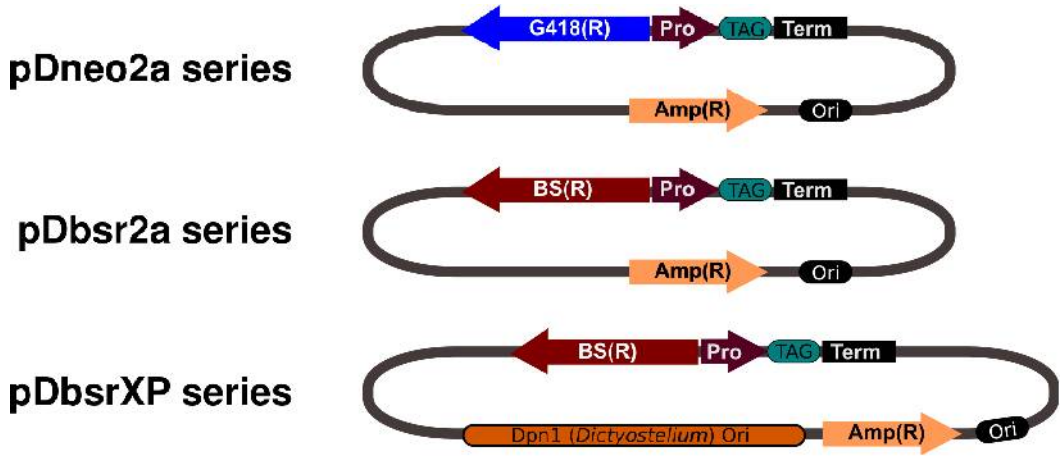


Fig. 3.2 **Schematic of the different vector backbones available.** Amp(R): bacterial ampicilin resistance cassette; Ori: bacterial origin of replication; G418(R): gene conferring resistance to Geneticin G418 in *Dictyostelium*; BS(R): gene conferring resistance to Blasticidin in *Dictyostelium*; Dpn1: *Dictyostelium* Dpn1 origin of replication, which allows extrachromosomal replication and maintenance of plasmids in *Dictyostelium.* Pro: a*ctin*6 promoter; TAG: epitope tag; Term: *actin*8 terminator.

Vector	Western blot	(Immuno) Fluorescence
pDbsrXP-HcpB-GFP	N.T.	yes
pDbsrXP-HcpB-RFP	N.T.	yes (Fig.3.4D)
pDneo2a-6xMYC-H2B	yes	yes (Fig. 3.3f)
pDneo2a-6xMYC-H2AX	yes	yes
pDneo2a-3xFLAG-H4	yes (Fig. 3.3a)	yes
pDneo2a-3xHA-H2AX	yes	yes
pDneo2a-3xHA-UBI	yes	N.T.
pDneo2a-3xFLAG-H3c	yes (Fig. 3.3a)	yes (Fig. 3.3d)
pDneo2a-HcpB-3xFLAG	yes (Fig. 3.3a)	yes (Fig. 3.3d)
pDneo2a-TAP-3xHA-H3c	yes	yes (Fig. 3.3e)
pDneo2a-AgoB-3xHA	yes (Fig.3.3b)	yes
pDneo2a-AgoB-6xMYC	yes (Fig.3.3c)	yes
pDneo2a-AgoB-GFP	N.D	yes
pDneo2a-AgoB-6xMYC-TAP	yes	yes
pDneo2a-6xMYC-DrnB	yes	yes
pDneo2a-6xMYC-DET1	yes (Fig. 3.31a)	yes (Fig. 3.31d)
pDneo2a-GFP-DET1	N.D.	yes (Fig. 3.31b)
pDneo2a-TAP-6xMYC-DET1	yes	N.T.
pDneo2a-GFP-DrnB	N.D.	N.T.
pDneo2a-TAP-6xMYC-DrnB	yes	yes
pDbsr2a-TAP-6xMYC-DrnB	yes	yes
pDneo2a-EriA-3xHA	yes (Fig. 3.3b)	yes (Fig. 3.3e)
pDneo2a-EriA-3xHA-TAP	yes (Fig. 3.4c)	yes
pDneo2a-HcpB-RFP	N.T.	yes
pDneo2a-SuvA-6xMYC	yes	yes (Fig. 3.6c)
pDneo2a-SuvA-mRFP	N.T.	N.D.
pDneo2a-mRFP-DET1	N.T.	N.D.
pDneo2a-GFP-H3v1	N.D.	yes (Fig. 3.12b)
pDneo2a-3xHA-H3v1	yes (Fig. 3.15a)	yes (Fig. 3.15b)
pDneo2a-3xFLAG-H3v1	yes (Fig. 3.15a)	yes
pDbsrXP-RFP-H3v1	N.T.	yes
pDbsrXP-HcpB-3xHA	N.T.	N.T.
pDbsrXP-RFP-H2AX	N.T.	yes (Fig. 3.12b)
pDbsrXP-3xHA-H2AX	yes (Fig. 3.10d)	N.T.
pDbsrXP-3xFLAG-H2AX	yes (Fig. 3.10c)	N.T.
pDbsrXP-RFP-H2B	N.T.	yes
pDbsrXP-H2B-6xMYC	N.T.	N.T.
pDbsrXP-H2B-3xFLAG	yes (Fig.3.3a)	N.T.
pDbsr2a-TAP-6xMYC-DrnB	yes	yes
pDneo2a-3xFLAG-UBI	yes	N.T.
pDbsrXP-3xHA-UBI	N.T.	N.T.
pDbsrXP-3xFLAG-UB	N.T.	N.T
pdneo2a-GFP-CulD	N.T	yes
pDbsrXP-3xHA-CulD	yes	yes

Table 3.2 **Expression vectors constructed during this study**. N.D.: expression not detected, N.T.: expression not tested.

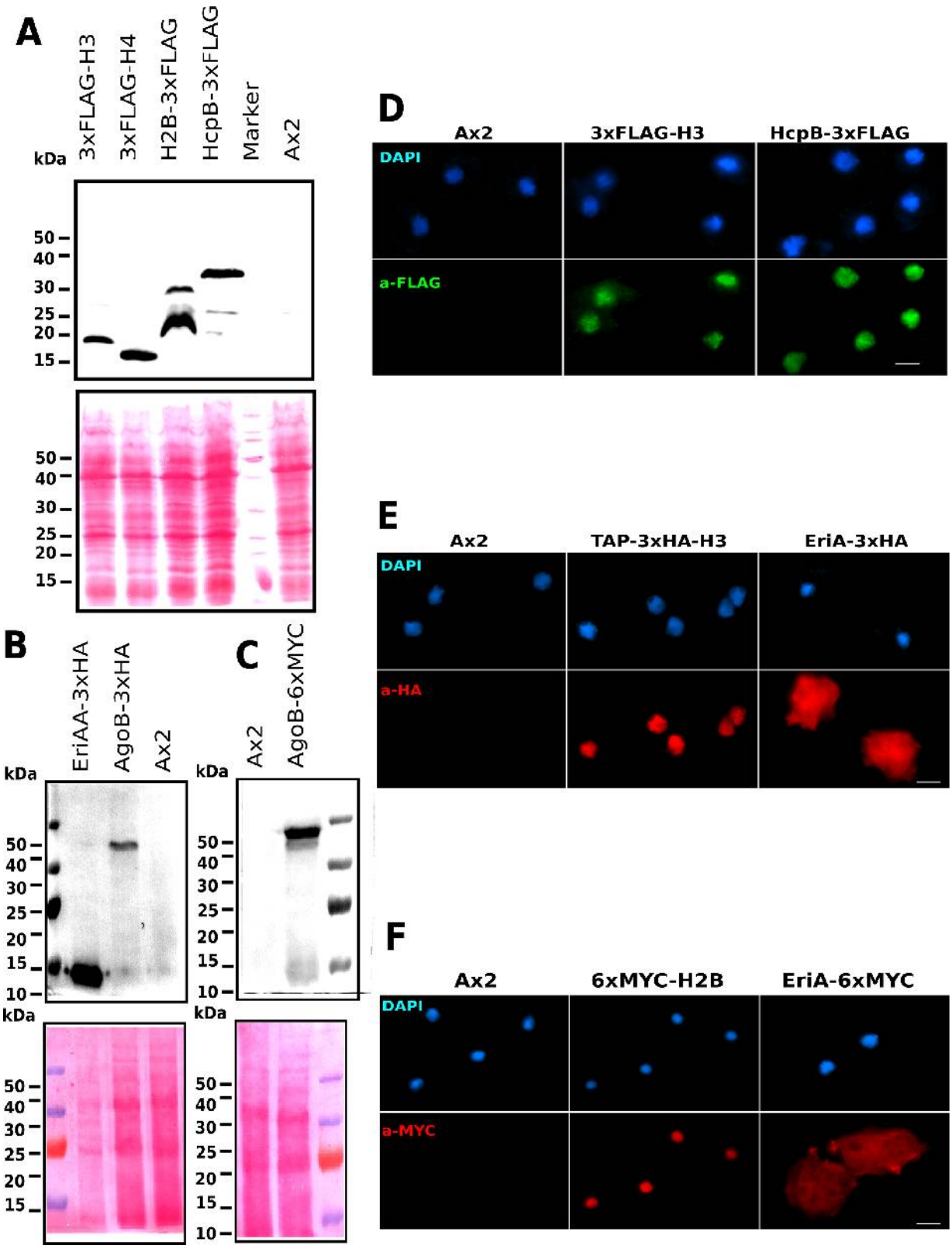


Fig. 3.3 **Validation of Expression Vectors. A:** Western blot on SDS-soluble protein extracts from *Dictyostelium* transformed with different proteins expressed with an N- or C-terminal 3xFLAG tag. **B:** Western blot on SDS-soluble protein extracts from *Dictyostelium* transformed with different proteins expressed with a 3xHA tag. **C:** Western blot on SDS-soluble protein extracts from *Dictyostelium* transformed with ArgonautB fused to a C-terminal 6xMYC tag. Lower panels show the membranes stained with Ponceau-S after western transfer to ensure even loading and transfer to the membrane. **D:** Immunofluorescence on fixed cells expressing N- or C-terminal 3xFLAG tag. **E:** Immunofluorescence on fixed cells expressing N- or C-terminal 3xHA tag. **F:** Immunofluorescence on fixed cells expressing N- or C-terminal 6xMYC tag. Scale bar = 5 µm.

3.1.4 Testing and Validation of the Vectors

To test the vectors and as part of other experiments, a number of genes of interest were cloned into the newly created vectors and checked of expression. Among the genes were *eriA* (DDB_G0283113, N and C-terminal fusion), *hcpB* (DDB_G0282987, N-terminal), *histone h2AX* (DDB_G0279667, C-terminal), *histone h2Bv3* (DDB_G0286509, N- and C-terminal), *histone h3c* (DDB_G0271092, C-terminal), *histone h4a* (DDB_G0277183, C-terminal), *ubiquitin* (DDB_G0282295, C-terminal), *argonautB* (DDB_G0290377, N-terminal) and *dicerB* (DDB_G0268410, C-terminal). These genes were PCR amplified from genomic DNA, cloned into vector pGEM-T-Easy or pJET. Genes were analysed to confirm the correct sequence, excised from the cloning vector and ligated into the desired expression vector. Expression constructs made during the course of this study are listed in table 3.2. After the constructs were confirmed to have the correct identity, they were transformed into *Dictyostelium* using electroporation and selected using G418 or Blasticidin. The resistant colonies were screened for expression of the transgene by western blot (Fig. 3.3a-c) or fluorescence microscopy. After subcloning, the lines were used for various experiments, such as immunolocalisation (Fig. 3.3d-f), immunoprecipitation (Fig. 3.4a,b), affinity purification (Fig. 3.4c) or chromatin immunoprecipitation. By using different selectable markers, cells can easily be super-transformed with a second transgene for colocalisation (Fig. 3.4d) or coimmunoprecipitation experiments.

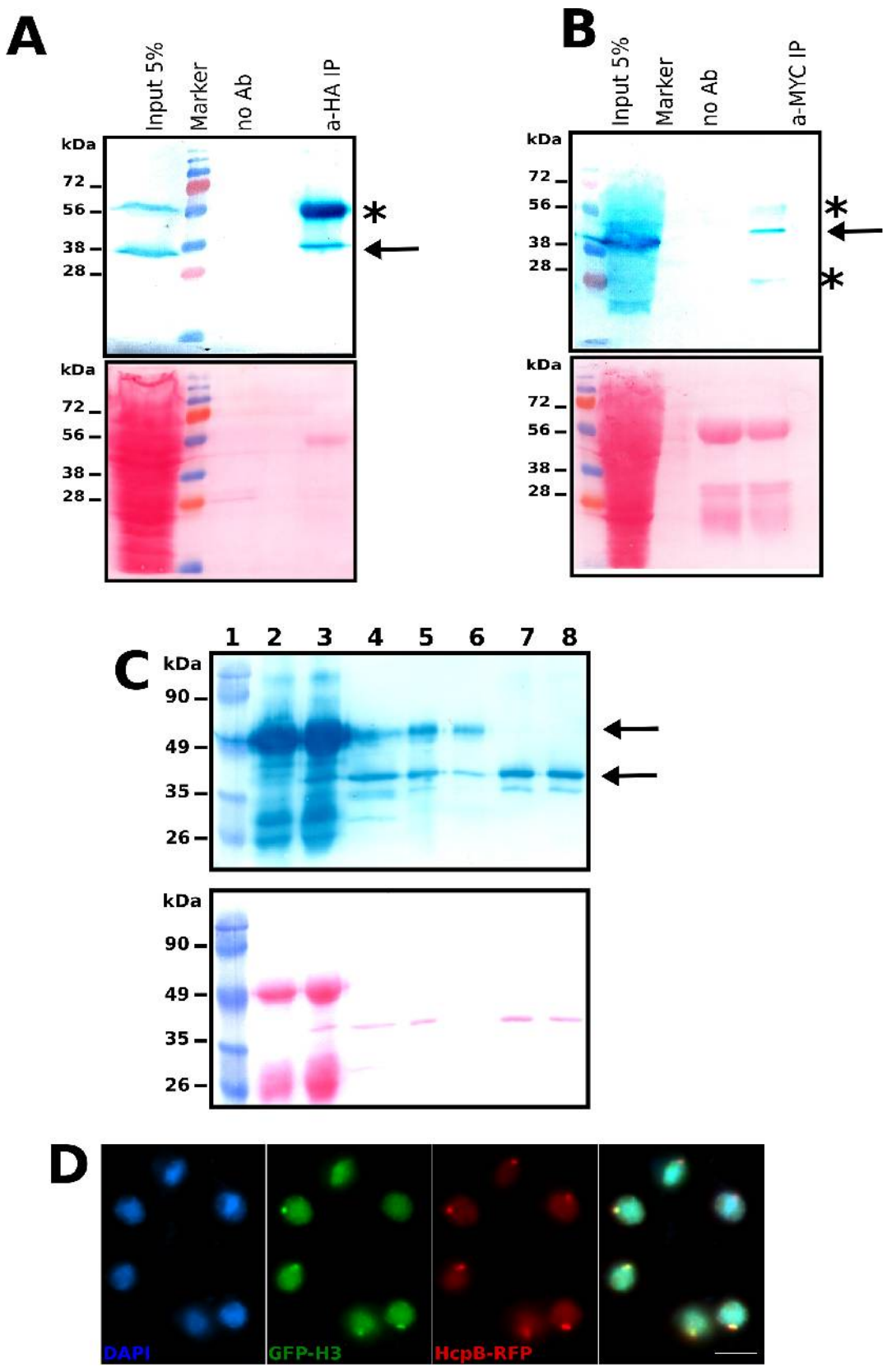


Fig. 3.4 **Validation of Expression Vectors. A:** Western blot showing immunoprecipitation of 3xHA tagged EriA. **B:** Western blot showing immunoprecipitation of 6xMYC tagged EriA. **C:** Tandem affinity purification of EriA-3xHA-TAP. Lane 1: MW marker, lane 2: IgG pre-cleavage, lane 3: IgG post-cleavage, lane 4: elutant, lane 5: CaM resin pre-elute, lane 6: CaM resin post-elute, lane 7: 1st Elution (2 mM EGTA), lane 8: 2nd elution (20 mM EGTA). **D:** Fluorescence images of fixed cells co-expressing GFP tagged histone H3 and RFP tagged HcpB. Arrows indicate epitope tagged protein, * indicates cross-reacting bands from the heavy and/or light chain of the antibody. Scale bar = 5 µm.

3.2 Nuclear organisation in Dictyostelium

As part of a wider study to understand the role of chromatin modifications in the epigenetic regulation of gene expression and development in *Dictyostelium*, the organisation of the nucleus in this species was examined.

3.2.1 Histone modifications

An alignment of *Dictyostelium* histone tails with those of yeast and humans shows that many of the residues subject known to be post-translationally modified are conserved in *Dictyostelium* (Fig. 3.5). It has already been shown that the heterochromatin associated H3K9me2 modification was present in this species and colocalises with the two *Dictyostelium* HP1 homologs, HcpA and HcpB. H3K9me2 (Kaller et al., 2006b; Kaller et al., 2007). The H3K4me (mono, di and tri) modification has also been detected in this species (Chubb et al., 2006).

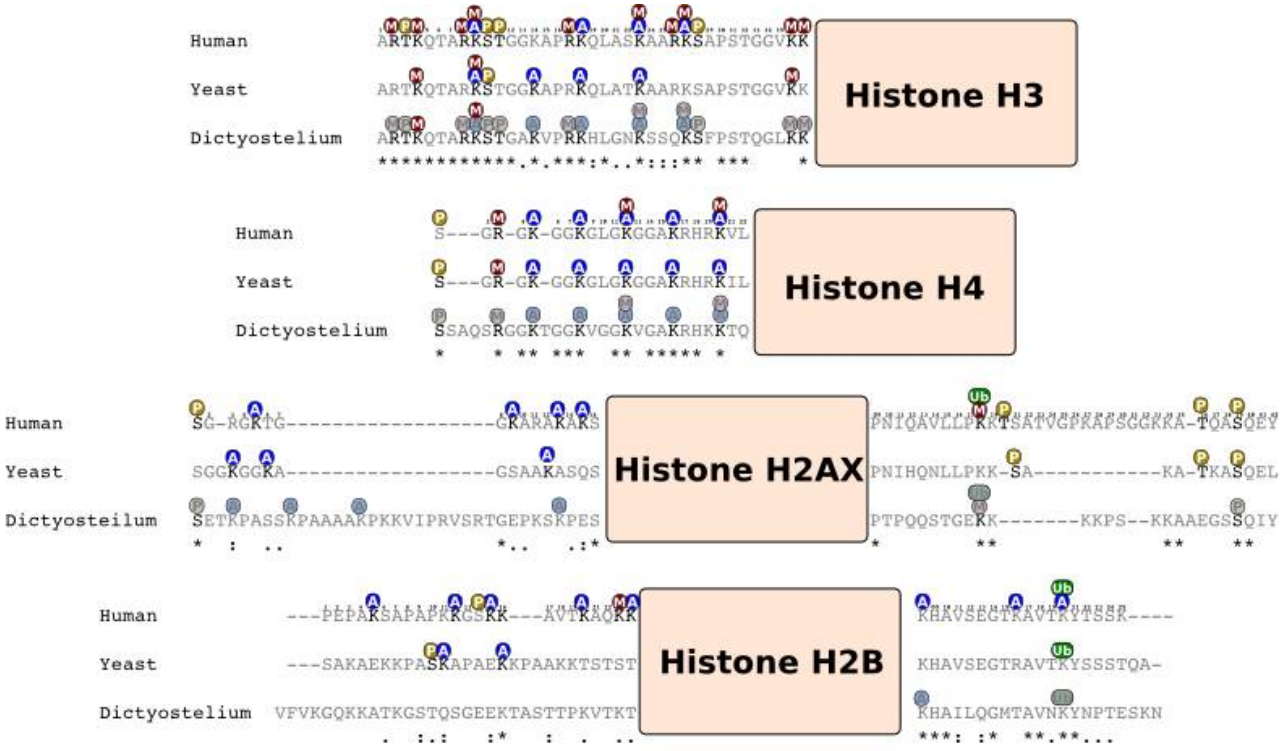


Fig. 3.5 **Post-Translational Histone Modifications**. Alignments of the N- and C-terminal tails of *Dictyostelium* core histones with those from yeast (*Saccharomyces cerevisiae*) and humans. Known post-translation modifications are indicated: M: methylation, A: acetylation, P: phosphorylation, Ub: ubiquitination. Possible post-translational modifications of *Dictyostelium* histone tails based on the conservation of the modified residues with yeast and humans are greyed out.

Histone Methylation

Methylation of Histone H3 at Lysine 4

Histone H3 methylation at lysine 4 occurs predominantly at the promoters of active genes and is catalysed by the Set1 histone methyltransferase (Bhaumik et al., 2007). Antibodies against H3K4me2 and H3K4me3 demonstrated that these modifications were distributed relativity evenly throughout the nucleus as has been previously described (Chubb et al., 2006). Double staining with an antibody against the heterochromatin marker H3K9me2 showed that H3K4me is excluded from the silent pericentromeric heterochromatin loci (Fig.3.6a).

Methylation of Histone H3 at Lysine 9

Methylation of Histone H3 at lysine 9 is a highly conserved histone modification generally associated with heterochromatin. It is present in most eukaryotes with the notable exception of the yeast *S. cerevisiae*. The enzyme primarily responsible for this modification is also conserved and known as Su(var)3-9 in *Drosophila*, Set3-9 in humans and Clr4 in *S. pombe* (Krauss, 2008). H3K9me2 has previously been detected in *Dictyostelium* (Kaller et al., 2006b). H3K9me is responsible for recruiting a number of chromatin binding proteins to heterochromatin such as HP1, which has two homologs in *Dictyostelium*, HcpA and HcpB. A BLAST search of the *Dictyostelium* genome revealed a single Su(var)3-9 homologue (DDB_G0269554, SuvA, M. Essid, Diploma thesis, 2004; Kaller et al., 2006a; Fig. 3.6b). *suvA* encodes a 1527 amino acid protein with an N-terminal region with no similarity to other known proteins while the C-terminus contains a pre-SET and SET domain with homology to Su(var)3-9 of *Drosophila*, Suv39H1 and H2 (human) and Clr4 (*S. pombe*) with e-values from $2e^{-18}$ to $1e^{-20}$. The *suvA* gene was cloned into the pDneo2a-6xMYC destination vector (Table 3.2) so that it was expressed with a C-terminal 6xMYC epitope tag. SuvA-6xMYC localised to a single large focus at the nuclear periphery and a few smaller foci reminiscent of H3K9me2 staining (Fig. 3.6c). An antibody against H3K9me3 partially colocalised with SuvA-6xMYC, suggesting that in *Dictyostelium* H3K9me is also catalysed by the SuvA enzyme. The anti-H3K9me3 antibody also labelled a band at 17 kDa, the expected molecular weight of histone H3 on a western blot, confirming its specificity (Fig. 3.6d). The H3K9me3 antibody gave a stronger signal than the anti-H3K9me2 antibody in both immunofluorescence and western blot experiments, however, this may simply reflect differences in antibody affinity, and it was not possible to determine the relative levels of H3K9me2 and H3K9me3. Attempts to knock out *suvA* to date have been unsuccessful (M. Essid, Diploma thesis, Kassel University, 2004), making it difficult to confirm its function. The failure to knock the gene out would suggest that it performs an essential function. Given that H3K9me binding proteins HcpA and HcpB are also essential, this would suggest that SuvA is indeed a histone H3K9 methyltransferase, as the loss of this mark would also abolish HcpA/B binding to chromatin. Western blotting demonstrated that nuclear the SuvA-6xMYC overexpression strain has increased levels of H3K9me2 and H3K9me3 compared to wild type, while H3K4 methylation remained largely unchanged (Fig. 3.6d), further suggesting that SuvA is a

H3K9 methyltransferase.

Methylation of histone H3 at lysine 27

The methylation of histone H3 at lysine 27 is another repressive mark that is catalysed by the Polycomb 2 (PCR2) complex in many organisms including metazoans, plants and *Giardia.* The *Dictyostelium* genome does not appear to contain any homologs of PCR2 complex constituents. While the lysine residue on histone H3 at position 27 itself is conserved in *Dictyostelium*, the flanking residues are not. Antibodies against H3K27me1, H3K27me2 and H3K9me3 failed to stain *Dictyostelium* nuclei (data not shown) suggesting that this modification is not present in *Dictyostelium*.

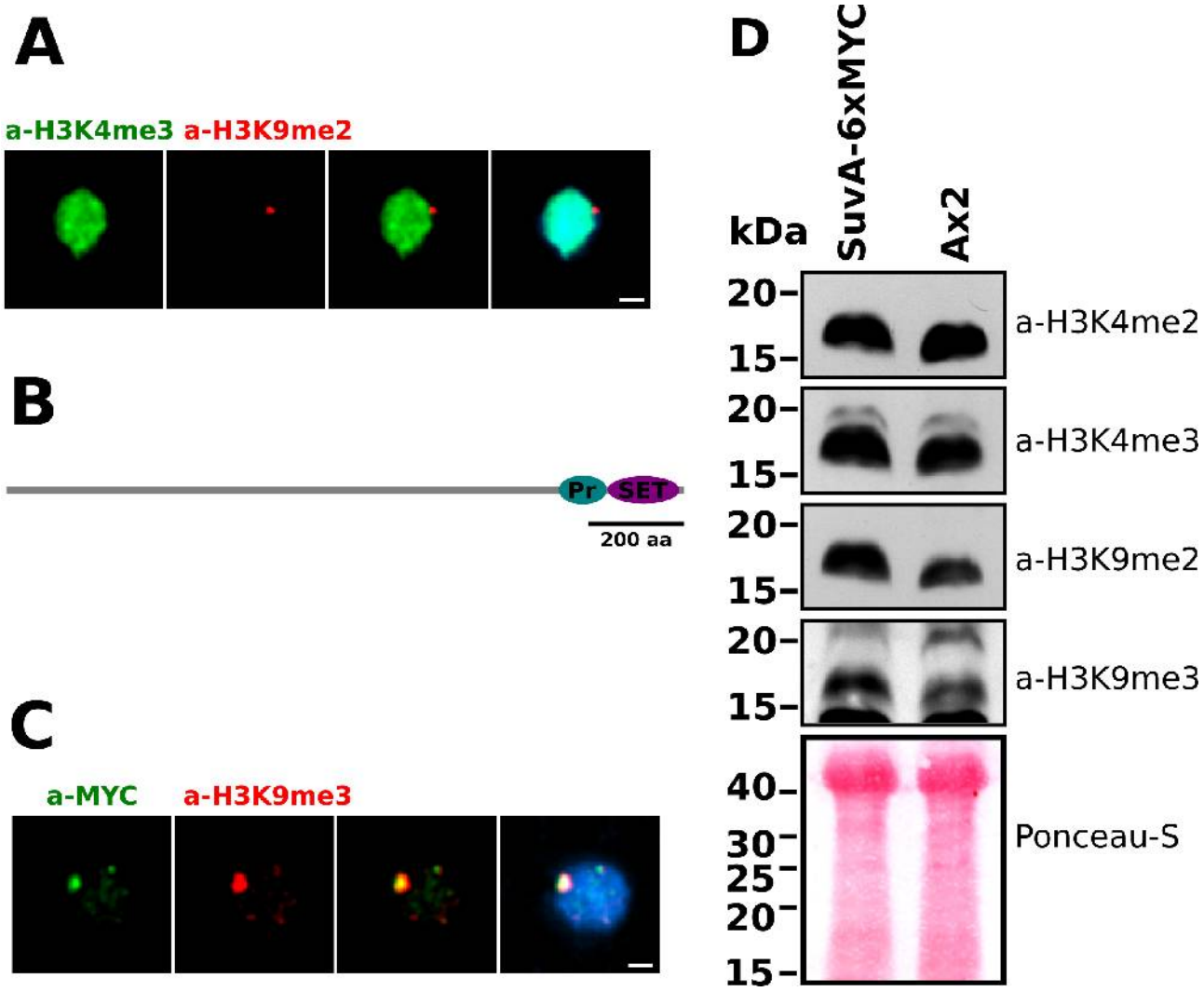


Fig. 3.6 **Characterisation of SuvA. A:** Immunofluorescence with antibodies against the euchromatin marker H3K4me3 and the heterochromatin marker H3K9me2 demonstrates that they occupy discreet nuclear territories. **B:** Schematic showing the domain organisation of the *Dictyostelium* SuvA protein. **C:** Immunofluorescence demonstrates that MYC-tagged SuvA colocalises with the H3K9me3, the histone modification it is thought to catalyse. **D:** Western blot with antibodies against different histone modifications on nuclear extracts of *Dictyostelium* over-expressing SuvA-6xMYC. The lower panels show the membranes stained with Ponceau-S after western transfer to ensure even loading and transfer to the membrane (the antibodies were bound to the same membrane sequentially with a stripping step in-between each antibody application). Scale bar = 1 µm.

Methylation of histone H3 at lysine 36

The histone methyltransferase SET2, which associates with elongating RNA pol II is responsible for H3K36 methylation which appears to be ubiquitous among eukaryotes (Bártová et al., 2008). A putative homolog of this protein has previously been annotated in *Dictyostelium* (DDB_G0648132, Kaller et al., 2006a). The residues surrounding this lysine on *Dictyostelium* histone H3 (in *Dictyostelium* this lysine is actually at position 39, but will be referred to as H3K36 for consistency with other organisms, see Annotations section) are well conserved with respect to other eukaryotes. Antibodies against H3K36me1 and H3K36me2 gave a speckled staining pattern throughout the nucleus (Fig. 3.7) similar to that of H3K4me (Fig. 3.6). This suggests that the role of the SET2 homologue in *Dictyostelium* is conserved with respect to that in other eukaryotes.

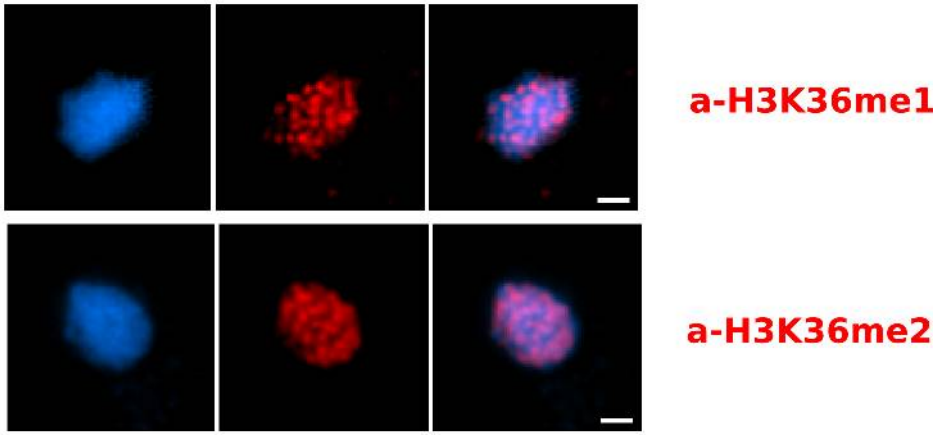


Fig. 3.7 **Histone H3K36 methylation**. Immunofluorescence on fixed cells with antibodies against methylated H3K36 shows that they are distributed throughout the nucleus. Scale bar = 1 µm.

Methylation of Histone H4 at lysine 20

Methylation of histone H4 at lysine 20 is a repressive mark found in plants, metazoans, *Plasmodium* and some fungi (Fuchs et al., 2006; Cui et al., 2008; Miao et al., 2006; Smith et al., 2008). The genome of *Dictyostelium* contains a number of putative histone methyltransferase in addition to the already identified SET1, SET2, SuvA and Dot1 homologs. However none of them has homology to the Suv(4-20) /SET8 histone methyltransferases responsible for methylation of histone H4 at lysine 20. While the N-terminal tail of *Dictyostelium* H4 is highly conserved with respect to other eukaryotes, the residues flanking the lysine 20 target site are not (Fig. 3.5). In agreement with this, antibodies against H4K20me1 and H4K20me2 failed to stain the nuclei (data not shown), further suggesting that this modification is not present in *Dictyostelium*.

Histone Acetylation

Acetylation of histone tails is generally associated with active genes and DNA repair. Antibodies against acetylated histone H3 and acetylated histone H4 display a punctate distribution throughout the nucleus (Fig. 3.8a) similar to what has been observed for histone H3K4me and H3K36me. One difference is that on mitotic nuclei histone H4ac is present on the nucleolar rDNA chromatin while

H3ac is not (white arrows in Fig. 3.8a). A number of putative histone acetyltransferases and deacetylases are present in the *Dictyostelium* genome. HatB (DDB_G0282143) and HdacC (DDB_G0280195; Sawarkar et al., 2009) were expressed as MYC tag fusions. Both the HatB and HdacC were nuclear localised with a distribution similar to H3ac and H4ac (Fig. 3.8b). Double labelling with anti H3K9me2 antibody shows that they are apparently excluded from the pericentromeric heterochromatin.

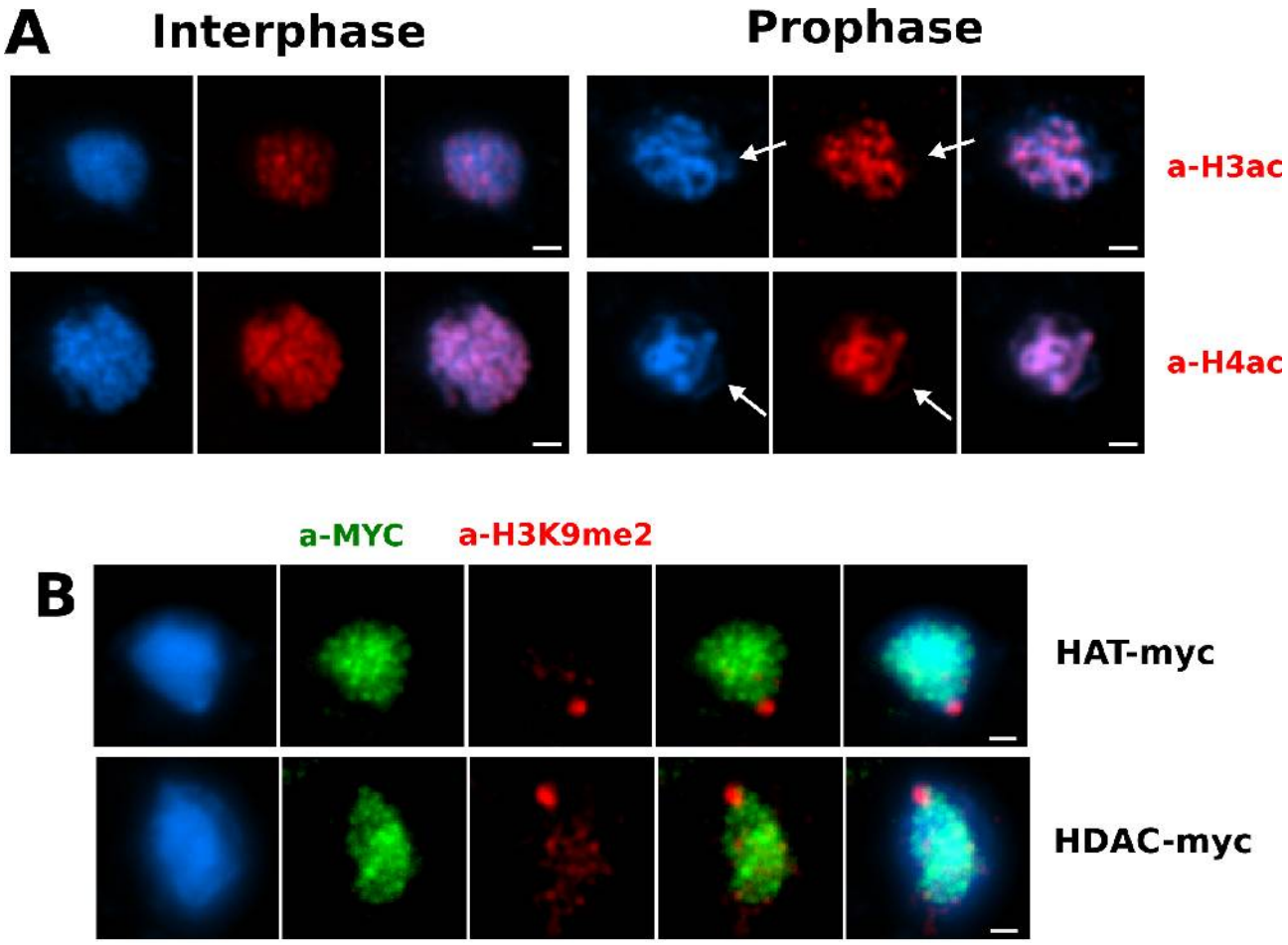


Fig. 3.8 **Histone Acetylation**. **A:** Immunofluorescence on fixed interphase and prophase cells with antibodies against acetylated histone H3 or acetylated histone H4. The arrows indicate non-chromosomal DAPI stained material thought to be rDNA. **B:** Immunofluorescence on fixed cells expressing MYC-tagged HAT or HDAC shows that they both are present in the euchromatin territory but fail to colocalise with the heterochromatin marker H3K9me2. Scale bar = 1 µm.

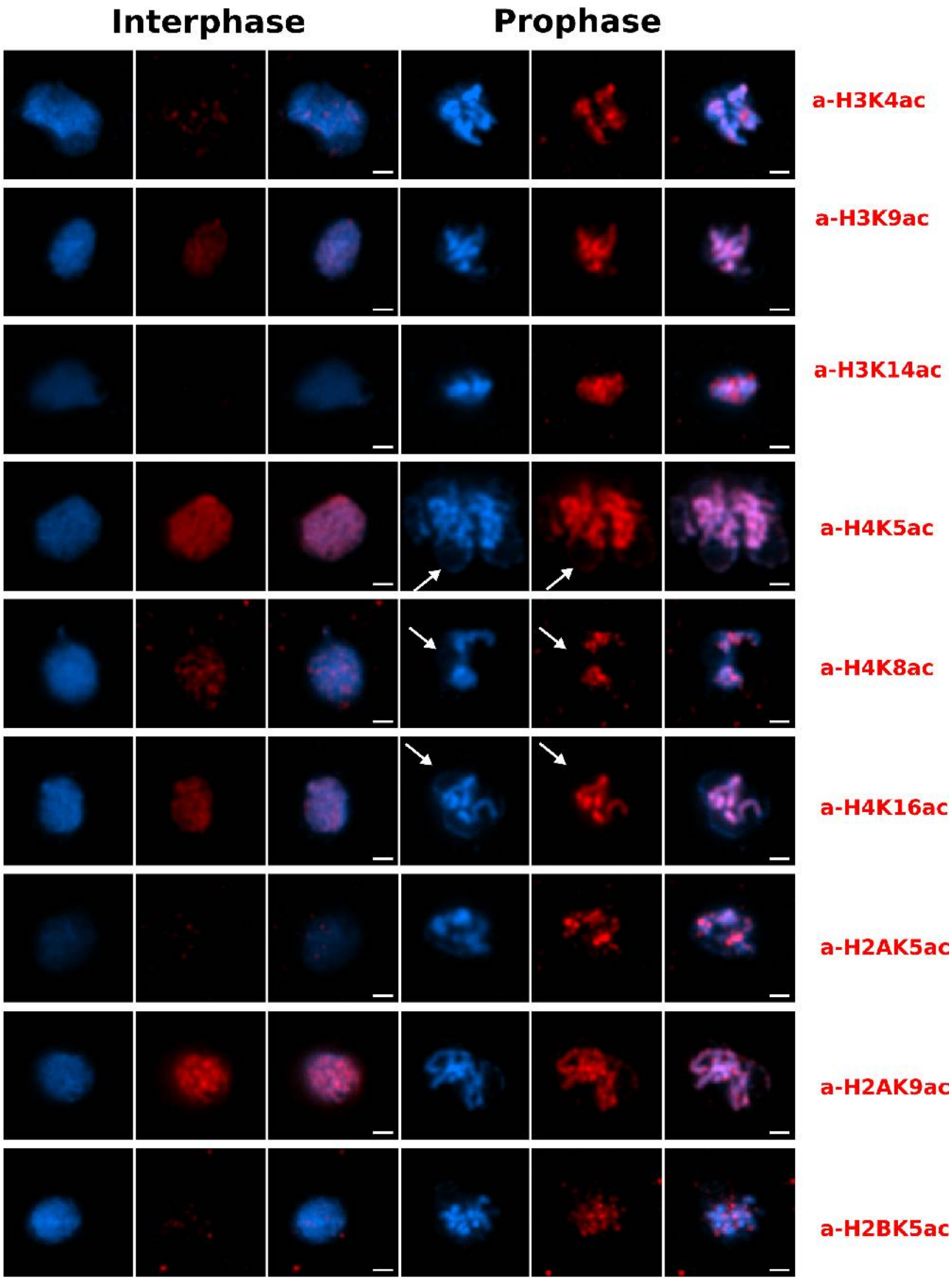


Fig. 3.9 **Histone Acetylation.** Immunofluorescence on fixed interphase and prophase cells with antibodies specific for different acetylated residues on the core histones. The arrows indicate non-chromosomal DAPI stained material corresponding to the rDNA. Scale bar = 1 µm.

Histone H3 acetylation

Acetylation of histone H3 at lysines 4, 9 and 14 is typically associated with gene transcription and DNA damage repair (Wang et al., 2008; Bhaumik et al., 2007). H3K9ac staining appeared to be similar to that of the H3ac, with increased levels apparent on mitotic chromosomes. Weak H3K4ac staining was present at approximately 8-10 puncta in interphase nuclei, while a significant increase was observed on mitotic chromosomes. H3K14ac was only detectable on mitotic chromosomes, suggesting that in *Dictyostelium* it may have a role in cell division (Fig. 3.9). Antibodies against H3K18ac and H3K27ac failed to give any signal, suggesting that these modifications may not be present in *Dictyostelium*.

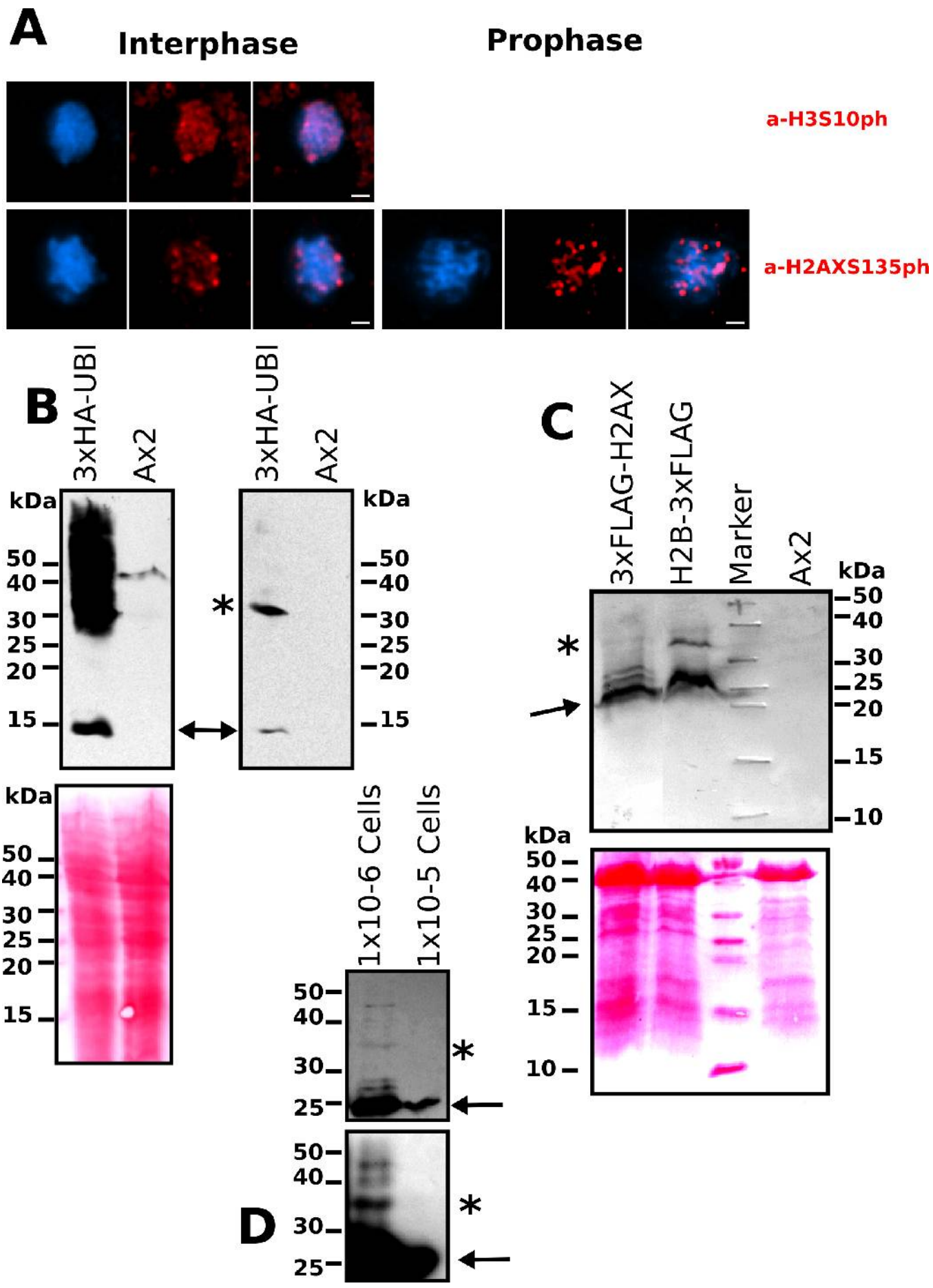
Histone H4 acetylation

The N-terminal tail of histone H4 is extensively acetylated at residues including lysines 5, 8, 12 and 16. These residues and their acetylation appear to be highly conserved throughout eukaryota, being present in plants, fungi and metazoa, as well as in the basal eukaryote *Plasmodium falciparum* (Miao et al., 2006). Antibodies against H4K5ac, H4K8ac, and H4K16ac displayed a punctate pattern throughout the euchromatin territory, similar to that of H3K4me and H3K36me, although fewer and more distinct spots were observed. In prophase cells H4K5ac but not H4K8ac or H4K16ac were present on the non chromosomal DNA thought to be the rDNA palindrome (Fig. 3.9, arrows)

Histone H2AX and H2B acetylation

Histones H2A and H2B are less highly conserved than H3 and H4 and their N-terminal tails in particular have little homology between *Dictyostelium*, fungi and metazoans, while the C-terminal tails are slightly more conserved (Fig. 3.5). An antibody against H2AK9ac labels the entire nucleus, including the rDNA palindrome (Fig. 3.9). While antibodies against H2AK5ac and H2BK5ac label mitotic chromatin but not interphase nuclei, suggesting that this acetylation occurs in a cell cycle dependent manner. These results must be interpreted with caution, as the low degree of conservation in the N-termini of *Dictyostelium* H2A and H2B means that they may be recognising some other epitope within the chromatin.

Fig. 3.10 **Histone Phosphorylation and Ubiquitination A:** Immunofluorescence on fixed interphase and prophase cells with antibodies specific for different phosphorylated residues on the core histones. **B:** Western blot on whole cell extracts of *Dictyostelium* over-expressing 3xHA-tagged mono-ubiquitin. The lower panels show the membranes stained with Ponceau-S after western transfer to ensure even loading and transfer to the membrane. The panel on the right represents a shorter exposure of that on the left. The arrows indicate a band at the size of unconjugated 3xHA-ubiquitin; the asterisk marks a band at the expected size of 3xHA ubiquitin conjugated to the core histone H2A or H2B. **C:** Western blot on nuclear extracts from *Dictyostelium* overexpressing 3xFLAG-tagged histone H2AX or histone H2B. The lower panels show the membranes stained with Ponceau-S after western transfer to ensure even loading and transfer to the membrane. The arrows indicate a band at the size of unconjugated epitope tagged histone; the asterisk marks a band at the expected size of ubiquitin conjugated to the epitope tagged histone H2A or H2B. **D:** Western blot on whole cell extracts of *Dictyostelium* overexpressing 3xHA-tagged H2AX. The panel on the right represemts a shorter exposure of that on the left. The arrows indicate the predicted size of unmodified 3xHA-H2AX and the asterisk indicates the expected size of the mono-ubiquitinated form. Scale bar = 1 µm.


A
Interphase
Prophase
a-H3S10ph
a-H2AXS135ph
B
3xHA-UBI
Ax2
kDa
50
40
30
25
20
15
C
3xFLAG-H2AX
H2B-3xFLAG
Marker
Ax2
10
1x10-6 Cells
1x10-5 Cells
D

Histone Phosphorylation

Phosphorylation of histones is generally associated with cell cycle regulation and DNA damage repair. Phosphorylation of histone H3S10 serves to antagonise binding of HP1 to H3K9me and has been implicated in the cell cycle dependent eviction of HP1 from heterochromatin (Johansen et al., 2006). HcpA and HcpB are lost from the centromeres during mitosis in *Dictyostelium*, suggesting that this mechanism may be conserved. Immunofluorescence with an antibody against H3S10ph shows that this modification is present in interphase cells. In some cases there is strong staining at a focus at the nuclear periphery that may correspond to the pericentromeric heterochromatin (Fig. 3.10). In response to DNA damage DNA-PK (DNA-protein kinase) is recruited to sites of DNA damage where it phosphorylates H2AX at its C-terminus. This serves to recruit the DNA damage repair machinery and this pathway is known to be conserved in *Dictyostelium* (Hudson et al., 2005). In vegetative *Dictyostelium* cells, not exposed to DNA damage agents, some H2AXS135ph was detectable and appeared to concentrate at several foci at the nuclear periphery (Fig. 3.10b).

Histone Ubiquitination

Ubiquitin is a small, highly conserved protein that acts as a post-translational modification by conjugation to other proteins via its C-terminal lysine residue. Ubiquitin can be conjugated to itself to form poly-ubiquitin chains, which target the protein for destruction by the 26S proteasome. Although ubiquitin is usually associated with this role, histones can also be monoubiquitinated, which will not target them to the proteasome. Instead, monoubiquitination on histones appears to serve as an epigenetic signal.

Monoubiquitination of histone H2B at lysine 123 on its C-terminal tail is catalysed by the Bre1/Rad6 ubiquitin-ligase complex. This modification is associated with active genes where it appears that H2BK123ub is necessary for Set1 to catalyse di and tri-methylation of H3K4me but not mono-methylation. *Dictyostelium* has a homologue of both Rad6 (DDB_G0275787) and Bre1 (DDB_G0274241). The C-terminus of H2B has a conserved lysine residue that could be the target of this complex (Fig. 3.5).

Anti-HA western blotting of cells expressing a 3xHA-Ubiquitin construct gave a characteristic smearing pattern (Fig. 3.10b). A lower exposure (right panel), indicated that the major ubiquitin conjugate is just over 30 kDa, the expected size of H2B(3xHA-Ubi) or H2AX(3xHA-Ubi). This suggests that one or both of these histones may be monoubiquitinated in *Dictyostelium*. To further investigate this possibility, western blotting was preformed on extracts from cells expressing H2B-3xFLAG or 3xFLAG-H2AX. Both lanes have a major band at 25 kDa, the expected size of H2B-3xFLAG and 3xFLAG-H2AX (Fig. 3.10c). For the tagged H2B lane a weaker band is present at approximately 33 kDa, the predicted weight of H2B-3xFLAG-Ubi. Approximately 10% of H2B appears to be ubiquitinated in *Dictyostelium*. This is similar to other species with compact genomes such as yeast and *Arabidopsis* (Osley, 2006; Cao et al., 2008; Dubin et al., 2008).

In metazoans the levels of H2BK123ub are low as only a small proportion of the genome is transcribed into protein coding RNA, instead the major ubiquitinated histone is H2A which is monoubiquitinated at lysine 119. This modification is catalysed by the polycomb repressive complex 1 (PCR1), and in female metazoans H2A on the inactive X-chromosomes is particularity heavily ubiquitinated (Osley, 2006). *Dictyostelium*, like yeast and plants, lacks the PCR1 complex, and an antibody against H2AK119ub (E6C5) gives no signal in any of these species (de Napoles et al., 2004). In spite of this, the target lysine and the residues flanking it are at least partially conserved (Fig. 3.5), suggesting that post-translational modification of this residue may occur in these species. Interestingly, it has recently been shown that as part of the DNA damage repair pathway, H2A can be ubiquitinated by a second complex containing the WD40 domain protein DDB1 (damaged-DNA binding protein 1) and the ubiquitin ligase adaptor Cullin4. DDB1 and Cullin4 homologs are highly conserved and found in all eukaryotes, including *Dictyostelium* where they are named RepE (DDB_G0286013, Alexander et al., 1996) and CulD (DDB_G0292794) respectively. *Dictyostelium* RepE is an essential protein with a role in DNA damage repair (Alexander et al., 1996).

3xFLAG-H2AX was present as a major band at 25 kDa, two additional bands are present at approximately 27 and 29 kDa which are too small to be a ubiquitinated species. The identity of these bands is unknown. A very faint band was observed at about 33 kDa (asterisk), which may be H2AXub. Nuclear extracts from cells expressing 3xHA-H2AX were run on a large polyacrylamide gel and western blotting preformed. A major band was present at 25 kDa, two slightly larger bands at 27 and 29 kDa were again observed. At longer exposures a band at 33 kDa was also clearly visible, possibly corresponding to 3xHA-H2AXub. The amount of the putative ubiquitinated H2AX is very low, corresponding to approximately 0.25% of the total 3xHA-H2AX pool.

3.2.2 Distribution of Histone Modifications at Genomic Loci.

Previously the presence of H3K9me2 has been described at the DIRS-1 and *skipper* retrotransposons. (Kaller et al., 2007), while it has been shown that H3K4 methylation at the 5' end of developmentally regulated genes increases as these genes are activated (Chubb et al., 2006). Chromatin immunoprecipitation was preformed using antibodies against H3K9me2, H3K9me3 and H3K4me3. As previously described, H3K9me2 was present at the DIRS-1 and *skipper* retrotransposons and H3K9me3 was also present at these loci.

A significant level of H3K4me3 was also present on both the *skipper* and DIRS-1 retrotransposons, suggesting that both may be transcribed by RNA pol II (Fig. 3.11a).This would be in agreement with data showing that the mRNA from *skipper* is easily detectable by northern blot (Kuhlmann et al., 2005).

HP1 was originally described as a H3K9me binding protein (Fanti and Pimpinelli, 2008) and it has been shown that HcpA and HcpB colocalise with H3K9me2 (Kaller et al., 2006b). However the affinity for H3K9me is rather weak and it appears that other factors are necessary for correct targeting of HP1 in vivo (Eskeland et al., 2007). Many HP1 isoforms do not colocalise with H3K9me, for example the plant homolog, LHP1 is excluded from the pericentromeric heterochromatin and is instead found within euchromatin territories where it appears to be targeted to H3K27me (Libault et al., 2005; Turck et al., 2007). A similar distribution is observed for the mammalian HP1 isoform ɣ (Minc et al., 2000). However H3K27me does not appear to be present in *Dictyostelium* and the localisation pattern of HcpA and HcpB appear similar to that of H3K9me2 (Kaller et al., 2006b). To see if HcpA and HcpB were present at the same loci as H3K9me2 their distribution was determined using chromatin immunoprecipitation (ChIP). ChIP was preformed on cells expressing HcpA-GFP or HcpB-GFP using an anti-GFP binding protein (Rothbauer et al., 2008). Both HcpA and HcpB bound to *skipper,* while only HcpB was enriched on DIRS-1. To a lesser extent HcpB was also found on the active, euchromatin *coronin* locus (Fig. 3.11b). Similar results were obtained for HcpB tagged with 3xHA, 6xMYC and HcpB-GFP precipitated with different antibodies (data not shown). These data suggest that in *Dictyostleium* HcpA and HcpB bind H3K9me decorated loci, but additional factors are required for targeting.

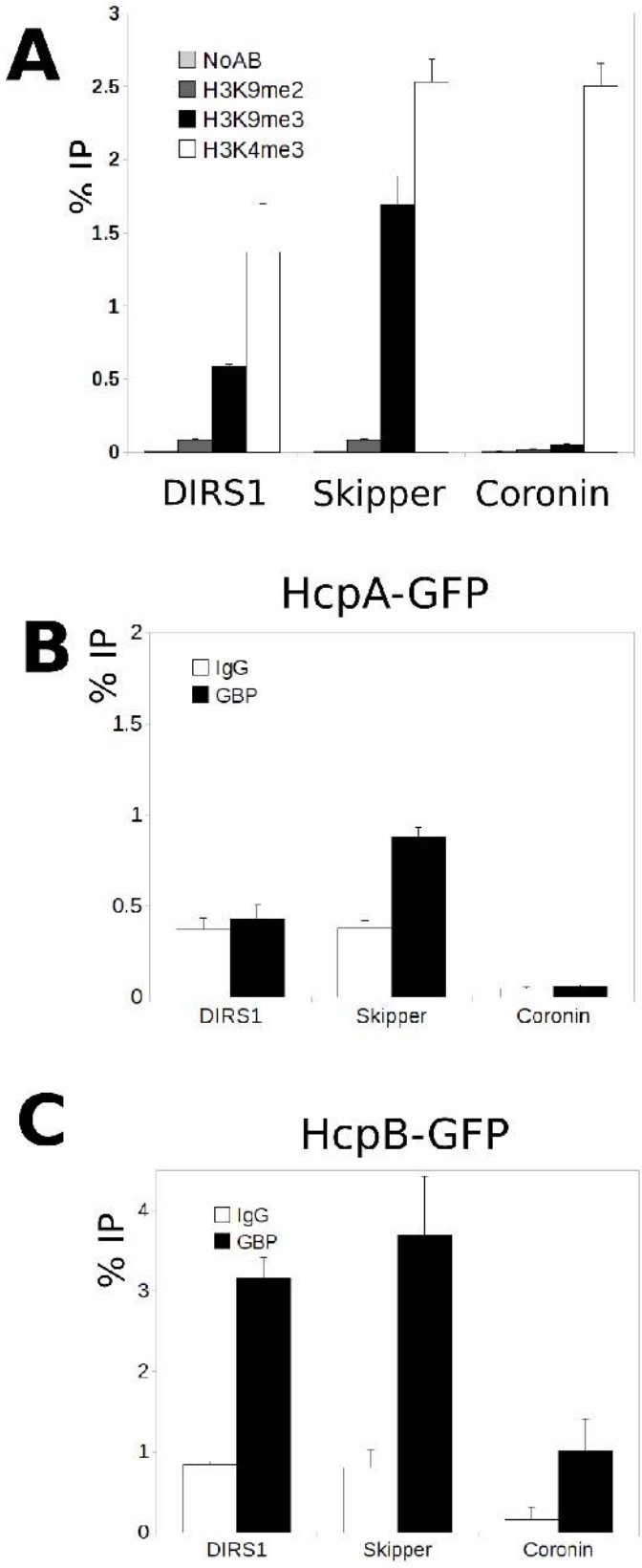


Fig. 3.11 **Distribution of Histone Post-Translational Modifications and Chromatin Binding Proteins at Different Genomic Loci. A:** Chromatin immunoprecipitation with antibodies against the heterochromatin markers H3K9me2 and H3K9me3 or the euchromatin marker H3K4me3 at different genomic loci. **B:** Quantification of chromatin immuonprecipitated together with GFP-HcpA. **C:** Quantification of chromatin immunoprecipitated together with GFP-HcpB. GBP: GFP binding protein. IgG: IgG negative control.

3.2.3 Centromeric Histone Variants

The centromeres of eukaryotic chromosomes are linked via the kinetochore complex to the cytoskeleton and are responsible for the correct separation and segregation of sister chromatids during mitosis and meiosis. Centromeric DNA sequences evolve rapidly and are thus not conserved between even closely related species. However, generally they consist of repetitive DNA sequences and transposons (Ekwall, 2007). In centromeric nucleosomes, histone H3 is replaced by the variant CenH3, which is thought to have a role in the recruitment of components of the kinetochore to the centromeres. CenH3 has a domain comprising alpha-helix 1 and loop 2 of the histone fold domain which is responsible for targeting it to the centromeres. This targeting domain co-evolves rapidly with the centromeric sequences, and thus CenH3 orthologs often have low homology.

3.2.4 Identification of *Dictyostelium* CenH3

Attempts to identify *Dictyostelium* CenH3 by BLAST using known CenH3 sequences from several species was not successful (in each case one of the core histone H3.3 variants H3a, H3b and H3c was found to be the *Dictyostelium* protein with the highest homology). Other groups have also reported similar results (A. Müller-Taubenberger, personal communication). In addition to the histone H3.3 variants H3a, H3b and H3c, two predicted proteins with a histone H3-like domain are present in the *Dictyostelium* genome. These proteins have been annotated as H3v1 and H3v2 (histone H3 variant 1 and 2).

The H3v2 is smaller than a typical histone H3 (96 vs.136 amino acids) and contains an intron. H3v1 is significantly larger than a typical histone H3 (619 vs. 136 amino acids) and contains a histone H3-like domain at the C-terminus (Fig. 3.12a). In an attempt to identify a *Dictyostelium* CenH3 ortholog H3c, H3v1 and H3v2, were expressed with an N-terminal GFP tag and their localisation was examined. GFP-H3c was distributed throughout the nucleus as would be expected for a core histone. GFP-H3v2 did not localise to the nucleus at all (data not shown). In interphase cells GFP-H3v1 was localised to a single focus (Fig. 3.12b) adjacent to the centrosome (Fig. 3.15), suggesting that it is the *Dictyostelium* ortholog of CenH3.

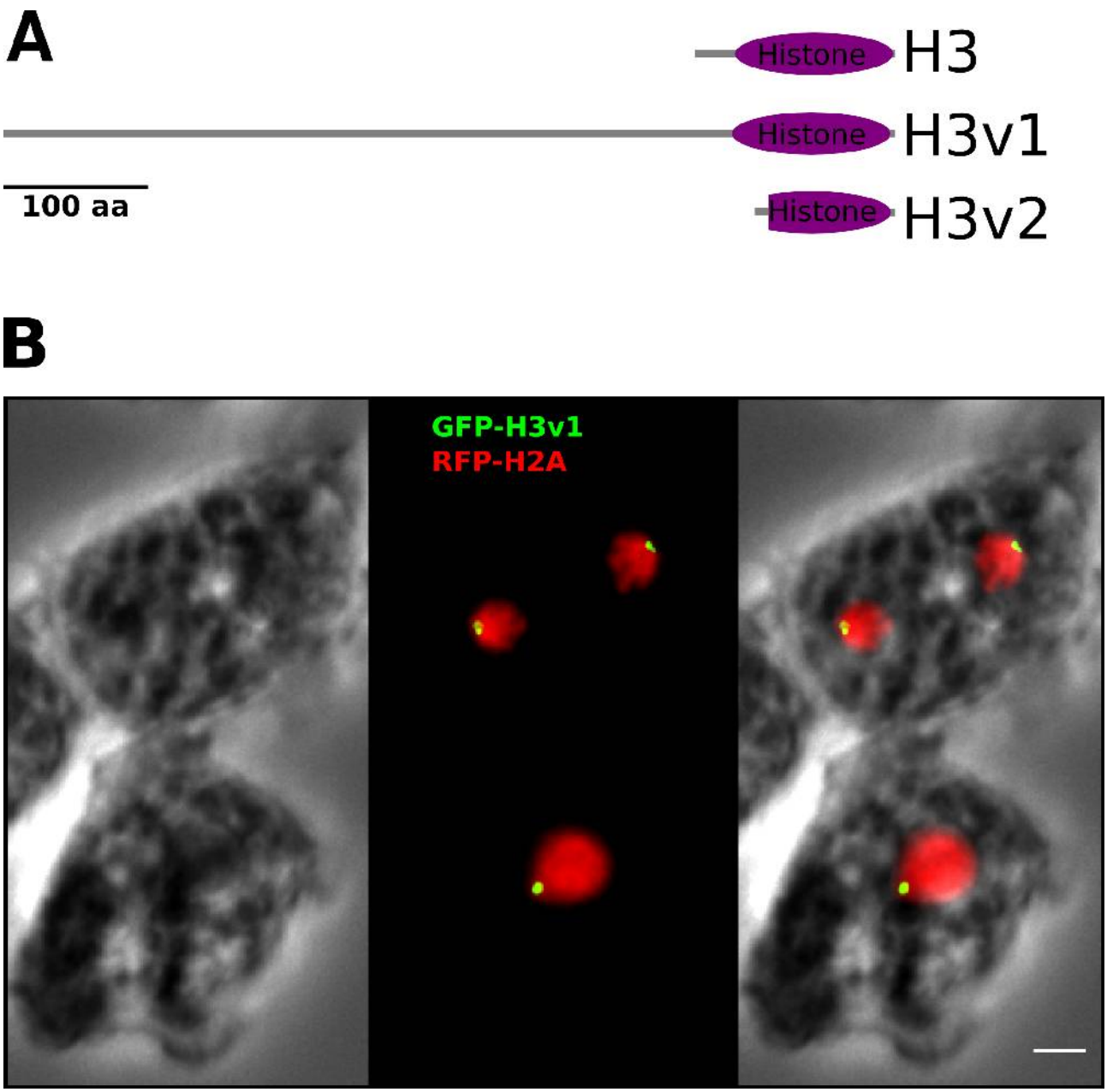


Fig. 3.12 ***Dictyostelium*** **Histone H3 Variants. A:** Schematic showing the domain organisation of the *Dictyostelium* histone H3 like genes. The conserved histone fold domain is shown in purple. as well as three almost identical histone H3 genes (H3a, H3b and H3c). The genome contains two additional genes with similarity to histone H3 (H3v1 and H3v2). **B:** Phase contrast and fluorescence images of interphase (bottom) and telophase (top) cell expressing GFP-tagged histone H3v1 together with the chromatin marker RFP-H2AX. Scale bar = 2 µm.

3.2.5 Analysis of H3v1

In addition to the C-terminal histone H3 domain, *Dictyostelium* CenH3 contains a large N-terminal domain with no homology to other known proteins. Long, unique N-terminal domains are found in many, but not all CenH3 proteins and have been proposed to serve to recruit components of the kinetochore complex to the centromeres (Malik et al., 2001). A BLAST search of *Dictyostelium* H3v1 against Genebank (November 2008) revealed that the most closely related protein is *Drosophila teissieri* Cid (CenH3, AAK20217). The similarity is restricted to the histone fold domain and is quite low ($4e^{-14}$). Compared to conventional histone H3, the histone fold domain of CenH3s

have a poorly conserved alpha-helix 2 and a longer loop 1, as this region serves as the targeting domain for recruitment to centromeres and is subject to strong evolutionary pressures (Henikoff et al., 2001). The histone H3 domain from H3v1 was aligned with histone H3 and CenH3 proteins from a number of other species (Fig. 3.13). Like the other known and putative CenH3 proteins, H3v1 has a poorly conserved alpha-helix 2. This region does however share some sequence homology with alpha-helix 2 of the putative CenH3 from *Entamoeba histolytica* and *Drosophila teissieri* Cid.

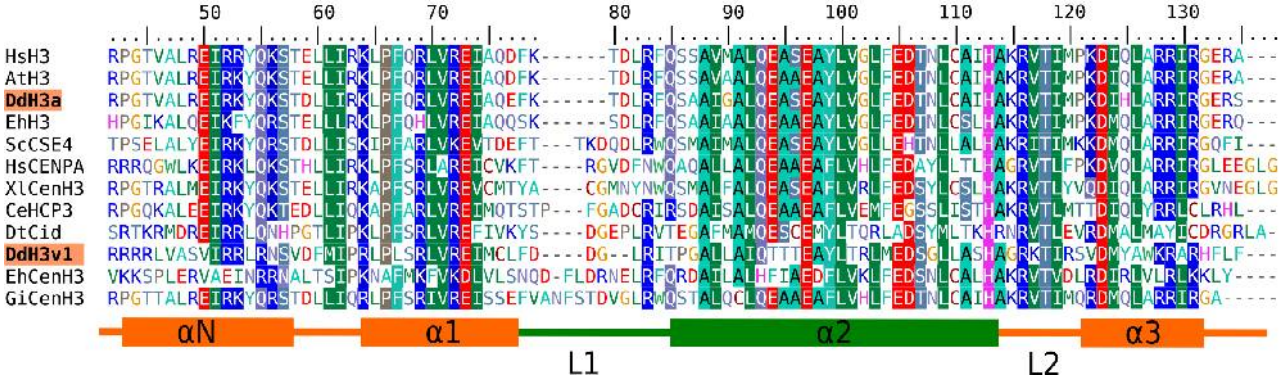


Fig. 3.13 **Alignment of Histone Domain of the Core Histone H3 and Centromeric Histone H3 from Different Species**. *Dictyostelium* H3a and H3v1 are highlighted in orange. Below the alignment is a schematic of the domain organisation of histone H3, with the N-terminal alpha-helix (αN), alpha-helixes 1-3 (α1-3) and loops 1 and 2 (L1, L2) indicated. L1 and α2, which have been shown to be responsible for targeting of CenH3 to the centromeres are shaded green. Hs: *Homo sapiens,* At: *Arabidopsis thaliana*, Dd: *Dictyostelium discodeum*, Eh: *Entamoeba hystolitica*, Sc: *Saccharomyces cerevisae*, Xl: *Xenopus laevis*, Ce: *Caenorhabditis elegans*, Dt: *Drosophila teissieri*, Gi: *Giardia intestinalis*.

However unlike all other CenH3 proteins examined, H3v1 does not have an extended loop 1 (Fig. 3.13). A phylogenetic tree was calculated using Bayesian methods (Huelsenbeck et al., 2001). While conventional H3 histones, including *Dictyostelium* H3a, H3b and H3c clustered close together, H3v1 was on a separate more dispersed branch with CenH3 from other species (Fig. 3.14).

3.2.6 Localisation of GFP-H3v1 Through the Cell Cycle

H3v1 was overexpressed with an N-terminal GFP, RFP, 3xFLAG, or 3xHA tag, and localisation was examined by immunofluorescence. Western blotting confirmed that the expressed protein was of the expected size (Fig. 3.15a). In all cases H3v1 localised to a single focus at the edge of the nucleus next to the centrosome during interphase (Fig. 3.15b) suggesting that the interphase centromeres remain clustered and attached to the centrosome as is seen in *S. cerevisiae*. This is in contrast to the situation in many metazoans, plants, *Giardia* and *S. pombe* which have dispersed centromeres during interphase. When clones expressing a high level of H3v1 were examined, in addition to the focus opposite the centrosome, the H3v1 fusion also accumulated in the nucleoli (Fig. 3.15c), a behaviour frequently observed for highly overexpressed nuclear proteins in *Dictyostelium.*

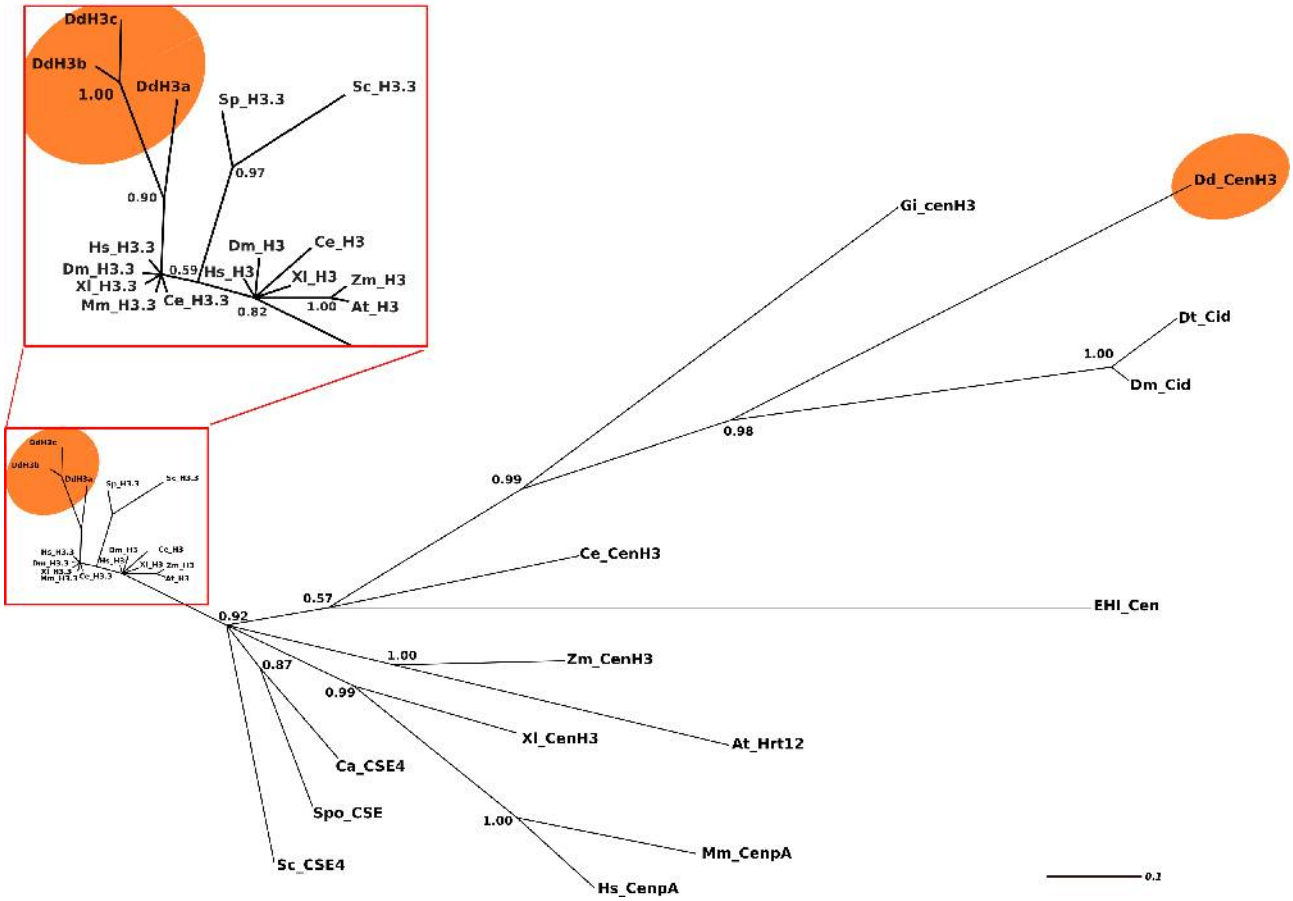


Fig. 3.14 **Histone H3 Phylogeny**. Phylogenetic tree of histone H3 and centromeric histone H3 from different species. Histones from *Dictyostelium* are indicated with an orange background, the insert shows the core histone H3s in more detail. Hs: *Homo sapiens,* At: *Arabidopsis thaliana*, Dd: *Dictyostelium discodeum*, EHI: *Entamoeba hystolitica*, Sc: *Saccharomyces cerevisae*, Xl: *Xenopus laevis*, Ce: *Caenorhabditis elegans*, Dt: *Drosophila teissieri*, Dm: *Drosophila melenogaster*, Gi: *Giardia intestinalis,* Zm: *Zea maize* Mm: *Mus musculus*, Ca: *Candida albicans,* Spo: *Schizosaccharomyces pombe.*

In contrast to metazoans, where overexpression of CenH3 leads to its misincorporation in non-centromeric regions of the chromosomes and neocentromere formation, in *Dictyostelium* overexpressed H3v1 does not appear to incorporate into loci outside of the centromeres.

The localisation of GFP-H3v1 during the cell cycle was examined in cells that had been fixed at different stages of the cell cycle and counterstained with antibodies against H3K9me3, tubulin or the centrosome marker DdCP224 (Gräf et al., 2000). As cells enter prophase the chromosomes begin to condense and up to six GFP-H3v1 foci were visible at the edge of the nucleus close to the centrosome (Fig. 3.16a and b). These six foci presumably correspond to the centromeres of the six chromosomes of the haploid *Dictyostelium* genome. When diploid cells were examined, up to 12 foci were visible (Fig. 3.16c). During metaphase the six GFP-H3v1 foci form a ring around the mitotic spindle and condense until they can no longer be clearly distinguished, the chromosomes also condense further. In anaphase and telophase cells the sister chromatids separated and a focus of GFP-H3v1 is localised at the leading edge of the chromosomes, immediately behind the centrosomes. Double labelling with anti-H3K9me3 shows that GFP-H3v1 has a very similar, but not

identical distribution pattern compared to this histone modification (Fig. 3.16b). In both interphase and telophase cells a cross-section through the nucleus shows that the centre of intensity of the GFP-H3v1 focus is slightly closer to the centrosome than that of H3K9me3 (Fig. 3.16d). This would suggest the presence of a core centromere domain containing H3v1 flanked by or interspersed with pericentromeric heterochromatin enriched in H3K9me3 as has been described for other species.

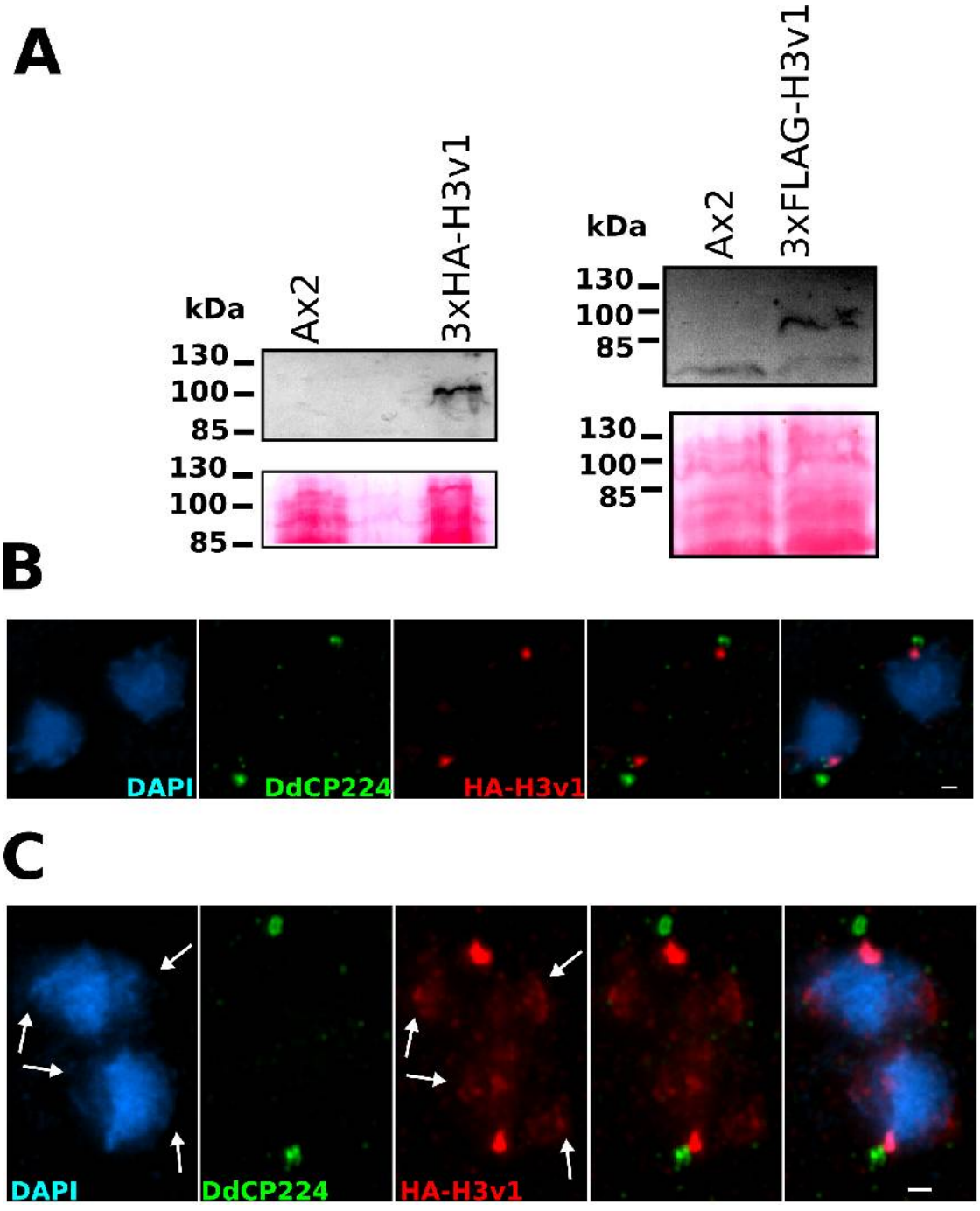


Fig. 3.15 **Expression of Epitope Tagged H3v1**. **A:** Western blot on SDS-soluble protein extracts from *Dictyostelium* transformed with 3xHA or 3xFLAG tagged H3v1. Lower panels show the membranes stained with Ponceau-S after western transfer to ensure even loading and transfer to the membrane. **B:** Immunofluorescence on fixed cells expressing 3xHA-H3v1 with an anti-HA antibody (red) and an antibody against the centrosome marker DdCP224 (green). **C** An example of a line where the epitope tagged H3v1 is expressed at a higher level, resulting in accumulation within the nucleoli (arrows) as well as at the centromeres. Scale bar = 1 µm.

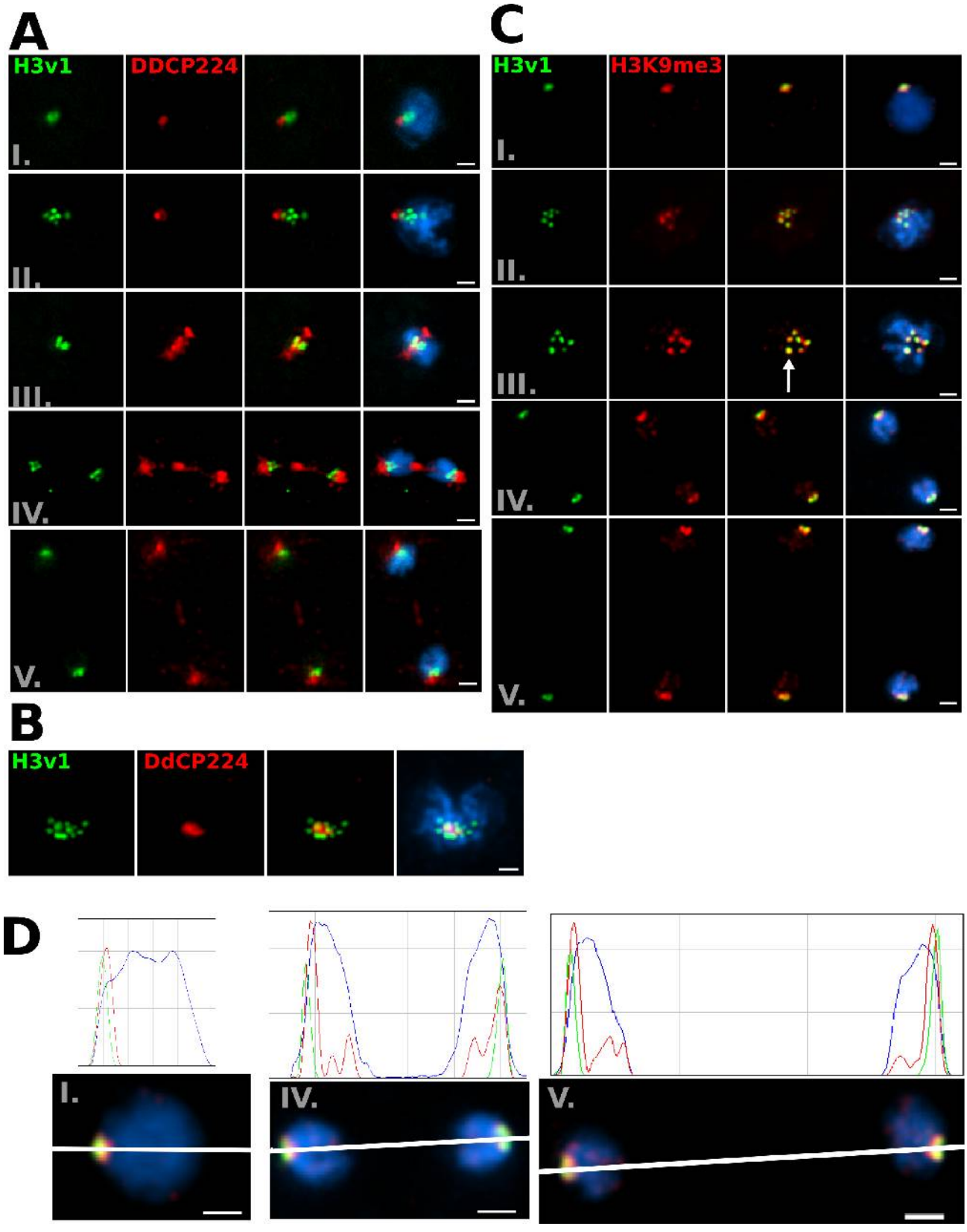
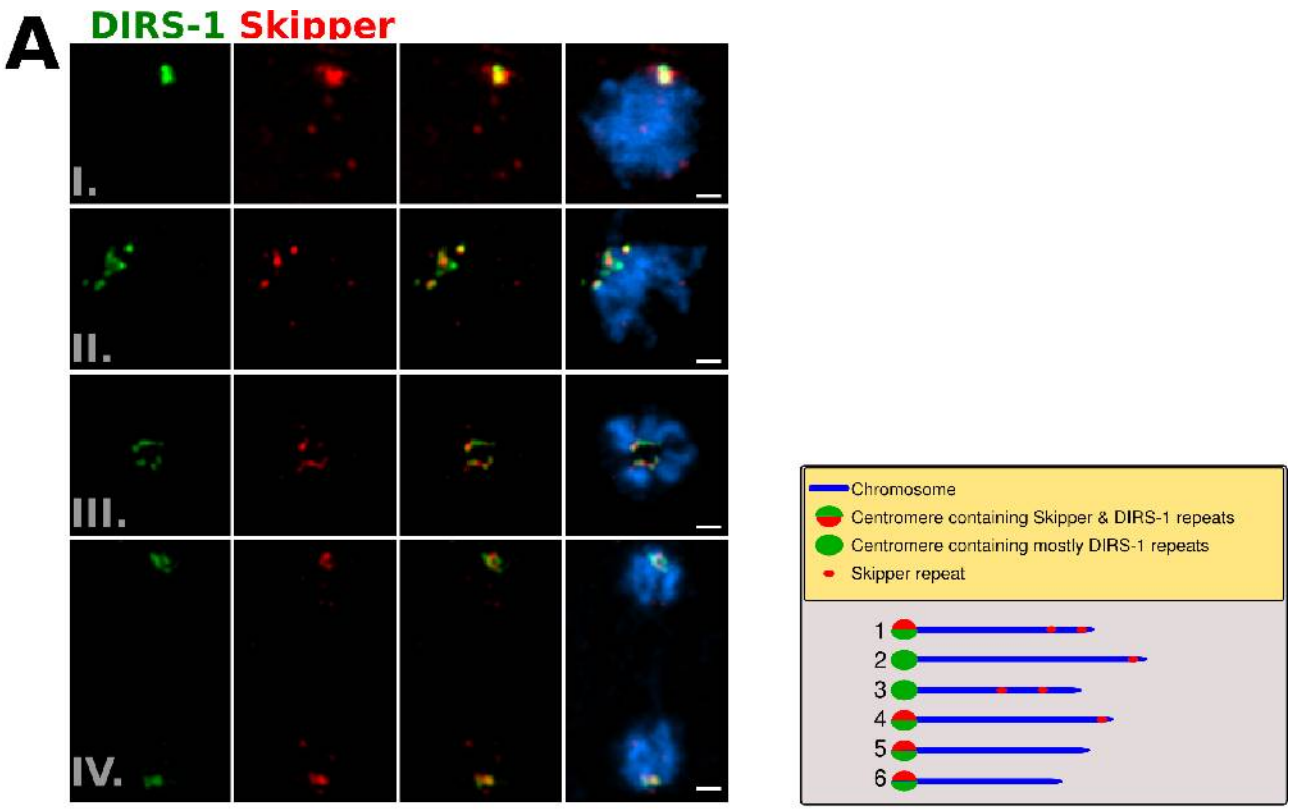


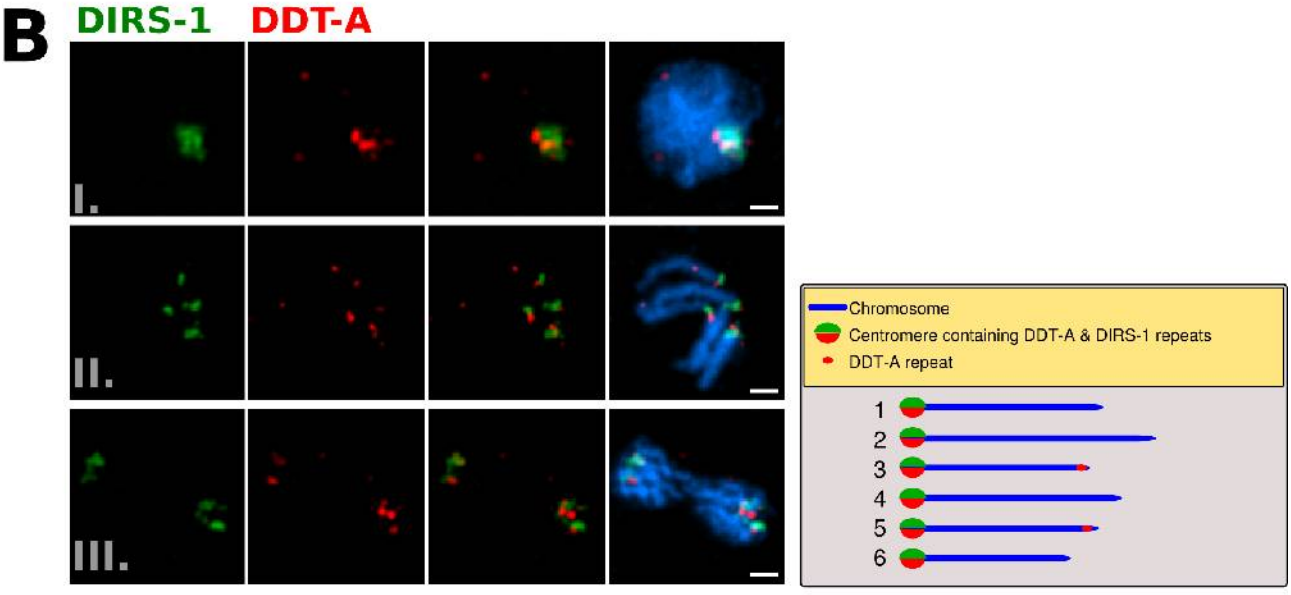
Fig. 3.16 **Centromere Behaviour during Mitosis**. The organisation of the centromeres was examined in fixed cells expressing GFP-H3v1. **A:** Cells immunostained with the centrosome marker DdCP224 at different stages of the cell cycle. **B:** Prophase diploid cell immunostained with DdCP224 and expressing GFP-H3v1 display up to 12 GFP-H3v1 label foci presumably corresponding to the 12 centromeres. **C:** Cells expressing GFP-H3v1 and labelled with the heterochromatin marker H3K9me3. The arrow indicates a centromere that appears to have two chromosomes attached. **D:** Cross sections through cells at different stages of the mitotic cycle show that GFP-H3v1 is slightly closer to the leading edge of the separating chromatids than the H3K9me3. I: interphase, II: prophase, III: metaphase, IV: anaphase, V: telophase. Scale bar = 1 µm.

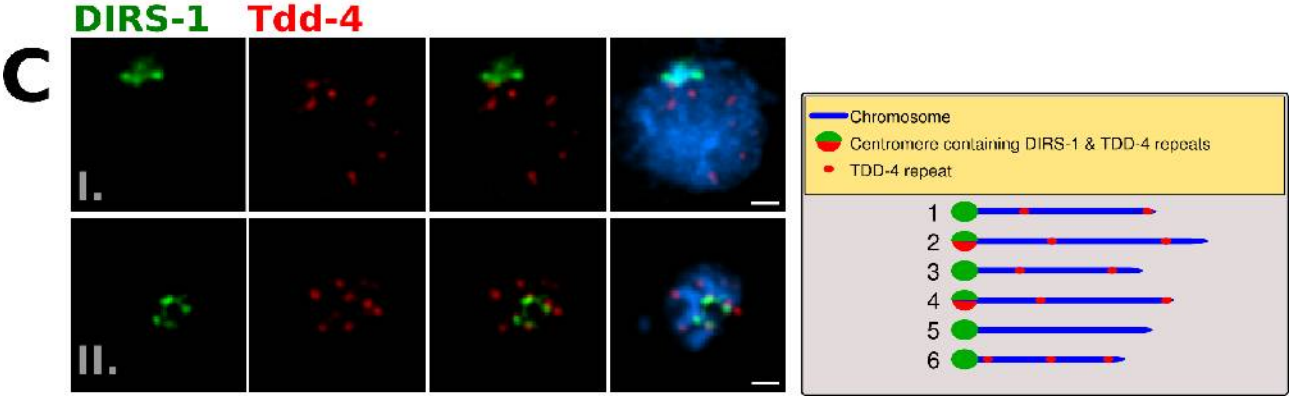
3.2.7 *Dictyostelium* centromeres contain transposons

Copies of DIRS-1, the most abundant retrotransposon in the *Dictyostelium* genome, have previously been proposed to act as centromeres as they appear to be present in six clusters, one on each chromosome (Loomis et al., 1995). DIRS-1 sequences are methylated (Kuhlmann et al., 2005) and are associated with the pericentromeric marker H3K9me2 (Kaller et al., 2007). Using fluorescence *in situ* hybridisation with a probe against DIRS-1 it has previously been reported that six foci are present in the nucleus of interphase cells (Eichinger et al., 2005). This is in contrast with the localisation data for the *Dictyostelium* CenH3 ortholog, H3v1 presented above and previously published data for the pericentromeric markers H3K9me2 and the HP1 homologs HcpA and HcpB (Kaller et al., 2006b), all of which suggest that at the resolution of light microscopy, the six centromeres cluster together in a single body. To resolve this dilemma, FISH was preformed using Cy3 or FITC-labelled PCR fragments or a plasmid containing a full length DIRS-1 clone (Y41, Cappello et al., 1984). All interphase cells display a single DIRS-1 focus at the nuclear periphery similar to that seen with GFP-H3v1. As cells entered prophase, up to six foci became visible. These condensed to a single focus in metaphase and then to a focus at the leading edge of the separating chromatids during anaphase and telophase (Fig. 3.17a). The distribution of the *skipper* retrotransposon, and the DNA transposons DDT-A and Tdd-4 was also examined. *skipper* is a 7 kB LTR retrotransposon of the Gypsy family present in about 50 copies in the *Dictyostelium* genome (Leng et al., 1998). The majority of *skipper* colocalises with DIRS-1 at the centromere clusters, while approximately five to nine additional small foci are present throughout the nucleus (Fig. 3.17a). Some, but not all of these small clusters are found at the nuclear periphery opposite the centromeres. Mapping of *skipper* onto the chromosomes shows that it is present at the distal ends (opposite ends from the telocentric centromeres) of chromosomes one, two and four. This would suggest that interphase chromosomes in *Dictyostelium* may be organised in a Rabl-like manner (Hochstrasser et al., 1986; Jasencakova et al., 2001), with the distal telomeres on the edge of the nucleus opposite the centromeres. The majority of DDT-A appears to be localised to the centromeres, with one to three small foci often present at the edge of the nucleus opposite the centromere cluster (Fig. 3.17b). Again as DDT-A is localised at the distal subtelomeric transposon clusters of chromosomes 3 and 4, this is indicative of Rabl-like organisation.

Fig. 3.17 **Localisation of Transposons. A:** Fluorescence *in situ* hybridisation (FISH) on cells at different stages of the cell cycle using a directly FITC-labelled probe for DIRS-1 and directly Cy3-labelled probe for *skipper*. **B:** FISH with probes FITC-DIRS-1 and Cy3-DDT-A. **C:** FISH with probes FITC-DIRS-1 and Cy3-Tdd-4. Proposed chromosomal distribution of the transposons according to genome sequencing data is indicated in schematics on the right. i: interphase, ii: prophase, iii: metaphase, iv: telophase. Scale bar = 1 µm.


A
DIRS-1 Skipper
I.
II.
III.
IV.
Chromosome
Centromere containing Skipper & DIRS-1 repeats
Centromere containing mostly DIRS-1 repeats
Skipper repeat
1
2
3
4
5
6


B
DIRS-1 DDT-A
I.
II.
III.
Chromosome
Centromere containing DDT-A & DIRS-1 repeats
DDT-A repeat
1
2
3
4
5
6


C
DIRS-1 Tdd-4
I.
II.
Chromosome
Centromere containing DIRS-1 & TDD-4 repeats
TDD-4 repeat
1
2
3
4
5
6

In contrast to DIRS-1, *skipper* and DDT-A, only a minor amount of Tdd-4 localised to the centromere cluster, instead it appeared to be present in 8-12 foci distributed along the chromosomes (Fig. 3.17c). These data are in good agreement with a recently published study which showed that DIRS-1, *skipper* and transposons of the DDT family are enriched at the proposed centromeres, but Tdd family members are not (Glöckner et al., 2009).

3.2.8 H3v1 colocalises with DIRS-1

DIRS-1 colocalises with both HA-tagged H3v1 and H3K9me3 (Fig. 3.18a and data not shown). During the cell cycle the two marks remain colocalised suggesting that the H3v1 may be incorporated in DIRS-1 containing chromatin. Due to the limited resolution afforded by microscopy based methods, ChIP was preformed on cells expressing GFP-H3v1 with an antibody against GFP. Significant enrichment of DIRS-1 was detected in chromatin containing H3v1 (Fig. 3.18b). *skipper* and two low copy number (non-transposon) loci located within the (peri)centromeric transposon cluster on chromosome one were also enriched but at lower levels than DIRS-1. In contrast the active genes *coronin*, *actin* and *rasG* were not enriched in H3v1 containing chromatin. These data provide direct evidence that the 200-300kB clusters of interspersed transposons at one end of each chromosome act as the centromeres in *Dictyostelium*.

3.2.9 Loading of H3v1 onto centromeres

Unlike the core histones, which are deposited in S-phase and replication independent variants such as histone H3.3 and H2AZ which are deposited largely in G-phase in a transcription dependent manner, the mechanism and timing of CenH3 deposition onto the centromeres has for a long time remained unresolved and subject to much speculation. Experiments in *Drosophila* and mammalian cells suggest that CenH3 loading occurs in anaphase and telophase/early G1 respectively (Schuh et al., 2007; Jansen et al., 2007). More recently it has been shown that the holiday junction repair protein HJURP is the chaperone responsible for *de novo* CenH3 deposition in mammals (Foltz et al., 2009; Dunleavy et al., 2009). However HJURP appears to be restricted to metazoans. In plants loading of CenH3 occurs in late G2 phase (Lermontova et al., 2006; Lermontova et al., 2007). Indirect evidence also suggests CenH3 loading in *S. pombe* occurs in S-phase or G2 (Takahashi et al., 2005).

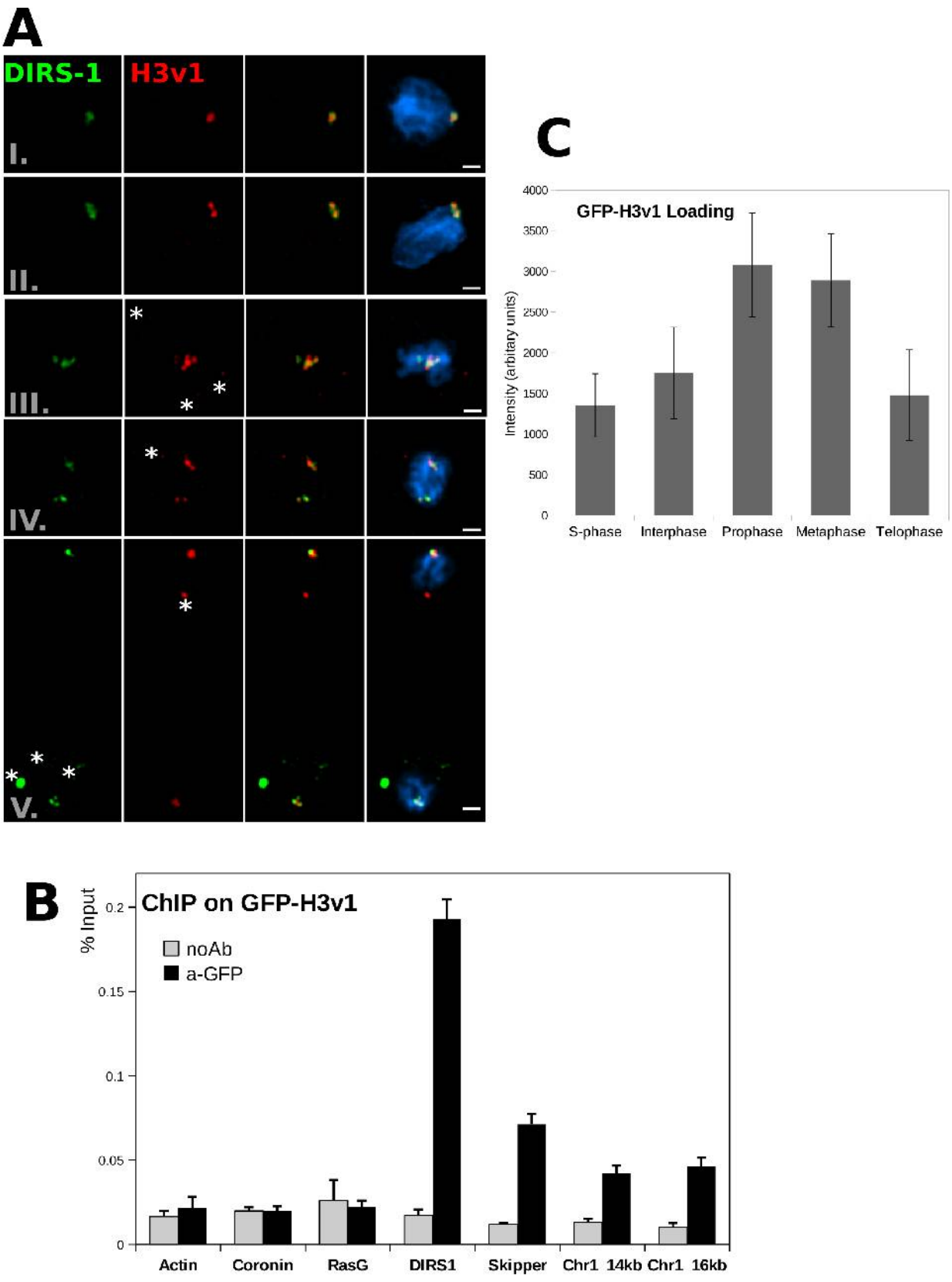
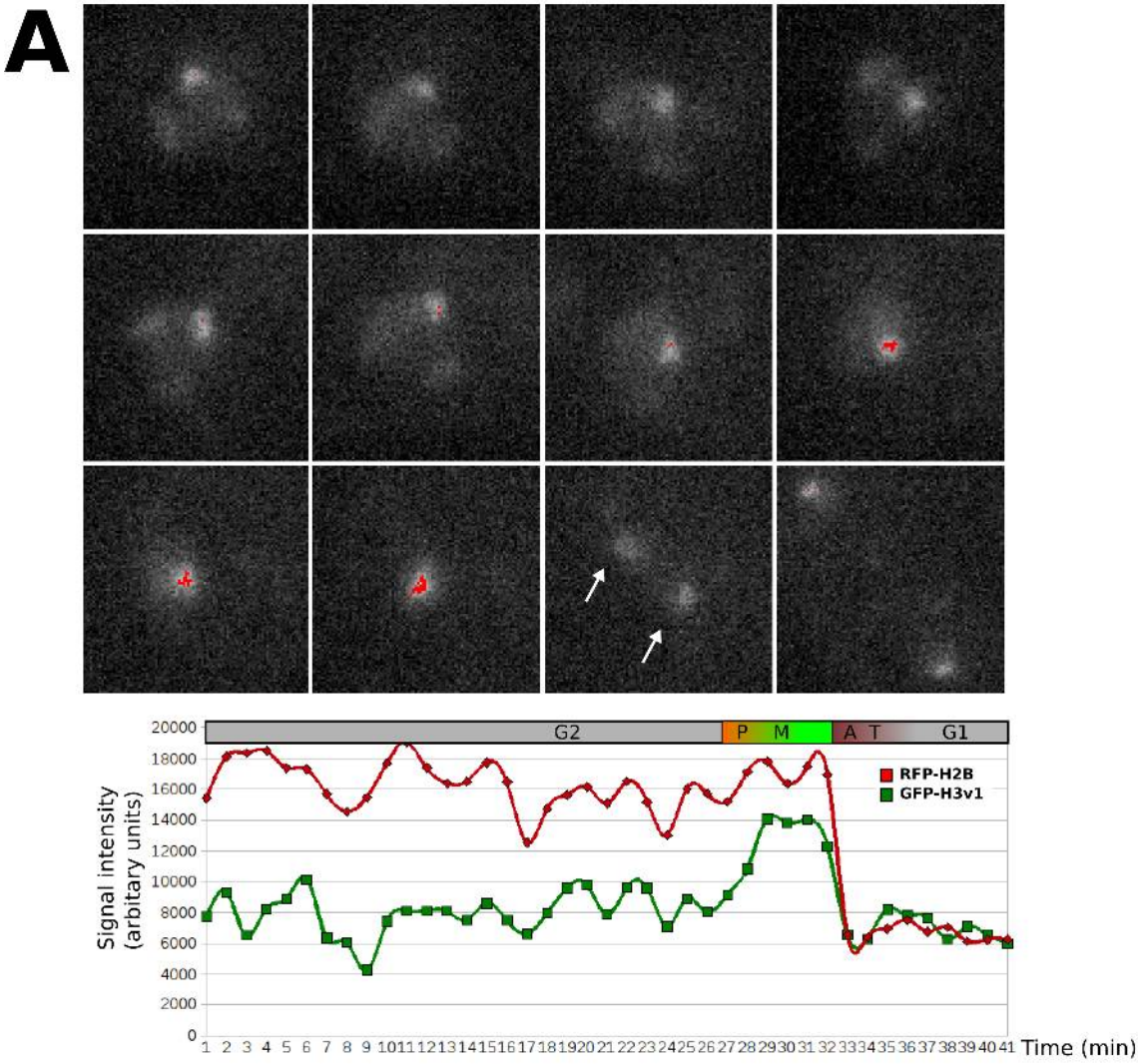


Fig. 3.18 ***Dictyostelium* Centromeres Contain DIRS-1. A:** Combined Immuno-FISH showing colocalisation of HA tagged H3v1 and DIRS1 retrotransposon. (I: interphase, ii: prophase, iii: metaphase, iv: telophase) asterisks indicates signals thought to be aspecific. Scale bar = 1 µm. **B:** ChIP of GFP-H3v1. Real time quantitative PCR was preformed with primers against the actively transcribed *actin*, *coronin* and *rasG* genes or the DIRS-1 and *skipper* retrotransposons, as well as two non-transposon sequences located on chromosome 1 within the proposed centromere regions. **C:** Cells co-expressing GFP-H3v1 and the cell cycle marker RFP-PCNA were fixed and the normalised intensity of the GFP-H3v1 signal at the centromeres at different stages of the cell cycle in cells was plotted.


A
G2
P
M
A
T
G1
RFP-H2B
GFP-H3v1
Signal intensity (arbitary units)
Time (min)
B
1
2
3
4
5
6
7
8
9
10
11
12
G2
P
M
A
T
G1
GFP-H3v1
RFP-H2A
Intensity (arbitary units)
Time (min)

Given *Dictyostelium* diverged from the opisthokont lineage shortly after the plant-animal split, determination of the CenH3 loading time in *Dictyostelium* may enable us to determine when loading of CenH3 occurs in ancestral eukaryotes and thus provide information on the evolution of the CenH3 loading mechanism. Cells expressing GFP-H3v1 and the cell cycle marker RFP-PCNA (Muramoto et al., 2008) were fixed and the intensity of the GFP at the centromere was measured at various stages of the cell cycle (Fig. 3.18c). After mitosis *Dictyostelium* cells enter S-phase almost immediately and in late S-phase a focus of RFP-PCNA is visible at the late-replicating heterochromatin (Muramoto et al., 2008). The intensity of GFP-H3v1 was low in late S-phase and not significantly higher in G2 (interphase). The intensity then doubled by prophase and remained constant in metaphase, then halved in telophase (where each daughter nuclei was measured separately). These data suggest that *de novo* CenH3 loading occurs in late G2 or at the G2-prophase transition.

Time lapse imaging was preformed on cells co-expressing GFP-H3v1 and a RFP tagged histone H2AX or H2B. Cells were tracked as they went through mitosis and the fluorescence intensity of GFP-H3v1 and the RFP-histone were measured every 60 or 20 seconds (Fig. 3.19a, b). The intensity of the signal from the core histone remains steady as cells enter mitosis and drops to approximately half the initial value in anaphase as the sister chromatids separate. In contrast the intensity of the GFP-H3v1 signal approximately doubles at the end of G2/ onset of prophase and then is halved in anaphase when the sister chromatids separate, indicating that loading of H3v1 occurs at the G2/prophase transition. In contrast to this, core histones (H2AX, H2B, H3, H4) are loaded onto the chromosomes during S-phase. To confirm that this is not the case for H3v1, GFP-H3v1 was co-expressed with RFP-PCNA and the cells were monitored by time-lapse as they exit mitosis and go through S-phase, which can be tracked using the RFP-PCNA marker. No increase in GFP-H3v1 intensity was observed during S-phase (Fig. 3.20). Due to the light sensitivity of *Dictyostelium* amoeba, it was not possible to track an individual cell through the entire cell cycle. Thusm it remains possible that some loading of H3v1 occurs during G2. However the strong increase in intensity observed at the G2/prophase transition strongly suggests that the bulk, if not all *de novo* loading of H3v1 onto the centromeres occurs at the onset of mitosis.

Fig. 3.19 **Loading of GFP-H3v1 onto Centromeres. A:** Time-lapse images of cells co-expressing GFP-H3v1 and RFP-H2B (not shown). Images were acquired every 60 seconds and the intensity of the GFP and RFP signals was plotted. Approximately 5 minutes prior to the separation of the sister chromatids (indicated by arrows) an increase in the intensity of the GFP signal was observed as evidenced by the appearance of values above an arbitrary threshold (marked red). **B:** Time-lapse images of a cell with three nuclei co-expressing GFP-H3v1 and RFP-H2AX. Images were acquired every 20 seconds and the overlay of the GFP and RFP signals from every third acquisition (every 60 seconds) is displayed. The GFP signal for one centromere at each time point (indicated with an arrow) is shown as an insert in grayscale. Signal intensities above an arbitrary threshold level are shaded red. Below the average signal intensities from the 3 nuclei averaged over 3 time points (1 min) are plotted together with the standard error. The bar above the charts indicates the stage of the cell cycle: G2, growth phase 2; P, prophase; M, metaphase; A, anaphase; T, telophase; G1, growth phase 1. Scale bar = 1 µm.

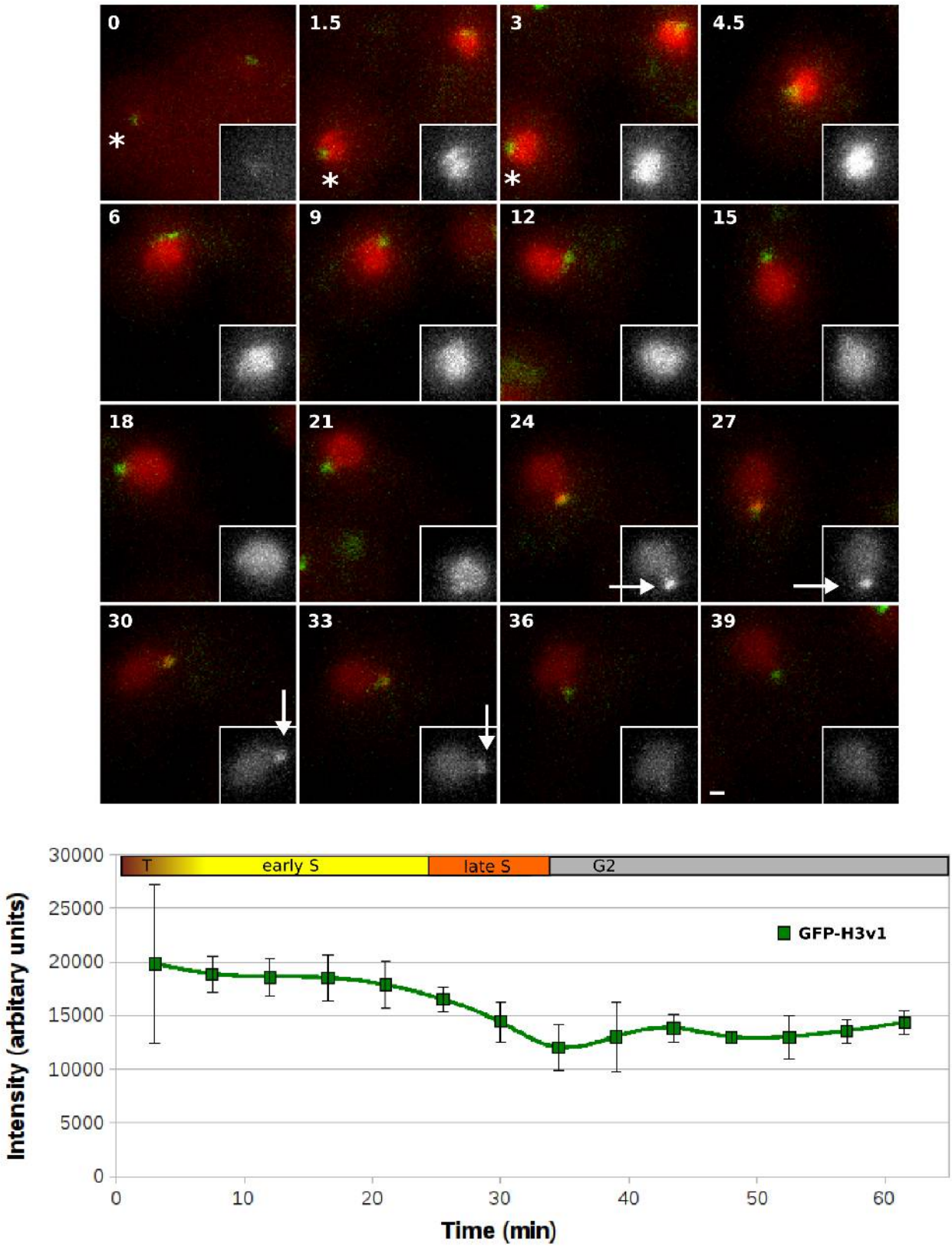


Fig. 3.20 **S-phase Behaviour of GFP-H3v1.** Time-lapse images of cells co-expressing GFP-H3v1 and RFP-PCNA starting from telophase. Images were acquired every 90 seconds and the overlay of the GFP and RFP signals is displayed. The time elapsed since the start of the acquisition is indicated in the upper left hand corner of each frame. The RFP signal for one centromere at each time point (indicated with an asterisk) is shown as an insert in grayscale. The strongly stained focus of RFP-PCNA characteristic of late S-phase is indicated with an arrow. The intensity of the GFP-H3v1 signal is averaged over 3 time points (4.5 min) and plotted below. The bar above the chart indicates the stage of the cell cycle: T, telophase; S, S-phase; G2, growth phase 2. Scale bar = 1 µm.

3.2.10 Telomeres

The *Dictyostelium* ribosome genes (rDNA) are present on extrachromosomal 88kB palindromes present at about 100 copies per cell (Emery and Weiner, 1981). The termini of these palindromes contain four almost perfect copies of a tandemly repeated 29 bp sequence followed by AG_{1-8} repeats reminiscent of telomeres in many eukaryotes (Emery and Weiner, 1981; Sucgang et al., 2003). *Dictyostelium* chromosomes appear to be telocentric and thus the proximal (centromere containing chromosome end) telomere consists of the centromeric transposon clusters with a partial copy of the extrachromosomal palindrome fused to the very terminus (Glöckner et al., 2009). The rDNA palindrome fused to each chromosome end has a different junction point (i.e. length), but in all cases appears to contain the terminal repeats, which presumably perform the telomere function for the chromosomes as well (Sucgang et al., 2003). The distal ends of the chromosomes appear to contain a small transposon cluster followed by a fragment of the extrachromosomal rDNA palindrome as is seen at the proximal telomere (Eichinger et al., 2005). These distal subtelomeric transposon clusters contain s*kipper*, DDT and Tdd transposons but not DIRS-1. FISH with probes against s*kipper* and DDT-A labelled several of the distal telomeres of metaphase cells, and foci at the edge of the nucleus opposite the centromeres in interphase, suggesting a Rabl like organisation (Fig. 17). To confirm this, it would be desirable to label the distal telomeres directly, however in *Dictyostelium* probes against the very ends would cross-hybridise with the extrachromosomal palindromes and the subtelomeric transposon clusters are non unique. Instead a series of tiling clones from the library used for sequencing the genome (Eichinger et al., 2005) spanning approximately 25 kB at the end of the gene coding regions adjacent to the distal telomeres of chromosomes one and two (generously provided by G. Glöckner) were used as probes. In FISH experiments probes against these distal subtelomeric regions of both chromosome one (Fig. 3.21a) and chromosome two (Fig. 3.21b) labelled a single focus that was usually on the opposite side of the nucleus to the centromeres, confirming that the chromosomes in *Dictyostelium* interphase cells are organised in a Rabl-like manner. In metaphase the probe against the subtelomeric region of chromosome two labelled the end of a single chromosome (Fig. 3.21b, lower panels) confirming its specificity.

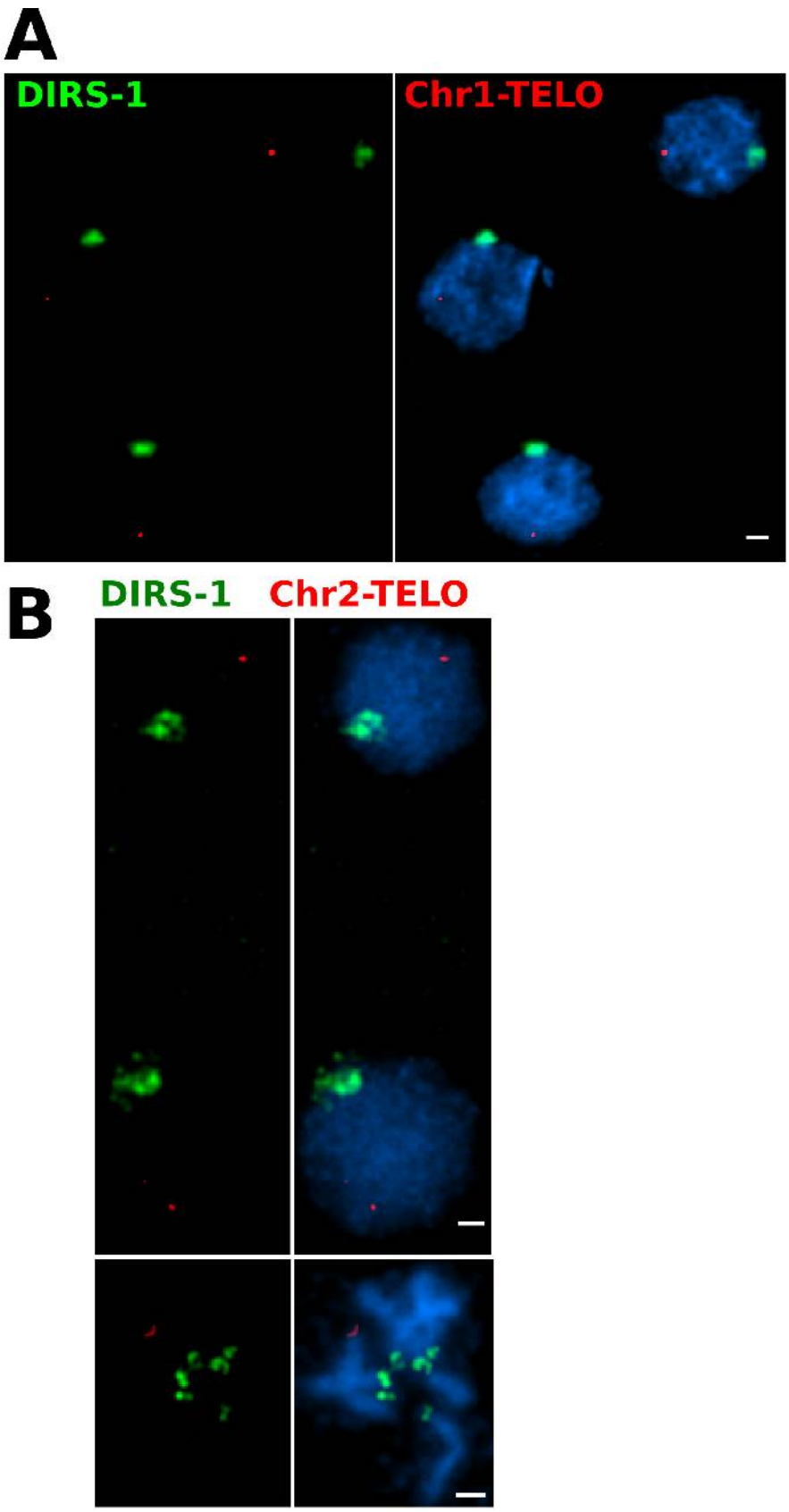


Fig. 3.21 **Telomere Organisation. A:** FISH with FITC-labelled probe against DIRS-1 and Cy3-labelled probe against the distal subtelomere region of chromosome 1. **B:** FISH with FITC-labelled DIRS-1 and Cy3-labelled probes against the distal subtelomere region of chromosome 2. An example of a metaphase cell is shown in the lower panel. Scale bar = 1 μm.

3.2.11 Extrachromosomal rDNA Palindrome

Early cytological studies found that *Dictyostelium* has seven chromosomes, one of which condenses slightly earlier than the others in prophase (Brody and Williams, 1974). However mapping studies identified only six linkage groups and it was subsequently shown that the seventh early condensing chromosome contains rDNA which exists on 88kB long extrachromosomal rDNA palindromes which are present at about one hundred copies per nucleus (Cockburn et al., 1978; Sucgang et al., 2003). During prophase a tleast some of these approximately one hundred copies of the palindrome appear to assemble into a pseudo-chromosome, hinting that they may segregate during mitosis in a manner analogous to true chromosomes. Analysis of late prophase and metaphase cells reveals up to seven DAPI stained bodies consisting of the six true chromosomes and the rDNA pseudo-chromosome. GFP-H3v1 or immunostaining against H3K9me3 revealed no more than six foci, suggesting that these marks are not present on the rDNA pseudo-chromosome (Fig. 3.16c). Consistent with this no H3K9me3 was detected on the rDNA palindrome using chromatin immunoprecipitation (Fig. 3.22a). Some H3K4me3 was detectable on the non-coding region of the palindrome and on the 26S ribosomal DNA, but these levels were significantly lower than those observed on either the active *coronin* locus or the *skipper* and DIRS-1 transposons. Interestingly we never saw a single chromosome not connected to a GFP-H3v1 focus. Instead the DAPI stained body presumed to be the rDNA pseudo-chromosome (indicated by arrow in Fig. 3.16c, panel III) always appeared to be in direct contact with one of the six true chromosomes. This could suggest that some kind of tethering interaction aids correct segregation of the rDNA palindrome. FISH experiments were preformed using a probe against the 26S rDNA. These results must be interpreted with caution as the denatured conditions used during the FISH procedure can alter the localisation of the small, 88 kB extrachromosomal rDNA palindromes (Sucgang et al., 2003). In interphase cells a dispersed dot-like pattern is present around the edge of the nucleus and in the half-moon shaped lobes that form the nucleoli (arrows, Fig. 3.22b) as has previously been described (Moerman et al., 1998). In prophase the elongated chromosomes are visible, while the 26S probe decorated thinner DAPI stained threads present in the nuclear remnants. In metaphase the probe again stained small DAPI threads interspersed between the six true chromosomes. In some cases they labelled a seventh chromosome like-body (Fig. 3.22b) and gave a characteristic pattern of four evenly spaced punctae. In anaphase and telophase the signal was associated with the separating chromatids but was quite dispersed. This suggests that either the pseudo-chromosome had started to decondense by this stage, or that the pseudo-chromosomes are more sensitive to disruption by the denaturation step of the FISH procedure at this stage of the cell cycle.

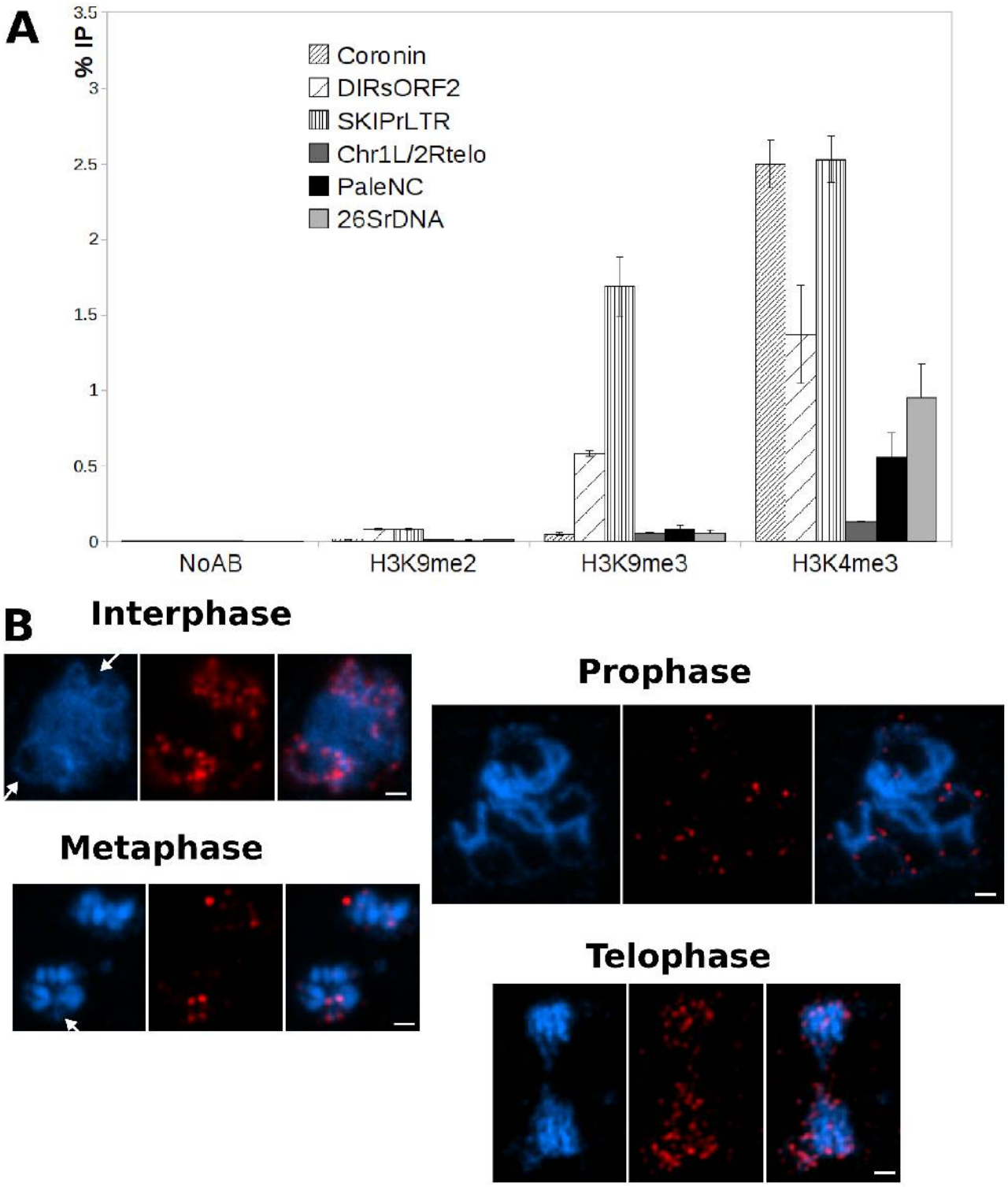


Fig. 3.22 **Telomeres and the Extrachromosomal rDNA Palendromes. A:** Chromatin immunoprecipitation with antibodies against the heterochromatin markers H3K9me2 and H3K9me3, or the euchromatin marker H3K4me3 at different genomic loci. **B:** FISH on cells at different stages of the cell cycle using a directly Cy3-labelled probe against the 26S-rDNA. Arrows indicate position of the nucleoli in interphase, and a seventh, chromosome-like body in metaphase. Scale bar = 1 µm.

3.3 Transgenes

3.3.1 Chromosomal Transgenes

Transformation of *Dictyostelium* with exogenous DNA containing a selectable marker results in the stable integration of the exogenous DNA sequence into the *Dictyostelium* genome (Nellen et al., 1984). Transformed vectors are thought to integrate at one or a few genomic loci as tandem arrays that may contain more than 100 copies of the vector. The copy number is heavily influenced by the type of selectable marker. Vectors containing the *nptI* or *nptII* genes conferring resistance to gentamicin (G418) typically integrate at approximately one hundred copies per cell. Vectors containing selectable markers conferring resistance to Blasticidin or the auxotrophic marker Thy-1 (Kalpaxis et al., 1991) typically integrate as single-copy or low copy insertions. The relationship between copy number and expression level is often non-linear. High copy transgenes are often expressed at a lower level than might be expected given their copy number. This suggests that transgene silencing or other epigenetic phenomena may reduce the expression levels of transgenes. To investigate this the organization of transgenes in the nucleus was examined. The copy number of independent lines transformed with a vector (pGEM-3Z containing a G418 resistance cassette) was determined by southern blotting (table 3.3 and data not shown). The localisation of transgenes of different copy numbers was then investigated using FISH. While the low copy number transgene clusters (containing up to 10 copies) were localised randomly within the nucleus (Fig. 3.23a) high copy-number transgene clusters were always at least partially colocalised with the centromere marker DIRS-1 (Fig. 3.23b). Similar results were obtained for a number of independent lines. These results suggest that either transgene insertion occurs preferentially at the centromeres or that transgene integration into the *Dictyostelium* genome occurs in a random manner, but that the locus at which the transgene has inserted is recruited to the pericentromeric heterochromatin. In order to resolve this question the localisation of the transgene insertion was examined through the cell cycle for line #15 (a G418 resistant vector containing an antisense construct designed to silence the gene *agoA*) which contains a single insertion of approximately 100 copies. In interphase it colocalises with the centromeric markers as has been observed for other high-copy transgene insertions (Fig. 3.24a)

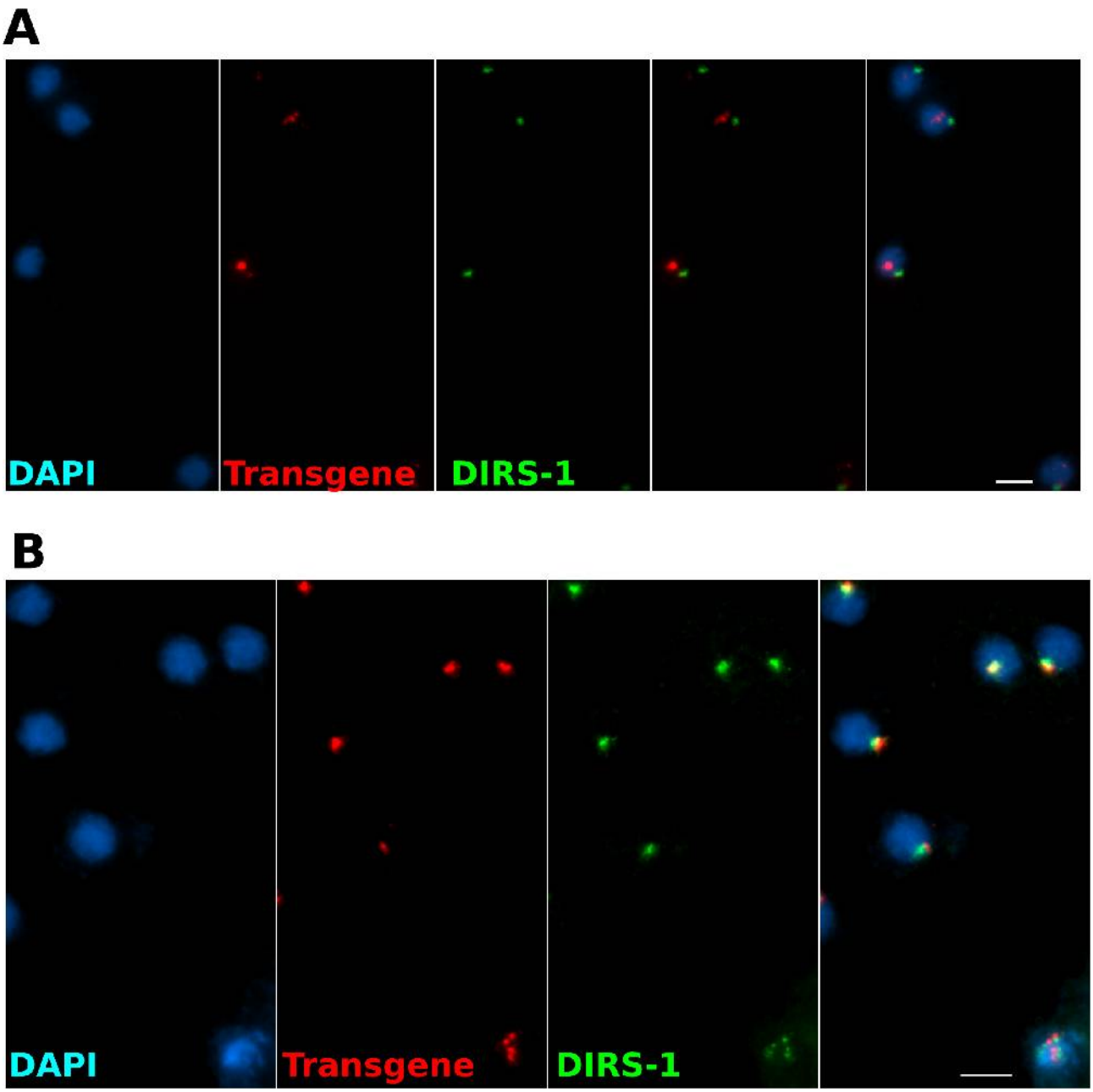


Fig. 3.23 **Localisation of Transgenes. A:** FISH on cells where a transgene of low copy number (approximately 10) has integrated using a directly FITC-labelled probe for DIRS1 and directly Cy3-labelled probe against the plasmid DNA in the transgene. **B:** FISH on cells where a transgene of high copy number (approximately 100) has integrated using a directly FITC-labelled probe for DIRS1 and directly Cy3-labelled probe against the plasmid DNA in the transgene. Scale bar = 5 µm.

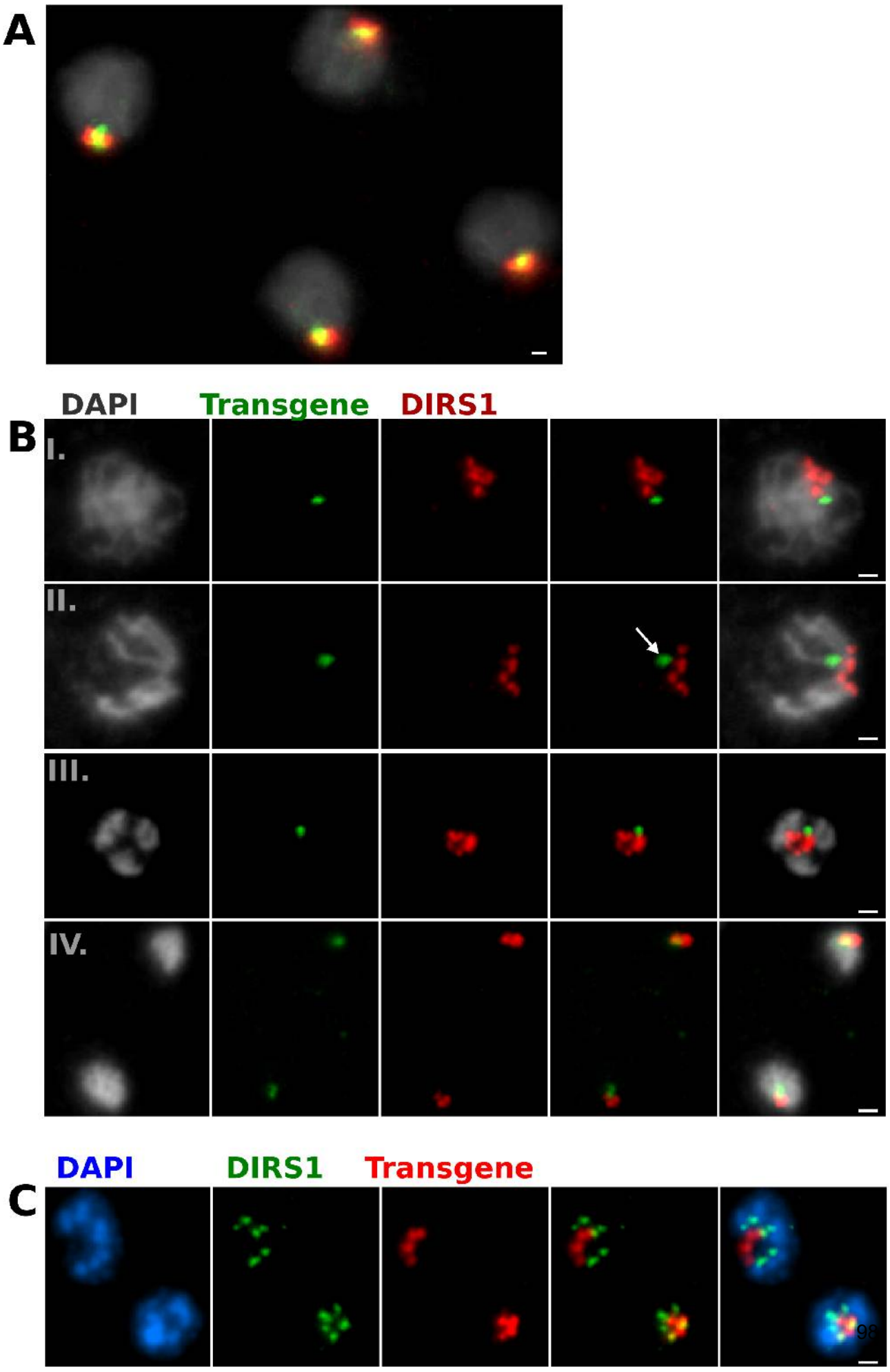
Line	Vector	Copy Number	Number of Insertions	Localisation
pG418-5	pGEM-G418	100	4	pericentromeric
pG418-8	pGEM-G418	100	?	pericentromeric
pLTR1	pGEM-LTR	10	?	euchromatin
agoAas15	pDneo2-agoAas	approx 100	1	pericentromeric
pGEM-E1	pGEM-G418	approx 100	1	pericentromeric
pGEM-E2	pGEM-G418	approx 100	2	pericentromeric
pGEM-E4	pGEM-G418	approx 100	1	pericentromeric
pGEM-E5	pGEM-G418	approx 100	1	pericentromeric
pGEM-F1	pGEM-G418	approx 100	2	pericentromeric
pGEM-F2	pGEM-G418	approx 100	2	pericentromeric
pGEM-F3	pGEM-G418	approx 100	4	pericentromeric
pGEM-F4	pGEM-G418	approx 100	3	pericentromeric
pISAR_A15	pISAR	variable	n/a	variable

Table 3.3 **Transgene Lines Examined**

As cells enter prophase the six DIRS-1 stained centromeres are visible and the transgene is visible as a discreet focus approximately 0.5 Mb along one of the chromosomes, indicating that it has not integrated into the centromeric region, but rather loops back to the centromeric region during interphase (Fig. 3.24b). As cells enter metaphase and the chromosomes become more compact the transgene locus no longer colocalises with the DAPI stained chromosomes, but rather appears to cluster around the microtubule spindle together with the other centromeres. During telophase the transgene locus trails slightly behind the centromeres (Fig. 3.24b). It has been previously reported that transformation by electroporation normally results in the transgene integrating into the genome at a single locus (Barth et al., 1998), however in over half the clones examined by FISH two to four different insertion sites were visible in metaphase cells (table 3.3, Fig. 3.24c). In mitotic cells it is evident that the transgene loci are clustering around the mitotic spindle in a manner analogous to the centromeres (Fig. 3.24c) suggesting that the transgene insertions may be acting as pseudo-centromeres. In order to investigate this possibility, the presence of different histone modifications on the transgenes was examined. In spite of being actively transcribed, significant levels of H3K9me2 was detected on the transgene (Fig. 3.25a), indicating that at least some copies are heterochromatic and silent. This would be consistent with the localisation to pericentromeric heterochromatin during interphase as observed in FISH experiments. ChIP using GFP tagged H3v1 also indicated that H3v1 was enriched on the transgene (Fig. 3.25b), consistent with its pseudo-centromere like behaviour in FISH experiments.

3.3.2 Extrachromosomal Transgenes

Many wild isolates of *Dictyostelium* contain naturally occurring plasmids that are nuclear localised (Noegel et al., 1985; Slade et al., 1990). Some of these plasmids such as Ddp1 and Ddp2 have been used to construct *Dictyostelium* transformation vectors that allow transgenes to be stably overexpressed without integration into chromosomal DNA (Chang et al., 1990; Levi et al., 2000; Veltman et al., 2009).


A
B
DAPI
Transgene
DIRS1
I.
II.
III.
IV.
C
DAPI
DIRS1
Transgene

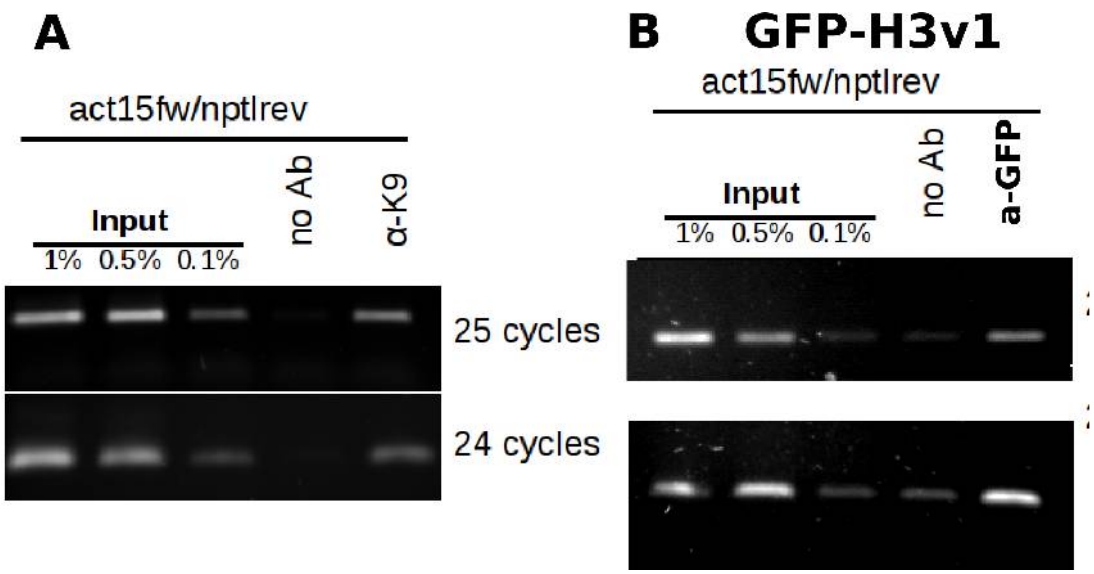


Fig. 3.25 **Distribution of Histone Modifications and Histone Variants on Transgenes. A:** Semi-quantitative PCR on chromatin immunoprecipitated with an antibody against the heterochromatin marker H3K9me2 indicates that this modification is present on the *nptI* resistance cassette present within the transgene. **B:** Semi-quantitative PCR on chromatin immunoprecipitated with an antibody specific for epitope tagged H3v1 indicates that this histone variant is present on the *nptI* resistance cassette present within the transgene.

Use of these extrachromosomal vectors allows for higher transformation efficiencies and often results in a higher level of expression compared to traditional integrating vectors.

The localisation of these extrachromosomal plasmids was examined by FISH. In contrast to transgenes integrated into the chromosome, the extrachromosomal transgenes did not localise to the pericentromeric heterochromatin, but were instead scattered throughout the nucleus (Fig. 3.26a). This result must be interpreted with caution as the denatured conditions used during the FISH procedure could potentially alter the localisation of small extrachromosomal elements (Sucgang et al., 2003). This would suggest that it is the tandemly repeated nature of the transgenes and not their copy number that causes them to be targeted to the pericentromeric heterochromatin.

Fig. 3.24 **Transgene Localisation During the Cell Cycle. A:** FISH on interphase cells where a transgene of high copy number (approximately 100) has integrated, apparently at a single locus. A directly Cy3-labelled probe for DIRS1 and directly FITC-labelled probe against the plasmid DNA in the transgene were used. **B:** The same line as in (A) at different stages of the cell cycle demonstrating the behaviour of the transgene throughout the cell cycle. I: interphase, II: prophase, III: metaphase, IV: telophase. The arrow indicates the position of the transgene along a prophase chromosome **C:** FISH on metaphase cells where a transgene of high copy number (approximately 100) has integrated, apparently distributed among four different loci. A directly FITC-labelled probe for DIRS-1 and directly Cy3-labelled probe against the plasmid DNA in the transgene were used. Scale bar = 1 µm

One transgenic line (Isar15) obtained using an integrating vector showed an unexpected localisation of the transgene. In contrast to all the other lines examined, it did not localise to a single focus, but rather showed a variable pattern around the edge of the nucleus (Fig. 3.26b). In metaphase cells some of the signal was not on the chromosomes but on small DAPI stained bodies (Fig. 3.26c). Genomic DNA was prepared from this line and a border fragment was recovered and sequenced (data not shown). The flanking sequence obtained was from a noncoding region of the extrachromosomal rDNA palindrome, about 300 bp from the central region of asymmetry (Sucgang et al., 2003). In order to confirm this result, FISH was preformed on this line with probes against the transgene and the 26S rDNA (Fig. 3.26d). Partial co-localisation of the transgene and the 26S rDNA was observed in both interphase and metaphase cells confirming that the transgene had integrated into the extrachromosomal rDNA palindrome. It is evident that only some of the approximately 100 copies of the extrachromosomal palindrome contain the transgene, while others give no signal with the transgene probe and thus appear to have the wild-type sequence. It has previously been shown that there is no sequence heterogeneity among the rDNA palindromes and it has been proposed that a “master copy” present on chromosome four is responsible for preventing heterogeneity (Sucgang et al., 2003) although the mechanism by which this would occur is unknown. These results suggest that some heterogeneity in the palindrome sequence can be tolerated and is stable to some extent (through development and the spore cycle, data not shown). This transgenic line may provide a useful tool for understanding the mechanism by which extrachromosomal palindromes are transmitted, replicated, and maintained free of sequence heterogeneity.

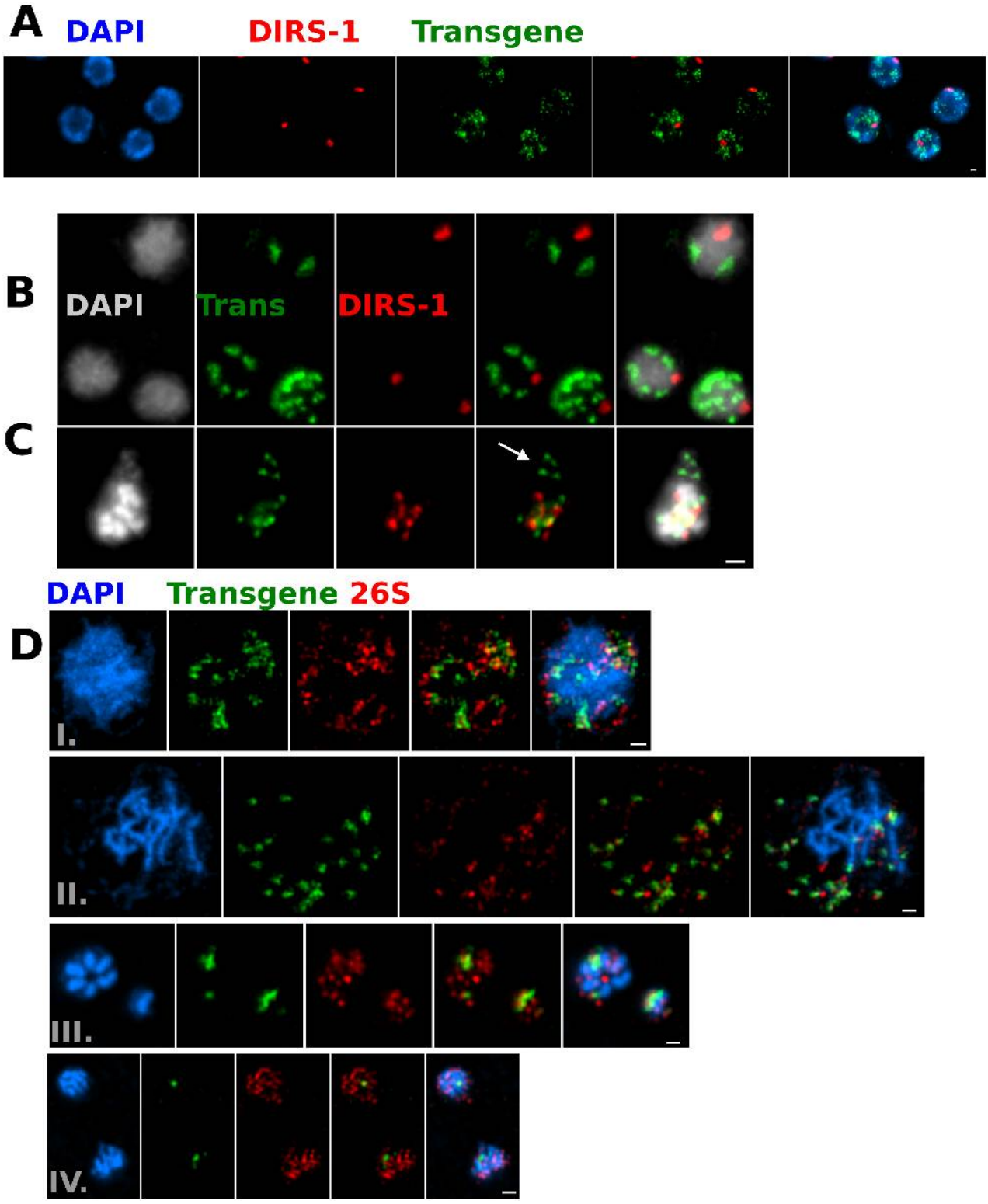


Fig. 3.26 **Behaviour of Non-Chromosomal Transgenes. A:** FISH on interphase cells transformed with a transgene contained on a plasmid together with a *Dictyostelium* extrachromosomal origin of replication (Dpn1). A directly Cy3-labelled probe for DIRS1 and directly FITC-labelled probe against the plasmid DNA in the transgene were used. **B:** FISH on interphase cells from a line transformed with an integrating transgene that displayed an unexpected behaviour, reminiscent of that seen in extrachromosomal transgene integrations. **C:** A metaphase cell from the same experiment as (B) shows that some copies of the transgene are present on small, non-chromosomal DAPI-stained bodies (arrow). **D:** FISH experiment on the line used in (B) and (C). A directly Cy3-labelled probe for the 26S-rDNA and a directly FITC-labelled probe against the plasmid DNA in the transgene were used. (i) interphase, ii: prophase, iii: metaphase, iv: telophase. Scale bar =1 µm

3.4 Development

3.4.1 Epigenetic Regulation of Developmental Genes

Loss of the histone methyltransferase Set1, which is responsible for methylation histone H3 at lysine 4 leads to mild developmental defects (Chubb et al., 2006). To further examine the role of histone modifications in developmental gene regulation the presence of H3K4me3, H3K9me2 and H3K9me3 at the promoters of the developmentally regulated genes *acaA, cotB, ecmA* and *rasG* was examined in vegetative cells. In agreement with previously published results high levels of H3K4me3 were detected at the promoter of the *rasG* gene which is expressed during vegetative growth (Robbins et al., 1992), but only low levels at the early development gene *acaA* (adenyl cyclase A), the pre-spore marker *cotB* or the prestalk marker *ecmA* (Fig. 3.27a) which are all silent in vegetative cells. Enrichment of H3K4me3 at *rasG* was comparable to that at the constitutively active *coronin* gene. Unexpectedly, neither H3K9me2 nor H3K9me3 were detected at the promoters of any of these silent loci, suggesting that unlike the situation in plants and animals, H3K9me may not be involved in maintaining facultative heterochromatin (silent genes that can be activated under different conditions, in different cell types or at different developmental stages). Chromatin immunoprecipitation was preformed on slugs after 16 hours of developmental with anti-H3K9me3 (Fig. 3.27b). H3K9me3 was present at DIRS-1 and s*kipper*, with the level at s*kipper* somewhat reduced compared to DIRS-1, which may be due to the previously described increase in *skipper* transcription during development (Kuhlmann et al., 2005). However, no significant enrichment at the inactive *rasG* gene was detected further suggesting that methylation of H3K9 is not involved in regulation of developmental gene expression in *Dictyostelium*.

3.4.2 Comparison of Histone Modification and Histone Modifying Enzymes

This analysis of histone modifications and histone modifying enzymes, although by no means exhaustive, suggest that many of the histone modifications necessary for developmental regulation in higher eukaryotes are not present in *Dictyostelium* (See table 1.2). However low levels of histone H2A ubiquitination were detected. This modification is associated with X-chromosome inactivation in mammals and in regulation of developmental and cell type specification in *Drosophila* where it acts to inhibit RNA pol II transcription (de Napoles et al., 2004; Stock et al., 2007; Zhou et al., 2008). While the PCR1 complex normally responsible for catalysing this modification is absent, *Dictyostelium* has a homolog of the damaged-DNA binding protein 1 (DDB1) called RepE (Alexander et al., 1996). DDB1 forms a complex with the E3 ligase adaptor Cullin4 that ubiquitinates H2A in response to DNA damage induced phosphorylation of H2AX (Kapetanaki et al., 2006). DDB1 also interacts with a chromatin binding protein Deetiolated1 (DET1) that has been implicated in regulation of development.

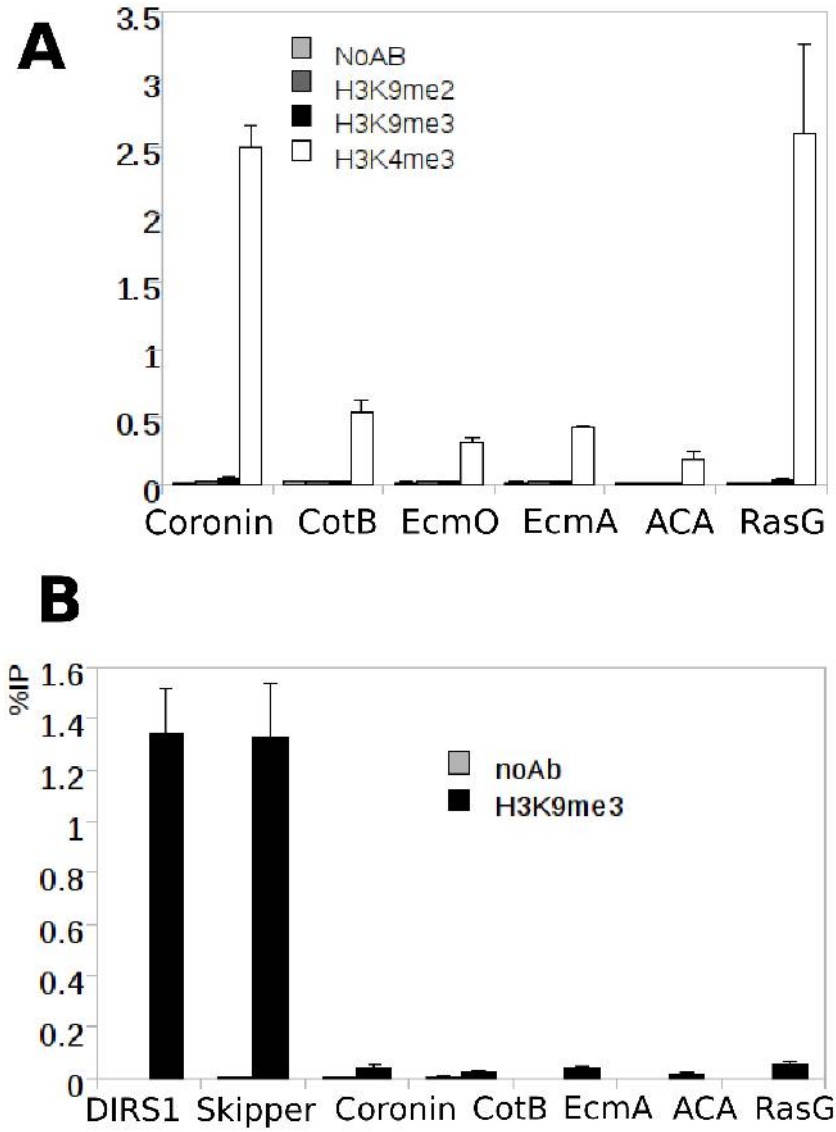


Fig. 3.27 **Histone Modifications at Developmentally Regulated Loci. A:** Chromatin immunoprecipitation on vegetative cells with antibodies against the heterochromatin markers H3K9me2 and H3K9me3 or the euchromatin marker H3K4me3 at different developmentally regulated loci. **B:** Chromatin immunoprecipitation on slugs 16 hours after the onset of development with an antibody against H3K9me3 at different developmentally regulated loci.

DET1 has a rather restricted evolutionary distribution, being found almost exclusively in plants and metazoans. However it is also present in *Dictyostelium*, suggesting that it may have a role in developmental regulation in this species.

3.4.3 *Dictyostelium* DET1

The approximately 60 kDa DET1 protein is nuclear localised and has no homology to any other known proteins (Pepper et al., 1994; Wertz et al., 2004). Based on sequence conservation between different species a DET1 domain of approximately 400 residues has been defined in the PFAM database (PF09737). A BLAST search against the predicted *Dictyostelium* proteome using

human DET1 as a query sequence revealed one protein (DDB_G0277075) with homology to human DET1 (Fig. 3.28a). However, the protein is significantly longer than the other DET1 homologs (1 462 amino acids compared to approximately 550 amino acids for other characterised DET1 proteins) and has been automatically annotated in Dictybase as having similarity of the mouse Sry (Sex determining region Y protein). A BLAST search of the Swissprot protein databank (http://www.expasy.ch, February 2007) with DDB_G0277075 as the query sequence gave hits against human, mouse ($1e^{-49}$), and *Arabidopsis* ($1e^{-45}$) DET1. DDB_G0277075 is located towards the distal end of chromosome 2 and encodes a protein of 1 462 amino acids and 169 kDa, it contains one short intron. Expressed sequence tags (ESTs) span part of the protein and confirm the presence of the intron. The putative *Dictyostelium* DET1 protein consists of a 500 amino acid region containing homopolymer stretches, followed by a region of 200 bp, homologous the N-terminal half of characterised DET1 proteins, a further 400 bp stretch of homopolymer sequences followed by another 250bp region homologous to the C-terminal half of characterised DET1 proteins (Fig. 3.28b). The mouse Sry protein also contains several large poly-glutamine stretches which would appear to account for the apparently incorrect annotation of the protein in Dictybase. DET1 has a very limited distribution and *Dictyostelium* appears to be one of the few species outside the metazoan and plant lineages where it is present (Fig. 3.28c). With the exception of *Dictyostelium*, DET1 homologs in all other species examined are small, with a continuous DET1 domain and no additional domains on the protein (data not shown).

3.4.4 DET1 Localisation

The det1 gene was cloned and inserted into the C-terminal cloning site of pDneo2a-6xMYC or pDneo2a-GFP and transformed into AX2. Western blotting confirmed that 6xMYC-DET1 was expressed and gave a band of the expected size (170 kDa; Fig. 3.31a). GFP-tagged DET1 localised to the nucleus in both live (Fig. 3.31b) and fixed cells (Fig. 3.31c), as has been previously described for DET1 in plants and *Drosophila* (Pepper et al., 1994; Berloco et al., 2001). Immunofluorescence on the MYC-tagged protein also demonstrated that the protein was present throughout the nucleus in a punctate pattern (Fig. 3.31d) and was not detectable on mitotic chromosomes indicating that it was displaced during the mitotic cycle (data not shown). DET1 was excluded from the nucleoli, however, it did accumulate to a high level at the periphery of the nucleoli. This was previously observed in *Arabidopsis* (M. Dubin, Masters thesis, The Open University, 2006) and suggests that DET1 may be involved in the regulation of the rDNA genes. Double immunostaining of MYC-DET1 with antibodies against H3K9me2 or H3K9me3 shows that DET1 appears to be largely excluded from the pericentromeric region when examined by widefield or confocal microscopy (Fig. 3.31d, e).

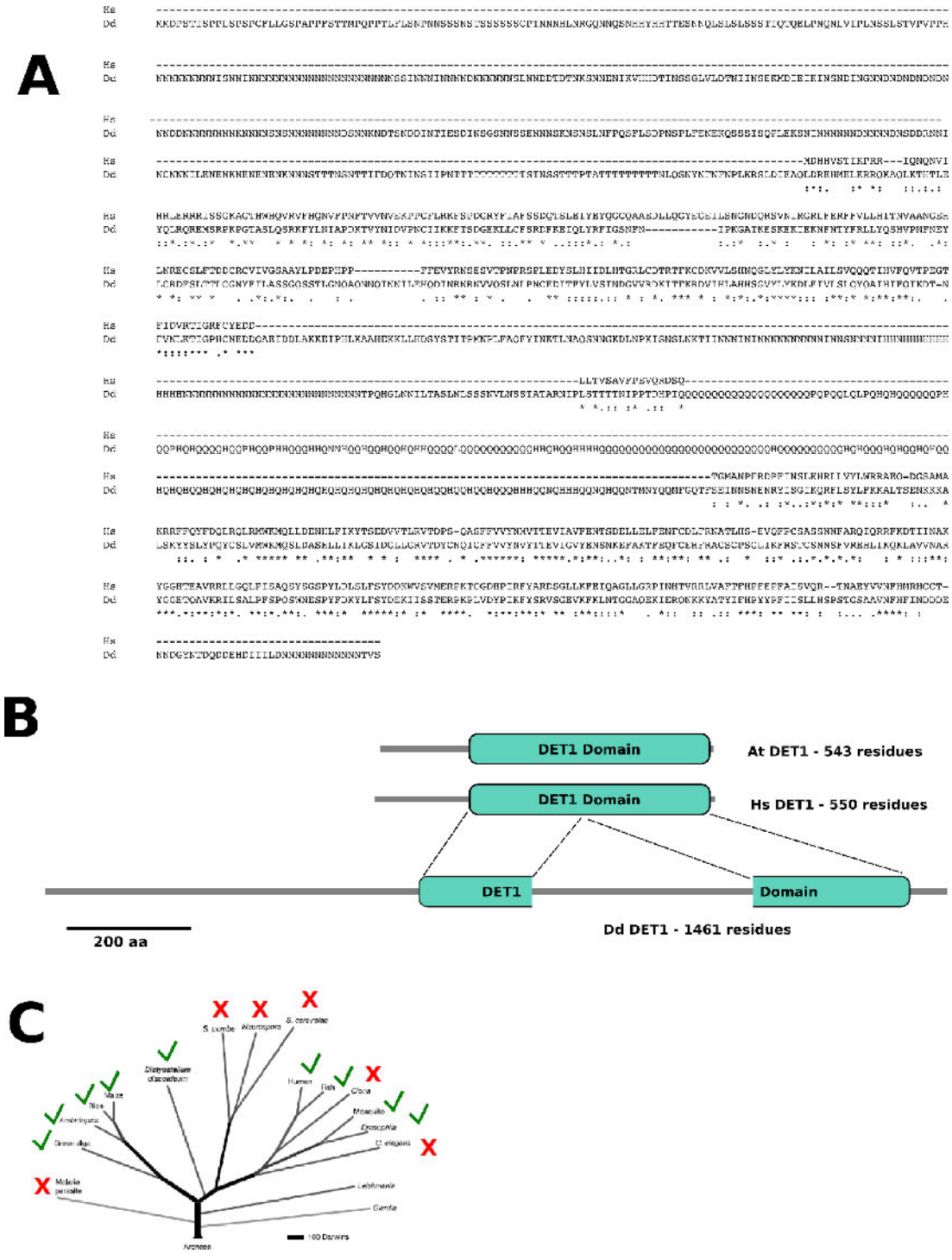


Fig. 3.28 **Putative *Dictyostelium* Homolog of DET1. A:** ClustalW alignment of human DET1 with the *Dictyostelium* homolog (DDB_G0277075). **B:** Schematic showing domain structure of DDB_G0277075 aligned with DET1 from human and *Arabidopsis.* **C:** Phylogram (adapted from Eichinger et al., 2005) of different Eukaryotic taxa, those containing a putative or confirmed DET1 homolog are indicated with a green tick, and those where it appears to be absent with a red cross.

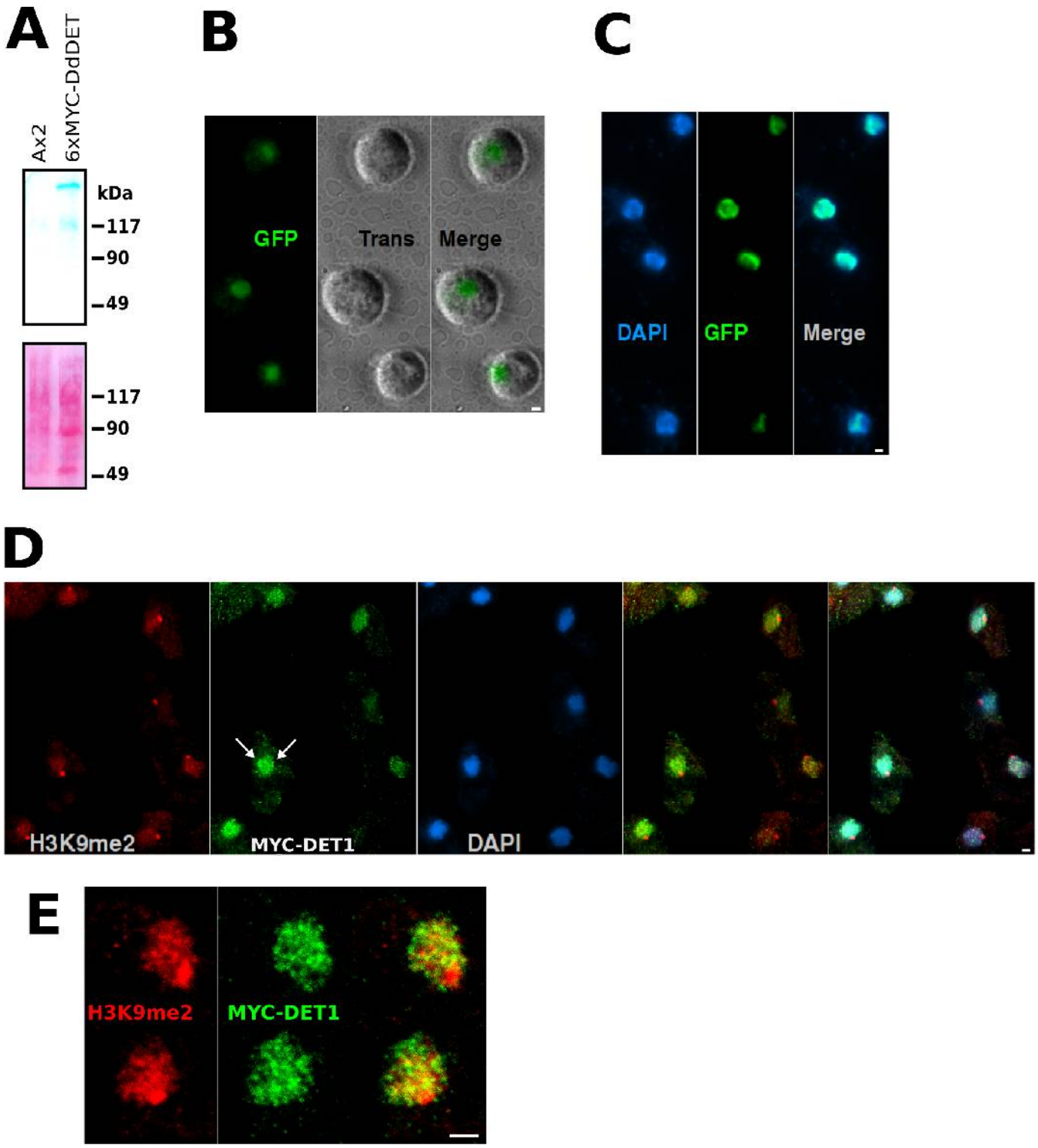


Fig. 3.29 **Expression of Epitope Tagged DET1**. **A:** Western blot on SDS-soluble protein extracts from *Dictyostelium* transformed with 6xMYC-DET1. Lower panels show the membrane stained with Ponceau-S after western transfer to ensure even loading and transfer to the membrane. **B:** Fluorescence microscopy on live cells expressing GFP tagged DET1. **C:** Fluorescence microscopy on fixed cells expressing GFP tagged DET1 stained with DAPI. **D:** Immunofluorescence on fixed cells expressing 6xMYC-DET1 with an anti-MYC antibody (green) and an antibody against the heterochromatin marker H3K9me2. **E:** Confocal image of cells from the same experiment as shown in panel (D). Scale bar = 2 µm.

3.4.5 Knockout of *det1*

A knockout construct was made by introducing a Blasticidin resistance cassette into the 5' half of the coding sequence of *det1* (Fig. 3.30a). This construct was then used to transform Ax2 cells. Three potential knockout lines were identified by PCR screening (data not shown). Two of the lines (8A, 9B) had an increased doubling time (12 hours compared to 10 hours for the wild type) when grown in liquid media, suggesting a mild growth or cell cycle defect (Fig. 3.31a). However the third clone 8D, displayed no increase in doubling time. When developed on phosphate agar plates clones 8A and 9B displayed a slight delay in the onset of development (Fig. 3.31b). In addition to the developmental delay, clones 8A and 9B (but not 8D) displayed enlarged aggregation territories and streaming could still be observed at 10 hours, while aggregation was complete in the Ax2 and 8D strain at 8 hours. Compared to wild type clones 8A and 9B formed fewer and larger slugs. The lower number of slugs appears to be partly due to the larger aggregation territories and partly because approximately half of the aggregates halt at the mound stage and do not develop any further. The spore forming ability of clones 8A and 9B was poor, but some spores were produced and these gave rise to viable amoeba after germination (data not shown). In an attempt to clarify whether the observed phenotypes are linked to the disruption of the *det1* locus or due to an unrelated genotype, a Southern blot was preformed on the 3 lines. The Southern blot confirmed that in all 3 lines the *det1* locus was disrupted (Fig. 3.30b). However, it was also evident that in line 8D, in addition to the disrupted locus a second wild type copy of the gene was present. This suggests that a duplication event had occurred and explained the lack of phenotype observed in this line. When grown on bacterial lawns, a growth defect was also observed, which could be rescued by super-transforming the knockout with a construct expressing 6xMYC-DET1, GFP-DET1 or TAP-MYC-DET1 (Fig. 3.32a). The developmental assay was repeated using wild type, the *det1*$^{-}$ mutant, and *det1*$^{-}$ complemented with either GFP alone (the empty vector), or GFP-DET1. In Ax2 and *det1*$^{-}$/GFP-DET1 the first mounds were visible at 5 hours of development, but not in the *det1*$^{-}$ strain nor in the det1^{-}/GFP strain, where aggregation onset appears to be delayed approximately 30 min with respect to wild type (Fig. 3.32b). As was previously observed the *det1*$^{-}$ mutant formed fewer and larger aggregates, many of which halted at this stage resulting in fewer spore-forming bodies. The *det1*$^{-}$/GFP-DET1 strain however, displayed a wild type phenotype confirming that the loss of the DET1 protein is responsible for the phenotype observed in the *det1*$^{-}$ mutant and that epitope tagged DET1 is functional.

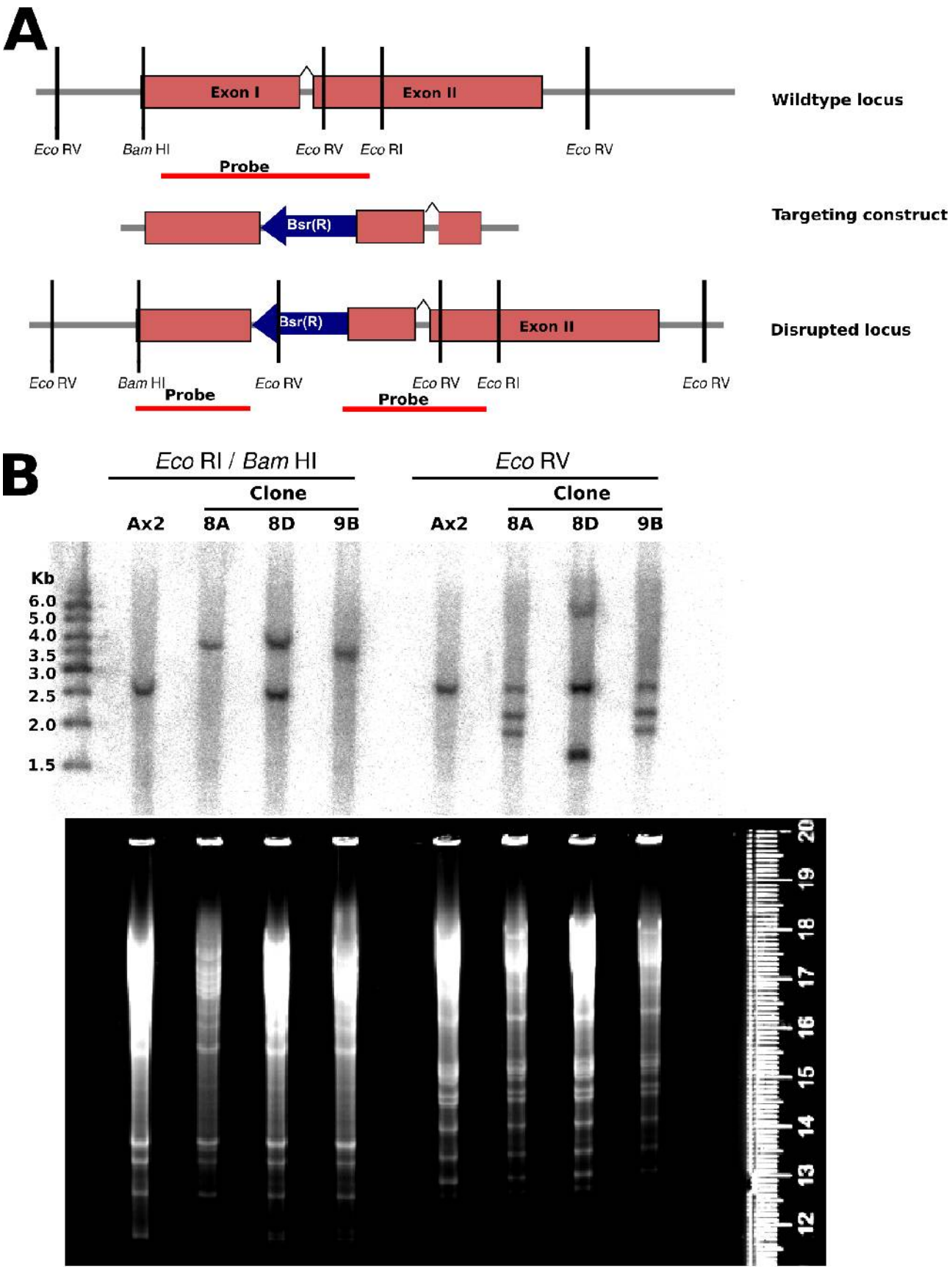


Fig. 3.30 **Disruption of the *det1* Locus. A:** Schematic of the *det1* locus, the targeting construct and the predicted organisation of the disrupted locus. The positions of restriction enzymes used in the Southern blot (panel B) are indicated and the probe used is shown in red. **B:** Southern blot on wild type (Ax2) and putative disrupted strains. The lower panel shows the ethidium bromide stained agarose gel prior to transfer.

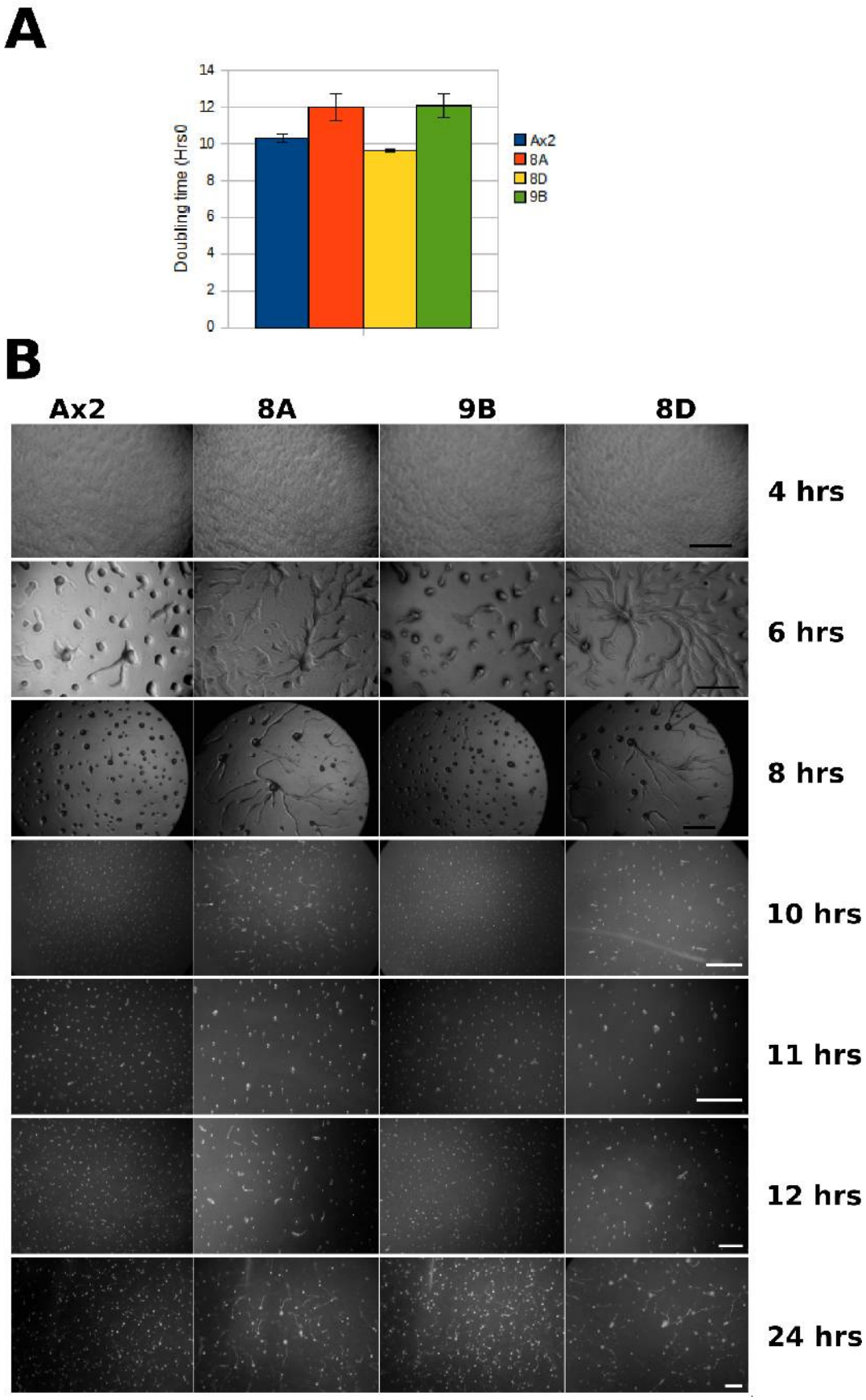


Fig. 3.31 **Phenotype of the *det1*⁻ Mutant. A:** Doubling time of Ax2 and putative disruption stains in HL5 shaking culture. **B:** Development of wild type and putative disruption strains on phosphate buffered agar at 22°C. Time after onset of starvation is indicated in hours. Black scale bar = 200 µm, white scale bar = 500 µm.

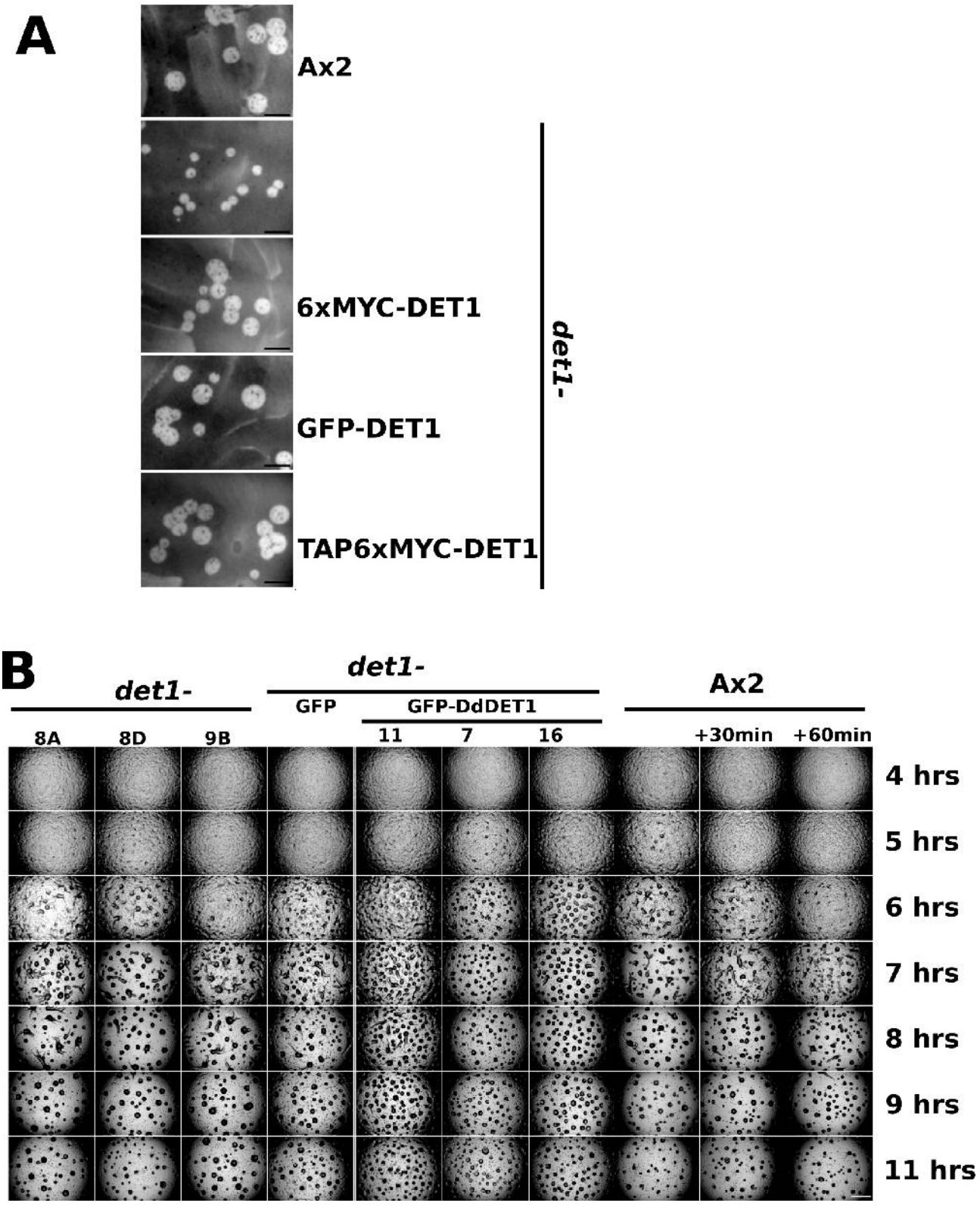


Fig. 3.32 **Complementation of *det1*⁻ Mutant**. **A:** Growth of Ax2, *det1*⁻ and *det1*⁻ complemented with different DET1 expression constructs on bacterial lawns. Pictures were taken after 72 hours growth at 22 C. **B:** Development of Ax2, *det1*⁻ and *det1*⁻ transformed with either GFP-DET1 or GFP alone on phosphate buffered agar at 22°C. Time after onset of starvation is indicated in hours except for the two columns on the extreme right, which where starved 30 min or 60 min later than the other strains. Black scale bar = 5 mm, white scale bar = 500 µm.

3.4.6 Role of DET1 in Cell Type Specification

Given that the *det1*$^{-}$ strain appears to display developmental defects and DET1 has been shown to have an important role in developmental regulation in other organisms, cell types specification was investigated in the *det1*$^{-}$ mutant. Ax2 and *det1*$^{-}$ (clone 8A) were transformed with the reporter constructs [cotB]:GFP and [ecmAO]:GFP. Lines expressing the reporter constructs were developed on phosphate agar for 17-24 hours in the dark and observed using a fluorescence microscope. In Ax2 the [cotB]:GFP reporter labelled the pre-spore cells comprising the posterior 80% of the slug while the [ecmAO]:GFP reporter labelled the anterior 20% of the slug comprising the pre-stalk cells, as well as giving a speckled pattern throughout the posterior pre-spore region due to the scattered ALCs (anterior like cells). Compared to wild type, *det1*$^{-}$ slugs expressed [ecmAO]:GFP more strongly, and the pre-stalk region was larger (the front 25-30% of the slug) than in Ax2 (Fig. 3.33a). [cotB]:GFP staining in the *det1*$^{-}$ mutant was much reduced, and absent in the anterior third of the slug, comprising the enlarged pre-stalk zone. In addition, staining was also absent in the posterior of most slugs (3.33b). As [ecmAO]:GFP did not appear to label these posterior cells their identity (e.g. cell type) remains unknown.

3.4.7 Analysis of Pre-Stalk Subtypes

The pre-stalk region of *Dictyostelium* contains several different zones with different pre-stalk subtypes (Williams, 2006). Given that the *det1*$^{-}$ mutant appeared to have an enlarged pre-stalk region, the relative composition of these different subtypes was examined. Ax2 and *det1*$^{-}$ were transformed with a reporter for PstB (pre-stalk B) cells ([ecmB]:LacZ), PstA cells ([ecmA]:LacZ), PstO cells ([ecmO]:LacZ) and the reporter [ecmAO]:lacZ (Early et al., 1993). Slugs were allowed to develop for the indicated lengths of time, then fixed and stained. The *ecmA* gene is expressed in almost all pre-stalk cells, but different cell types express it using either the distal part of the promoter ([ecmO] or the proximal part ([ecmA]. These cell types are referred to as PstO and PstA respectively. *ecmB* is expressed in the PstB cells which are present at the base of the pre-stalk zone in migrating slugs and will form the basal disk and lower cup in mature culminants. A small region within the PstA zone also expresses *ecmB* and this region is called the PstAB zone.

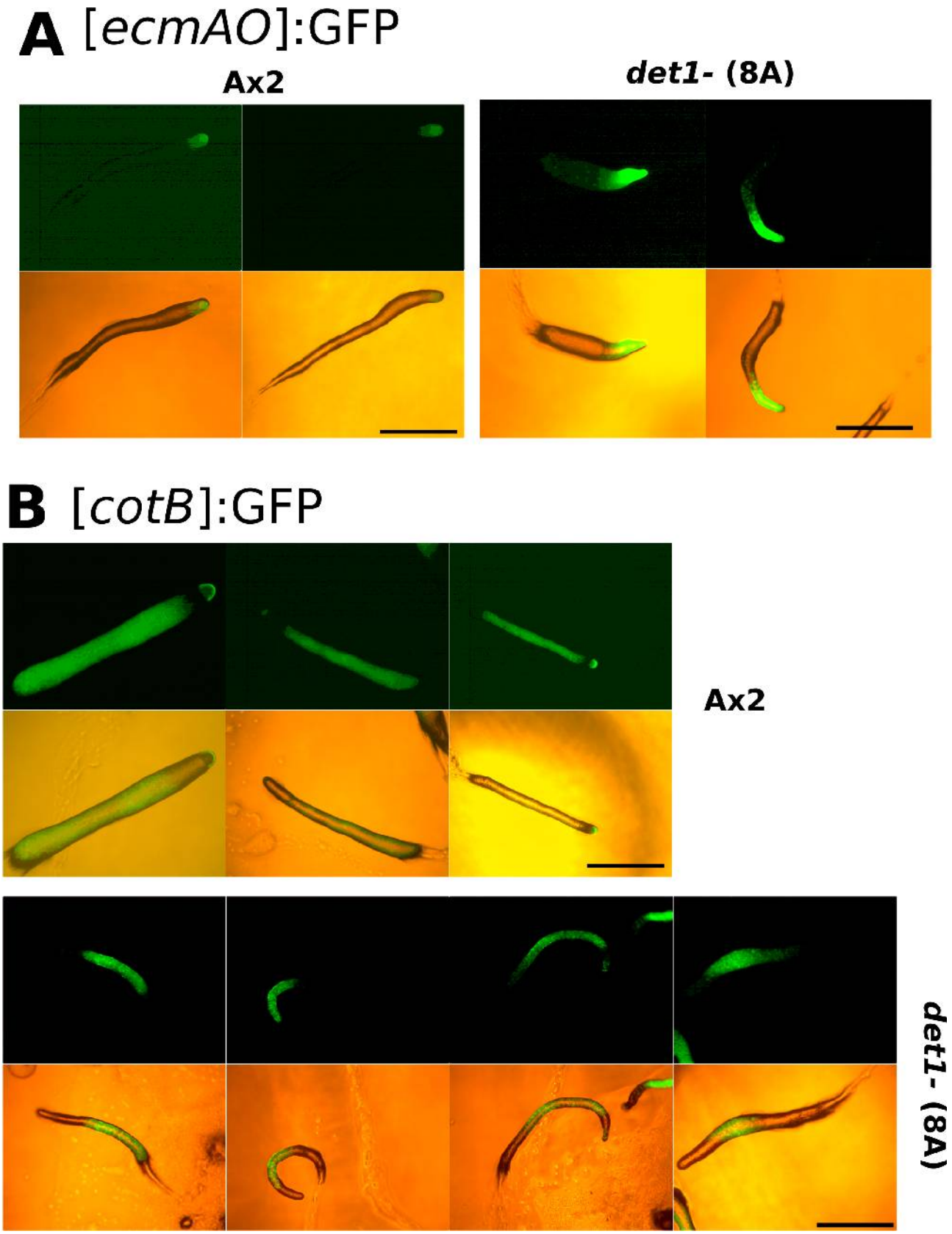


Fig. 3.33 **Developmentally Regulated Gene Expression. A:** Fluorescence microscopy of wild type or *det1*$^{-}$-slugs transformed with the pre-stalk reporter [ecmAO]:GFP 17 hours after the onset of starvation. **B:** Fluorescence microscopy of wild type or *det1*$^{-}$ slugs transformed with the pre-spore reporter [cotB]:GFP 17 hours after the onset of starvation. Scale bar = 500 µm.

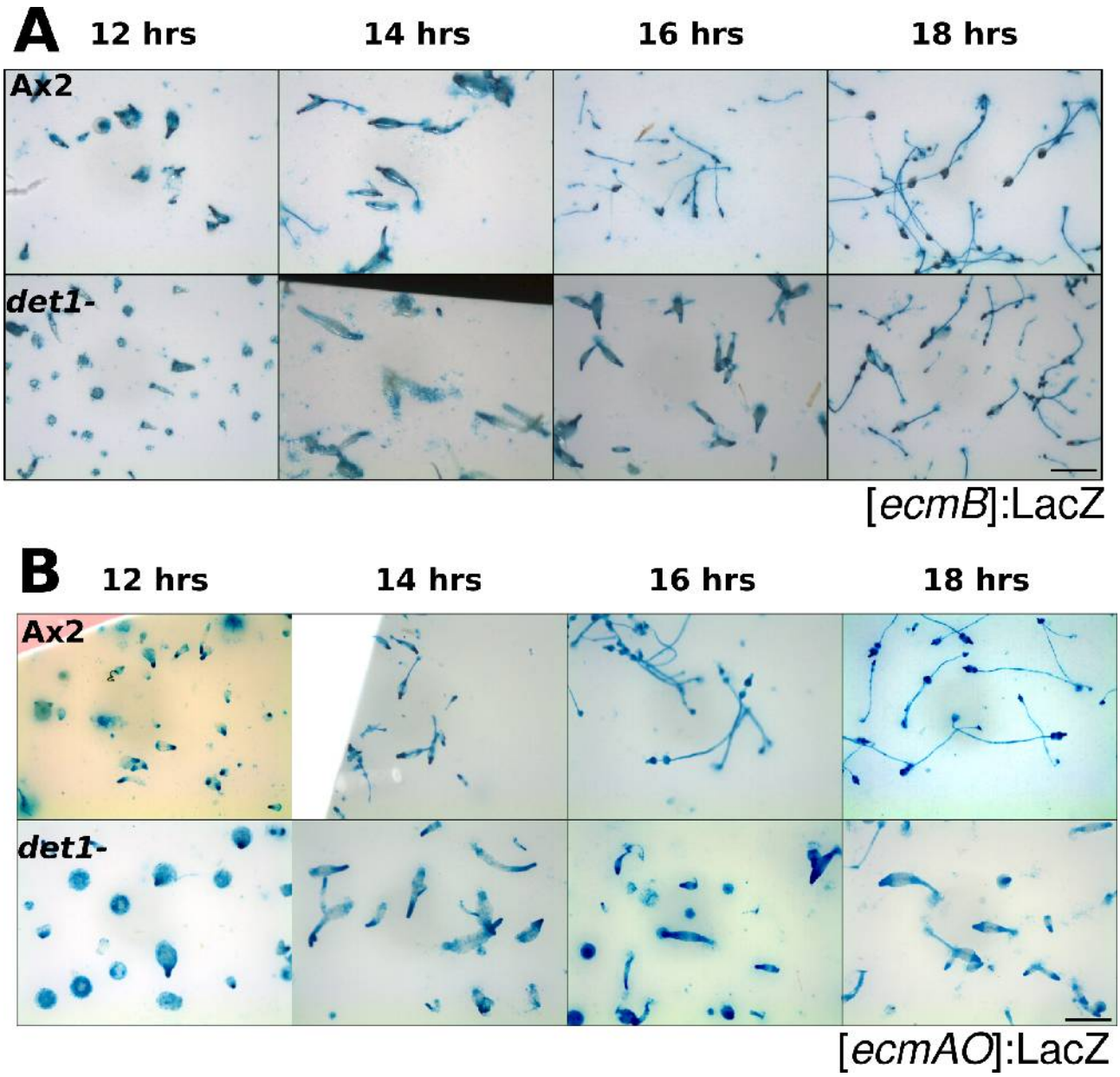


Fig. 3.34 **Effect of DET1 on the Formation of Pre-Stalk Subtypes. A:** wild type or *det1*$^{-}$ slugs transformed with the pre-stalk subtype reporter [ecmB]:LacZ were fixed and stained with Beta-Gal at the indicated times after the onset of starvation. **B:** wild type or *det1*$^{-}$ slugs transformed with the pre-stalk subtype reporter [ecmAO]:LacZ were fixed and stained with Beta-Gal at the indicated times after the onset of starvation. Scale bar = 500 µm

Compared to Ax2, [ecmB]:lacZ staining in the *det1*$^{-}$ mutant appeared to be weaker (Fig. 3.34a). It was also apparent that after 16-18 hours the mutant has a developmental delay of approximately four hours compared to the wild type. The [ecmAO]:lacZ marker labels the entire pre-stalk region, i.e. the PstA, PstO cells and PstAB cells (Fig. 3.34b). As previously observed for the [ecmAO]:GFP construct, the zone expressing [ecmAO]:LacZ was expanded in the mutant compared to the wild type. Although variable, it typically stained the front 25-30% of the slug instead of the front 20% in the wild type. Staining of the slug posterior and also within the pre-spore region is also stronger in the *det1*$^{-}$ mutant. Looking at the [ecmA]:LacZ reporter which contains the proximal portion of the

ecmAO promoter, it was apparent that this promoter is expressed to a much higher level in the *det1*$^{-}$ mutant (Fig. 3.35a). Some scattered staining was visible well into the pre-spore zone and at the slug posterior rear-guard region. By 16 hours strong staining of the emerging stalk was observable in the *det1*$^{-}$ mutant, while PstA staining of the stalk was weak to non-existent in wild type. Compared to Ax2, [ecmO]:LacZ expression in the *det1*$^{-}$ mutant appeared to be weaker and more diffuse (Fig. 3.35b). In most *det1*$^{-}$ slugs the PstO zone was larger than that of wild type, but staining was weaker. Also the staining did not end abruptly at the pre-spore region, but continued throughout the slug, suggesting an increased number of ALCs (anterior like cells), which became more abundant toward the posterior of the slug. The *det1*$^{-}$ mutant slugs are generally much larger than the wild type slugs, probably due to the enlarged aggregation territory observed earlier. Also many of the *det1*$^{-}$ mutant aggregates were still arrested at the mound stage 18 hours after the initiation of development. These arrested mounds showed a rather homogeneous staining of the [ecmAO]:lacZ, [ecmO]:lacZ and [ecmA]:lacZ markers, indicating that they were able to differentiate into pre-stalk and pre-spore cells. However, for some reason they were unable to sort within the mound to form the anterior pre-stalk zone necessary for migratory slug formation. These results suggest a relative increase in the number of the PstA cells and a reduction in size of the pre-spore region.

Immunostaining was preformed on slugs with an antibody against the SP35 pre-spore protein (EB4, PsvA, Hildebrandt et al., 1991; Hildebrandt and Nellen, 1992). In Ax2 the antibody labelled the pre-spore cells in a rather uniform manner. In contrast, in the *det1*$^{-}$ mutant the pattern was quite variable from slug to slug (Fig. 3.35c), with some cells displaying expression over the entire length of the slug. In many slugs however it was largely absent from the anterior third of the slug which corresponds to the enlarged pre-stalk region and was also absent from the posterior of the slug as was observed for the [cotB]:GFP line.

3.4.8 Genomic Targets of DET1

The expression patterns of the cell type markers suggest that the *det1*$^{-}$ mutant has defects in cell type proportioning and patterning. This phenotype is reminiscent of the *dimB*$^{-}$ mutant in Ax2 (Zhukovskaya et al., 2006). DimB is a homolog of c-Jun in mammals and HY5 in plants, both of which are targeted for degradation by a ubiquitin ligase complex containing the DET interacting protein COP1 (Constitutive Photomorphogenisis 1, Wertz et al., 2004; Saijo et al., 2003). DimB is known to bind directly to the *Dictyostelium ecmA* promoter, while DET1 is known to bind the promoters of developmentally regulated genes in *Drosophila* (Berloco et al., 2001) and plants (M. Dubin, Masters thesis, The Open University, 2006). To see if DET1 also binds to developmentally regulated genes in *Dictyostelium* ChIP was preformed on vegetative cells expressing GFP-DET1 in the *det1*$^{-}$ background (Fig. 3.36). No enrichment of DET1 was detected on DIRS-1, consistent with its lack of co-localisation with heterochromatin markers H3K9me2 and H3K9me3 (Fig. 3.29d, e).

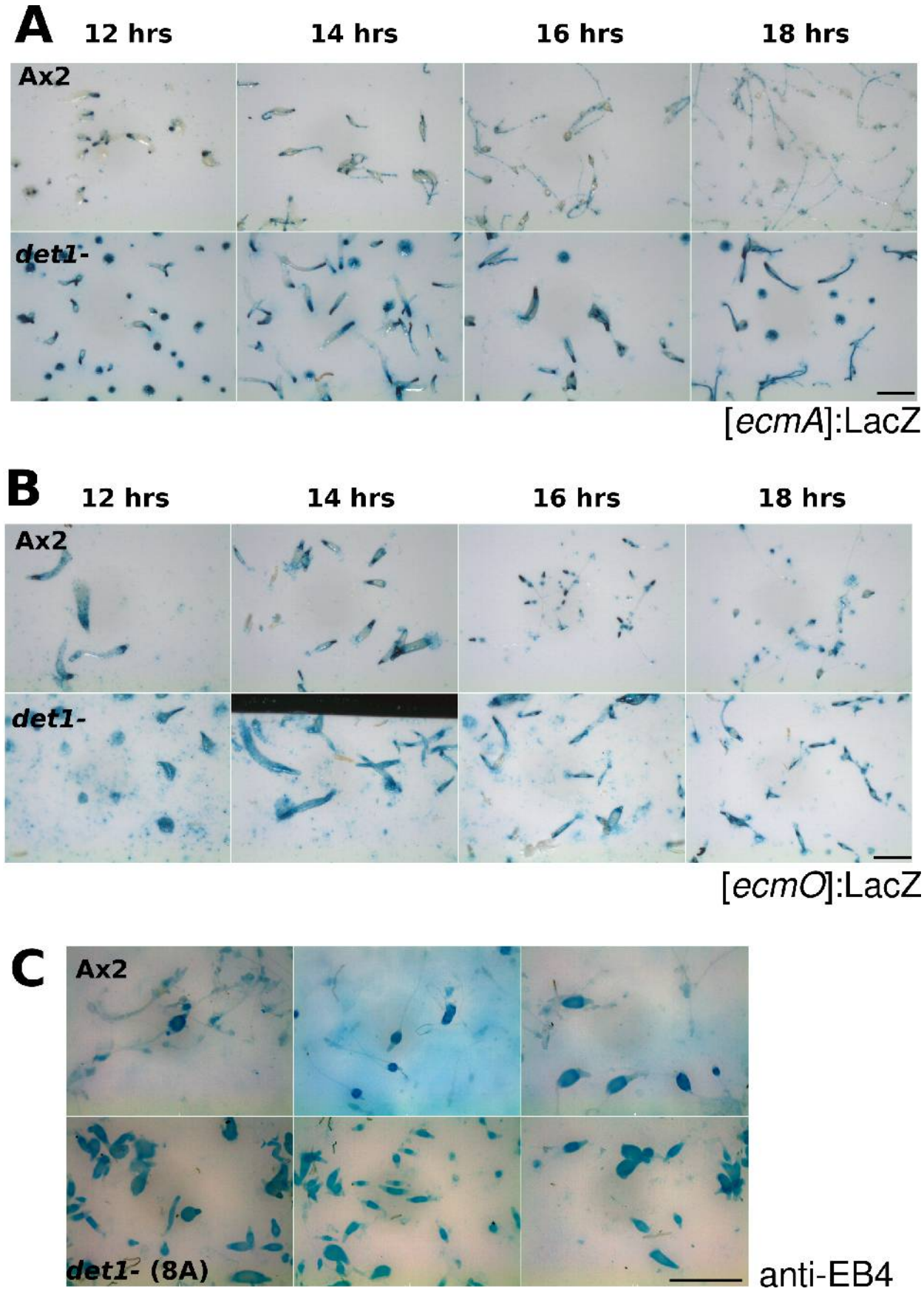


Fig. 3.35 **Regulation of Cell-Type Formation. A:** Wild type or *det1*$^{-}$ slugs transformed with the pre-stalk subtype reporter [ecmA]:LacZ were fixed and stained with Beta-Gal at the indicated times after the onset of starvation. **B:** Wild type or *det1*$^{-}$ slugs transformed with the pre-stalk subtype reporter [ecmO]:LacZ were fixed and stained with Beta-Gal at the indicated times after the onset of starvation. **C:** Wild type or *det1*$^{-}$ slugs fixed and stained with an antibody against the pre-spore marker PsvA (EB4). Scale bar = 500 µm.

Enrichment was also not detected at the constitutively active c*oronin* gene. However the promoters of *cotB*, *acaA*, and the proximal *ecmA* promoter were enriched. DET1 was present on the promoter of the developmentally regulated *psvA* gene, but not on exon 2 of the coding region. The *psvA* gene also contains a second promoter in exon 3, which drives antisense transcription (Hildebrandt et al., 1992). High levels of DET1 were also detected at this promoter. These data suggest that the miss-regulation of *cotB*, *ecmA* and *psvA* (*EB4*) observed is in the *det1*$^{-}$ mutant is probably due to the loss of DET1 from their promoters, rather than an indirect effect due to a general miss-regulation of development. A slight enrichment of DET1 was also detected at a non-coding region within the extrachromosomal rDNA palindrome, but not at the active 26S rDNA locus. This may explain the enrichment of GFP-DET1 observed at the nucleolar periphery (Fig. 3.29d).

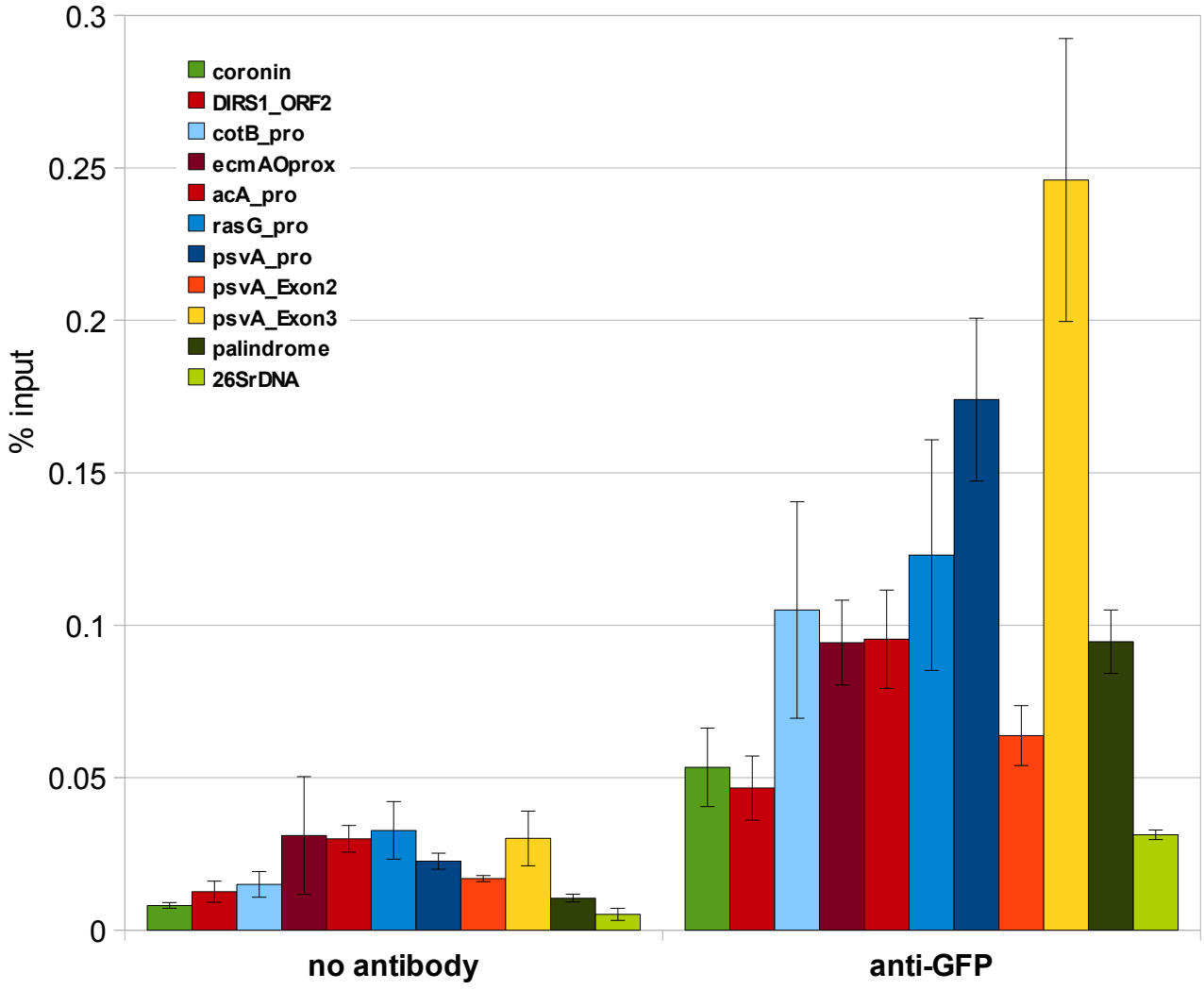


Fig. 3.36 **Genomic Targets of DET1.** Real-time PCR on chromatin precipitated from vegetative cells expressing GFP-DET1 with an antibody against GFP with primers against different genomic loci.

4 Discussion

4.1 Cloning Vectors

A series of expression vectors that allows proteins of interest to be overexpressed with an N-or C-terminal fusion tag were made. In contrast to many existing *Dictyostelium* expression vectors, no additional amino acids are introduced at either the N or the C-terminus of the protein (which could interfere with protein folding/activity). A short glycine/serine polylinker is used between the tag and the protein to increase the chances to obtain soluble fusion proteins and ensure the tag is accessible. Integrating vectors conferring resistance to Geneticin and Blasticidin allow for high and low levels of expression respectively. An extrachromosomal vector conferring Blasticidin resistance allows super-transformation of existing Geneticin resistant lines so that the two proteins of interest may be expressed at similar levels. The vectors allow proteins of interest to be expressed with GFP, RFP, 3xHA, 3xFLAG, 6xMYC or TAP tags.

The use of standardised cloning sites facilitates transfer of a gene of interest into the different versions of the expression vectors, thus combining advantages of SSR vectors such as Gateway vectors without the disadvantages such as the additional N- and C-terminal residues and long and highly charged polylinker sequences. In summary over 20 proteins have been cloned so far using this system, and in all cases, the protein was successfully expressed using one of the tag/resistance cassette combinations available (this thesis; Sawarkar et al., 2009; B. Boesler, B. Foeldesi and V. Maximov; personal communications). The resulting constructs were successfully used for a number of experiments, including protein localisation, immunolocalisation, western blotting, immunoprecipitation and chromatin immunoprecipitation, demonstrating the versatility of the system.

4.2 Nuclear Organisation and Histone Modifications

Double immunolabelling experiments demonstrated that pericentromeric heterochromatin and euchromatin occupy discrete territories in *Dictyostelium* nuclei. In addition to the previously described di-methylation of histone H3 at lysine 9 within pericentromeric heterochromatin, extensive tri-methylation of this residue was also detected. H3K9 methylation appears to be catalysed by the SuvA histone methyltransferase. SuvA is a member of the Su[var]3-9/Clr4p/DIM5 subfamily of H3K9 methyltransferases which are unique to the unikont linage (Krauss 2008). Unlike most members of the Su[var]3-9-like family, SuvA lacks the chromodomain, that at least for the human SUV39H1 is required for methylation activity and binding to HP1 (Yamamoto and Sonoda, 2003). It appears that this chromodomain was acquired in animals after the Amoebozoa-opisthokont split, although it has also been lost independently multiple times in the opisthokont linage, including from *Neurospora* DIM5 (Krauss, 2008). Su[var]3-9-like family members catalyse

H3K9 methylation exclusively in constitutive heterochromatin, and appear to have no activity against facultative heterochromatin or euchromatin (Krauss, 2008). Consistent with this, epitope-tagged SuvA localisation was largely restricted to the pericentromeric heterochromatin and the euchromatin territory appears largely devoid of H3K9 methylation. Nor was H3K9 methylation detected on any protein coding genes by chromatin immunoprecipitation.

Using antibodies specific for different histone modifications, H3K4me2, H3K4me3, H3K36me1 and H3K36me2 were detected but not the repressive modifications H3K27me and H4K20me. Extensive acetylation of histones H2A, H2B, H3 and H4 was also detected. Histone acetylation was observed throughout the euchromatin territory. The level of acetylation at a number of residues; H3K4ac, H3K14ac, H2AK5ac and H2BK5ac either increases, or was only detectable on mitotic chromosomes, suggesting that they may have a role in the regulation of mitosis. It has previously been shown that H3K4ac levels at the centromeres are regulated in a cell cycle dependent manner and that this is required for mitotic progression (Eot-Houllier et al., 2008).

An antibody against H3S10ph detected this modification throughout the nucleus with higher levels at a focus that may represent the pericentromeric heterochromatin, suggesting that its role in regulating HP1 binding to H3K9me (Johansen et al., 2006; Zhang et al., 2006) may be conserved in *Dictyostelium.* In response to DNA damage, DNA-PK (DNA-protein kinase) phosphorylates H2AX in the chromatin flanking the damaged DNA. Low levels of H2AXS135ph were detected at a number of small foci around the nuclear periphery, consistent with previous reports demonstrating the presence of this modification in this species (Hudson et al., 2005).

Phosphorylation of H2AX at serine 135p serves to recruit the DNA damage repair machinery including a DDB1 containing protein complex which catalyses the ubiquitination of H2A (Kapetanaki et al., 2006). RepE, a homolog of DDB1 is an essential protein in *Dictyostelium* (Alexander et al., 1996). Low levels of a higher molecular weight species of H2AX, which may correspond to the ubiquitinated form, were detected. This suggests that a RepE containing complex also catalyses the ubiquitination of H2AX in *Dictyostelium*.

4.2.1 Histone modifications at genomic loci

In addition to H3K9me2 and 3, a significant level of H3K4me3 was detected on both the *skipper* and DIRS-1 retrotransposons, suggesting that both may be transcribed by RNA pol II. This is in agreement with data showing that the mRNA from *skipper* is easily detectable by northern blot (Kuhlmann et al., 2005). While long DIRS-1 transcripts are barely detectable in wild type cells, more than half of a *Dictyostelium* small-RNA library consists of DIRS-1 derived sequences (Hinas et al., 2007). Also DIRS-1 transcripts are present at a high level in the RNA directed RNA polymerase C (*rrpC*) knockout (Martens et al., 2002), suggesting that DIRS-1 is indeed transcribed at a reasonably high level, but in wild type cells the RNAs are rapidly converted to siRNAs due to

RrpC activity.

HcpA and HcpB were also enriched on the retrotransposons *skipper* and DIRS-1. While both showed a similar distribution by fluorescence microscopy, HcpB but not HcpA was enriched at DIRS-1. While comparing relative enrichment between different ChIP experiments is problematic, the enrichment values suggest that HcpB may bind to chromatin more strongly than HcpA.

4.2.2 Centromeric Histone H3 and Centromeres

In addition to the histone H3.3 variants H3a, H3b and H3c, two additional genes in the *Dictyostelium* genome encode proteins with histone H3-like domains. These proteins have been annotated as H3v1 and H3v2 (histone H3 variant 1 and 2). The H3v2 is smaller than a typical histone H3 (96 vs. 136 amino acids) and contains an intron, a feature usually absent from histone genes encoding replication dependent histones (histones deposited on chromatin in a replication independent manner sometimes have introns, for example H3.3 in *Arabidopsis,* or the proposed *Dictyostelium* H2A.Z gene). H3v1 is longer than a typical histone H3 (619 vs 136 amino acids) and contains a histone H3-like domain at the C-terminus. The histone H3 fold of H3v1 shares some similarity with CenH3 from *Drosophila* family members. This probably represents convergent evolution. In contrast to all other CenH3 orthologs examined, the loop-1 of the H3v1 histone fold is not longer than that of a conventional histone H3. This suggests that a longer loop-1 may not be essential for targeting of CenH3. GFP or epitope tagged H3v1 colocalises to a single focus with heterochromatin marker H3K9me3 during interphase. During mitosis up to six foci are visible which partially colocalise with H3K9me3. During anaphase and telophase H3v1 is slightly closer to the leading edge of the separating chromatids, suggesting the existence of distinct core centromere and pericentromeric heterochromatin territories.

It has been proposed that the DIRS-1 retrotransposon acts as the centromeres in *Dictyostelium* (Loomis et al., 1995). Eichinger et al., showed that DIRS-1 localises to 6 distinct foci in the interphase nucleus (Eichinger et al., 2005). In FISH experiments it was observed that all of DIRS-1 and most of the *skipper* and DDT-A but not the Tdd-4 transposons colocalise at a single focus at the nuclear periphery. The discrepancy between these results and the previously published data can probably be attributed to the use of indirect detection of the probe (using a fluorophore-conjugated antibody to detect the digoxygenin-labelled probe), which in our hands also resulted in quite a punctuate appearance. No signal from the DIRS-1 probe was ever observed elsewhere in the nucleus, suggesting that it is exclusively localised to the centromeres. It has also been shown that in interphase nuclei, HcpA, HcpB and H3K9m2 localise to a single focus adjacent to the centrosome that shows behaviour reminiscent of centromeres (Kaller et al., 2006b; Kaller et al., 2007). Immuno-FISH experiments demonstrated that DIRS-1 colocalises with H3K9me3 and H3v1. As cells entered prophase, up to six DIRS-1 foci became visible. These condensed to a single

focus in metaphase and then to a focus at the leading edge of the separating chromatids during anaphase and telophase. This pattern appeared identical to what was observed for GFP-H3v1. The results suggest that DIRS-1 and/or *skipper* either form the core centromere or the pericentromeric heterochromatin. When ChIP was preformed to precipitate H3v1 associated chromatin, DIRS-1 was more highly enriched compared to *skipper*. This is in contrast to ChIP preformed against H3K9me3 and H3K4me3, where *skipper* is more highly enriched than DIRS-1. This suggests that H3v1 containing nucleosomes assemble preferentially on DIRS-1 sequences and thus that DIRS-1 acts as the core centromere. Presumably other copies of DIRS-1 and/or *skipper* act as the pericentromeric heterochromatin.

Time-lapse experiments and experiments on fixed cells showed that H3v1 is loaded onto the centromeres at the onset of mitosis. Given that CenH3 loading in plants also occurs at the start of mitosis, this suggests that this is the timing of CenH3 loading in ancestral eukaryotes and that an alternative loading mechanism during anaphase involving the HJURP protein (Dunleavy et al., 2009; Foltz et al., 2009) has evolved in metazoans after the amoebozoa-animal split and probably after the divergence of the fungal linage (Takahashi et al., 2005).

Probes against the distal telomeres of chromosome 1 and chromosome 2 localised opposite to the clustered centromeres in the interphase nucleus, suggesting that the interphase nucleus is organised in a Rabl-like manner. This organisation of the nucleus is reminiscent of the situation in *S. cerevisiae*, where the centromeres also remain clustered and attached to the MTOC and the telomeres localised to the opposite side of the nucleus (Jin et al., 2000).

4.2.3 rDNA-Palindrome

The *Dictyostelium* rDNA genes are organised on extrachromosomal rDNA palindromes present at approximately 100 copies per nucleus. These palindromes cluster together to form a pseudo-chromosome during mitosis. This pseudo-chromosome does not seem to contain H3v1 suggesting an alternative mechanism is used to ensure its segregation during mitosis. It also appears to lack most of the histone modifications examined, including H3K9me2,3 H3K4me2,3 H3K36me as well as most of the histone acetylation marks examined, except for H4K5ac and H2AK9ac, suggesting that these two marks may be deposited by complexes that are not associated with RNA pol II.

4.3 Transgenes

High copy number transgenes were found to colocalise with the pericentromeric heterochromatin during interphase. This appears to be due to some mechanism causing the locus where the transgene has integrated to loop out and to come into contact with the pericentromeric heterochromatin. The mechanism by which this occurs is as yet unknown, but it may be triggered by the presence of large tracts of repetitive DNA as this looping was not observed on low copy

transgenes (less than 10 copies). During mitosis the transgenes are localised around the mitotic spindle in a similar manner to DIRS-1, suggesting that the transgenes may be acting as pseudo-centromeres.

The pericentromeric marker H3K9me2 and core centromere marker H3v1 were enriched on high copy-number transgenes. One transgene examined was found to have integrated into the extrachromosomal rDNA palindrome instead of the chromosomal DNA. In spite of being present at a similar copy number compared to the integrating chromosomal transgenes, they did not localise to the pericentromeric heterochromatin. Transgenes on extrachromosomal plasmids, present at approximately 100 copies per nucleus also did not localise to the pericentromeric heterochromatin. Together, this strongly suggests that it is the repetitive nature of high copy transgenes, and not their copy number, which causes them to behave in a centromere-like manner. The presence of heterochromatic marks on these high copy transgenes may partially explain their relatively low expression levels (for their copy number).

Apart from the centromeres, the *Dictyostelium* genome appears to be largely devoid of large tracts of repetitive elements. The distal subtelomere transposon clusters appear to be quite small (15-30 kb) and instead of chromosomal arrays of rDNA genes as found in most eukaryotes, *Dictyostelium* rDNA genes are dispersed on many small extrachromosomal elements, each containing only two copies of the rDNA genes. A possible explanation for this is that large transposon clusters or other repetitive structures such as rDNA arrays occurring in *Dictyostelium* may form pseudo-centromeres, as was observed for the transgenes, these could eventually lead to chromosome breakage and genome instability. However, no chromosomal breakages were observed in the transgenic lines, and in telophase, the transgenes were observed to lag behind the centromeres, suggesting they are only weakly connected to the MTOC. A possible explanation for the failure of transgenic lines to form true bicentric chromosomes is that the centromeric histone H3v1 has lower affinity for the transgene, than for DIRS-1. This may explain why small clusters of retroelements other than DIRS-1 are tolerated at loci outside the centromeres (e.g. the distal subtelomere transposon clusters) in *Dictyostelium* but not DIRS-1 itself. Alternatively, the transgenes may not recruit the entire apparatus required for centromere function.

4.4 Epigenetic Regulation of Development

In order to examine the possible role of epigenetic factors in the regulation of development, the presence of histone modifications on promoters of developmentally regulated genes was examined. In vegetative cells high levels H3K4me3 were detected at the promoter of the *rasG* gene which is expressed during vegetative growth (Robbins et al., 1992). Only low levels of H3K4me3 were present on the promoters of the early development gene *acaA*, the pre-spore marker *cotB* and the pre-stalk marker *ecmA* , all of which are silent in vegetative cells. The

enrichment of H3K4me3 at *rasG* was comparable to that at the constitutively active *coronin* gene. H3K9me2 and H3K9me3 were not detected on the promoters of any of the developmentally regulated genes which are silent during vegetative growth (*acaA, cotB* and *ecmA*). Chromatin immunoprecipitation was preformed on slugs at the 16 hour developmental stage with an antibody against H3K9me3. Compared to vegetative cells the amount of H3K9me3 on *skipper* was somewhat reduced while the amount on DIRS-1 was largely unaffected. This may be due to the previously described increase in *skipper* transcription during development (Kuhlmann et al., 2005). However no H3K9me was detected at the promoter of the inactive *rasG* gene, nor at the promoters of any of the other developmentally regulated genes examined.

The failure to detect H3K9 methylation at the promoters of any of the developmentally regulated loci (with the exception of *skipper* retrotransposon), suggests that unlike the situation in plants and animals, H3K9me is not involved in maintaining facultative heterochromatin (silent genes that can or will be activated under different conditions/ cell type or developmental stage). This result is in good agreement with the immunostaining data and consistent with the identification of SuvA as a member of the constitutive heterochromatin-specific Su[var]3-9-like family of histone methyltransferases.

4.4.1 Additional Chromatin Factors Involved in the Regulation of Development?

The *Dictyostelium* genome was examined for other chromatin binding/modifying proteins that may have a role in development. The DET1 protein is of particular interest as it is only found in a limited set of the crown group of eukaryotes, almost all of which are multicellular. It is essential for correct developmental regulation in *Drosophila* and *Arabidopsis* were it has been shown to bind the promoters of developmentally regulated genes. The molecular mechanism by which DET1 functions is still not clear, but it appears to act as a substrate adaptor for Cullin based ubiquitin ligase. Its putative interacting partner in *Dictyostelium*, CulD has not been characterised, but two other cullins, CulA and CulB are essential for proper development in this species (Mohanty et al., 2001; Wang et al., 2002).

4.4.2 DET1

Dictyostelium DET1 contains large homopolymer stretches found in many *Dictyostelium* proteins and is significantly larger than DET1 homologs present in other eukaryotes. However the DET1 domain itself appears to be well conserved. Loss of DET1 results in a slight decrease in growth rate of vegetative cells. DET1 was overexpressed with an N-terminal GFP or 6xMYC tag as C-terminal tags have previously been shown to adversely affect DET1 function, at least in *Arabidopsis* (Schroder et al., 2002). When transformed into *det1*$^{-}$ cells the overexpressed DET1 fusion protein rescued the mutant phenotype, suggesting that it is functional. Epitope tagged DET1

appeared evenly distributed in the nucleus but double labelling experiments showed that it was largely excluded from the H3K9me2 stained loci, suggesting it is exclude from constitutive heterochromatin. Stronger staining of DET1 was apparent at the rim of some nucleoli (but not in the nucleoli themselves). This result is consistent with previous observations showing that DET1 was enriched at the nucleolar periphery in *Arabidopsis* (M. Dubin, Masters thesis, Open University, 2006). Moreover, the finding that DET1 binds the non-coding region region of the rDNA palindrome, which was also found to localise to the nucleolar periphery in FISH experiments supports this result.

4.4.3 Role of DET1 in Regulating *Dictyostelium* Development

The *det1*$^{-}$ knockout displays a relatively mild phenotype in early development, the onset of aggregation is delayed approximately 30 minutes compared to the wild type, and the aggregation territories are enlarged, suggesting a possible defect in quorum sensing (Jain et al., 1992). At the mound stage up to 50% of the mounds (which vary in size more than those of wild type cells, but on average are significantly larger) arrest at this stage and do not proceed any further in development. The other mounds continue to form slugs that are often larger than wild type slugs, possibly as a result of the enlarged aggregation territories. These eventually form fruiting bodies with approximately 4 hours delay compared to wild type. The mutant fruiting bodies often have longer stalks than wild type, but the spore masses are not significantly larger than those of wild type in spite of the larger slug size. This indicates that the mutant may have an altered ratio of stalk-to-spore cells.

Compared to Ax2, *det1*$^{-}$ slugs expressing GFP under control of the pre-spore *cotB* promoter, showed a reduced zone of expression, with the expression being limited to the central region of the slug (approximately 50% of the cells), in contrast to Ax2 where the posterior 80% of the cells are labelled. In *det1*$^{-}$ slugs transformed with the pre-stalk marker [ecmAO]:GFP the pre-stalk zone was larger than that of wild type. In order to further examine this patterning defect, cells were transformed with LacZ reporter genes under the control of different promoters specific for different pre-stalk cell subtypes. Among the pre-stalk subtypes the PstB zone was largely unaffected. However the PstA zone was greatly enlarged and expression of *ecmA* from the proximal part of the promoter ([ecmA]:LacZ) was much stronger in the mutant than wild type. In contrast the PstO zone, where *ecmA* is expressed from the distal part of the promoter, the level of expression appears weaker than in Ax2 (Fig. 4.1). The border with the pre-spore zone was more diffuse and many [ecmO]:LacZ expressing cells are present in the pre-spore zone, in particular towards the posterior of the slug. As the posterior of the *det1*$^{-}$ slugs fail to express *[cotB]:GFP* or stain with an antibody against the SP35 protein (PsvA/EB4). It seams that these are likely to be pre-stalk like or anterior-like cells. Loss of DET1 appears to shift the ration of pre-spore:pre-stalk cells from 4:1 in wild type to approximately 3:2, or perhaps even 1:1 in *det1*$^{-}$ slugs.

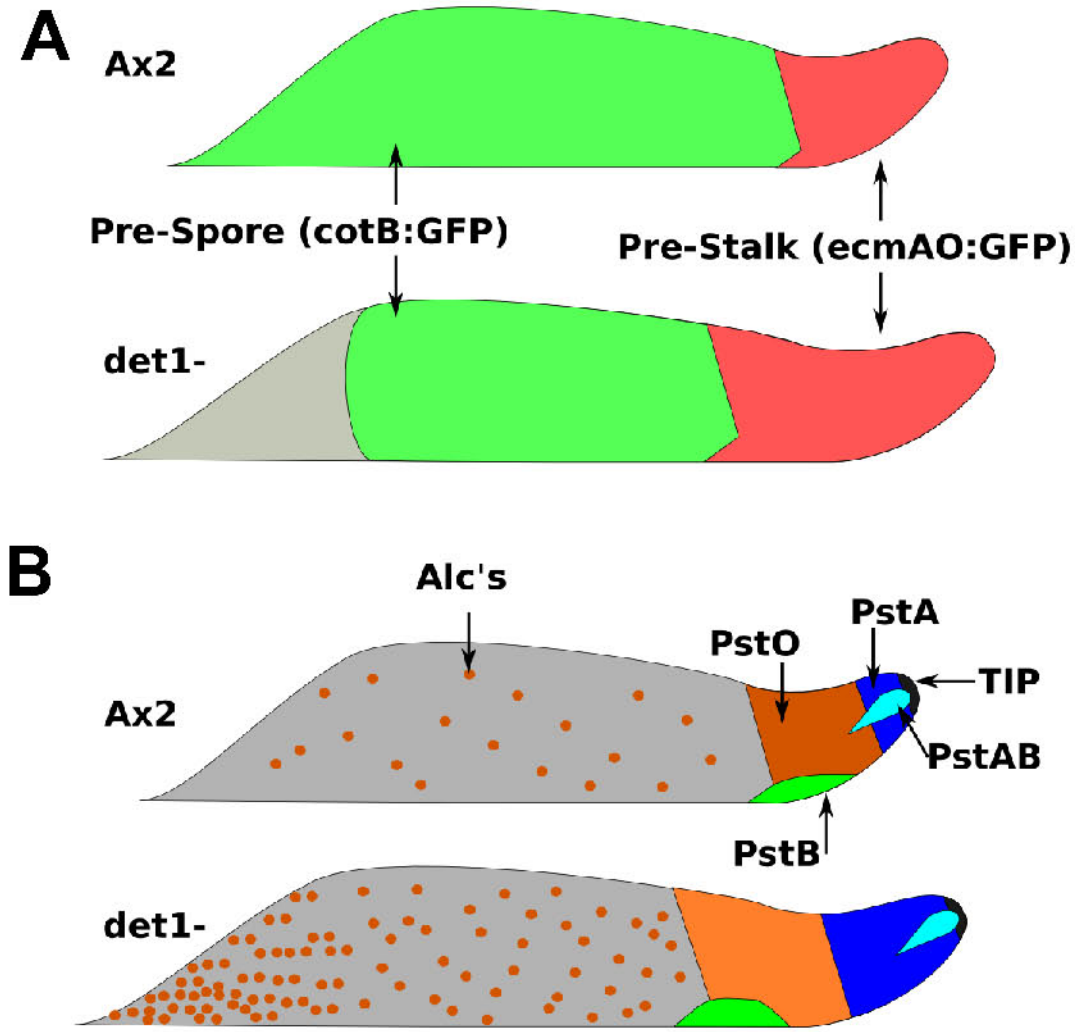


Fig. 4.1 **Effect of DET1 on *Dictyostelium* Development. A:** Schematic showing the observed distribution of the pre-spore marker [cotB]:GFP (in green) and the pre-stalk marker [ecmAO]:GFP (in pink) in wild type and *det1*⁻ slugs. **B:** Schematic showing the observed distribution of the pre-spore sub-types in wild type and *det1*⁻. Alc: anterior like cell.

DIF-1 is secreted by pre-spore cells (which are themselves DIF-1 insensitive) and causes cells, which would otherwise become pre-spore cells to enter the pre-stalk pathway (Brookman et al., 1987). DIF-1 induces nuclear accumulation of the bZIP transcription factor DimB which together with DimA and MybE regulates expression of the *ecmAO* gene from the distal (ecmO) part of the promoter while the proximal (ecmA) part of the promoter is regulated by another, DIF-1 independent pathway. The *det1*⁻ mutant expresses [ecmO]:LacZ in more cells, but the level of expression seems to be weaker than in wild type cells. In other organisms DET1 appears to act as a transcriptional repressor, so it seems probable that the increase in the percentage of pre-stalk cells in the *det1*⁻ mutant is due to the inability to repress the pre-stalk genes, as opposed to excessive DIF-1 production, which would presumably lead to high levels of expression. This is similar to plants, where the *det1*⁻ mutant is unable to maintain the repressed state of light regulated

genes in the dark, and then fails to fully activate them in the light (Schroder et al., 2002). The bZIP transcription factor DimB is a homolog of *Arabidopsis* HY5 and of c-Jun in mammals. In *Arabidopsis* DET1 binds HY5 regulated promoters, while in mammals a DET1 containing complex catalyses c-Jun degradation (Osterlund et al., 2000; Wertz et al., 2004; M. Dubin, Masters Thesis, The Open University, 2006).

Enrichment of DET1 was detected at the promoters of a number of developmentally regulated genes such as *acaA*, *ecmA*, *cotB* and *psvA*, but not the constitutively active *coronin* gene, nor the DIRS-1 retrotransposon. This demonstrates that DET1 may regulate these loci directly by modifying chromatin at their promotors or by modulating transcription factor activity and/or abundance at these promoters.

The developmental delay, asynchronous development and poor spore-forming ability observed in the *det1*$^{-}$ cells is reminiscent of that observed when histone hyperacetylation is induced by treating cells with HDAC inhibitors (Sawarkar et al., 2009). Given that histone acetylation has been shown to inhibit DET1 binding to chromatin (Benvenuto et al., 2002), it is plausible that developmental phenotype observe with HDAC inhibitor treatment may be due, at least in part, to impaired DET1 binding to its target genes.

5 Future Directions

Recently another set of cloning vectors for *Dictyostelium* were described (Veltman et al., 2009). Advantages of this system included the availability of Hygromycin resistant extrachromosomal vectors, and the ability to express two different transgenes on the same vector. Adapting the vectors described in this thesis to include these features would be relativity easy, and would further improve their versatility.

This study together with mass-spectrometric analysis has revealed the presence of a number of histone modifications in *Dictyostelium*. Of particular interest are a number of residues which appear to be acetylated only during mitosis. The possible role of these modifications in the cell cycle could be studied by mutating these residues in the endogenous histone and/or characterising the enzymes thought to be responsible for catalysing these modifications.

The characterisation of the centromeric histone H3 variant presented here, together with the findings that high copy transgene display centromere-like behaviour provides an attractive model to study centromere formation and behaviour. Experiments could focus on determining the requirements for the transgenes to act as true centromeres. Possible approaches include adapting the transgene sequence so that H3v1 has higher affinity for it. This might be achieved by of introducing the core centromere sequence from another species, together with expressing H3v1 mutated to have the targeting domain from this species. Another approach would be to artificially induce small RNAs against the transgene similar to those observed for DIRS-1 (using a hairpin or antisense construct). Finally, the presence of different proteins recruited to these transgenes could be studied using the recently developed PICh (proteomics of isolation chromatin segments) method (Dejardin and Kingston, 2009).

Further study of DET1 should provide a better understanding of how development is regulated. *Dictyostelium* is an attractive candidate for these studies due to the ease of molecular manipulation in this species and because in contrast to other species where DET1 has been studied the *Dictyostelium det1*$^{-}$ mutant is viable. Possible experiments include examining the targeting of DET1 to different loci in different mutant backgrounds (for example *dimB*$^{-}$ or *dimA*$^{-}$). The targeting of these and other transcription factors could also be examined in the *det1*$^{-}$ background. It would also be interesting to determine the targets of the DET1 associated ubiquitin ligase activity(s). Finally epistatis experiments should be carried out to determine whether or not the phenotypes observed in HDAC inhibitor treated cells are linked to those of the *det1*$^{-}$ mutant.

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