The Role of the Ino80 Chromatin Remodelling Complex Subunit Ies6 in Maintaining Genome Stability

A dissertation submitted for the degree of Doctor of Philosophy

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May 2010

To my family

Declaration

This dissertation is the result of my own work carried out at the MRC Genome Damage and Stability Centre at the University of Sussex between October 2007 and May 2010. This thesis is the result of my own work and includes nothing that is the outcome of work carried out in collaboration, except where specifically indicated and clearly stated in the text. I declare that this dissertation and the work presented herein have not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Acknowledgements

First and foremost I wish to thank my supervisor, Jessica Downs. I am deeply grateful for her support, unbeatable optimism and enthusiasm, as well as her kindness and humour both in and out of the laboratory. I would also like to thank all members past and present of the Downs group, especially Anna Chambers, Nicola Gardner, Megan Healy, Ola Zaid and Pete Brownlee for their support and friendship and the enjoyable time I have spent with them in the lab.. I am particularly grateful to both Nicola and Anna for their friendship and willingness to put up with my quirks (I doubt the buffers will remain in order of decreasing pH) and Anna again for her patience and inexhaustible laboratory knowledge! I also wish to thank all members of write-up area G4.14 for their friendship and support. I am further grateful to my second supervisor, Alan Lehman for his guidance and support, as well as Matt Neale for his advice, friendship and early morning 6.[53] Clapham-to-Lewes commuter camaraderie. Thanks also to Eva Hoffman and Carl X for their help with the tetrad dissection.

I also wish to thank both Nigel Savery, who is largely to blame for all this and Mark Dillingham for their enthusiastic introduction to the DNA repair research field and their support over the years, as well as all members of D.40 at the University of Bristol.

I am also grateful to Marie-Claire Giraud, Christine Etave and Anna Pasquer for their support over the years.

Thank you to a number of people who have been supportive and helpful over the last few months: thank you for the support and understanding from everyone at 45OQ, especially Jos Ramage and Hazel Atkin, as well as the interest and support from the gentlemen of Long Ditton Cricket Club. I also wish to thank Jon Faulkner who provided the support of an entire regiment. I am further grateful to Catherine Millington-Sanders for caring as much as she does.

Thank you to my closest friend, Rachael Middleton, who although far away has made herself close through many small gestures and to Laura Capra, my close friend of many years.

Importantly, thank you to the many families in which I have been at home: the Familles Duchenne, Janin and Traeger, who all treated me as one of their own. Special thanks also to Laura Traeger and Marie Janin, as well as in loving memory of Anita Traeger.

I am extremely grateful to Allan and Barbara Ormerod for their generosity, kindness and friendship and I am forever indebted to Allan, who proofread the entire thesis.

I cannot thank Thomas Ormerod enough for his support, understanding and patience over the last few months, but I also wish to thank him for his loving friendship and kindness.

Finally, I owe a great deal of this achievement to my family. Thank you to John Lee, not only for the Sunday afternoons spent with a chemistry set brewing pH indicator from cabbage, but also for his support, guidance and friendship over the years. I am grateful to my granddad, Willibald ("Dododo") Langefeld, the never-faltering foundation of our small family, for his loving support of his granddaughters and in loving memory of my grandmother, Ilse ("Nana") Langefeld, a truly special lady, who is sorely missed, but lovingly remembered. Thank you also to my sister Annabel Fenwick, who is the family sunshine and a cherished friend. Finally, but most importantly, I am forever indebted to my loving mum, Brigitte Lee, who does so much for her daughters. Thank you very much.

Abstract

The *S.cerevisiae* Ino80 chromatin remodelling complex is known to be involved in the DNA damage response at double strand breaks and has more recently also been shown to play a role at stalled replication forks. The many functions of this remodellor are likely to be mediated by different subunits of the complex. Interestingly, strains harbouring a deletion of the *IES6* subunit are hypersensitive to hydroxyurea and fail to stabilise stalled replication forks, phenocopying strains lacking the catalytic subunit, *INO80*, indicating a role for Ies6 within the complex's response to DNA damage. Although largely uncharacterised, Ies6 contains a YL1 domain, which is a putative DNA binding domain. *In vitro* DNA binding gel shift assays with recombinant Ies6 showed that this protein does possess DNA binding activity. Recombinant Ies6 bound both Holliday Junction and Y-fork DNA, as well as linear duplex DNA, displaying a small but reproducible preference for the two branch-structured DNAs. Recombinant Ies6 containing mutations in the protein's C-terminal YL1 domain were generated and a quadruple mutant, *ies6*-T119A/K122A/S127A/T129A, exhibited significantly reduced DNA binding activity compared to the wild-type protein. The importance of the DNA binding activity was investigated *in vivo*, and, in contrast to the wild-type strain, the DNA binding mutant of *IES6* failed to complement the deletion strain's HU-hypersensitivity. Interestingly, overexpression of Top3 or Cdk1, but not Top2 or Clb2 also rescued the *ies6* deletion strain's HU-hypersensitivity to near wild-type levels. Further investigation revealed that Ies6 is also required for the maintenance of correct cellular ploidy, as in the absence of *IES6*, cellular ploidy is seen to increase prior to a drift towards unregulated ploidy and aneuploidy, which are hallmarks of cancer. Notably, the protein's ability to bind DNA correlated with its ability to maintain cellular ploidy. We therefore propose that the Ino80 chromatin remodelling complex Ies6 subunit plays an important role in the maintenance of genomic stability.

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Abbreviations

53BP1	p53 binding protein 1
5-FOA	5-fluoro-orotic acid
Act1	actin
ADP	adenosine diphosphate
Amp	ampicillin
AP site	abasic site
APS	ammonium persulphate
Arp	actin related protein
ARS	autonomously replicating sequence
Asf	anti-silencing function
AT	ataxia-telengiectasia
ATM	ataxia-telengiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telengiectasia and Rad3-related
ATRIP	ATR interacting protein
BER	base excision repair
Bik1	bilateral karyogamy defect
bp	base pair

Brca 1	breast cancer susceptibility type 1 protein
BRCT	BRCA1 C-terminal
BSA	bovine serum albumin
Caf1	chromatin assembly factor 1
CEN	centromere
Cdc6	cell division cycle 6
Cdk1	cyclin dependent kinase 1
CHD	chromodomain,helicase, DNA binding
ChIP	chromatin immunoprecipitation
Chk1	checkpoint kinase 1
CIP	calf intestine phosphorylase
Crt1	constitutive rnr transcription 1
Cse4	chromosome segregation 4
Csm3	chromosome segregation in meiosis 3
C-terminus	carboxyl terminus
D.melanogastor	*Drosophila melanogastor*
Ddc1	DNA damage checkpoint 1
DDR	DNA damage response
D-loop	displacement loop
DMSO	dimethyl sulfoxide

Dna2	DNA synthesis defective 2
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNase1	deoxyribunuclease 1
dNTP	deoxyribonucleotide triphosphate
ds/ss DNA	double-/single-stranded deoxyribonucleic acid
DSB	double strand break
DSBR	double strand break repair
DTT	dithiothreitol
Dun1	damage uninducible 1
E.coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
Exo1	exonuclease 1
FACS	fluorescence activated cell sorting
FACT	facilitates chromatin transcription
FHA	forkhead-associated
H.sapiens	*Homo sapiens*
HAT	histone acetyltransferase
HDAC	histone deacetylase

HMG	high mobility group
HR	homologous recombination
HRP	horseradish peroxidise
HU	hydroxyurea
Ino80	inositol-requiring protein 80
IPTG	isopropyl β-D-1-thiogalactopyranoside
IR	ionising radiation
ISWI	imitation switch
LB broth	Luria Bertani broth
MBP	maltose binding protein
Mcm	mini chromosome maintenance
MCS	multiple cloning site
Mec 1	mitosis entry checkpoint 1
MEF	mouse embryonic fibroblast
MMS	methyl methane sulphonate
MNase	micrococcal nuclease
MOPS	3-(n-morpholino) propanesulfonic acid
Mrc1	mediator of the replication checkpoint 1
Mre11	meiotic recombination 11 homologue
Mw	molecular weight

Nbs1	nijmegen breakage syndrome 1
NER	nucleotide excision repair
NHEJ	non-homologous end joining
N-terminus	amino terminus
NuA4	nucleosome acetyltransferase of histone H2A/H4
ORC	origin recognition complex
ORF	open reading frame
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pfam	database of protein families
PIKK	phosphatidylinositol kinase-related kinase
poly(dIdC)	poly(deoxyinosinic-deoxycytidylic)
PMSF	phenylmethyl sulphonyl fluoride
Rad51	radiation sensitive 51
RFB	replication fork barrier
RFC	replication factor C
RNase	ribonuclease
RNR	ribonucleotide reductase
RPA	replication protein A

rpm	revolutions per minute
RSC	remodels the structure of chromatin
S.cerevisiae	*Saccharomyces cerevisiae*
S.pombe	*Schizosaccharomyces pombe*
SC	synthetic complete
SD	synthetic drop-out
SDS	sodium dodecyl sulphate
SDSA	synthesis-dependent single-strand annealling
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGD	Saccharomyces genome database
Sgs1	slow growth suppressor1
SSA	single-strand annealling
SWI/SNF	switch mating type/sucrose non-fermenting
Swr1	SWI/SNF-related protein
TCA	trichloroacetic acid
Tel1	telomere maintenance 1
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tof1	topoisomerase I interacting factor
TopBP1	topoisomerase binding protein

Tris	2-amino-2hydroxymethyl-propane-1,3-diol
UV	ultraviolet
v/v	volume per unit volume
w/v	weight per unit volume
X-GAL	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside
Xrs2	X-ray sensitive 2
YEATS	Ynl107 (*S.cerevisiae* YAF systemic name), ENL (eleven-nineteen leukaemia) protein, AF9 (human leukaemogenic protein), Transcription factor (TF) IIF small subunit
YPAD	yeast extract, peptone, adenine, dextrose
YY1	Yin-yang 1

1.1 DNA damage repair pathways are multifold and are crucial for protection of genomic stability

Cells are continuously exposed to DNA damage

Cells are continuously exposed to exogenous sources of DNA damage, such as ionising radiation (IR), or ultra-violet light (UV). In addition to these exogenous sources, endogenous cellular metabolism also has the potential to induce DNA damage.

Broadly, DNA damage can be grouped into two categories depending on whether the damage has occurred to the DNA bases, or the DNA backbone itself. DNA base damage such as O^6-methylguanine, in the form of reduced, oxidised or fragmented bases can be caused by IR or reactive oxygen species caused for example by UV. UV further has the potential to cause specific DNA base damage in the form of thymidine dimers and (6-4) photoproducts. Carcinogenic chemicals, as well as chemotherapeutic agents can form base adducts, thus generating a further form of DNA base damage (Sancar et al., 2004) Enzymes involved in DNA metabolism constitute an endogenous source of DNA damage by erroneous metabolism. Damage to the DNA backbone itself poses an even greater threat to the cell and arises as single-strand or double-strand DNA breaks. These can be generated by exogenous sources, such as IR, γ-rays or certain chemicals, but also arise throughout cellular metabolism, both by erroneous activity from enzymes involved in DNA metabolism and as legitimate intermediates of certain DNA metabolic pathways, such as homologous recombination (Sancar et al., 2004).

Cells are thus exposed to a huge spectrum of potential DNA damaging sources, many of which they are not able to evolve protective mechanisms for (e.g. IR) and which have the potential to cause a variety of very different damage to the cellular DNA content. Avoidance of genomic instability with its deleterious effects both on unicellular and multicellular organisms is of paramount importance and explains why cells have evolved a whole repertoire of specialised pathways to take care of repairing the inflicted damage as faithfully as possible.

Pathways for the repair of damage to DNA bases

Damage to DNA bases is usually repaired either by base excision repair (BER) or nucleotide excision repair (NER). In BER, the damaged base is recognised and removed by a specific gylcosylase, leaving an abasic site (AP site). In humans, the Apn1 (*S.cerevisiae*)/APE1 endonuclease (*H.sapiens*) recognises the AP site and recruits either Polβ or RFC/PCNA/Polδ/ε (DNA polε in budding yeast) for gap filling of a single nucleotide (short patch) or 2-10 nucleotides (long patch), respectively. Ligation of short and long patches is mediated by Ligase 3/XRCC1 and Ligase1, respectively. Ligation is mediated by DNA ligase Cdc9 in budding yeast. In contrast to the specific recognition of different damaged bases in BER, NER generically recognises more bulky, helix distorting DNA base damage. Recognition occurs by XPC, which recruits TFIIH, XPA and RPA to the site of damage. The helicase activities of TFIIH, XPB and XPD create a 20-30bp bubble around the site of damage and the damaged oligonucleotide is removed by sequential cleavage at the 3' and 5' sites by XPG and XPF/ERCC1, respectively, and the oligonucleotide is released. This is achieved by Rad 2 and Rad1-Rad10 in budding yeast. The gap is next filled by PCNA/DNA pol δ/ε and ligation is mediated by DNA ligase 1 (*S.cerevisiae* Cdc9) (Caldecott, 2007; Lindahl and Wood, 1999).

DNA double strand breaks

DNA double strand breaks (DSBs) are potentially the most deleterious DNA damage that cells can encounter and may be caused by an exogenous source, such as Ionising radiation (IR) or may arise endogenously, for example as a by-product of replication stress resulting in a collapsed replication fork. DSBs constitute a particular danger for the cell, as the free DNA ends generated by the break have the potential to recombine with a repetitive sequence on another chromosome. Furthermore, the free DNA ends are substrates for non-homologous end-joining (NHEJ) between non-homologous ends. Such aberrant DNA processing can lead to

gross chromosomal rearrangements, such as inversion, duplication, translocation or deletion, which are hallmarks of cancer (Aguilera and Gomez-Gonzalez, 2008).

Cells faced with a DSB must therefore be able to halt progression of the cell cycle, not only to avoid the propagation of incorrect genetic information to their progeny, but also to allow for the activation of the appropriate DNA repair pathway, the recruitment of the repair pathway's components and time for processing of the damage by the repair factors.

This molecular response portrays many hallmarks of a traditional cellular signalling pathway: Initially, the damage must be recognised by a "damage sensor". The binding of the sensor to the damage, analogously to the binding of a ligand to a receptor in a classic metabolic cell signalling pathway then elicits a cascade of downstream cellular events. This signalling is achieved by the so-called signal transducers and allows for amplification of the signal, as well as accounting for the resulting, pleiotropic cellular changes. These changes in the cellular household are achieved via "effector components" of the signalling pathway, permitting the cell to react and adapt to the initial signalling stimulus.

The MRX (MRN) complex senses DSBs

The following events in DSB repair take place in S/G2 phase of the cell cycle, in which DSB repair by HR predominates, whereas NHEJ is the preferred repair pathway in G1 (Ira & Foiani 2004).

The primary DSB "sensor" in the *S.cerevisiae* cellular response to a DSB is the MRX complex (MRN in *H.sapiens*). The complex consists of two highly conserved subunits, Mre11 and Rad50, as well as Xrs2 (*S.cerevisiae*) or Nbs1 (*H.sapiens*) The MRX complex recognises and binds DNA ends, thereby acting as an "end-bridging" or "tethering" molecule, keeping the two broken ends in close proximity (Chen et al., 2001; de Jager et al., 2001).

MRX is partially responsible for the next fundamental step in the processing of the DSB, although there is some redundancy with other nucleases, such as Exo1. Indeed, the complex resects the DSB ends in a 5'-3' manner, thereby generating a stretch of single-stranded DNA (ssDNA). The ssDNA generated by the MRX complex and other nucleases is coated with replication protein A (RPA) and together, the MRX complex and the RPA-coated ssDNA recruit the key players of the downstream signalling cascade, Mec1 (ATR) and Tel1 (ATM) (Alani et al., 1992; Harrison and Haber, 2006; Krogh and Symington, 2004; Nakada et al., 2004; Rouse and Jackson, 2002a, b).

The DNA damage checkpoint is mediated by the checkpoint kinases Mec1 (ATR) and Tel1 (ATM)

Mec1 and Tel1 are both members of the phophatidylinositol 3' kinase-like kinase (PIKK) family and are pivotal to the DSB response as they are key to linking recognition of DNA damage with signal transduction due to their kinase activity. This family of kinases phosphorylate protein substrates as opposed to lipids (Norbury and Hickson, 2001) on a conserved motif consisting of a serine or threonine residue followed by a glutamine (S/T-Q) (Abraham, 2004).

The importance of the Mec1 kinase is highlighted by the inviability of the *mec1* deletion strain (Desany et al., 1998). This is mirrored in human cells, where ATR is also an essential gene (Abraham 2001) and disruption of ATR leads to embryonic lethality (Brown and Baltimore, 2000; de Klein et al., 2000). The link to human disease further underlines the importance of both these kinases: indeed ATM-deficient mammalian cells are sensitive to ionising radiation and mutations in ATM and ATR have been identified as the genetic cause of the diseases ataxia-telengiectasia (AT) and Seckel syndrome, respectively (O'Driscoll et al., 2003; Savitsky et al., 1995). Both diseases are characterised by distinct clinical features, however AT is

importantly characterised by a definite cancer-predisposition, whilst a possible Seckel syndrome-related cancer predisposition remains unclear.

Correct spatiotemporal recruitment of these kinases is an important part of the coordination of the DNA damage checkpoint and in the case of Mec1, is achieved by an RPA-ssDNA signal as well as the use of the kinase binding partner, Ddc2 (ATRIP) (Cortez et al., 2001; Lisby et al., 2004; Paciotti et al., 2000; Rouse and Jackson, 2002b). Ddc2 and Mec1 are likely to form a functional complex, as deletion of *DDC2* mimics deletion of *MEC1* (Paciotti et al., 2000). It was thought likely for Ddc2 to play a role in the recruitment of the complex and in 2003 it was demonstrated that the recruitment of Mec1 is indeed achieved by recognition and binding of Ddc2 to RPA-coated ssDNA, which allows for the recruitment of the Mec1-Ddc2 complex (Zou and Elledge, 2003). This also accords the ssDNA generated at sites of damage a fundamental role as a damage signal, which is corroborated by its ubiquity in the early stages of many different types of DNA damage.

The recruitment of Tel1 (ATM), the second DNA damage kinase is mediated by the MRX (MRN) complex bound to DNA via a direct interaction with the MRX (MRN) subunit Xrs2 (Nbs1) (Lisby et al., 2004; Nakada et al., 2003; Usui et al., 2001). Tel1 in budding yeast seems to play less of a crucial role than its human orthologue, ATM, but analogously to ATM it is not an essential gene. Strains harbouring a deletion of *TEL1* are however not significantly sensitive to DNA damaging agents, whereas their human counterparts are highly IR-sensitive (Aylon and Kupiec, 2004). Strains lacking both *tel1* and *mec1* are however more sensitive to DNA damaging agents than the *mec1* deletion alone (Aylon and Kupiec, 2004), suggesting there is some redundancy in the two kinases' targets. It is the MRX complex bound to the DNA ends of DSBs that is responsible for recruiting Tel1, which interacts with the Xrs2 subunit of the complex (Falck et al., 2005; Nakada et al., 2005; Usui et al., 2001). The reason for the more extensive function of

ATM in human cells compared to budding yeast Tel1 may be due to the fact that DNA ends generated at DSBs persist for longer in an unressected form in human cells, thus generating a lenghtier ATM activation signal via MRX (Garber et al., 2005). In budding yeast, Tel1's primary function is in regulating telomeres (Greenwell et al., 1995).

Traditionally, the DNA damage kinases are thought to respond to different types of DNA damage, with ATM being activated following IR-induced DSBs and ATR activation occurring in response to UV-induced DNA damage and replication stress (Abraham, 2001). Although it was expected that the kinases, as they respond to different types of damage, activated the DNA damage checkpoint independently of each other (Abraham, 2001), it was more recently shown that there exists a certain level of crosstalk between the ATM and ATR DNA damage response activation pathways (Jazayeri et al., 2006). Indeed, following IR-induced DSBs, MRN recruits ATM and resects the DNA, thus recruiting RPA. This in turn recruits and activates ATR, leading to a collaborative activation of the DNA damage response (Jazayeri et al., 2006). This crosstalk however is unique to the S and G2 phases of the cell-cycle and is lost following use of CDK inhibitors (Jazayeri et al., 2006). This is likely to function analogously in budding yeast, as Mec1 activation of the DNA damage response is regulated throughout the cell cycle by CDK activity and is limited to S/G2 (Ira et al., 2004; Pellicioli et al., 2001).

The checkpoint clamp Ddc1-Mec3-Rad17 (Rad9-Hus1-Rad1) and clamp loader Rad24-RFC (Rad17-RFC)

A further complex involved in the recognition of DSBs and recruitment of downstream signalling proteins is the budding yeast Ddc1-Mec3-Rad17 complex. The orthologous human complex is composed of Rad9, Hus1 and Rad1, hence the complex is termed "9-1-1 complex". These three subunits display limited homology to PCNA and do form a sliding clamp analogously to PCNA (Dore et al., 2009; Venclovas and Thelen, 2000). RPA has been shown to

be important in the recruitment of the complex to a DSB (Lisby et al., 2004; Zou et al., 2003), but extensive resection is not required (Nakada et al., 2004). Indeed, the clamp is loaded at ss/dsDNA junctions, which are present at breaks even after minimal resection (Majka and Burgers, 2003; Zou et al., 2003). Recruitment of the Ddc1-Mec3-Rad17 complex does however require Rad24 (Lisby et al., 2004), which forms a complex with the Rfc2-5 subunits in the place of Rfc1. This Rad24-RFC (Rad17-RFC in humans) takes on the role of a clamp loader for Ddc1-Mec3-Rad17 onto the DNA, analogously to the loading of PCNA by RFC onto DNA (Majka and Burgers, 2003; Zou et al., 2003). Aside from the recruitment of substrates for phosphorylation and therefore signal transduction by Mec1, the checkpoint clamp may also play a role in the actual recruitment and activation of Mec1 (Barlow et al., 2008).

Downstream checkpoint signalling is mediated by Rad9 (53BP1), Chk1 (Chk1) and Rad53 (Chk2)

Mec1 and Tel1, the DNA damage checkpoint kinases are responsible for the downstream signalling cascade, composed of adaptor proteins and transducing kinases following the detection of DNA damage (see Figure 1.1.1). Rad9 has been found to be such an "adaptor protein". Dimerisation of the Rad9 protein mediated by its C-terminal BRCT motifs is required for its checkpoint function. The protein is a main target of the checkpoint kinases, resulting in its hyperphosphorylation (Emili, 1998). The hyperphosphorylated form of Rad9 is recognised and bound by one of the effector kinases, Rad53, via its FHA domains. Rad9 therefore acts as a mediator or recruitment platform, bringing the effector kinase into proximity of the PIKKs, resulting in activation and hyperphosphorylation of Rad53 (Durocher et al., 2000). The 9-1-1 complex has also been implicated in the recruitment of both Rad9 and Rad53 (Emili, 1998). Rad9 is also thought to be important for activation of the second effector kinase, Chk1, although Chk1 does not seem to contain domains capable of specifically recognising

hyperphosphorylated Rad9 and the exact mechanism of Chk1 activation is unclear (Harrison and Haber, 2006).

The effector kinases, Chk1 and Rad53, are also serine-threonine kinases and following their activation by Mec1-and/or Tel1-dependant hyperphosphorylation, are responsible for the relaying of the DNA damage checkpoint response to regulatory proteins of the cell cycle machinery and the execution of a global DNA damage response (Figure 1.1.1) (Norbury and Hickson, 2001; Sancar et al., 2004). This leads, for example, to a prolonged arrest of the cell cycle via stabilisation of Pds1 and maintenance of CDK acitivity, as well as the induction of damage-inducible genes, for example the ribonucleotide reductase genes (Harrison and Haber, 2006). Furthermore, the DNA damage signalling cascade also leads to the establishment of a distinct chromatin environment surrounding the DSB site (for a more extensive discussion see section 1.4).

In human cells, there are two proteins in the ATM/ATR pathway which contain tandem BRCT domains: 53BP1 and BRCA1, which are likely to function homologously to Rad9 (S.cerevisiae). Chk1 and Chk2 are the human orthologues of budding yeast Chk1 and Rad53, respectively. The mammalian DNA damage response is however more complex than the response seen in budding yeast. There is a larger number of proteins involved in the DNA damage response and some, such as p53, have no orthologue in budding yeast, thus adding further intricate layers of complexity to the cells response and the organism's survival strategy to DNA damage (Norbury and Hickson, 2001). The importance of many of these mammalian proteins is highlighted by their link to cancer, such as the link of Brca1 and Brca2 mutations to hereditary breast cancer (Lynch et al., 2008; Silva et al., 2008).

Organism and cell cycle dependent preference for DBS repair pathways

Cells have two repair pathways at their disposal for the repair of DSBs: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is the more faithful repair pathway, compared to NHEJ and requires a homologous template. Preference of one pathway over another does not simply reflect differences between organisms, but also relies on the availability of a homologous template, which is dependent on both ploidy and the stage of the cell cycle (Richardson and Jasin, 2000). Accordingly, HR is preferentially employed in the S- and G2-phases of the cell cycle, whilst NHEJ is the main repair pathway in G1 (Ira et al., 2004; van Gent et al., 2001). In yeast, HR is by far the preferred pathway and NHEJ is down-regulated to avoid the generation of genomic instability (Lieber et al., 2003). In humans however, NHEJ plays a far more prominent role, due to the risk of generation of genomic instability being lower due to the extensive intergenic regions of the genome, as well as the physical impracticality of performing a homology search across the entire genome (Lieber et al., 2003).

Repair by homologous recombination

The basic model for homologous recombination is highly conserved throughout all organisms and is centred on four reaction steps: initiation, homologous pairing, DNA heteroduplex extension and finally resolution (see Figure 1.1.2).

The initiation step is important for the generation of ssDNA at the break site onto which the recombinase Rad51 can be loaded. This is achieved by the action of a nuclease, such as MRX, which generates 3’ ssDNA tails at break sites (see Figure 1.1.2) (Kowalczykowski, 2000; Stracker et al., 2004). The resected DNA is bound by RPA (see Figure 1.1.2), which both protects it from nucleolytic degradation, as well as eliminating secondary structure inhibitory to recombination (Alani et al., 1992). Next, with the help of recombination mediators, Rad51 is loaded onto the ssDNA, replacing the previously bound RPA and forming a nucleoprotein

filament along the length of the ssDNA (see Figure 1.1.2). Rad51 is the eukaryotic orthologue of bacterial RecA and forms similar helical filaments on the DNA to its bacterial counterpart (Conway et al., 2004). Rad51 loading requires mediators such as Rad52, which displaces RPA prior to loading Rad51 (Song and Sung, 2000). Rad55 and Rad57 are also classified as mediators and are likely to act by stabilising the filament formed (Hays et al., 1995). Formation of this filament is essential for the ensuing "homology search" of the ssDNA with any dsDNA available, as the nucleoprotein filament constitutes the actual active species of the Rad51 protein. The mechanism of the homology search is unclear, but is probably based upon simple and passive diffusion along, and continuous comparison of, two DNA molecules following a random collision, with the ssDNA being employed to confer specificity to the search (Kowalczykowski, 2000; Krogh and Symington, 2004). Once the homologous DNA has been located, the two molecules undergo synapsis between the two DNA molecules and strand exchange (see Figure 1.1.2) (Sung and Robberson, 1995). Joint formation involves the invasion of the ssDNA and displacement of one of the strands from the homologous duplex thereby generating a D(displacement)-loop (see Figure 1.1.2). A further member of the Rad52 epistasis group and a dsDNA-dependent ATPase of the Swi2/Snf2 family, Rad54, facilitates heteroduplex extension (Heyer et al., 2006). The invading 3' end of DNA is used as a primer for synthesis of DNA, using the undamaged, homologous DNA as a template.

These events generate a complex DNA molecule requiring resolution, for which two models have been proposed (see Figure 1.1.2). In the DSB repair model, a further, Rad52 epistasis group-mediated strand invasion event occurs (Aylon and Kupiec, 2004), leading to the formation of a double Holliday Junction (Holliday, 1964, 1974). Holliday Junction resolution is a semi-conservative process (Symington, 2002) and is mediated by Yen1/Gen1 in budding yeast and humans, respectively (Ip et al., 2008; West, 1996). In the second model, termed synthesis-dependent strand annealing (SDSA), only one strand invasion occurs and the invading strand is

displaced from the homologous template following DNA synthesis allowing it to re-anneal to its original complimentary strand and act as a template for DNA synthesis (Haber et al., 2004; Symington, 2002).

DSBs occurring in regions of repetitive DNA may be repaired by a further HR-based repair pathway termed single-strand annealing (SSA). This repair process relies on the direct annealing of the ssDNA tails generated following the break, thus foregoing the need for the full complement of HR proteins (Sung and Klein, 2006; Symington, 2002).

In higher eukaryotes the basic mechanism of DSB repair by HR is highly conserved, however due to a far larger number of mediator proteins, the actual execution of the molecular events is much more complex: Rad51 alone has numerous paralogues (Rouse and Jackson, 2002a).

DSB repair by non-homologous end-joining

The main DNA damage sensor in the NHEJ pathway of DNA repair is the Ku complex, which in eukaryotes consists of the Ku70-Ku80 heterodimer (Downs and Jackson, 2004). The Ku complex binds to the DNA ends in a DNA sequence-independent manner, thereby fulfilling both a molecular tethering function and protecting the DNA ends from spurious nuclease activity and loss of genetic information (Downs and Jackson, 2004; Mimori and Hardin, 1986). In budding yeast, the MRX complex is also recruited to the DNA ends (Daley et al., 2005). There is no requirement for extensive homology, as is the case for repair by HR, but the NHEJ pathway does display a preference for repair at regions of microhomology (Lieber et al., 2003). Following the alignment of the DNA ends, they are rejoined by Lig4/Dnl4 (H.s. DNA ligase IV) and Lif1 (H.s. XRCC4) (for review see (Daley et al., 2005; Downs and Jackson, 2004; Lieber et al., 2003)). In higher eukaryotes, the Ku complex also recruits the DNA-PKcs, serine-threonine kinases to the site of the break, as well as the Artemis nuclease, which may allow for a small

amount of nucleolytic processing at the DNA ends (Lieber et al., 2003; Moshous et al., 2001).Although no orthologues of DNA-PKcs or indeed the Artemis nuclease have yet been identified in lower eukaryotes, such proteins may exist and it is probable that the mechanism of repair by NHEJ is highly conserved across these organisms.

1.2 Replication and its role in maintaining genomic integrity

Replication and the maintenance of genome integrity

During cellular S-phase, the entire cellular genome must be faithfully copied once by the process of replication in order to be passed on to the cell's progeny. Replication is a complex DNA metabolic process mediated by large multi-enzymatic complexes and indeed DNA is at its most vulnerable throughout S-phase (Aguilera and Gomez-Gonzalez, 2008). Problems occurring during replication are not only liable to the generation of DNA damage as ssDNA nicks, DSBs or aberrant DNA structures but may also give rise to more widespread chromosome rearrangements, translocations and the general genome instability (Aguilera and Gomez-Gonzalez, 2008; Branzei and Foiani, 2009, 2010; Mizuno et al., 2009; Paek et al., 2009). Replication therefore has the potential to be a threat to genome integrity and indeed inappropriate replication or response to problems occurring during replication contributes to cancer (Kastan and Bartek, 2004). It is therefore not surprising that an intricate and highly conserved cellular network exists to coordinate replication and repair.

Replication is initiated by origin licensing and firing

DNA replication commences from so-called origins of replication, which in yeast are constituted of 3-4 repeats of a 10-15bp sequences contained within a region of 100-150bp. This includes the ARS element (Autonomously Replicating Sequence), which is both highly-conserved and essential, as well as less conserved sequences called B-elements (Bell and Dutta, 2002). These sequences are bound by the origin recognition complex (ORC), composed of six subunits (Orc1-6), which lead to a demarcation of the site for later initiation of DNA synthesis (Bell and Stillman, 1992; Rao and Stillman, 1995; Rowley et al., 1995). It is thought

that ORC binds A-rich sequences within the A and B elements and that its binding requires binding though not hydrolysis of ATP (Austin et al., 1999; Bell and Stillman, 1992; Chesnokov et al., 2001; Klemm et al., 1997). Timing and regulation of ORC binding varies across organisms, however in *S.cerevisiae* (and in *S.pombe*), it is believed that ORCs constitutively bind origin sites throughout the cell cycle (Aparicio et al., 1997; Ogawa et al., 1999; Santocanale and Diffley, 1996; Tanaka et al., 1997). Cdc6, a member of the AAA+ ATPase family (Neuwald et al., 1999), along with Cdt1, are the next proteins to fulfil an important role in the assembly of the pre-replication complex (pre-RC) (Bell and Dutta, 2002; Zegerman and Diffley, 2009). Recruitment of Cdc6 relies on the presence of ORC and is in turn responsible for the "licensing", that is the loading of the Mcm2-7 helicases in an inactive form (Aparicio et al., 1997), for which ATP binding and hydrolysis are thought to be important (Elsasser et al., 1996; Perkins and Diffley, 1998; Weinreich et al., 1999). The amount of available Cdc6 is regulated throughout the cell cycle as in S-phase it undergoes ubiquitin-dependent degradation following phosphorylation by the S-CDK protein complex, the catalytic subunit of which is Cdk1, a protein belonging to the cyclin-dependent protein kinase family of proteins which are key regulators of cell cycle progression (Diffley, 2004). Upon entry into S-phase, the pre-RC must be converted into a bi-directional replication fork (Zegerman and Diffley, 2009). This requires both Mcm10, which is also required for replication progression at later stages, as well as Cdc45 and Dpb11, thought to play a role in loading the replicative polymerases (Aparicio et al., 1999; Masumoto et al., 2000; Zou and Stillman, 2000). The transition to active S-phase DNA replication lies under the control of the kinases Cdc7/Dbf4 and S-CDK. In budding yeast, the presence of ORC is necessary for the recruitment of Cdc7/Dbf4 (Pasero et al., 1999), which is thought to phosphorylate the Mcms (Lei et al., 1997; Weinreich and Stillman, 1999). In *S.cerevisiae*, S-CDK activity is an absolute requirement for the initiation of DNA replication (Bell and Dutta, 2002) and phosphorylates both Sld2 and Sld3, leading to the recruitment of Dpb11(Zegerman and Diffley, 2007).

Replication is achieved by a multi-subunit protein complex, the replisome

The replisome leaving the origin following origin licensing and firing is a large multi-protein complex (see Figure 1.2.1). The polα/primase component of the complex is pivotal for initiation of DNA synthesis from the replication fork as it synthesises a short RNA primer which it extends, with a small stretch of DNA (Waga and Stillman, 1998). Polα/primase synthesises the initial primer for the leading strand, as well as all lagging strand primers. Polα however lacks processivity and dissociates rapidly from the DNA (Murakami and Hurwitz, 1993). Replication factor C (RFC) is a further important component of the complex; it binds to primer-template junctions, or nicks in duplex DNA and loads proliferating cell nuclear antigen (PCNA) at these sites (Cai et al., 1996; Lee et al., 1991; Tsurimoto and Stillman, 1990, 1991). PCNA is a trimeric complex, which encircles the DNA and acts as a processivity factor for the replicative polymerases δ and ε (Bauer and Burgers, 1988; Bravo et al., 1987; Prelich et al., 1987a; Prelich et al., 1987b; Tan et al., 1986). Further components that are found in the replication fork are the Rad27 nuclease, RNaseH1 and the Mcm replicative helicases. DNA synthesis thus proceeds following primer synthesis by DNA polα/primase and polymerase switching to polδ and ε, mediated by RFC/PCNA (Tsurimoto et al., 1990; Tsurimoto and Stillman, 1991; Waga and Stillman, 1994) and proceeds processively on the leading and discontinuously between Okazaki fragments on the lagging strand. Exactly how the activities of polδ and ε are distributed are unclear, though both seem necessary for replication to occur and recent work favours a model in which polδ and polε operate on the lagging and leading strands respectively (Nick McElhinny et al., 2008). Okazaki fragment removal, or maturation, is achieved by the concerted action of Rad27 (hFen1), RNaseH1 and Dna2 and the gaps are filled by the replicative polymerases prior to ligation by DNA ligase1 (Waga and Stillman, 1998).

Barriers to replication fork progression can lead to replication fork stalling

Replication proceeds in the above described manner and must ensure that the entire genome duplication is achieved and mediated by the replication forks from all origins fired during S-phase. This in itself is no small task, however it is made considerably harder by the presence of naturally occurring replication fork barriers (RFBs) and fragile sites (Labib and Hodgson, 2007). RFBs can be found at certain DNA loci, such as the rDNA sequences in budding yeast, where they ensure replication occurs in the same direction as transcription (Labib and Hodgson, 2007). The tRNA genes have been identified as so-called "fragile sites" in *S.cerevisiae*, which lead to a slowing of replication (Admire et al., 2006) and an increase in the generation of recombinogenic structures due to the presence of inverted repeats in the DNA sequence (Lemoine et al., 2005). It must also be taken into account that the DNA a replication fork must travel along is coated with protein complexes which the replicative helicases must contend with to allow successful passage of the replication fork and the DNA itself may adopt secondary structures inhibitory to replication fork passage (Labib and Hodgson, 2007; Zegerman and Diffley, 2009). Furthermore, replication forks may be slowed or stalled due to the action of exogenous DNA damaging agents or replication inhibitors. Exogenous agents lead to the stalling of replication forks either by inhibition of the replicative helicases by strand cross -linking or bulky DNA adducts. Moreover, exogenous agents may cause replication forks to stall by inhibition of the replicative polymerases, which may occur by small DNA adducts (induced by MMS, for example), by nucleotide-depletion (induced by HU) or by competitive inhibition (with for example aphidicolin) (Zegerman and Diffley, 2009) (see Figure 1.2.2).

Stalled replication forks activate the S-phase checkpoint

When replication forks encounter damage and the replisome remains intact, following removal of the "road block", replication may restart. However, encountering damage may also lead to

replication fork disassembly and collapse, generating further DNA damage (see Figure 1.2.2) (Aguilera and Gomez-Gonzalez, 2008). This can arise by conversion of ssDNA strand nicks to DSBs. Uncoupling of leading and lagging strand synthesis commonly results in fork reversal, thus generating Holliday Junction or "chicken foot" DNA structures that the cell must process to restart recombination and avoid DNA damage and loss of genetic information (see Figure 1.2.2) (Aguilera and Gomez-Gonzalez, 2008). Stalled replication forks are liable to fork collapse and thus the generation of DSBs thereby acting as a signal for checkpoint activation and the downstream cellular signalling cascade. A further important mechanism of checkpoint activation is thought to be via the generation of tracts of ssDNA coated with RPA (Zegerman and Diffley, 2009). These may occur due to continued unwinding by the replicative helicases following stalling of the polymerase (Byun et al., 2005; Walter and Newport, 2000). The ssDNA-RPA acts to recruit Mec1 and Rad24/9-1-1 (see previously), as well as Dpb11 (TopBP1), which binds both the 9-1-1 and Mec1 via Ddc2 and is important for Mec1 activation (Kumagai et al., 2006; Majka and Burgers, 2007; Mordes et al., 2008a; Mordes et al., 2008b; Wang and Elledge, 2002). Mec1 checkpoint activity leads to downstream activation of Rad53 via hyperphosphorylation of mediator proteins, leading to a coordinated cellular response to enable repair of the damage and allow replication fork restart.

The S-phase checkpoint is mediated by Mrc1 and Tof1/Csm3

Although Rad9, the key mediator of the DNA damage signalling cascade acts as a checkpoint mediator in response to replication fork stalling, it is thought to play far less of a role than it does at DSBs outside of S-phase (Alcasabas et al., 2001). Indeed, the mediator of the replication checkpoint fulfilling an analogous role to Rad9 in the DSB response is Mrc1 (Alcasabas et al., 2001) and has been shown to be required for S-phase in replication stress but does not function in the G2/M DNA damage checkpoint (Alcasabas et al., 2001). The protein is

phosphorylated both by Mec1 and Rad53 in response to replication stress (Alcasabas et al., 2001; Osborn and Elledge, 2003) although it is not sure how this is mediated, as no direct interaction between the two proteins has been confirmed (Branzei and Foiani, 2009). In mammals however, a direct interaction has been confirmed for Claspin and Chk1, which is essential for Chk1 phosphorylation (Kumagai and Dunphy, 2000, 2003; Yoo et al., 2006). Furthermore it has been shown that Mrc1 plays a role in replication independent of the replication checkpoint and is associated with the replication fork during normal S-phase (Katou et al., 2003; Osborn and Elledge, 2003) and binds as a late component following initiation (Osborn and Elledge, 2003), interacting both with Cdc45 and the MCM helicases (Katou et al., 2003). Indeed, the presence of Mrc1 at the replication fork is required for normal rates of fork progression (Osborn and Elledge, 2003; Szyjka et al., 2005). A further protein, Tof1 and its binding partner Csm3 have been shown to play a similar role and are also both present at the replication fork during normal S-phase, as well as playing a role in the replication checkpoint (Katou et al., 2003). In *mrc1* or *tof1* deletion strains, uncoupling of the polymerase and the helicases is observed, leading to tracts of unreplicated DNA, which may play a role in the activation of the checkpoint, as well as inhibiting fork restart, suggesting these proteins play a role in stabilisation of paused forks (see Figure 1.2.3) (Katou et al., 2003). Interestingly Tof1 and Csm3 are important for replication fork stabilisation at RFBs, where Mrc1 is dispensable (Calzada et al., 2005; Szyjka et al., 2005). More recently, a further interaction of Mrc1 with the replisome component polε has been described (Lou et al., 2008), which may explain the destabilisation of polε at stalled replication forks in *mrc1* deletion strains, as well as the uncoupling effect between polymerase and helicases observed in these mutants (Lou et al., 2008).

The S-phase checkpoint activates a transcriptional response in yeast

The effector kinases activate a number of cellular pathways in response to replication fork stalling and as part of the S-phase checkpoint. It has been suggested that eukaryotic cells, analogously to bacteria, mount an "SOS transcriptional response" (Fry et al., 2005). In yeast, Mec1 and Rad53 phosphorylate and activate the Rad53-related kinase Dun1 (Allen et al., 1994; Huang et al., 1998; Zhou and Elledge, 1993). Dun1 in turn phosphorylates Crt1, a transcriptional repressor of a variety of yeast repair genes (Huang et al., 1998). Phosphorylated Crt1 is no longer able to bind to the X-box upstream of these genes, thus leading to their transcriptional upregulation (Huang et al., 1998). In human cells, the transcription factor E2F-1 is phosphorylated by the checkpoint kinases (Lin et al., 2001b; Stevens et al., 2003) and also leads to increased expression of proteins involved in DNA replication and repair (Polager et al., 2002; Ren et al., 2002). Although numerous genes have been reported to be up-or downregulated in response to DNA damage, the significance of these changes are still unclear and their contribution to the recovery of stalled forks may possibly be very little, as the inhibition of protein synthesis does not affect the survival of budding yeast after treatment with hydroxyurea (Branzei and Foiani, 2009; Zegerman and Diffley, 2009).

The cellular nucleotide household genes are upregulated following activation of the S-phase checkpoint in yeast

The ribonucleotide reductase (RNR) is a multi subunit complex, composed of the two subcomplexes Rnr1 and sometimes Rnr3, as well as Rnr2-Rnr4 and is responsible for the rate-limiting step in dNTP synthesis. Maintenance of the correct dNTP pool for each of the four nucleotides is critical and regulation of RNR activity is achieved by multiple mechanisms including direct inhibition, transcriptional regulation and redistribution between the cytoplasm and the nucleus. In normal S-phase, RNR activity increases both in yeast and human cells (for

review see (Nordlund and Reichard, 2006). A further downstream effect of the S-phase checkpoint is upregulation of the RNR and thus an increase in available nucleotides, which is mediated by the Dun1 kinase. The phosphorylation of Dun1 by Rad53 and subsequent inhibition of Crt1 mediated by its Dun1-dependent phosphorylation (see above) leads to the inhibition of Crt-repression of the *RNR2*, *RNR3* and *RNR4* genes, thus allowing for an increase in RNR complex (Huang et al., 1998). Activated Dun1 further phosphorylates Sml1, the inhibitor of the Rnr1 subunit, thus targeting it for degradation and leading to a rise in available Rnr1 (Zhao and Rothstein, 2002). Lastly, Dun1 also phosphorylates Dif1, again targeting it for degradation, which increases Rnr2-Rnr4 retention in the nucleus, by an as yet unclear mechanism (Lee et al., 2008). In fission yeast, RNR is regulated somewhat analogously to budding yeast, however a protein known as Spd1 fulfils the functions of both Sml1 and Dif1 (Liu et al., 2003; Nordlund and Reichard, 2006). This part of the checkpoint response does not seem to be conserved in human cells, as dNTP levels do not seem to display a similar increase following DNA damage (Hakansson et al., 2006).

The S-phase checkpoint inhibits further (late) origin firing

A further downstream effect of S-phase checkpoint activity mediated by the checkpoint kinases is the inhibition of further origin firing following DNA damage and replication fork stalling, a conserved event across both budding and fission yeast as well as human cells (Kim and Huberman, 2001; Maya-Mendoza et al., 2007; Santocanale and Diffley, 1998). The mechanism by which origin firing is inhibited still remains uncertain, but is likely to involve inhibition of the kinases responsible for the transition to active S-phase. In budding yeast, Dbf4 is phosphorylated by Rad53, causing both inhibition of Cdc7 kinase activity as well as the dissociation of Cdc7/Dbf4 from the chromatin (Pasero et al., 1999; Weinreich and Stillman, 1999) and phosphorylation of Dbf4 is conserved in fission yeast and human cells (Matsuoka et

al., 2007; Takeda et al., 1999). The significance of inhibition of further origin firing is unclear and is the focus of numerous speculations, such as an increase in the time accorded to the cell for repair, the maintenance of correct dNTP pool levels and even the protection of vulnerable chromosomal structures such as the late-replicating budding yeast telomeres (Zegerman and Diffley, 2009).

Crucially, the S-phase checkpoint is responsible for stabilisation of stalled replication forks

The most important downstream effect of the S-phase checkpoint is the stabilisation of stalled replication forks in order to allow resumption of replication following DNA damage repair. This cellular process again relies on the checkpoint kinases. Although the exact mechanism for replication fork stabilisation remains unclear, Mec1 and Rad53 have been shown to share responsibility for fork stabilisation through seemingly independent pathways (Cobb et al., 2003; Lucca et al., 2004). Deletion of these kinases leads to dissociation of the replisome at the stalled fork, termed replication fork "collapse" (Cobb et al., 2003; Lucca et al., 2004). Collapsed replication forks not only cause incomplete replication of the genome, but are also liable for processing into pathological recombinogenic DNA structures giving rise to further damage the cell must process (Sogo et al., 2002). Replication fork stabilisation must therefore address both intact replisome retention and inhibition of the generation of aberrant DNA structure. Indeed replisome stabilisation by retention of the polymerases is seen to be dependent on Mec1 and the RecQ helicase Sgs1 (Cobb et al., 2003). Rad53 on the other hand seems to exert its function in fork stabilisation via phosphorylation-dependent inhibition of Exo1 nuclease, which has been speculated to destabilise stalled forks by mechanisms such as Exo1-dependent replication fork resection and generation of ssDNA causing further, sustained checkpoint activation (Morin et al., 2008).

1.3 Changes in cellular ploidy alter cell physiology and contribute to genomic instability and cancer

Genome duplication in S-phase is controlled by origin licensing and firing dynamics

In S-phase the cell's entire genome must be faithfully copied once and only once. This process is ensured by replication from multiple licensed origins (see section 1.2). However replication originating from multiple random origins leaves the cell facing the so-called "random completion problem", that is the cell must ensure no unreplicated DNA is present at time of entry into mitosis (Zegerman and Diffley, 2009). This problem is thought to be most likely solved by "origin redundancy" (for review see (Hyrien et al., 2003)), with many more potential origins being present on the genomic DNA than are actually required during S-phase (Santocanale and Diffley, 1996; Woodward et al., 2006; Wyrick et al., 2001). This leads to the presence of two types of origin: those which are active, which fire after licensing in G1 and those which are replicated passively, termed "dormant" (Zegerman and Diffley, 2009), although pre-RC's are formed at both types of origins in late M and G1 (Santocanale and Diffley, 1996; Woodward et al., 2006; Wyrick et al., 2001). This system requires the disassembly of the pre-RC at passively replicated origins to avoid re-replication thereby returning the origin to its previous, unlicensed status (Laskey and Harland, 1981; Santocanale and Diffley, 1996). The mechanism of removal of the pre-RC concomitantly with replication fork passage remains unclear, but has been shown to cause brief replication fork pausing (Wang et al., 2001). Once fired or passively replicated, origins cannot be re-licensed prior to the cell undergoing mitosis. This is regulated by CDKs, with a decrease in CDK levels during late M/early G1-phase promoting origin licensing, whereas an increase in CDK levels towards the end of G1 is not only inhibitory to origin licensing but also promotes the initiation of replication (Blow and Dutta, 2005).

DNA over-replication leads to an increase in cellular ploidy

Origin licensing and firing must be strictly controlled to ensure accurate and complete genome replication and misregulation of these molecular processes can give rise to DNA over-replication. DNA over-replication, leading to an increase in ploidy, can occur by a number of distinct mechanisms: re-replication, endoreduplication, mitosis failure and gene amplification (see Figure 1.3.1). In re-replication, origins erroneously fire more than once, leading to a continuous increase in cellular DNA content and so-called "partial ploidy". Endoreduplication is characterised by multiple S-phases, as origin licensing and firing is no longer coupled to passage through mitosis. Origins however still only fire once per cycle, giving rise to discrete increases in cellular DNA content. Incorrect mitosis may also lead to discrete increases in cellular DNA content but are independent of origin licensing (see Figure 1.3.1). Furthermore, specific re-replication of certain chromosomal regions may occur, leading to DNA over-replication known as "gene amplification" (for review see (Arias and Walter, 2007; Edgar and Orr-Weaver, 2001; Porter, 2008).

Polyploidy occurs commonly in plants and lower eukaryotes in nature

Although in general prokaryotes and some unicellular eukaryotes contain a haploid genome and most eukaryotes are diploid, polyploidy can be observed relatively commonly in nature. Indeed there is evidence to suggest that polyploidisation by genome duplication is one of the mechanisms contributing to evolutionary diversity (Ohno et al., 1968). This allows the duplicated genes to "drift", that is accumulate mutations without any detrimental effect to the organism itself and potentially even to the organism's advantage (Ohno et al., 1968; Thorpe et al., 2007). Many plant species commonly contain polyploid cells and use increased ploidy to their evolutionary advantage (for review see (Doyle et al., 2008).

However polyploidy can also occur stably in lower eukaryotes, such as fish and amphibians (Comai, 2005; Ohno et al., 1968). An increase in ploidy is less well tolerated in higher eukaryotes and has indeed been linked to spontaneous human abortions (Comai, 2005; Eiben et al., 1990; Storchova and Pellman, 2004) and is therefore a much rarer event compared to plants. However, in somatic cells of highly proliferative tissue, such as the liver and bone marrow, polyploidy is indeed observed. The best characterised example of this is the megakaryocyte, a specialised blood cell of the bone marrow, whose extreme increase in ploidy correlated with the ability to bud off sufficient anucleated thrombocytes, thus mediating blood clotting (for review see (Ravid et al., 2002). A link between polyploidy and stress and ageing is also seen in the increase of polyploid hepatocytes in liver regrowth following damage or metabolic stress, as well as with hepatocyte age and senescence (Gupta, 2000), therefore implicating polyploidy in a variety of pathological processes (Storchova and Pellman, 2004).

Natural occurrence of increased ploidy may be explained by possible advantages polyploidy confers to the cell

In general, an increase in ploidy may confer three types of advantage to an organism: Firstly, it has been suggested that some polyploid cells may display higher fitness than their non-polyploid counterparts, bestowing upon them a greater chance of survival. This has been suggested to function somewhat analogously to “hybrid vigour”. Secondly, polyploidy may also confer upon the cell the possibility of asexual reproduction. Finally and possibly of most importance is the gene redundancy associated with polyploid cells. This not only allows the cell to escape from the effects of deleterious but recessive alleles, but also plays an important role evolutionarily (Ohno et al., 1968), allowing for gene diversification of the redundant allele, whilst ensuring survival by virtue of the original copy (for review see (Comai, 2005)).

Changes in cellular ploidy alter cell physiology and are deleterious to a number of cellular processes

Whilst some polyploid cells may display greater fitness and survival, an increase in ploidy also brings with it a number of problems for many cellular processes. An increase in ploidy is usually associated with an increase in cellular size (Galitski et al., 1999), however not all cellular components increase proportionately following endoreduplication and polyploidy, generating potentially deleterious alterations in ratios of cellular components. Further problems occurring in polyploid cells are likely due to alterations in gene expression. A study comparing gene expression in yeast strains with increasing ploidy but conserved mating type found both genes which were ploidy-induced and genes which were ploidy-repressed (Galitski et al., 1999). Although expression levels of most genes did not vary, some of the genes identified in the screen help explain the morphological changes observed with an increase in ploidy in budding yeast, as for example the gene encoding the rho-like GTPase Gic2 is ploidy-repressed. Gic2 is involved in actin organisation both in initiation of budding (bud site selection and bud emergence, as well as shmoo formation) and cellular polarisation. Hand in hand with an increase in cellular size, the authors further observed greater elongation in the strains with increased ploidy, but did not observe any changes in cellular growth rate. The study suggests two possible mechanisms explaining alteration of gene expression: firstly, although there is no increase in the ratio of certain genes over others in endoreduplicated cells, a general increase of genes may be sensed by transient pairing of homologous chromosomes, which has been implicated in alteration of gene expression. Secondly, whilst the chromatin and thus nuclear size double, the nuclear envelope only increases to a lesser extent and the alteration of this ratio may lead to changes in the concentration of gene expression regulatory factors within the nucleus. Furthermore, these cells suffer from problems passaging through mitosis (and meiosis). Importantly, it has been shown that mitotic loss of chromosomes is a possible outcome of such mitotic difficulties in polyploid cells, generating aneuploid daughter cells

(Mayer and Aguilera, 1990) and it is thought that these mitotic difficulties often arise from problems with correct spindle formation (for review see (Comai, 2005; Storchova and Pellman, 2004)). Polyploid budding yeast also display greater sensitivity to certain DNA damaging agents compared to wild-type, suggesting genomic instability as one of the hallmarks of increased ploidy. These alterations in cell physiology which go in hand with an increase in ploidy thus highlight the importance of taking cellular ploidy into account experimentally.

Insights into genetic constraints of polyploidy in yeast

The significance of ploidy-specific physiology was further highlighted by the phenomenon of ploidy-specific lethality in budding yeast, which was first described by Lin et al. (Lin et al., 2001a): following the deletion of *BIK1*, which encodes a microtubule and kinetochore binding protein in haploids and diploids, which remain viable, as well as triploids and tetraploids, which are highly sensitive and inviable, respectively. The same laboratories recently took their investigation further and by modification of the budding yeast mating type locus were able to generate synthetic tetraploids for screening a large proportion of the yeast deletion library for ploidy-specific lethality (Storchova et al., 2006). Astonishingly, the genes identified in this screen were found to belong to one of three specific groups: genes involved in homologous repair of DNA damage, genes involved in the establishment and cohesion of sister chromatids and a subset of genes important for the mitotic spindle. The authors also confirmed the result was not due to alterations in gene or protein expression levels. The fact that all three groups are involved in the maintenance of genomic stability makes this result even more interesting and the authors conclude genomic instability thus to be the most significant of the physiological changes due to increased ploidy in budding yeast. Animal cells require functional HR machinery for survival, a trait not shared by haploid or diploid budding yeast (Krogh and Symington, 2004). The dependence of polyploid yeast on the HR pathway is therefore likely to

reflect an increase in DNA damage arising throughout S-phase, which may be due to an increase in spontaneous damage, or due to problems replicating an increased amount of DNA with the same temporal constraints as wild-type yeast. Indeed, both an increase in Ddc1-foci generated in S-phase and their possible persistence into mitosis as well as increased spontaneous DNA damage were observed with increased ploidy in this study, although no cell cycle progression defect or increased dependency on the replication proteins Cdc6, Cdc7 and Pol32 was noted. The authors' investigation into the subset of genes displaying ploidy-specific lethality involved in mitosis reveals that these genes are in particular involved in correct chromosome segregation. Indeed, in accordance with their previous data regarding the cell cycle progression, they observe no mitotic delay. However they observe normal cytoplasmic and spindle microtubule dynamics, as well as kinetochore attachment to microtubules. The mitosis-involved ploidy-specific lethal genes do however include Ipl1 (Aurora B in humans), whose action increases bipolar over syntelic kinetochore attachments as well as checkpoint activation upon sensing kinetochores not under tension. Further ploidy-lethal genes involved in mitosis are proteins which respond to abnormal kinetochore-microtubule attachments, such as Bub1, Bub3 and Sgo1. The authors go on to show a ploidy-dependent increase in syntelic and monopolar kinetochore attachments corresponding with increased chromosome loss by non-disjunction, using a non-replicating conditional di-centric minichromosome. The study suggests that the altered spindle geometry resulting from a doubling in cell volume and of the spindle pole body, which is not mirrored by a double in other cellular components, such as spindle length, may lead to increase in the frequency of syntelic attachments. This defect may be compounded by defects in sister chromatid cohesion – which makes up the third group of specific ploidy-lethal genes.

Increased cellular ploidy contributes greatly to genomic instability

As Storchova *et al.* point out, all three groups of specific ploidy-lethal genes identified contribute to maintenance of genomic stability and indeed increased genomic instability may be one of the main characteristics of polyploid budding yeast. Their study also confirms a correlation between increased yeast ploidy and an increase in chromosome loss, as previously reported in the literature (Mayer and Aguilera, 1990). They also observe an increase in gene conversion frequency between homologous chromosomes following an increase in ploidy; however they do not observe an increased rate of mutation. Others have reported increased ploidy to be mirrored by a slight but significant increase in gross chromosomal rearrangements (Huang and Koshland, 2003), one of the hallmarks of genomic instability (Aguilera and Gomez-Gonzalez, 2008). Furthermore, polyploid budding yeast are hyper-sensitive to DNA damaging agents such as HU or MMS, as well as microtubule poisons compared to wild-type, but show comparable sensitivity to wild-type upon exposure to osmotic stress, UV or oxidative damage (Andalis et al., 2004; Storchova et al., 2006).

Although the genetic screen performed by Storchova et al. has certain limitations, such as its inability to take metabolic effects into account as it was performed on rich media and the fact that the effect of essential genes (particularly important for genes involved in replication for which a role in polyploidy is highly plausible) (Thorpe et al., 2007), it has none-the-less advanced the understanding in the ploidy field significantly. Furthermore, the groups of genes identified in budding yeast, may prove to be interesting for ploidy-related investigation in mammalian cells (Thorpe et al., 2007), seeing as changes in ploidy are also a hallmark of cancer.

Polyploidy may be a precursor of aneuploidy, a hallmark of cancer

Many cancer cells display altered ploidy, with some displaying triploidy or tetraploidy, such as in some epithelial tumours (Storchova and Pellman, 2004). However the most common alteration of ploidy characterising cancerous cells is aneuploidy, that is loss of chromosomes (Rajagopalan and Lengauer, 2004). This was in fact already observed in 1891 by Hansemann and suggested as to play a role in malignancy by Boveri in 1914 (Boveri, 1914; Hansemann, 1891). Although aneuploidy is seen to be one of the most common features of cancer cells, it still remains unclear whether aneuploidy is cause or effect (Duesberg and Rasnick, 2000; Matzke et al., 2003; Nigg, 2002; Rajagopalan and Lengauer, 2004; Storchova and Pellman, 2004). Exactly how aneuploid cancer cells arise also remains a matter of debate, with two proposed mechanisms. One mechanistic view is that successive mutational "hits" of both "caretaker" and "gatekeeper" genes lead to progressive genomic instability and thus progressive chromosome loss and aneuploidy (see Figure 1.3.2). An alternative mechanism has been suggested by which cells first experience an increase in ploidy, followed by chromosome loss (see Figure 1.3.2). The latter model is supported both by data from yeast and human cancer predispositions, such as Barrett's oesophagus (Reid et al., 1996). In yeast, increased ploidy correlated with a significant increase in chromosome loss and loss of one chromosome further corresponded with an increased likelihood of further chromosome loss (Mayer and Aguilera, 1990). Increased ploidy in yeast leads to increased genomic instability, which may be explained by a plethora of effects arising from the increase in ploidy, such as incorrect spindle apparatus, microtubule functioning or chromosome pairing, incomplete replication of the entire genome or altered gene expression (Mayer and Aguilera, 1990). In humans, Barrett's oesophagus is a disease of the epithelium lining the oesophagus and although considered pre-malignant, it renders the individual at high risk of developing oesophageal cancer. Individuals thus undergo continuous monitoring by gastroscopy and gastroscopic biopsies, making these samples useful for the study of cancer progression. Investigation of such samples revealed

that progression to cancer was not only shown to correlate with increased aneuploidy, but also correlated with an increase in ploidy, followed by progression to aneuploidy and a progression to high-grade dysplasia and genomic instability (Reid et al., 1996). Interestingly, out of the multiple components implicated in failed cytokinesis, leading to tetraploidisation of human cells as a precursor to aneuploidy, both the DNA damage response and delayed replication have been highlighted, as they may lead to inhibition of the pathways required for chromosome segregation and successful cytokinesis (King, 2008).

1.4 Chromatin

Eukaryotic DNA is complexed with histones to form chromatin

Due to the need both for compaction and for protection, in the eukaryotic nucleus DNA is present complexed to a number of small proteins termed histones. This DNA-protein complex is known as chromatin and is made up of units termed nucleosomes (Kornberg, 1977). Each nucleosome consists of two copies of the four core histones, H2A, H2B, H3 and H4, thus forming an ocatmeric protein complex around which the DNA can be wrapped.

Histones are small (between 11 and 18kDa in *S.cerevisiae*), highly basic and positively charged proteins. Although the four core histones, H2A, H2B, H3 and H4 show relatively low primary structure homology (Arents and Moudrianakis, 1995), a conserved 3-diomensional structural motif, the "histone-fold" has been identified in each of the core histones. This structural motif has been shown to be crucial for histone-histone contacts and thus histone pair formation (Arents and Moudrianakis, 1995; Davey et al., 2002; Luger et al., 1997). Due to the presence of two helix-strand-helix motifs, it has been suggested the histone fold may have arisen via tandem duplication (Arents and Moudrianakis, 1995). Interaction between histone fold domains, also known as the globular domains, thus allows the dimerisation of H2A and H2B and the tetramerisation of H3 and H4 (Arents et al., 1991; Arents and Moudrianakis, 1995; Luger et al., 1997) as well as the final octameric histone association. It is this tripartite association of a single H3-H4 tetramer, flanked by two H2A-H2B dimers which allows the formation of the core nucleosomes, around which the DNA can be wound (see Figure 1.4.1).

The core nucleosome interacts with around 146bp of DNA, leading the DNA to be wound across the protein in 1.65 flat, left-handed superhelical turns (Luger et al., 1997). The H3-H4 tetramer bind the central region of the DNA, with each H2A-H2B dimer making contact with

the DNA either side (Luger et al., 1997). Each histone also contains “histone-fold extensions”, structural elements adjacent to the histone fold, though still integral to the core protein, which are responsible for further protein-DNA contacts (Luger et al., 1997). These protein-DNA contacts are primarily mediated by interactions between the main chain atoms of the histones and the DNA phosphodiester backbone, thus leading to a tight, but unspecific interaction between the DNA and the proteins (Luger et al., 1997) (see Figure 1.4.1).

These crystallographic data (see Figure 1.4.1) (Davey et al., 2002; Luger et al., 1997) support the visualisation of DNA in 1974, described to have the appearance of a “nodular fibril” or of “beads-on-a-string”, with the DNA regularly forming a spheroid structure (Olins and Olins, 1974). In the same paper the authors suggest higher orders of DNA compaction are likely to occur given the spatial constraints of the eukaryotic nucleus compared to the amount of DNA. Indeed, this initially observed “beads-on-a-string” basic organisation of DNA and protein does form higher order structures, though there is as not yet strict consensus to the exact nature of the final compacted structures (Campos and Reinberg, 2009; Tremethick, 2007). The linker histone, H1, facilitates the transition to higher order structures (Campos and Reinberg, 2009; Catez et al., 2006). The linker histone not only binds and protects the linker DNA (Noll and Kornberg, 1977; Richmond et al., 1984), but also forms further contacts to the nucleosomes. This linker histone-nucleosome binding leads to stabilisation of the nucleosomes and induces a small conformational change, making higher orders of chromatin compaction possible (Catez et al., 2006). It is likely that higher-order chromatin forms a higher-order solenoid-type structure, though the precise folding and interactions between nucleosomes remain to be elucidated (Robinson et al., 2006; Tremethick, 2007). Furthermore, *in vivo* compaction not only relies on the linker histone, but post-translational modifications of histones, such as hypoacetylation of the histone H4 tail (Dorigo et al., 2003) and cation-chromatin binding, which acts both directly by leading to DNA electrostatic neutralisation and further chromosome condensation, and indirectly by inhibition of TopoII (Strick et al., 2001) and which

are thought to play an important role (Campos and Reinberg, 2009; Tremethick, 2007). The eukaryotic cell's ability to form chromatin and indeed to form further intricate higher-order chromatin states of varying degree thus fulfils its requirements with regards to packaging the DNA in the nucleus, generation of a protected environment for its genetic information and variable chromosome condensation throughout the cell cycle.

Chromatin structure is integral to DNA metabolism

One of the advantages of the tight DNA packaging is the protection it offers the DNA against damage and unwanted enzymatic activity. However all wanted DNA metabolism must take place within the chromatin context. Therefore in certain situations such as replication or transcription, the chromatin structure must be altered to allow greater accessibility to the DNA. Alteration of chromatin structure can be achieved by the use of histone variants, covalent modification of histones and chromatin remodelling. Chromatin modification thus can lead to changes in accessibility, the set-up of specialised chromatin regions and play a role in the recruitment of effector proteins.

Histone variants replace canonical core histones often marking specialised chromatin sections

One of the multiple ways for the cell to generate distinct or specialised chromatin regions is by the replacement of one of the core histones with a so-called "histone variant". Such histone variants display variations in the primary amino-acid sequence when compared to the core histone (Talbert and Henikoff, 2010). Many of these histone variants are conserved across eukaryotes, thus ascribing them an important and conserved role in the definition of distinct or

specialised chromatin domains, although many species also contain organism-specific histone variants, likely to play a more specialised role (Talbert and Henikoff, 2010).

An example of a conserved histone variant is H3 variant CENP-A in humans or Cse4 in yeast, which is specifically found in nucleosomes at centromeres. Specialised centromeric nucleosomes are therefore postulated to possess the ability to form unique higher-order chromatin structures allowing kinetochore assembly. The biochemical composition of centromeric CENP-A/Cse4 nucleosomes remains unclear, as does their structure, although an amino acid substitution is thought to weaken the histone-DNA interaction at the DNA entry/exit point from the nucleosomes (for review see (Black and Bassett, 2008; Campos and Reinberg, 2009; Talbert and Henikoff, 2010)).

The conserved histone variants of the canonical histone H2A, especially variants H2A.Z and H2AX are of greater pertinence to the DNA damage response and genomic stability. The H2A.Z (Htz1 in yeast) variant of core histone H2A is well conserved both in higher and lower eukaryotes (Campos and Reinberg, 2009). Determination of the crystal structure of a nucleosome containing H2A.Z-H2B dimers demonstrated no gross rearrangements of nucleosome structure or DNA wrapping and binding (Suto et al., 2000). However the authors conclude that incorporation of the H2A.Z variant leads to slight destabilisation of the interface between H2A.Z-H2B and the H3-H4 tetramer, as well as slight changes in surface residues, thus offering the potential for the recruitment of specific interacting partners, as well as the existence of a metal-binding pocket (Suto et al., 2000). Nucleosomes containing the Htz1/H2A.Z variant are found flanking nucleosome-free regions at transcription start sites in both yeast and humans, where they are thought to increase RNA polymerase recruitment (Adam et al., 2001; Hardy et al., 2009). In yeast, *HTZ1* is not an essential gene and its deletion leads to a global decrease in transcription (Zhang et al., 2005). It has also been postulated that Htz1 is located in a manner to delineate heterochromatin and thus antagonise silencing

(Meneghini et al., 2003).The exact role of the Htz1/H2A.Z variant is unclear as it has been implicated both in gene activation and gene silencing (see (Altaf et al., 2009) for review), but its function may be mediated by its change in nucleosome stability and a difference in turnover rate compared to nucleosomes containing H2A (Suto et al., 2000). Furthermore, loss of this variant histone in mammalian and yeast cells is characterised by chromosomal mis-segregation (Faast et al., 2001). Further evidence linking the H2A.Z histone variant to the maintenance of genomic stability lies in the hyper-sensitivity of *htz1* deletion strains in yeast to a variety of DNA damaging conditions, such as UV irradiation (Mizuguchi et al., 2004).

H2AX is a further mammalian variant histone of H2A and interestingly shares greater homology with yeast H2A than the canonical mammalian H2A (Downs et al., 2000). Mammalian H2AX and yeast H2A both contain a conserved PIKK consensus site in their C-terminal domains, phosphorylation of which constitutes one of the key components of the DNA damage response, leading to an alteration of chromatin structure and increased accessibility and repair ((Downs et al., 2004; Downs et al., 2000), considered in more detail further down).

A "histone code" of covalent modifications allows for specific chromatin alteration and set-up of distinct chromatin regions

A further and impressively versatile mechanism of chromatin alteration, which can both generate a change in chromatin structure as well as act as a specific recruitment platform is achieved by covalent chemical modification of histones. Aside their globular, "histone-fold" domain, histones contain long N- and C-terminal tail domains, which account for ~25% of their mass and protrude from the nucleosomes (Luger et al., 1997). These protruding histone tails, as well as the histone cores, are the site of covalent modification such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation (Wolffe and Hayes, 1999). These

modifications impact upon both histone-histone and histone-DNA contacts, thereby changing the chromatin structure and DNA accessibility (for review of structural changes upon modification see (Wolffe and Hayes, 1999)). However such an array of histone “markers” and potential marker combinations, coupled with their ability to act as specific recruitment platforms for chromatin modifying activities allowing the cell to set up distinct and distinctive regions of chromatin and DNA metabolic activity has led to the proposal that these modifications may be used as a “histone code”, which can be read by the cell and may be the origin of a concerted cellular response to chromatin alteration (Jenuwein and Allis, 2001; Strahl and Allis, 2000). The most well-studied histone modification is that of histone lysine acetylation by HAT (Histone acetylase) complexes. Acetylation of lysine residues counteracts the positive charge on the unmodified lysine and is thus thought to alter histone-DNA contact and contribute to the generation of a more open chromatin structure. This process plays an important role in regulation of transcription. In accordance with this, many transcriptional activators and repressors contain HAT and HDAC (histone deacetylase) activities, respectively. Histone acetylation is further associated with the maturation of newly replicated chromatin: acetylated H3 and H4 are incorporated into newly synthesised chromatin and progressively lose their acetylation marks as the newly assembled chromatin matures. Histone mono- di- or tri-methylation is achieved by residue-specific histone methyltransferases that aside their role in epigenetics, have been associated in numerous cellular processes, such as being involved in transcription most likely by recruiting other factors, as well as heterochromatin formation and thus heterochromatic gene silencing. Histone demethylases usually also demonstrate specificity towards unique methylated lysine residues (Bhaumik et al., 2007).There are many histone modifications implicated in the DNA damage response and recently, in addition to recruitment of Rad9 (53BP1) by methylated H3K79 (Wysocki et al., 2005), methylation of H4K20 has been ascribed a role in DNA repair by acting as a further recruitment platform for Rad9 (53BP1) (Kilkenny et al., 2008)(for review see (Bhaumik et al., 2007; Strahl and Allis,

2000)). A further histone modification to play a pivotal role in the DNA damage response and DNA repair is phosphorylation (discussed below), as well as playing an important role in cellular division.

Further chromatin alteration is mediated by chromatin remodelling complexes

Further alteration of histone-DNA interactions within nucleosomes can be achieved by the activity of large ATP-dependent chromatin remodelling enzymes (Downs et al., 2007; Lusser and Kadonaga, 2003). Such changes in protein-DNA interaction are mediated by a variety of mechanisms, such as nucleosome sliding, partial or complete octamer removal or histone exchange within the octamer, replacing a canonical histone with the appropriate histone variant (Downs et al., 2007; Lusser and Kadonaga, 2003). (for a full discussion see chromatin remodelling section, 1.5)

Chromatin involvement in cellular processes

Chromatin at DSBs

Chromatin modification is thus an integral component and vital part of the molecular response to cellular events. In the case of DNA damage by DSB, passive chromatin would merely hinder the repair process, blocking access to the damaged DNA. Chromatin modification thus plays an active role in the facilitation of DNA damage repair. Indeed in the case of DNA damage leading to a DSB, phosphorylation of yeast histone H2A (H2AX in humans) is one of the earliest and most robust signalling events (Downs et al., 2000). Phosphorylation of H2A was shown to be dependent on the PIKK kinases Mec1 and Tel1 in budding yeast (Downs et al., 2000). Analagously, in human cells, ATM and ATR have been found to be responsible for H2AX

phosphorylation in response to DSBs and replication stress, respectively (Burma et al., 2001; Ward and Chen, 2001). DNA-PK has also been shown to phosphorylate H2AX and although it may aid the DNA damage response (Stiff et al., 2004), it is believed to play a more important role in apoptosis (Mukherjee et al., 2006).

H2A phosphorylation in budding yeast is not only an extremely rapid, but also a very wide-spread response and has been shown to spread up to 10-20kb from the break site by ChIP with a specific phospho-S129-H2A antibody in a time-frame of minutes (Downs et al., 2004; Shroff et al., 2004). Interestingly, the ChIP signal in the 1-2kb surrounding the DSB itself was found to be very low in both studies (Downs et al., 2004; Shroff et al., 2004), with the greatest enrichment observed at about 3-5kb from the break (Shroff et al., 2004). It has been postulated that the low ChIP signal directly adjacent to the DSB may be due to the presence of a large number of repair factors directly at the DSB, or a possible occlusion of the antibody's recognition site by other DNA-damage induced histone modifications such as phosphorylation of S122 of H2A in budding yeast (Foster and Downs, 2005; Harvey et al., 2005), as the kinases Mec1 and Tel1 have been confirmed to be present at the DSB lesion itself (Nakada et al., 2003; Rouse and Jackson, 2002b).

The DNA damage kinases are members of the PIKK family which recognise an S/T-Q consensus sequence (see section 1.1). Interestingly, an SQ-E consensus sequence is found contained within the C-terminal tail of many H2A histones (Downs et al., 2000) and S129 of this sequence on budding yeast H2A has indeed shown to be the target of the DNA damage response kinases Mec1 and Tel1 (Downs et al., 2000). Correspondingly, phosphorylation of the C-terminal H2AX S139 in humans has also been observed in response to ionising radiation (Rogakou et al., 1999; Rogakou et al., 1998). The importance of the DNA damage-dependent phosphorylation of the SQ-E/D motif on H2A and H2A variant histones is highlighted by its conservation across species; indeed it is phosphorylated in budding and fission yeasts, *Drosophila melanogaster,*

Xenopus laevis, mice and human cells (Downs et al., 2000) (Madigan et al., 2002; Nakamura et al., 2004; Redon et al., 2002; Rogakou et al., 1999; Rogakou et al., 1998; Shroff et al., 2004).

Mutation of the budding yeast SQ-E/D motif leads to a marked sensitivity of the mutant strains to DNA damaging agents which induce DSBs, such as MMS and phleomycin, though not UV irradiation (Downs et al., 2000) . Furthermore, yeast genetics suggest the motif plays the larger part of its role in the DNA damage response facilitating repair by NHEJ, rather than HR (Downs et al., 2000). The authors also confirm that unlike strains harbouring a deletion of the Mec1 kinase, strains containing mutated C-terminal SQ-E/D motifs neither impinge on the cell's transcriptional response to DNA damage nor display a defect in the G2/M checkpoint, thus ascribing phosphorylation of this motif both a more specific as well as a solely functional role in the DNA damage response (Downs et al., 2000), although more recently, it has been found that phosphor-S129 H2A also contributes to the G1/S cell cycle checkpoint by recruiting Rad9 (Javaheri et al., 2006).

Similarly to yeast cells, $H2AX^{-/-}$ MEFs (mouse embryonic fibroblasts), display slow proliferation and accumulate non-dividing cells, which are observed to be phenotypically very close to $ATM^{-/-}$, $Ku70^{-/-}$ or $Ku80^{-/-}$ MEFs (Celeste et al., 2002). Furthermore, although H2AX is not seen to be essential, metaphase spreads of $H2AX^{-/-}$ MEFs confirm the presence of chromosomal aberrations and significantly increased genomic instability (Bassing et al., 2002; Celeste et al., 2002). Further to being implicated in NHEJ in mammalian cells, H2AX has also been implicated in HR (Celeste et al., 2002; Xie et al., 2004). However, analogously to results from budding yeast, $H2AX^{-/-}$ MEFS are not checkpoint deficient (Celeste et al., 2002). At the level of the whole organism, $H2AX^{-/-}$ mice display a marked increase in sensitivity to induction of DNA damage and male $H2AX^{-/-}$ mice are infertile (Celeste et al., 2002). Although $H2AX^{-/-}$ mice display only a modest cancer predisposition (Bassing et al., 2002; Celeste et al., 2002), this increases dramatically with the concomitant homozygous deletion of p53 (Bassing et al., 2003; Celeste et

al., 2003a). Tumourigenicity in these mice is seen to be dose-dependent with the severity of the phenotype correlating with increased loss of H2AX, i.e. $H2AX^{+/-}p53^{-/-}$ mice display an intermediate phenotype compared to $H2AX^{+/+}p53^{-/-}$ and $H2AX^{-/-}p53^{-/-}$ (Bassing et al., 2003; Celeste et al., 2003a). These results together with the *in vitro* observations of increased genomic stability (Bassing et al., 2003; Celeste et al., 2002) and the observed action of H2AX as a suppressor of oncogenic translocations (Bassing et al., 2003) highlight the importance of H2AX and ascribe it a role as a dose-dependent tumour suppressor (Zaid and Downs, 2005).

The importance of budding yeast H2A and mammalian H2AX in response to DNA damage in the form of DSBs has been clearly demonstrated, however its precise mechanism remains less clear and several mechanisms have been proposed. Covalent modification of H2A/H2AX with a negatively charged phosphate has the potential to bring about a change in binding affinity and therefore a structural change within the nucleosomes, either with regards to histone-histone or histone-DNA interaction, especially as the H2A C-terminal tail is proposed to be positioned close to the DNA entry/exit point of the nucleosomes (Luger et al., 1997). Aside a possible effect at the level of the nucleosomes, covalent modification of the H2A/H2AX C-terminal tail by phosphorylation may also have an effect on the organisation of chromatin into higher-order structures (Chambers and Downs, 2007). This hypothesis is corroborated by the increased sensitivity *in vivo* of chromatin harbouring H2A containing a phosphomimic at its C-terminus compared to wild-type chromatin to micrococcal nuclease (MNase) suggesting a possible relaxing of the chromatin structure (Downs et al., 2000).

Given its role in the prevention of genomic instability and oncogenic translocations as well as its likely participation in NHEJ, it has been suggested H2AX may be involved in the recruitment of proteins which function to tether the broken DNA end, thus occluding the recombinogenic DNA ends (Zaid and Downs, 2005). Furthermore, a key hallmark of the DNA damage response is the rapid recruitment of DNA damage signalling and repair factors to the site of damage and

this recruitment has been linked to H2A/H2AX phosphorylation (for review see (Downs et al., 2007)). Thus, in mammalian cells, H2AX has been shown to be responsible for the formation of IR-induced DSB foci, hothouses of DNA repair (Bekker-Jensen et al., 2006; Paull et al., 2000). However a number of the initial DNA damage recognition factors, such as Nbs1, BRCA1 and 53BP1 are not affected by loss of H2AX and still localise to sites of damage (Celeste et al., 2003b). Overall this suggests a role for H2AX as a recruitment and retention platform for many of the DNA damage signalling and repair factors downstream from the initial recognition event and a role in spatio-temporal sustention of the response (Celeste et al., 2003b; Downs et al., 2007; van Attikum and Gasser, 2005).

There are a number of further histone modifications which play a role in mediating the DNA damage response and it is likely more are yet to be identified. Both acetylation and methylation of H3 play a role in the DNA damage response (Downs et al., 2007; Masumoto et al., 2005). H3K56, is acetylated in newly synthesised histones and then rapidly removed after deposition, however upon DNA damage this acetylation is maintained in a Rad9-and Mec1-dependent manner (Masumoto et al., 2005). Acetylation is mediated by Rtt109 in budding yeast and also requires histone chaperone Asf1 (Collins et al., 2007; Das et al., 2009; Driscoll et al., 2007; Masumoto et al., 2005)). Methylation of H3 occurs at multiple residues, but it is methylation of K79, which is important for the DNA damage response. In *S.cerevisiae*, H3K79 methylation, leading to the recruitment of Rad9 is mediated by Dot1 and requires previous H2BK123 ubiquitylation by the Rad6-Bre1-Lge1 complex (Giannattasio et al., 2005; Wysocki et al., 2005). The importance of this modification is underlined by its contribution to the recruitment of 53BP1 (S.c. Rad9) in human cells (Cao et al., 2002; DiTullio et al., 2002) via the tudor domains of 53BP1, which also binds H2AX. As methylation of H3K79 does not seem to increase following DNA damage, it has been postulated that as part of the DNA damage response and alteration of chromatin structure, intrinsically methylated H3K79 may be increasingly exposed to its interacting partners (Huyen et al., 2004). Additionally, methylation

of H4K20 has been ascribed a role in DNA repair by acting as a further recruitment platform for Rad9 (53BP1) (Kilkenny et al., 2008).

Chromatin at replication forks

Replication is a further DNA metabolic process that must be dealt with in the context of chromatin. Indeed, the replication fork cannot progress through the chromatin barrier and nucleosomes are disassembled both ahead and behind the travelling replication fork (Gasser et al., 1996; Ransom et al., 2010; Sogo et al., 1986), however the mechanism by which this is mediated is unclear and may rely not only on the passage of the fork, but possibly also on chromatin remodelling complexes and histone chaperones (Ransom et al., 2010). Nucleosome disassembly first requires the removal of the two H2A-H2B dimers (Jackson, 1988), which is possibly mediated by FACT (facilitates chromatin transcription), which in budding yeast interacts with RPA (VanDemark et al., 2006) and in humans with the MCM complex (Tan et al., 2006), although other chaperones such as Nap1 have also been implicated (for review see (Ransom et al., 2010)). Once the two H2A-H2B dimers have been removed, removal of the more stable H3-H4 tetramer is likely to be mediated by the histone chaperone Asf1 (Groth et al., 2007a).

Nucleosome reassambly following fork passage is thought to be initiated by Caf-1 (Chromatin assembly factor) dependent H3-H4 deposition both of “recycled”, parental and newly synthesised H3-H4 (Annunziato, 2005; Groth et al., 2007b). In yeast, all newly synthesised H3-H4 is acetylated on H3K56, recognised by CAF-1 (Masumoto et al., 2005; Verreault et al., 1996), which localises to the replication fork via its interaction with PCNA (Krude, 1995; Shibahara and Stillman, 1999). Acetylation of H3K56 is mediated by Rtt109 in budding yeast and also requires histone chaperone Asf1 (Collins et al., 2007; Das et al., 2009; Driscoll et al., 2007; Masumoto et al., 2005). Caf-1 in turn has far greater affinity for K56 acetylated H3 over the unacetylated form thus stimulating nucleosome reassembly following replication fork

passage (Li et al., 2008). Following deposition, H3K56 is gradually deacetylated (Masumoto et al., 2005).

Chromatin disassembly and reassembly is also crucial to many DNA repair processes and the same molecular machinery is largely involved in both cases (Ransom et al., 2010). For repair to occur, the DNA must be accessible, which requires nucleosome removal. Furthermore, the generation of RPA-coated ssDNA at sites of damage confirms nucleosome removal, due to concomitant DNA resection at sites of damage (Chen et al., 2008; Ransom et al., 2010). Chromatin reassembly following repair is thought to occur analogously to that following replication fork passage (Ransom et al., 2010). Interestingly however, although DNA damage is repaired in the absence of Asf1 and Caf1 (Linger and Tyler, 2005; Ramey et al., 2004) , the DNA damage checkpoint persists if these histone chaperones are inactivated (Chen et al., 2008; Kim and Haber, 2009)), leading to the hypothesis that reassembly of chromatin following DNA may signal the completion of DNA repair (Ransom et al., 2010). In human cells it was recently shown that H3K56 is a less common mark of newly synthesised histones than in budding yeast, but may be functionally replaced by H4K5K12 diacetylation (Jasencakova et al., 2010). In the same study, the authors observe an increase of Asf1 on chromatin, likely complexed with histones and MCM proteins upon replication stress and an increase in histones “stored” by Asf1 following replication stress and blockage of histone deposition. They also correlate increased ssDNA generation over 1.5h following treatment with HU with an increase in parental chromatin modifications on histones bound by Asf1. This is followed by a concomitant decrease of Asf1-associated “parental” histones following replication restart, thus favouring a role for Asf1 in histone “recycling”.

1.5 Chromatin remodelling and the Ino80 chromatin remodelling complex

Chromatin remodelling and Chromatin remodellors

Packaging of the DNA not only allows the cell to package its genetic information tightly and afford it maximal protection from both exogenous and endogenous sources of damage, but also confers a further level of regulation to cellular processes relying on DNA, such as replication and transcription. Chromatin is integral to DNA metabolism and thus mechanisms must exist to allow essential processes such as replication and repair to take place within the chromatin environment, for example removal of nucleosomes ahead of replication forks and nucleosome deposition following replication fork passage, as well as regulation of DNA accessibility must be ensured. Aside from use of variant histones, leading to a change in nucleosome structure and histone modifications, capable of both altering chromatin conformation and recruiting chromatin and DNA modifying activities, chromatin remodellers play an important role in chromatin modification. Chromatin remodellors are large, ATP-dependent remodelling complexes (Clapier and Cairns, 2009; Lusser and Kadonaga, 2003), which alter protein-DNA interactions by nucleosome sliding, partial or complete octamer removal, or exchange of core and variant histones within the histone octamer (Clapier and Cairns, 2009; Downs et al., 2007; Lusser and Kadonaga, 2003).

Broadly, there are four families of chromatin remodellors, as defined by their core ATPase subunit. All share common features and all employ ATP hydrolysis to mediate chromatin remodelling. However each family of chromatin remodellors also fulfils a unique role due to the presence of unique subunits within the complex and/or insertion domains with the ATPase subunit. Furthermore, many chromatin remodellor subunits contain specialised domains, allowing further specialisation and diversity of activity. However all display DNA- and nucleosome-binding activity, DNA-dependent ATPase activity, the ability for DNA translocation

and all contain subunits capable of interacting with further chromatin modification factors (Clapier and Cairns, 2009). It is thought that the combination of recognition and binding domains allows for the recruitment of these large remodelling complexes, as the binding constants for single domains range from nanomolar to micromolar (Clapier and Cairns, 2009). The multitude of potential interactions also poses the question as to whether such domain-specific interactions may also function to modulate the remodelling complex's activity (Clapier and Cairns, 2009).

ISWI family

The ISWI family of chromatin remodellors was originally identified in *Drosophila melanogaster,* however members of this chromatin remodelling family have subsequently been identified both in human and budding yeast. In contrast to most chromatin remodellors, which are large, multi-subunit complexes, ISWI family chromatin remodellors usually contain only 2-4 subunits (Clapier and Cairns, 2009). ISWI chromatin remodellors play a role both in gene silencing and gene activation and have also been implicated both in chromosome organisation by influencing the generation and maintenance of higher-order chromatin structures and in replication, as it may facilitate the progression of replication forks through heterochromatin (for reviews see (Corona and Tamkun, 2004; Mellor and Morillon, 2004).

CHD family

The CHD family of chromatin remodellors (chromodomain, helicase, DNA binding) was first identified in yeast, but homologous complexes have since also been identified in other organisms including humans (Clapier and Cairns, 2009). These complexes are composed of 1-10 subunits, with the catalytic subunit harbouring the defining two tandemly-arranged

chromodomains at its N-terminus (Clapier and Cairns, 2009). The complexes localise to highly transcriptionally active regions of more "open" chromatin and are believed to play a role in maintaining chromatin in a transcriptionally active state, as well as possibly playing a role in transcriptional elongation (for review see (Marfella and Imbalzano, 2007).

SWI/SNF family – Rsc and SWI/SNF

Most eukaryotes contain two SWI/SNF (switching defective/sucrose non-fermenting) family complexes, with distinct ATPase subunits, which in budding yeast are SWI/SNF and RSC (Remodels structure of chromatin) (Clapier and Cairns, 2009; Mohrmann and Verrijzer, 2005) and which indeed share a number of homologous subunits.

SWI/SNF's chromatin remodelling activity was first identified in its role in the regulation of gene transcription (Owen-Hughes and Workman, 1994) and was subsequently shown to be involved in nucleosome sliding, alteration of DNA-protein interactions and histone eviction (Whitehouse et al., 1999). Strains harbouring a deletion of the catalytic subunit snf2 or of snf5 are hypersensitive to the DNA damaging agents bleomycin and HU, but show no defects in end-joining assays, suggestive of a role for SWI/SNF in DSB repair by HR (Chai et al., 2005). Both these subunits have also been shown to localise to HO-induced DSB templates, further corroborating this hypothesis; they are first detected 40 minutes after break induction and protein levels at the break site increase over the following 4 hours (Chai et al., 2005). The authors also suggest SWI/SNF may be involved in the synapsis step of DSB repair by HR, as the yeast deletion strains are defective for synapsis (Chai et al., 2005). However more recent work by the Peterson lab proposes that the requirement for SWI/SNF at DSBs for the initial HR synapsis step may be unique to gene conversion at the MAT locus, which requires HR with a heterochromatic homologous donor (Sinha et al., 2009). Similarly, in mammalian cells, inhibition of SWI/SNF complexes leads to increased DNA DSB sensitivity and decreased repair,

as well as a defect in γH2AX establishment and focus formation (Park et al., 2009; Park et al., 2006; Peng et al., 2009). This role in DSB repair was seen to be independent both of the PIKKs and the complexes role in transcription, as neither the expression of H2AX, nor of the PIKKs was seen to be affected (Park et al., 2006). These results suggest a more direct role for the SWI/SNF remodellors at DSBs which may possibly involve increasing access for phosphorylation of H2AX (Park et al., 2006) . Whilst the SWI/SNF remodellors do not affect transcription of H2AX or the PIKKs (Park et al., 2006), it is important to remember that they nonetheless contribute to the cellular DDR via their role in transcription, indeed they have been shown to increase transcription of Rnr3 following DNA damage (Sudarsanam et al., 2000).

The second member of the SWI/SNF family of chromatin remodellors is the RSC complex, comprising 15 subunits in budding yeast, many of which have conserved homologues in mammalian cells. Many chromatin remodellor subunits contain specialised domains, allowing further specialisation and diversity of activity: bromodomains are particularly prevalent in the SWI/SNF family of remodellors recognise and bind acetylated lysines. In RSC, particularly, multiple bromodomains are present, which may signal a function on cooperative acetylated lysine binding (Clapier and Cairns, 2009). A further layer of specificity is added by the ability of some bromodomains to recognise specific acetylated lysine residues (Clapier and Cairns, 2009). The Rsc1 and Rsc2 subunits of RSC also contain bromo-adjacent-homology domains, which may further contribute to histone recognition and binding (Onishi et al., 2007). RSC has also been implicated in the DNA damage response, as yeast harbouring *sth1* (the catalytic subunit), as well as *rsc1* and *rsc2* subunit mutations display sensitivity to DNA damaging agents (Bennett et al., 2001; Chai et al., 2005; Kent et al., 2007; Shim et al., 2005). However mutations in these subunits do not seem either to affect checkpoint activation or to be involved in the transcriptional regulation of the DNA damage response (Angus-Hill et al., 2001; Shim et al., 2005). Contrary to SWI/SNF, a role for RSC in NHEJ has been suggested and indeed, Sth1

recruitment is dependent on Mre11, Rsc30 and the yeast Ku proteins (Shim et al., 2005). Like SWI/SNF, RSC has also been implicated in the repair of DSBs by HR (Chai et al., 2005). However in contrast to SWI/SNF, the catalytic subunit, Sth1 appears at the site of the DSB 10 minutes after break induction by ChIP (Chai et al., 2005), displaying comparable kinetics to the DNA damage kinases Mec1 and Tel1, as well as MRX (Chai et al., 2005; Lisby et al., 2004). Furthermore, aside from its early role in DSB repair by HR, suggested by the temporal dynamics of the complex's recruitment, *rsc2* mutant strains were defective in the ligation of DNA products resulting from resolved Holliday Junctions, thus suggesting a further, late role for the complex in DSB repair (Chai et al., 2005). Mutant *rsc2* yeast strains, analogously to mammalian cells, display decreased H2A phosphorylation following damage throughout the cell cycle (Liang et al., 2007), as well as displaying decreased ssDNA generation at the break site and decreased DNA-damage induced cohesion (Liang et al., 2007). Following the observation of a synthetic genetic interaction between *rsc2* and *tel1* and *mec1*, the same authors further show decreased Mec1 and Tel1 recruitment to DSB sites by ChIP in *rsc2* mutant strains, leading to a decrease in Rad9 and Rad53 recruitment (Liang et al., 2007). Importantly, it has been shown that RSC functions to slide nucleosomes adjacent to DSBs (Kent et al., 2007; Shim et al., 2007; Shim et al., 2005) and that this activity correlated with the complex's ability to promote the DNA damage signalling via the presence of phosphorylated H2A (Kent et al., 2007; Shim et al., 2007; Shim et al., 2005).

Ino80 family and Ino80

The Ino80 family of chromatin remodellors includes Ino80 and Swr1 and is characterised by the presence of a split ATPase domain in the catalytic subunit of the complex as well as the presence of Rvb1 and Rvb2 proteins, which show homology to bacterial RuvB.

Swr1

The SWR chromatin remodelling complex is composed of 14 subunits, with Swr1 being the catalytic subunit (Mizuguchi et al., 2004). Both actin and the actin-related proteins Arp4 and Arp6 belong to the SWR complex. SWR shares some of its subunits with the Ino80 complex (Arp4, Rvb1 and Rvb2) but also shares further subunits (Arp4, Swc4 and Yaf9) with the NuA4 histone acetyltransferase (Mizuguchi et al., 2004). Interestingly, in yeast, deletion of *swr1* leads to a similar phenotype to deletion of the histone variant *htz1*, both displaying sensitivity to DNA damaging agents such as MMS or UV-irradiation (Downs et al., 2004; Kobor et al., 2004; Mizuguchi et al., 2004), whereas the double mutant does not display increased sensitivity, suggesting an epistatis between the two genes (Downs et al., 2004). Furthermore, comparison of the transcriptional profile of the deletion strains revealed a 40% overlap in the genes regulated by these proteins (Mizuguchi et al., 2004). *In vitro*, SWR displays Swr1-mediated nucleosome-dependent ATPase activity (Mizuguchi et al., 2004). Further *in vitro* studies have also shown the complex to specifically interact with phosphorylated H2A (Downs et al., 2004). As for its *in vivo* role, it is thought that SWR is responsible for the replacement of the H2A-H2B dimer within the nucleosome with the variant histone Htz1-H2B dimer (Auger et al., 2008; Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). In humans, the TIP60 complex is thought to fulfil the role of SWR and is interestingly, by virtue of its subunit composition, thought to be an amalgamation of SWR and NuA4 (Auger et al., 2008; Doyon et al., 2004).

Ino80

Yeast Ino80 was first investigated by Ebbert et al who identified an *ino80-1* mutant as defective in the activation of inositol/choline responsive genes (Ebbert et al., 1999). They also observed an inability of the mutant strain to grow on media lacking inositol (Ebbert et al., 1999). The protein product was identified as a 1489 amino-acid polypeptide, of which the C-terminus displays significant identity (more than 30%) to the Snf2/Swi2-like ATPases, although

the proposed ATPase domain contains a spacer (Ebbert et al., 1999). The putative ATPase domain contains the conserved GXGKT sequence (Walker et al., 1982), which was proposed to be the nucleotide binding site, with the lysine of the conserved sequence making contact with the phosphate groups of the nucleotide (Ebbert et al., 1999; Story and Steitz, 1992). Indeed in Snf2, mutation of the corresponding lysine to arginine leads to loss of ATPase activity (Laurent et al., 1993). Analogously, the authors of this study found they could complement the ino80-1 mutation with full-length *INO80* on a plasmid, however introduction of full-length harbouring a lysine-to-arginine mutation at K737 was unable to rescue the ino80-1 mutant (Ebbert et al., 1999). The authors further observed Ino80 to be part of a large complex by gel-filtration (Ebbert et al., 1999). Furthermore, the complex also has a pleiotropic effect on gene expression (Ebbert et al., 1999). It was thus proposed that this complex might be considered a new chromatin remodelling complex (Ebbert et al., 1999). These observations were followed up by Shen et al, who purified the Ino80 complex by gel-filtration and identified 12 polypeptides (Shen et al., 2003): Ino80, Act1, Arp4, Arp5, Arp8, Rvb1, Rvb2, Taf14, Nhp10, Ies1 and Ies3. Since, this has been extended to 15 polypeptides, as Ies2, Ies4, Ies5 and Ies6 only associate with the complex at lower salt (Shen et al., 2003). Interestingly, whereas all other components associate with the complex with a 1:1 stoichiometry, Rvb1 and Rvb2 both associated with the complex with 6:1 stoichiometry, reminiscent of the bacterial RuvB, which forms a double hexamer (Shen et al., 2000).

The Ino80 complex is a large, multi-subunit remodelling complex

The versatility of chromatin remodellors is a direct result of their existence as large-multi-subunit complexes, with different subunits being responsible for distinct roles or activities of the complex. Observing the variety of subunits associated with chromatin remodellors, it is interesting to note that actin and Arps 4-9 (actin-related proteins), members of the actin

superfamily (Muller et al., 2005), are conserved subunits of a variety of chromatin remodelling complexes, thus hinting at an important and conserved role for these proteins (Cairns et al., 1998; Downs et al., 2004; Doyon et al., 2004; Galarneau et al., 2000; Gottschalk et al., 2008; Ikura et al., 2000; Kobor et al., 2004; Mizuguchi et al., 2004; Peterson et al., 1998; Shen et al., 2000; Zhao et al., 1998). In contrast to Arps 1-3 & 10, Arps 4-9 are predominantly nuclear and their integration into chromatin remodelling complexes across species suggests they fulfil a conserved role in chromatin metabolism (Blessing et al., 2004; Boyer and Peterson, 2000; Olave et al., 2002). All Arps contain a conserved ATP-/ADP binding domain, thus conferring upon them the potential for a conformational change, and thus a mechanism of regulation (Boyer and Peterson, 2000; Frankel and Mooseker, 1996). However comparison of Arp domains suggests that nuclear Arps may only bind ATP weakly or possibly even by a novel mechanism (Muller et al., 2005). Although nuclear Arps seem to be incorporated into chromatin remodelling complexes as monomers with a 1:1 stoichiometry, they are often found to be present in pairs of two distinct Arps (Farrants, 2008). Arp5 and Arp8 have been identified as part of the ino80 complex and have been shown to play an important role in the complex's functioning. Indeed, although both Arp5 and Arp8 are dispensable for the maintenance of the complex's integrity, loss of Arp8 causes loss of both Arp4 and actin (Shen et al., 2003). Swr1 on the other hand contains Arp6, which is thought to play an important role in the facilitation of histone variant exchange (Krogan et al., 2003; Mizuguchi et al., 2004; Wu et al., 2005). Arp7 and Arp9 are interestingly unique to fungi (Muller et al., 2005) and are integral to the SWI/SNF complexes, though it would seem they are of greater importance in the SWI/SNF complex compared to RSC (Blessing et al., 2004; Chen and Shen, 2007; Muller et al., 2005). Importantly, Arp4, which is found as a subunit of Ino80 and Swr1, (as well as NuA4), has been shown to bind phosphorylated H2A in response to DNA damage (Downs et al., 2004), giving rise to the hypothesis that it may act as a recruitment module for these complexes to the sites of DNA

damage (Downs et al., 2004), thus forming a further link between covalent histone modification and chromatin remodelling by "reading" the histone DNA repair code.

Further subunits of the Ino80 complex (see Figure 1.5.1), such as Taf14, as well as Rvb1 and Rvb2, are also shared with other remodelling complexes. Aside from its role in the Ino80 complex, Taf14 also associates with TFIID, TFIIF, SWI/SNF and RSC (Kabani et al., 2005). The protein contains a YEATS domain, which, in budding yeast, is found in three proteins: Taf14, Yaf9, a component of the NuA4 chromatin modifying complex and Sas5, part of the SAS silencing complex. Budding yeast have been shown to require at least one of these three proteins for cell survival (Zhang et al., 2004). The YEATS domain is not important for these proteins' association with the respective protein complexes (Schulze et al., 2010) and has been ascribed a role in the negative regulation of cell growth in budding yeast (Schulze et al., 2010). Although the exact role of Taf14 remains unclear, its association with a number of complexes involved in DNA metabolism suggests it may act as a link between a variety of chromatin activities and a common cellular process, possibly via its YEATS domain and its involvement in growth regulation (Schulze et al., 2010).

The Rvb1 and Rvb2 proteins identified as integral to the Ino80 complex (Shen et al., 2000), have been shown to be essential for cellular viability (Qiu et al., 1998). Rvb1 and Rvb2 are members of the AAA+ family of ATPases (Hanson and Whiteheart, 2005) and there is limited homology between both Rvb proteins and bacterial RuvB which is involved in Holliday Junction resolution in bacteria (West, 1996). The Rvb proteins are required for the chromatin remodelling activity of the Ino80 complex, but do not seem to play a role in recruitment of the complex to promoters *in vivo* (Jonsson et al., 2004). They are also responsible for the recruitment of a further subunit, Arp5 to the Ino80 complex (Jonsson et al., 2004).

Nhp10 (non-histone protein 10), an HMGB (non-sequence specific high mobility group) protein co-purifies with the Ino80 complex and so far has not been identified as a component of any

other complexes (Shen et al., 2003). HMGB proteins contain HMG boxes, which are DNA binding motifs, which often display a preference for distorted DNA (Ohndorf et al., 1999). Indeed, *in vitro*, Nhp10 has been shown to bind DNA, with a slight preference for DNA containing "loops", consisting of tandem pairs of mismatches, however the protein does not display a significant preference for Holliday Junction DNA (Ray and Grove, 2009). The same authors also showed that Nhp10 binds either blunt DNA ends or DNA ends with A-T containing overhangs, thus protecting the DNA from exonucleolytic, but not endonucleolytic processing *in vitro* (Ray and Grove, 2009).

Finally, the Ino80 complex also contains 6 Ino80-specific subunits, known as Ies1-6 (Ino-eighty-subunit). Ies2, 4, 5 & 6 only co-purify with the complex under low salt conditions (Shen & Wu 2003). These subunits remain relatively uncharacterised and as Ies1 and Ies3-5 do not seem to be evolutionarily conserved they may play a budding-yeast specific role in regulating Ino80 complex activity (Bao and Shen, 2007).

The Ino80 complex contains many evolutionarily conserved protein subunits

The importance of the Ino80 complex is highlighted by its evolutionary conservation across species (Table 1.5.1). Indeed, both homologous complex subunits to budding yeast Ino80 and further complex subunits conserved between other organisms have been identified in *S.pombe, Drosophila melanogaster, Arabidopsis thaliana* and *Homo sapiens* (Fritsch et al., 2004; Hogan et al., 2010; Jin et al., 2005; Klymenko et al., 2006). Subunits highly conserved across the species aside from the Ino80 ATPase are the Rvb AAA+ ATPase proteins, identified as Rvb1/Rvb2 in S.pombe, pontin 52/reptin 52 in D.melanogaster and TIP49/TIP48 in human cells. Further conserved subunits are Arp4 (BAF53A in human cells), Arp5, Arp8 and Actin. Additionally, Ies2 (PAPA-1 in human cells) and Ies6 (Fritsch et al., 2004; Hogan et al., 2010; Jin et al., 2005; Klymenko et al., 2006) are highly conserved subunits, suggesting these form the

"core Ino80 complex". *S.pombe* Ino80 complex also contains an HMG-box-containing, nhp10-like protein, as well as a Taf14 homologue (Hogan et al., 2010). Each of the complexes also contains subunits unique to each species, suggesting the existence of species-specific regulatory mechanisms (Table 1.5.1). The presence of Zn-finger transcription factors in *S.pombe*, *D.melanogastor* and mammalian cells is also of interest and is thought to possibly play a role in the regulation of transcription by the complex (Morrison and Shen, 2009). Thus the Iec1 protein identified in *S.pombe* contains a C2H2 zinc-finger motif, similar to that of the GLI-Krüppel family of transcription factors. The polycomb group pleiohomeotic (PHO), part of *D.melanogastor* Ino80 and YY1, which is integral to mammalian Ino80 (Cai et al., 2007; Wu et al., 2007), are also members of the GLI-Krüppel transcription factor family (Cai et al., 2007; Hogan et al., 2010; Klymenko et al., 2006; Wu et al., 2007). These three proteins further display similarity at the level of a seven amino-acid "HTGEKP(F)" motif (Hogan et al., 2010). So far no evidence for an orthologous protein in *S.cerevisiae* has been found (Hogan et al., 2010).

Recent work on the YY1 subunit of the human Ino80 complex makes these subunits particularly interesting: Yin Yang-1 (YY1) is a GLI-Krueppel zinc-finger polycomb group (PcG) transcription factor, which was identified as a subunit simultaneously by two groups (Cai et al., 2007; Wu et al., 2007). It plays a role both in the transcriptional activities of the Ino80 complex, as well as its role in DNA damage (Cai et al., 2007; Wu et al., 2007). Indeed, the Conaway lab demonstrated a role for the human Ino80 complex as an essential co-activator of YY1-regulated genes, which are involved in such essential processes as cell growth and cell cycle regulation, as well as differentiation and apoptosis (Cai et al., 2007; Wu et al., 2007). However it remains to be elucidated whether activation involves Ino80 chromatin remodelling following recruitment to the promoter by YY1, or whether Ino80 chromatin remodelling facilitates YY1 binding (Cai et al., 2007). Of further interest is the role the YY1 has been shown to play in the maintenance of genome stability (Wu et al., 2007). In this study the authors find that mice embryonic fibroblasts (MEFs) lacking YY1 display changes in ploidy, as well as

aberrant chromosomes structures, a hallmark both of cells deficient in DNA repair, usually by homologous recombination, as well as cancer . The authors further find increased sensitivity to DNA damaging agents in human cells in the absence of YY1, which is epistatic with the absence of Ino80. As they also observe both the G1/S and G2/M checkpoints to be intact in these cells, the DNA damage sensitivity is likely to result from a DNA repair deficiency (Wu et al., 2007). Furthermore, YY1 deficient cells display a decreased recombination rate in recombination assays (Wu et al., 2007). The authors hypothesise that YY1, which co-purifies with TIP49A/TIP49B may act analogously to RuvA, although there is a lack of sequence homology. YY1 however binds DNA and displays a preference for structured DNAs, such as Holliday Junctions and Y-structures, supporting this hypothesis and a role for YY1 in the maintenance of genomic stability (Wu et al., 2007).

The Ino80 complex is involved in DNA metabolic pathways

In vitro, Ino80 possesses DNA or nucleosome-dependent ATPase activity, ~95% of which can be ascribed to the catalytic subunit as determined by comparison to the K737R mutant (Shen et al., 2000). Further studies found a 3'-5' helicase activity present in the purified complex, which again is dependent on the catalytic subunit but which may rely on contributions from the Rvb proteins (Shen et al., 2000). The Ino80 complex binds DNA (Shen et al., 2003) and also displays the ability to mobilise, or slide nucleosomes, although the precise mechanism by which Ino80 remodels chromatin remains unclear (Shen et al., 2003). The human Ino80 complex is also able to catalyse the hydrolysis of ATP in a DNA- or nucleosome-dependent manner (Jin et al., 2005). Although stimulated by the presence of DNA, the stimulation was greater in the presence of nucleosomes, but not affected by the presence of free histone octamers (Jin et al., 2005). Human Ino80 complex is also able to slide nucleosomes *in vitro* and, analogously to yeast, would seem to preferentially slide nucleosomes from DNA ends towards a more central

position (Jin et al., 2005; Shen et al., 2003). *In vivo*, yeast strains harbouring a deletion of *ino80* are seen to be sensitive to DNA damaging agents, such as HU, MMS, UV-irradiation and IR (Shen et al., 2003). *S.pombe* deletion strains of *ino80* are inviable, however fission yeast deletion strains of one of *arp8*, *ies2*, *ies6* or *iec1* are also seen to be sensitive to HU, bleocin, and UV irradiation, whereas strains harbouring a deletion of *NHP10* do not display DNA-damage sensitivity (Hogan et al., 2010). The DNA-damage sensitivity in the absence of Ino80 complex subunits therefore constitutes a link between a chromatin remodellor and the DNA damage response, which may be mediated indirectly by transcriptional effects or directly via chromatin remodelling activity at sites of damage (Shen et al., 2000).

The Ino80 complex at DNA double strand breaks

Ino80 is likely to play a role in the DNA damage response

Although it had been established that the Ino80 complex plays a role in the DNA damage response, it remained to be established whether this role was mediated by its activity in transcriptional activation or by a more direct mechanism, possibly via chromatin remodelling at the site of damage. Indeed, investigation into the Ino80 complex' role in transcription following DNA damage found the global transcription profile to remain approximately unchanged, with regards to genes involved in DNA repair, HR and NHEJ (Mizuguchi et al., 2004; Morrison et al., 2004). A further study which not only investigated changes in the *ino80* deletion strain, but also the *arp8* and *arp5* deletion strains, as the *ino80* deletion is not viable in all genetic backgrounds, found there to be no changes in DNA repair or checkpoint genes in the deletion strains compared to the wild-type in absence of damage and this remained the case following exposure to MMS (van Attikum et al., 2004). The same authors also followed the induction of genes involved in DNA repair, DNA processing and cell cycle regulation and found their induction to be normal in the mutant strains compared to the wild-type (van

Attikum et al., 2004). The authors do note that the ino80 deletion strain fails to induce Mrc1 and Tof1 to normal levels, whereas the *arp5* and *arp8* deletion strains behave normally (van Attikum et al., 2004). This highlights the difficulty of comparison between yeast genetic backgrounds and strains harbouring deletions of different complex subunits. Both studies confirm that induction of RNR1 and RNR3 is normal in the respective Ino80 subunit deletion strains (Morrison et al., 2004; van Attikum et al., 2004). In addition to the fact that the Ino80 complex does not seem to play a role in the transcriptional regulation of the DNA damage response, strains harbouring an *ino80* deletion display a normal cell cycle arrest following treatment with HU (Morrison et al., 2004). The checkpoint in *ino80*, *arp5* and *arp8* deletion strains is further confirmed to be intact by the normal Rad53 phosphorylation following treatment with MMS (van Attikum et al., 2004).

The Ino80 complex localises to DSBs

Evidence for a direct role in the DNA damage response to DSBs was obtained by three groups, all using the HO endonuclease system allowing for the generation and maintenance of a single DSB in budding yeast. Indeed, myc-tagged Ino80, Arp5 and Arp8 accumulate at DSBs and the surrounding chromatin by ChIP (van Attikum et al., 2004). In this study their recruitment was detected at 0.4kb and 1,6kb from the break site. The earliest time point for detection was found to be 1h and detection peaked at 4h (van Attikum et al., 2004). The Shen group obtained similar results using Flag-tagged Ino80, Arp5 and Arp8 and found increased signal at 30 min post DSB-induction (Morrison et al., 2004). In a further study, Arp4 was also ChiPped to directly at the DSB site, as well as at 1.5kb from the break site, again with maximal detection at 4h (Downs et al., 2004). In this study, Arp4 is detected up to 10kb each side of the break, which corroborates data detecting Ino80, Arp5 and Arp8 up to 9kb each side of the break (Morrison

et al., 2004), although it must be remembered that Arp4 is a subunit of multiple chromatin modifying complexes (such as NuA4) (Downs et al., 2004).

Phosphorylated H2A plays an important role in the recruitment of the Ino80 complex to DSB

One of the earliest responses to DNA damage is the phosphorylation of H2A at S129 in budding yeast and phosphorylation of S139 of the histone variant H2AX in mammals (see above). It was therefore proposed that phosphorylated H2A might play a role in the recruitment of DNA repair factors, including chromatin remodelling complexes (see Figure 1.5.2) (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004). It was previously shown that the Ino80 complex co-purifies with the core histones (Mizuguchi et al., 2004) and repetition of these experiments revealed the complex also co-purifies with phosphorylated H2A, in a DNaseI, RNase A and ethidium bromide-resistant manner, suggesting this interaction is not mediated by nucleic acid (Morrison et al., 2004). Under the same conditions, the SWI/SNF complex did not associate with phosphorylated H2A, thus confirming the physiological relevance of the co-purification (Morrison et al., 2004). Further experiments were carried out *in vivo* by the Gasser group, who first confirmed the presence of phosphorylated H2A at the HO break site and checked that although appearance of phosphorylated H2A is dependent on the damage kinases (Shroff et al., 2004), it was not dependent on the presence of Ino80, Arp5 or Arp8 (van Attikum et al., 2004). However upon mutation of H2A at S129, there was a significant decrease in Ino80 complex presence at the DSB site (van Attikum et al., 2004). Similarly, deletion of the DNA damage kinases, Mec1 and Tel1 lead to a significant decrease in Ino80 complex recruitment to sites of DSBs (Morrison et al., 2004). These experiments are further corroborated by phosphorylated H2A peptide pull-down assays with the Ino80 complex (Downs et al., 2004).

The mechanism of interaction between INO80 and phospho-H2A remains unclear

Although the recruitment of the Ino80 complex to sites of DNA damage has been confirmed and been shown to be dependent upon phosphorylation of H2A, the exact nature of the interaction between the complex and the phosphorylated histone and the identity of the subunit mediating this interaction remain controversial. Co-purification experiments in *arp8* deletion strains showed wild-type levels of association with phosphorylated H2A, whereas the same experiments in a strain lacking *NHP10*, a further complex subunit unique to Ino80 showed a significant decrease in the levels of phosphorylated H2A co-purifying with the Ino80 complex (Morrison et al., 2004). The authors also observed a decrease in the recruitment on the Ino80 complex to sites of DSBs by ChIP in the *nhp10* deletion strain (Morrison et al., 2004) and thus conclude that Nhp10, a unique subunit of the Ino80 complex is the "recruitment module" to DSBs via its interaction with phosphorylated H2A (Morrison et al., 2004), although loss of Nhp10 does not affect remodelling activity (Shen et al., 2003). However the same authors previously demonstrated that *nhp10* deletion strains are not sensitive to the DNA damaging agent HU, which might be expected should Nhp10 be key to the recruitment of the Ino80 complex (Shen et al., 2003), a finding confirmed in fission yeast, in which the corresponding *nhp10* deletion strain is neither sensitive to HU, bleocin or UV irradiation (Hogan et al., 2010). In a further study, recruitment of multiple chromatin remodellors or chromatin modifying complexes is ascribed to Arp4 as in the authors' hands, recombinant Arp4 is observed to specifically interact with phosphorylated H2A *in vitro* (Downs et al., 2004). Furthermore, the same authors detect a decrease both in the recruitment of the NuA4 complex, as well as the Ino80 complex by ChIP at sites of DSBs in an *arp4* mutant strain (Downs et al., 2004). These results, combined with the DNA damage sensitivity of the *arp4* mutant would argue perhaps for a communal role for Arp4 in recruitment of chromatin modifying complexes to DSB sites. The precise mechanism of Ino80 recruitment to sites of DSBs and the

subunits involved thus remain to be elucidated and the process may well be mediated by a number of subunits and interactions with the chromatin surrounding the break.

The Ino80 complex is likely to be important for double strand break repair by homologous recombination

The sensitivity of the Ino80 complex deletion strains and the recruitment of the complex to DSBs clearly afford the complex a role in the processing and repair of such damage, however the complex's actual function at sites of damage still remains controversial and unclear. The DNA damage sensitivity profile of the *ino80*, *arp5* and *arp8* deletion strains with their hypersensitivity to MMS and HU closely resemble those of strains harbouring deletions of components of the *RAD52* HR repair pathway (van Attikum et al., 2004). Furthermore, double deletion strains of Ino80 subunits and proteins involved in HR display a synthetic sick phenotype upon exposure to DNA damaging agents (Morrison et al., 2004). Both in the *arp8* deletion strain, as well as a strain harbouring mutations of the S129 of H2A, a decrease in the generation of ssDNA at the DSB site is observed by PCR (van Attikum et al., 2004). As deletion of Arp8 abolishes the complex' chromatin remodelling activity but the Rvb proteins remain associated with the complex, the authors conclude that the decrease in ssDNA generation is due to a lack of chromatin remodelling by the Ino80 complex (van Attikum et al., 2004). The generation of ssDNA is a critical step in the DDR, responsible for the recruitment of a number of downstream repair factors which might explain the deletions strains' inability to repair and thus sensitivity to DNA damaging agents (van Attikum et al., 2004). The authors also point out that they find similar kinetics between the generation of ssDNA and Ino80 recruitment, further supporting the hypothesis for the involvement of the complex in the generation of ssDNA, potentially aiding resection and therefore the recruitment of downstream factors, however DNA damage recognition factors, such as the MRX complex are, due to their fast recruitment

kinetics, unlikely to be affected by Ino80 chromatin remodelling (van Attikum et al., 2004). This has further been proposed to be due to a role for Ino80 in evicting histones at the DSB (Tsukuda et al., 2005; Tsukuda et al., 2009; van Attikum et al., 2007). Subsequently, the Gasser group proposed that removal of variant histone H2A.Z and phosphorylated H2A , as well as the core histones by the Ino80 complex at sites of damage facilitates both the binding of the Mre11 nuclease and thus the MRX complex, as well as yKu80 (van Attikum et al., 2007). They further observe a decrease in Mec1-Ddc2 recruitment following the decrease in histone eviction in the *arp8* deletion strain, as well as a decrease in Rad53 phosphorylation, which stands in contrast to the normal checkpoint activation as monitored by Rad53 phosphorylation they previously observed in the *ino80*, *arp5* and *arp8* deletion strains (van Attikum et al., 2007; van Attikum et al., 2004). Furthermore, the Osley group report that resection is normal compared to wild-type in an *arp8* deletion strain and that nucleosome eviction by the Ino80 complex is dependent on MRX and required for Rad51 ability to displace RPA and thus the initiation of repair by homologous recombination (Tsukuda et al., 2005). In a following study however, but now using a diploid yeast strain, the authors surprisingly not only observe a defect in the initial strand invasion event of DSB repair by HR in the *arp8* deletion strain, but also propose a role for the Ino80 complex in later stages of DSB repair by HR, controlling gene conversion tract length (Tsukuda et al., 2009). Finally and adding to the controversy, a further group has shown that the observed chromatin disassembly correlated and is coupled with DNA resection and the fact histones do not bind ssDNA (Chen et al., 2008). In this study whilst they observe a significant defect in both resection and chromatin disassembly in an *mre11* deletion strain, the defect is very subtle in both regarding resection and chromatin disassembly in an *arp8* deletion strain (Chen et al., 2008). Thus although Ino80 has been shown to play an important role in the repair of DSBs the complex's precise mechanism of action remains to be elucidated.

Ino80 at stalled replication forks

Although the precise action of the Ino80 complex at DSBs is still unclear it nonetheless plays a role in the DDR to DSBs. More recently, it was proposed the complex may also play a role in replication, in particular at stalled replication forks, due to the hypersensitivity to HU of a number of Ino80 subunit deletion strains (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008).

The Ino80 complex localises to origins of replication

Following ChIP on chip experiments of Ino80 and Arp5 across four budding yeast chromosomes, both subunits were enriched at sites of initiation of DNA replication (mainly at early origins), as well as at tRNA genes, which represent natural replication fork pausing sites (Shimada et al., 2008). Following release of the yeast into S-phase under fork stalling conditions using HU, an increase in Ino80 and Arp5 occupancy at origins was observed (Shimada et al., 2008). This is further confirmed by yeast genome-wide ChIP-chip performed in another study, in which Ino80 is seen to be recruited to ARS sequences during S-phase and displays a slight preference for early-firing origins (Falbo et al., 2009). Further ChIP experiments with the Ino80 subunit and Polε upon release of yeast into S-phase under HU-induced fork stalling conditions show accumulation of both the polymerase and Ino80 not only at the origin but also at 4kb from the origin, where stalled forks might be expected, leading to the suggestion Ino80 may itself associate with the replication fork (Shimada et al., 2008). The authors also observed an increased presence of the Ino80 subunit at late firing origins over time in HU-induced replication fork stalling conditions although in these circumstances firing from such origins is inhibited (Shimada et al., 2008). An additional study also observed an increase in Ino80 at early-firing origins upon release from G1 into S-phase, as well as a concomitant decrease of Ino80 ChIP signal at the origin and an increase at sites distal to the

origin, mirroring the ChIP signal from PCNA during S-phase progression, providing further evidence for an association of Ino80 with the replication fork (Papamichos-Chronakis and Peterson, 2008). In a separate study, the Nhp10 subunit was also seen to be increased at the same sites as Pol1 by ChIP, again supporting the hypothesis that Ino80 may interact with the replication fork (Vincent et al., 2008).

Replication forks collapse in the absence of the Ino80 complex

Indeed, the importance of these findings is highlighted *in vivo* not only by the hypersensitivity of Ino80 complex subunit deletion strains to chronic HU exposure (Papamichos-Chronakis and Peterson, 2008), but also by their sensitivity and inability to recover from acute exposure to HU (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008). This high lethality rate in the face of acute replication stress indicates that replication forks collapse in the absence of a functional Ino80 complex and suggest a possible role for the chromatin remodelling complex in S-phase progression (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008).

The Ino80 complex is likely to play a role in S-phase progression

The afore mentioned ChIP-chip experiments suggest the Ino80 chromatin remodelling complex plays a role in initiation of replication, however its association with the replication fork and the hypersensitivity to acute HU exposure suggest it may also play a role in S-phase progression. Indeed, by fluorescence-activated cell sorting (FACS), it was seen that cells lacking *INO80* possess slower kinetics of replication compared to wild-type cells (Papamichos-Chronakis and Peterson, 2008). Although there is a delay in initiation in the *ino80* deletion strain compared to the wild-type, this delay is not sufficient to explain the slow S-phase kinetics and delayed G2 entry. In addition, activation of the Cdc7 kinase is also seen to be comparable in the deletion

and wild-type strains (Papamichos-Chronakis and Peterson, 2008). This difference is exacerbated upon addition of HU, as in contrast with the wild-type cells, which complete S-phase, albeit with slower kinetics (160 min as opposed to 60 min in absence of HU), cells lacking *INO80* permanently arrest in a partially replicated state, even after 200 min as seen by persistent Rad53 phosphorylation (Papamichos-Chronakis and Peterson, 2008). This is confirmed in the study by the Gasser group, which also observes the persistence of unreplicated DNA in both the *ino80* and *arp8* deletion strains compared to wild-type in the presence of HU, although the latest time point assessed in this work is 75 min following release from HU (Shimada et al., 2008). A third study further implicates the Ino80 complex in replication fork progression as a decrease in rate of replication fork progression is observed in an *isw2 nhp10* double deletion strain (Vincent et al., 2008). Both studies further investigate the effect of the absence of the Ino80 complex on S-phase progression by 2D gel electrophoresis (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008). In one, upon release into HU, no bubble arcs, typical of initiation, nor X-structures, reflecting stalled forks were observed over a 4h time course, however initiation had taken place as confirmed by PCR of the DNA adjacent to the origin, leading the authors to conclude that forks stall rapidly upon encountering replication stress in the absence of the Ino80 complex (Papamichos-Chronakis and Peterson, 2008). Further evidence for a continuous role for the Ino80 complex throughout S-phase was obtained by use of a temperature-inducible ino80-degron: loss of Ino80 during S-phase in the absence of HU led to a reduction in S-phase kinetics, whereas in the presence of HU led to permanent arrest of replication (Papamichos-Chronakis and Peterson, 2008). In the second study, initiation and some elongation was observed over the time of 1h, by the presence of bubble arcs and Y-arcs, respectively, however in the *ino80* and *arp8* deletion strains, these structures persisted (although the authors note the absence of a “cone” signal characteristic of collapsed replication forks) and contrary to the wild-type replication did not go to completion (Shimada et al., 2008).

Similar results have been found in a study investigating human Ino80: indeed, although cells lacking Ino80 showed normal entry into S-phase, they displayed impaired S-phase progression and a low rate of DNA synthesis (Hur et al., 2010). Furthermore, human Ino80 colocalised with PCNA by immunofluorescence, leading the authors to propose that human Ino80, analogously to its yeast counterpart may also associate with replication forks (Hur et al., 2010). This colocalisation was seen to be dependent on the presence of a functional Ino80 ATPase, that is the complex' ability to remodel chromatin (Hur et al., 2010).

The Ino80 complex has been implicated in the DNA damage tolerance response

Recently, another study of Ino80 in replication used 2-D gel electrophoresis and found bubble arcs in both the wild-type and *arp8* deletion strain, which the authors interpret to mean both strains are proficient in the maintenance of arrested forks following replication stress (Falbo et al., 2009). However in this study the only time point investigated following synchronous release into HU was 1h. This is in contrast to the afore mentioned studies in which replication has gone to completion in the wild-type strain by 1h (Shimada et al., 2008) and in which far less replication intermediates are resolved by 2-D gel electrophoresis in the mutant strains (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008). Again no cone signal, indicative of replication fork collapse was seen (Falbo et al., 2009). The authors also propose that Ino80 is not required for recovery of replication forks upon removal of HU as observed by FACS data not published (Falbo et al., 2009). The authors thus conclude that the HU-sensitivity in the absence of the Ino80 complex is not a direct result of defective S-phase progression or replication fork maintenance following HU-induced replication stress (Falbo et al., 2009). Instead, this study observes a defect of the *ino80* deletion strain to process stalled replication forks caused by acute MMS exposure in S-phase, as seen by S-phase specific induction of phosphorylated H2A foci (Falbo & Shen 2009). Furthermore, the authors observe a decrease in

PCNA ubiquitylation, both in the deletion strain and in the presence of the catalytic-dead Ino80-K737A mutant, as well as a decrease in the Rad18 ChIP signal at origins following acute MMS exposure in S-phase. They also observe a decreased ChIP signal for Rad51 at origins following acute MMS exposure and note the absence of Rad51-dependent recombination intermediates characteristic of stalled replication fork processing as assessed by 2-D gel electrophoresis (Falbo et al., 2009). Based on these data, the authors conclude that Ino80, by virtue of its chromatin remodelling acitivity plays an early role in the DNA damage tolerance pathway, rather than in S-phase progression or the stabilisation of stalled replication forks, as it impinges both on the Rad6 and Rad51 pathways allowing processing of blocked replication forks (Falbo et al., 2009).

The Ino80 complex, S-phase and the establishment of sister chromatid cohesion

Interestingly, a further study observed hypersensitivity of the *arp8* deletion mutant to the microtubule-destabilising drug benomyl, suggesting the *arp8* deletion strain is defective either in kinetochore function or sister chromatid cohesion (Ogiwara et al., 2007). The centromeric association of kinetochore components was normal in the *arp8* deletion strain, however the mutant strain was defective in sister chromatid cohesion both in centromeric regions and on chromosomal arms (Ogiwara et al., 2007). Furthermore, ChIP experiments demonstrate that cohesion factors are still able to bind in the absence of Ino80, which thus does not play a role in the recruitment of such factors (Ogiwara et al., 2007). The authors also observe the association of the Ino80 complex with the replication fork, especially at early origins upon treatment with HU, as seen in the above mentioned studies (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008) and speculate that the presence of Ino80 may influence the association of sister chromatid cohesion factors such as PCNA and Ctf18 with the replication fork. In support of this, they do observe some variation in the presence of these

factors in the mutant strains compared to the wild-type strain (Ogiwara et al., 2007). Finally, the authors contemplate the possibility that Ino80 may be critical for the correct modulation of the replication fork's chromatin environment thus impinging on the establishment of sister chromatin cohesion (Ogiwara et al., 2007).

The Ino80 complex may be involved in chromosome segregation in mitosis

In a recent study, the human Ino80 complex was found to be associated with microtubules during mitosis, as well as with α-tubulin, as seen by co-IP, an interaction seen to increase during mitosis compared to asynchronous cells (Hur et al., 2010). Cells lacking Ino80 were observed to be unable to correctly assemble microtubules in mitosis leading to mitotic defects, including failure to correctly separate chromatids followed by an abortive anaphase thus leading to an increase in cellular ploidy (Hur et al., 2010). Furthermore, mitotic spreads of cells lacking Ino80 displayed both changes in ploidy, as well as structural chromosomal abnormalities, allowing the authors to conclude that Ino80 is important for the maintenance of genomic stability via a role in correct chromosome segregation (Hur et al., 2010).

The Ino80 complex may play a role in the checkpoint or in checkpoint adaptation following DNA damage

Purification of the Ino80 complex from cells treated with MMS compared to untreated cells led to the identification of the DNA-damage dependent phosphorylation of Ies4, which was shown to be due to Mec1 and Tel1 by genetic analysis, on serine residues at the protein's N-terminus (Morrison et al., 2007). Phosphorylated Ies4 was shown not to affect the actual repair process; however a modified "phosphomimic" Ies4 displayed decreased viability when exposed to DNA damaging agents and increased checkpoint activity as seen by increased and persistent Rad53

phosphorylation (Morrison et al., 2007). Furthermore, genetic analysis of a strain harbouring these two proteins revealed these two proteins to act in a compensatory manner in the replication checkpoint response (Morrison et al., 2007). The authors also observed a decrease in S-phase progression even in the absence of DNA damage in this double mutant strain (Morrison et al., 2007). The authors suggest three possible explanations for their observations i) chromatin remodelling by the Ino80 complex may mediate Tof1's role in sister chromatin cohesion in postreplicative repair ii) chromatin remodelling by the Ino80 complex may allow for the recruitment of checkpoint proteins and iii) checkpoint proteins may interact directly with the Ino80 complex (Morrison et al., 2007). The Ino80 complex has also been implicated in the checkpoint adaptation. In the presence of a persistent DSB, whilst over time wild-type cells adapt and escape the checkpoint, thus progressing through the cell cycle with unrepaired DNA damage, cells harbouring a mutant Ino80 are unable to adapt and continue to display high levels of checkpoint activation, monitored by Rad53 phosphorylation (Papamichos-Chronakis et al., 2006).

Further work is needed to settle controversies as well as to more clearly define and gain mechanistic insight into the multiple roles the Ino80 complex is likely to play in S-phase, replication and the avoidance of genomic instability throughout these cellular events. However the above studies clearly demonstrate the importance of Ino80 in the maintenance of genomic stability in S-phase and highlights its intriguing multi-facetted nature.

1.6 Perspectives

Ies6 a unique, uncharacterised subunit of the Ino80 complex

The Ino80 complex contains six Ino80-specific subunits, known as Ies1-6 (Ino-eighty-subunit). Ies2, 4, 5 & 6 only co-purify with the complex under low salt conditions (Shen et al., 2003). This subset of subunits remains relatively uncharacterised and as Ies1 and Ies3-5 do not seem to be evolutionarily conserved, they may play a fungal specific role in regulating Ino80 complex activity (Bao and Shen, 2007). Ies2 and Ies6, however, are conserved across species including humans, suggesting they play an important, evolutionarily conserved role in the complex's activity. It is therefore of interest to investigate Ies6 in budding yeast in an attempt to elucidate its function within the Ino80 complex.

Ies6 is a putative DNA binding protein

Ies6 is a small (166 amino acids), basic protein (pI = 11.05), which remains uncharacterised, aside from its association with the Ino80 complex under low salt conditions (Morrison & Shen 2003). The protein does however contain a C-terminal YL1 domain (SGD, Pfam). This domain is homologous to the YL1 protein, first identified in 1995, which was thought to be a putative DNA binding protein and transcription factor and localised to the nucleus (Horikawa & Oshimura 1995). More recently the mammalian YL1 protein was identified as a subunit of both the TRRAP/TIP60 and the SRCAP (similar to budding yeast SWR1) complex where it interacts with an uncharacterised zinc-finger protein (Cai et al., 2005). The ability of this domain to bind DNA still remains unclear, however its presence within the Ies6 protein leads to the hypothesis that Ies6 may possess DNA binding activity. Although a number of proteins found within the Ino80 complex have been implicated in the recruitment of Ino80 to chromatin (Downs et al.,

2004; Morrison et al., 2004) and have been shown to possess the ability to bind DNA, it seems reasonable that the complex' interaction with chromatin and DNA is mediated by numerous contacts and indeed possible that each of these chromatin/DNA-interacting subunits may be fulfilling specialised roles of recruitment, recognition, retention, interaction and modulation. Furthermore, the apparent absence of a budding yeast homologue of the mammalian YY1 protein makes it possible for one of the yeast subunits to fulfil a similar role (see table 1.5.1), which not only involves DNA binding activity but also the recognition of structured DNA. In light of these facts, this study investigated the potential DNA binding activity of the Ies6 subunit of the Ino80 chromatin remodellor.

Does Ies6 contribute to the maintenance of genomic stability?

Aside its role in inositol metabolism and transcription for which it was originally identified, the Ino80 complex has, over the last few years, been identified and recognised as playing a crucial role in the maintenance of genomic stability. The complex has not only been ChIPped to DSBs, where it plays a role in the repair of the DNA damage, but more recently, the complex has also been shown to be associated with replication forks, where it is critical to replication fork stability in times of replication stress. Furthermore, the Ino80 complex has also been implicated in checkpoint adaptation, sister chromatid cohesion and correct chromosome segregation, all processes involved in preserving genomic stability. The Ino80 complex is a large, multi-subunit complex and it is reasonable to assume that some of the subunits may play selective roles in the different aspects of Ino80's involvement in such a variety of cellular pathways. Considering the above, the purpose of this study was to investigate the phenotype associated with the loss of Ies6, the protein's possible DNA binding activity and the relationship between observed phenotype and the possible DNA binding activity, with the aim

of elucidating the function of the Ies6 subunit within the Ino80 complex and its role in the maintenance of genomic stability.

2.1 DNA Manipulation

Plasmids used in this study

Plasmid	Organism	Source	Features
pGEM-T-Easy	Bacteria	Promega	Contains 3' T overhangs compatible for PCR products with 3' A overhangs; multiple cloning site (MCS) within LacZ gene provides blue/white screening of recombinants; T7 and SP6 promoter sequences flank the MCS; *Amp*^{R} gene; f1 origin
pET-15b	Bacteria	Novagen	Expression vector that provides N-terminal 6-histine tag; Thrombin cleavage site for N-terminal tag removal; T7 promoter and terminator sequences flank the MCS; *Amp®* gene; pBR322 origin
pMAT10	Bacteria	(Peränen et al., 1996) gift from Marko Hyvönen	Expression vector that provides N-terminal 6-histidine tag followed by MBP; Thrombin recognition site for N-terminal double tag removal; T7 and SP6 promoter sequences flank His-MBP and the MCS; *Amp*^{R} gene; pBR322 origin

pRS413	Yeast and Bacteria (shuttle vector)	Stratagene	Yeast centromeric (CEN6) plasmid; HIS3 gene; MCS flanked by T7 and T3 promoter sequences; *Amp*^{R} gene, f1 and ColE1 origins
pRS416	Yeast and Bacteria (shuttle vector)	Stratagene	Yeast centromeric (CEN6) plasmid; URA3 gene; MCS flanked by T7 and T3 promoter sequences; *Amp*^{R} gene, f1 and ColE1 origins
pBG1805	Yeast and Bacteria (Yeast overexpression vector)	Open Biosystems	Yeast 2 micron plasmid; URA3 gene; yeast ORF under the control of the *GAL1-10* promoter; C-terminal tandem affinity tag; *Amp*^{R} gene, derived from pRSAB1234 (Gelperin et al., 2005)

Nucleic acid quantitation

Nucleic acids were quantified by optical density analysis using a UV spectrophotometer (Eppendorf Biophotometer) and UV cuvettes (Fisher). The sample's absorbance at 260nm was measured and the concentration determined using the following calculation: 1 absorbance unit at 260nm is equivalent to 50µg/mL dsDNA or 33µg/mL of ssDNA.

The molecular weight of the DNA could then be determined using the following equations:

Mw of dsDNA = number of base pairs x [(665 g/mol)/base pair]

Mw of ssDNA = number of bases x [(325 g/mol)/base]

Restriction enzyme DNA digestion

DNA restriction digest reactions were performed using 1-20µg DNA, a final concentration of 1 x enzyme buffer and 1µg/10µL final volume restriction enzyme (unless otherwise stated all enzymes were supplied by NEB). Reactions were incubated at 37°C for 3h or as recommended by the manufacturer. For vectors requiring de-phosphorylation, 1µL calf intestine phosphorylase (CIP)/50µL final volume (NEB) was added for the final hour of incubation at 37°C, prior to enzyme inactivation and DNA purification by phenol-chloroform extraction and ethanol precipitation or agarose gel electrophoresis of the reaction for analysis or gel purification by gel extraction.

Phenol-chloroform extraction and precipitation of DNA

DNA restriction digests were made up to a final volume of 200µL with sterile water. An equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1) was added and the reactions vortexed prior to centrifugation at 13.000 r.p.m. for 5 min. The aqueous top layer was removed and transferred to a clean microfuge tube. 20µL 3M NaAc pH 5.2 and 440µL ice-cold 100% ethanol were added, the sample was gently mixed and subsequently incubated at -20°C for 30 min. Next, the sample was centrifuged at 13.000 r.p.m. for 15 min and the resulting supernatant discarded. The pellet was washed with 1mL ice-cold 70% ethanol before a further centrifugation at 13.000 r.p.m. for 5 min. The resulting pellet was air-dried and finally resuspended in 15-50µL sterile water and stored at -20°C.

Polymerase chain reactions (PCRs)

PCR reactions were typically performed in a final volume of 100µL and set up as below:

Reagent	Amount
DNA template	0.1-10ng plasmid DNA or 200-1000ng yeast genomic DNA
10 x Thermo Pol Buffer (NEB)	1 x
dNTPs (Fermentas)	100µM each of dATP, dCTP, dGTP, dTTP
Oligonucleotide primers	500nM each
Pfu DNA polymerase*	1µL/100µL final volume
Taq DNA polymerase*	0.5µL/100µL final volume

*Pfu and Taq DNA polymerases were purified in the laboratory and appropriate concentrations for PCR reactions were determined empirically.

To ensure high-fidelity PCR for cloning, Pfu DNA polymerase was used. Taq polymerase was added to the reaction to enhance processivity. All PCRs were performed in a TC 300 thermocycler (Techne) and typical cycling parameters were set as below:

Step	Number of cycles	Temperature	Time
1	1	95°C	30s
2	30	95°C 52-56°C 68°C	30s 1 min 2min/1000 bases amplified
3	1	68°C	5 min
4	1	10°C	Hold

A-tailing

PCR products for ligation into pGEM-T-Easy were A-tailed according to manufacturer's instructions (Promega) prior to ligation into pGEM-T-Easy.

Ligation reaction

All ligations were performed using restriction enzyme-digested, de-phosphorylated, phenol-chloroform-extracted and ethanol-precipitated vector and compatibly digested and gel-purified insert from pGEM-T-Easy and T4 DNA ligase (NEB) according to manufacturer's instructions.

Cloning

Gene sequences were amplified from yeast genomic W303 DNA using primers complimentary to the gene and/or surrounding sequence and standard PCR conditions. The PCR product was analysed by agarose gel electrophoresis prior to A-tailing, subcloning into pGEM-T-Easy and final cloning into the desired vector.

Site-directed mutagenesis

Site-directed mutagenesis of *IES6* was performed by two sequential PCR reactions using nested primers and the wild-type sequence cloned into a plasmid as the template. In the first PCR reaction, the N-terminal and C-terminal portions to the site for mutagenesis were generated, using primers covering the mutagenic site and primers to the N- or C-terminus of the gene, as well as a plasmid containing the wild-type gene sequence. The second PCR reaction used the

so-generated products as a template for generating the final, full-length gene PCR product. The final PCR product was then cloned into the required vector via pGEM-T-Easy (see Figure 2.1).

Agarose gel electrophoresis

Plasmids and DNA fragments were visualised by agarose TAE (40mM Tris acetate, 1mM EDTA, 0.0011% (v/v) glacial acetic acid) gel electrophoresis, on 1% or 2% (w/v) agarose gels with 0.5µg/mL ethidium bromide for fragments greater or less than 1kb, respectively. DNA samples were supplemented with 6 x loading buffer (50mM Tris, pH 8.0, 10mM EDTA, pH 8.0, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF). Gels were electrophoresed at 90 - 120V in 1 x TAE until DNA fragments were sufficiently resolved and fragment size could be established by comparison with 500ng of 1kb or 100bp (both NEB) gene ruler. DNA was visualised and photographed using a UV light source.

DNA purification from gels

DNA was extracted from agarose gels subsequently to sufficient resolution by agarose gel electrophoresis to allow fragment separation using either a QIAquick Gel Extraction (Qiagen) or a Nucleospin Extract II (Machery-Nagel) kit, according to manufacturer's protocols. In the final step, the DNA was eluted with 15-30µL of sterile water.

DNA sequencing

Plasmids generated in this study were sequenced at GATC Biotech (http://www.gatc-biotech.com/en/index.html). Plasmids and primers were provided to the company at 30-100ng/µL and 10pmol/µL, respectively in a final volume of 30µL (sufficient for 8 reactions).

Polyacrylamide gel analysis of oligos (protocol from Qiagen)

Analysis of the purity of oligonucleotides to be used for Gelshift assays was performed on a 15% acrylamide/urea gel using a stock of 40% acrylamide/bis-acrylamide (29:1) (Severn Biotech) and final concentrations of 0.48g/mL gel urea and 1 x TBE (89mM Tris-borate, 50mM EDTA, pH 8.0). Gels were pre-run in 1 x TBE buffer at 200V for 30 min. Samples were prepared by addition of 200pmol oligonucleotide in 2µL sterile water to 1.25 x formamide loading buffer (90% (v/v) formamide, 11.1mM EDTA, pH 8.0) and heating at 95°C for 2 min before being chilled on ice. Samples were loaded onto the gel together with a marker lane containing xylene cyanol FF and bromophenol blue in order to follow the migration of the oligonucleotides, as xylene cyanol FF and bromophenol blue co-migrate with 30 and 9-10 base oligonucleotides, respectively. Subsequent to sample loading, the gel was electrophoresed for 1.5h at 200V. Following oligonucleotide resolution, the gel was stained in 0.02% methylene blue solution (0.5mg methylene blue/1mL solution in 1 x TBE) for 30 min with gentle rocking. The gel was then destained with multiple washes in sterile water and scanned.

Generation of radioactively-labelled DNA duplex, Y-fork and Holliday Junction

Radioactively-labelled DNA duplex, Y-fork and Holliday Junction were generated following a published protocol (Rass and West, 2006). Briefly, oligonucleotide -01 was radio-labelled with

γ-^{32}P ATP using PNK and was subsequently purified on a G-50 spin column (Amersham). The labelled oligonucleotide was then used in an annealing reaction containing an excess of the required cold oligonucleotides. The structures were then electrophoresed on a 12% native polyacrylamide gel and the relevant bands excised and eluted into TMgN buffer (10mM Tris-Cl, pH8.0, 1mM $MgCl_2$, 50mM NaCl) at 4°C overnight.

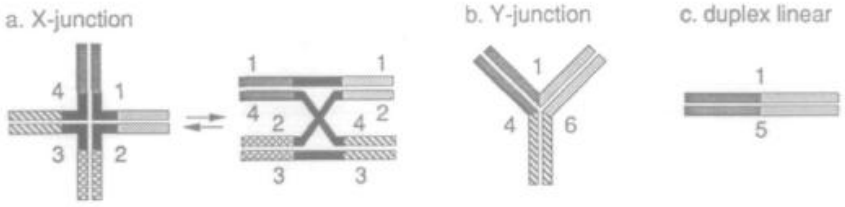

Figure 2.1.2 Holliday Junction, Y-fork and duplex DNA structures generated for investigation of MBPIes6's DNA binding activity, reproduced from (Lloyd and Sharples, 1993)**.**

Plasmids constructed for this study

No.	Name	Vector	Primers	Features
1	pET-15b ies6	pET-15b (see above)	#7 & #8	*IES6* open reading frame cloned from yeast genomic DNA (W303a strain); 5' and 3' ends include *NcoI* and *XhoI* restriction sites, respectively, subcloned via pGEM-T-Easy

2	pET-15b ΔF44ies6	pET-15b (see above)	#9 & #8	*IES6* coding sequence, N-terminally truncated at amino acid 44, 5' and 3'ends include *NcoI* and *XhoI* sites, respectively, subcloned via pGEM-T-Easy
3	pMAT10 ies6	pMAT10 (see above)	#10 & #8	*IES6* coding sequence cloned from yeast genomic DNA (W303a strain); 5' and 3' ends include *NcoI* and *XhoI* restriction sites, respectively, subcloned via pGEM-T-Easy
4	pMAT10 ST ies6	pMAT10 (see above)	#10,#8#, #11 & #12	Site-directed mutagenesis, using #7 as template was employed to generate the mutant *IES6* sequence, which was subcloned as above
5	pMAT10 TK ies6	pMAT10 (see above)	#10,#8, #13 & #14	Site-directed mutagenesis, using #7 as template was employed to generate the mutant *IES6* sequence, which was subcloned as above
6	pMAT10 TKST ies6	pMAT10 STies6 (plasmid 4, see above)	#10, #8, #13 & #14	Site-directed mutagenesis, using #8 as template was employed to generate the mutant *IES6* sequence, which was subcloned as above

7	pRS413 ies6	pRS413 (see above)	#15 & #16	*IES6* coding sequence and promoter and terminator sequences cloned from yeast genomic DNA (W303a strain) into pGEM-T-Easy and subcloned into pRS413 via *NotI* sites of pGEM-T-Easy
8	pRS413 ST ies6	pRS413 (see above)	#15, #16, #11 & #12	Site-directed mutagenesis, using #11 as template was employed to generate the mutant *IES6* sequence, which was subcloned via pGEM-T-Easy using the *NotI* sites as above
9	pRS413 TK ies6	pRS413 (see above)	#15, #16, #13 & #14	Site-directed mutagenesis, using #11 as template was employed to generate the mutant *IES6* sequence, which was subcloned via pGEM-T-Easy using the *NotI* sites as above
10	pRS413 TKST ies6	pRS413 STies6 (plasmid 8, see above)	#15, #16, #13 & #14	Site-directed mutagenesis, using #12 as template was employed to generate the mutant *IES6* sequence, which was subcloned via pGEM-T-Easy using the *NotI* sites as above

11	pRS416 ies6	pRS416 (see above)	#15 & #16	*IES6* coding sequence and promoter and terminator sequences cloned from yeast genomic DNA (W303a strain) into pGEM-T-Easy and subcloned into pRS413 via *NotI* sites of pGEM-T-Easy
12	pRS416 ST ies6	pRS416 (see above)	#15, #16, #11 & #12	Site-directed mutagenesis, using #11 as template was employed to generate the mutant *IES6* sequence, which was subcloned via pGEM-T-Easy using the *NotI* sites as above
13	pRS416 TK ies6	pRS416 (see above)	#15, #16, #13 & #14	Site-directed mutagenesis, using #11 as template was employed to generate the mutant *IES6* sequence, which was subcloned via pGEM-T-Easy using the *NotI* sites as above
14	pRS416 TKST ies6	pRS416 STies6 (plasmid 12, see above)	#15, #16, #13 & #14	Site-directed mutagenesis, using #12 as template was employed to generate the mutant *IES6* sequence, which was subcloned via pGEM-T-Easy using the *NotI* sites as above

2.2 Bacteria

Bacterial strains used

The following bacterial strains were used for DNA manipulation or for protein expression in this study:

Bacterial Strain	Source	Genotype	Use
XL1-Blue	Stratagene	*rec*A1 *end*A1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' *pro*AB *lac*I^{q}ZΔM15 *Tn*10(Tet^{R})]	DNA cloning
Rosetta (DE3)	Novagen	F-*omp*T *hsd*SB(rB-mB-) *gal dcm* (DE3) pRARE2 (Cam^{R})	Protein expression

Bacterial culture, quantification and storage

Bacterial cells were cultured in sterile Luria Bertani broth (LB) (10g/L Bacto Tryptone, 5g/L yeast extract, 10g/L sodium chloride, pH 7.0) or on LB plates (LB media supplemented with 5g/L agar), at 37°C. Liquid cultures were grown with shaking. Plasmid selection was ensured by supplementing the LB media with the appropriate antibiotic(s). Ampicillin, kanamycin and chloramphenicol were used at a final concentration of 100µg/mL, 50µg/mL and 34µg/mL, respectively. Bacterial growth was followed and the number of cells was quantitated by spectrophotometric analysis of the optical density of the culture at an absorbance at 600nm. Short-term storage of bacterial strains occurred on LB plates kept at 4°C. For long-term storage, saturated, liquid cultures were stored at -80°C in 30% (v/v) glycerol.

Preparation of rubidium chloride-competent *E.coli* cells

A 2mL sterile LB starter culture of *E.coli* cells was grown overnight at 37°C with shaking, following which, 0.5mL of the starter culture was used to inoculate 100mL sterile fresh LB broth. The cells were then grown to an absorbance at 600nm of 0.5. The cells were chilled on ice prior to collection by centrifugation at 10K for 5 min (SS34 rotor, Sorvall RC6). Next, the cells were resuspended in 30mL ice-cold buffer B1 (30mM potassium acetate, 50mM manganese chloride, 100mM rubidium chloride, 10mM calcium chloride, pH 5.8, 15% (v/v) glycerol) and incubated at 4°C on ice for 90-120 min. The cells were again harvested by centrifugation at 5K for 5 min (SS34 rotor, Sorvall RC6). The pellet was then gently resuspended in 4mL buffer B2 (10mM 3-(n-morpholino) propanesulfonic acid (MOPS), pH 7.0, 10mM rubidium chloride, 75mM calcium chloride, 15% (v/v) glycerol) and 60µL dimethyl sulfoxide (DMSO) were added. Finally, the cells were aliquoted (50-200µL) into pre-chilled microfuge tubes, flash-frozen in liquid nitrogen and stored at -80°C.

Transformation of rubidium chloride-competent *E.coli* cells

Rubidium chloride-competent cells were thawed on ice and then 50µL competent cells per transformation were added to pre-chilled microfuge tubes containing between 50ng – 1µg of the DNA (plasmid or ligation product) to be transformed on ice. The cells and DNA were incubated on ice for a minimum of 30 min. Next they were heat-shocked at 42°C for 45s before being chilled on ice for 3s. 500µL sterile LB broth was now added to each transformation prior to incubation at 37°C with shaking for a minimum of 45 min. The transformations were next briefly centrifuged and the supernatant removed. The cells were gently resuspended in 100µL sterile LB broth before being plated onto LB agar plates supplemented with the appropriate antibiotic(s) for plasmid selection. For blue-white screening using pGEM-T-Easy, the plates were further supplemented with 0.4M of isopropyl β-D-1-thiogalactopyranoside (IPTG) and

40µg/mL of 5-bromo-4-chloro-3indolyl-β-D-galactopyranoside (X-gal). The plates were incubated at 37°C overnight.

Plasmid isolation from *E.coli* cells

A single colony was used to inoculate 5mL sterile LB broth, supplemented with the appropriate antibiotic(s) and grown at 37°C with shaking overnight. The cells were harvested by centrifugation and plasmid DNA was isolated from the cells using either a QIAprep Spin Miniprep (Qiagen), or a Nucleospin Plasmid (Machery-Nagel) kit, as per manufacturer's instructions. In both cases, the plasmid was eluted in 50µL sterile water in the last step.

2.3 Yeast

Yeast strains used

The following yeast strains were used in this study as background host strains for the generation of mutant yeast strains for phenotypic analysis:

Yeast strain	Source	Genotype
BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
Δies6 BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YEL044W::KanMx4
Δies1 BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YFL013C::KanMx4
Δies2 BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YNL215W::KanMx4
Δies3 BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YLR052W::KanMx4
Δies4 BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YOR189W::KanMx4*
Δies5 BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YER092W::KanMx4
BY4743 MAT a/α	Euroscarf	Mat a/α *his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*

Δarp8 BY4741 MAT a	Euroscarf	Mat a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YOR141C::KanMx4
BY4743 *IES6/ies6* MAT a/α	This study	Mat a/α *his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 IES6/ies6*
Δino80 BY4733 MAT α	Kind gift from Xuetong Shen (Shen et al., 2000)	MAT α *his3Δ200 leu2Δ0 met15Δ0 ura3Δ0 trp1Δ63* YGL150C::*TRP1*

Yeast culture, quantification and storage

Sterile, rich yeast media (YPAD) (see below) was used for yeast cultures, when plasmid selection was not required. However when selection was required during yeast growth, the yeast was cultured in synthetic drop-out (SD) media (recipe from Steve Elledge's laboratory, see below). This ensured selection of plasmids containing the yeast marker genes *HIS3*, *LEU2*, *TRP1* or *URA3*, by growing cultures in SD media lacking histidine (SD/-his), leucine (SD/-leu), tryptophan (SD-trp) or uracil (SD/-ura), respectively. In the case of marker plasmids containing the *URA3* marker gene it was also possible to select against the plasmid, by growing cultures on synthetic complete (SC) media (synthetic media supplemented with all four amino acids, but containing uracil at a reduced concentration relative to SD media), and containing 1g/L fluoro-orotic acid (5-FOA). Yeast growth was followed and the number of yeast cells was quantitated by spectrophotometric analysis of the optical density of the culture at an absorbance at 600nm. Short-term storage of yeast strains occurred on agar plates kept at 4°C. For long-term storage, saturated, liquid cultures were stored at -80°C in 30% (v/v) glycerol.

Yeast sporulation

Diploid yeast strains were initially patched onto pre-sporulation plates and incubated at 30°C for 2d. Following a further patching onto pre-sporulation plates and a further 2d incubation at 30°C, the patched yeast were used to set up liquid, 2mL sporulation cultures. The sporulation cultures were first incubated at 30°C with shaking for 2d, after which they were incubated at 18°C with shaking and monitored daily for tetrad formation by microscopy.

Yeast dissection

Tetrad dissection was kindly performed by Dr Eva Hoffman.

Yeast media

Rich yeast media (YPAD; liquid):	10g/L yeast extract
	20g/L peptone from casein
	0.1g adenine
	2% (w/v) glucose
Rich yeast media (YPAD; solid),	
as above, but supplemented with:	5g/L agar

Yeast synthetic drop-out media:

1 x YNB:	6.7g/L Difco yeast nitrogen base without amino acids
1 x drop-out solution:	87mg/100ml THUL drop out mix, 10µL 10M NaOH per 100mL
glucose:	final 2% (w/v) glucose
for agar plates:	10g/350mL H_2O per final 500mL SD agar
1 x HIS:	20 µg/mL
1 x LEU:	60 µg/mL
1 x TRP:	40 µg/mL
1 x URA:	20 µg/mL
THUL drop-out mix (final concentration in media):	40µg/mL adenine
	20 µg/mL L-arginine
	100µg/mL L-aspartic acid
	100µg/mL L-glutamic acid
	20µg/mL L-methionine

THUL drop-out mix (final concentration in media):	50µg/mL L-phenylalanine
	375µg/mL L-serine
	200µg/mL L-threonine
	30µg/mL L-tyrosine
	150µg/mL L-valine
	30µg/mL L-lysine
FOA plates:	1 x YNB
	1 x drop-out solution
	100µL/L 10M NaOH
	2% (w/v) glucose
	2% (w/v) agar
	1 x TRP
	1 x HIS
	1 x LEU
	0.6 x URA
	1g/L 5-FOA
G418 (final concentration)	200µg/mL

Yeast pre-sporulation plates:	20g peptone/L
	10g yeast extract/L
	5% (w/v) glucose
	20g/L agar
Sporulation media (liquid):	1% potassium acetate
	0.005% zinc acetate
	1 x relevant amino acids

Yeast genomic DNA isolation

Yeast cells from a 10mL sterile, liquid culture were harvested by centrifugation at 3000 r.p.m for 5 min. The yeast cells were next washed with 0.5mL of sterile water, repeatedly harvested by centrifugation as previously and then resuspended in 200µL breaking buffer (2% Triton-X-100, 1% SDS, 100mM NaCl, 10mM TrisCL pH 8.0, 1mM EDTA). 200µL phenol-chloroform (phenol-chloroform-isoamyl alcohol mixture (25:24:1)) 200µL acid-washed glass beads were added prior to mechanical disruption of the yeast cells. The supernatant was transferred to a clean microfuge tube and vortexed following the addition of 200µL 1x TE. The sample was centrifuged at 13K for 5 min. The clear, aqueous layer was transferred to a clean microfuge tube and 1mL 100% ethanol added. Following a further centrifugation at 13K for 3 min, the supernatant was removed and the sample was resuspended in 400µL 1 x TE. 30µg RNaseA were added and the sample was incubated at 37°C for 15 min. Next, 5µL of 7.5M ammonium acetate and 1mL 100% ethanol were added. The sample was mixed by inversion and centrifuged at 13K for 3 min. The supernatant was removed and the pellet was air-dried and finally resuspended in 100µL 1 x TE.

Transformation of yeast cells

Yeast cultures were grown to an absorbance at 600nm of 0.5 in 10mL sterile rich, or SD liquid media. The cells were harvested by centrifugation at 3000 r.p.m. for 5 min and washed with 1mL sterile water, before repeated centrifugation. The cells were washed in 1mL fresh 1 x TE/ 1 x LiOAc (10mM Tris, pH 8.0, 1mM EDTA, pH 8.0, 100mM LiOAc) and harvested as previously and the cells were resuspended in 100µL 1 x TE/ 1 x LiOAc. Approximately, 0.2 - 1µg of the DNA to be transformed, as well as 50µg of Salmon sperm DNA from testes (Sigma) were added to 50µL of the yeast cell suspension. 300µL fresh PEG4000 solution (40% (w/v) PEG, 1 x TE, 1 x LiOAc) were added and the transformation mix was incubated at 30°C with shaking for 30 min. Following the incubation, the cells were heat-shocked at 42°C for 15 min and subsequently harvested by brief centrifugation (5s at 3000 r.p.m.), resuspended in 100µL 1 x TE and spread on sterile, rich or selective agar plates. The plates were incubated at 30°C until colonies were clearly visible.

Yeast strain construction

Yeast strains generated for this study were constructed either by transformation of host yeast cells with plasmids or by transformation of the host yeast cells with a PCR integration product (see transformation of yeast cells). Selection for the plasmid or integration construct was achieved by growth on sterile, selective, SD media. Correct integration of PCR product was further confirmed by PCR using genomic yeast DNA as a template (see isolation of genomic yeast DNA and section 2.1, PCR).

Recovery of plasmid DNA from yeast

Recovery of plasmid DNA from yeast was adapted from (Singh and Weil, 2002) essentially as follows: cells from a 5mL overnight yeast culture were harvested by centrifugation and resuspended in 300µL Qiagen Miniprep buffer P1. Cell lysis occurred by mechanical disruption 98 x 20s ribolyser). Next, 300µL Qiagen buffer P2 was added and following mixing by inversion, the samples were incubated at room temperature for 10min. 420µL Qiagen buffer N3 were added and the sample was again mixed by inversion prior to centrifugation at 13.000 r.p.m. for 10 min. From this point, the Qiagen Miniprep kit was used as per manufacturer's instructions (including extra wash). In the final step, the DNA was eluted using 30µL sterile water. The entire elution was transformed into rubidium-competent XL1-blue *E.coli*. Colonies arising from this transformation were again used for plasmid DNA extraction (as above) and analysed by restriction digest and agarose gel electrophoresis (as above).

Chronic HU exposure (spot tests)

To investigate sensitivity to hydroxyurea (HU), yeast strains were grown to an absorbance at 600nm of 0.5 and diluted to an absorbance at 600nm of 0.2. Serial five-fold dilutions of each strain were plated on YPAD or yeast minimal media with or without varying concentrations of HU. The plates were incubated at 30°C until colonies were clearly visible and then photographed.

Acute HU assay

Wild-type (BY4741), *arp8* and *ies6* deletion strains were grown to an absorbance at 600nm of 0.5 in YPAD and arrested in G1 by the addition of α-factor (5µg/mL). Cells were then washed

three times with rich media before cultures were split and released either into pre-warmed YPAD, or pre-warmed YPAD with 0.2M HU. At 2, 4 and 6h post-release, 100µL of yeast culture diluted to an absorbance at 600nm of 0.001 were plated onto rich media without HU an incubated at 30°C for 2-3 days, after which the strains' ability to form colonies was scored. The experiment was performed in triplicate and error bars represent the standard deviation.

Fluorescence activated cell sorting (FACS)

Yeast strains were grown to an absorbance at 600nm of 0.5 and 1.5mL culture were centrifuged, resuspended in 1mL ice-cold 70% ethanol and incubated at 4°C overnight. Cells were next harvested by centrifugation and resuspended in 0.5mL 1mg/mL RNase A in 50mM TrisCl pH7.5 and incubated at 37°C for 4h. Following a further centrifugation, cells were resuspended in 0.5mL proteinase K in 50mM TrisCl pH7.5 and incubated at 50°C for 1h. Finally, cells were harvested by centrifugation and resuspended in FACS buffer (200mM TrisCl pH7.5, 200mM NaCl, 78mM $MgCl_2$) and stored at 4°C. 30µL cells were added to 1mL 50µg/mL propidium iodide in 50mM TrisCl pH7.5 and samples were sonicated (7s using a microtip at 20%). Finally, samples were analyzed using a FACS Calibur machine and CellQuest®.

Yeast whole cell extract protein preparation

Yeast cells were grown to the desired absorbance at 600nm and cells from 1.5mL yeast culture were harvested by centrifugation. 5 pellet volumes 20% (v/v) TCA (trichloroacetic acid) and 1 pellet volume acid-washed glass beads were added. Next, the cells were lysed by mechanical disruption in a ribolyser (FastPrep FP120, B10101 Thermo Savant, 2 x 30s bursts, with a 30s interval) and the resulting lysate transferred to a clean microfuge tube. The sample was centrifuged at 13.000 r.p.m. for 15 min at 4°C and the resulting supernatant discarded. The

protein pellet was twice washed with 1mL acetone, followed by centrifugation at 13.000 r.p.m for 1 min. Finally, the pellet was air-dried and resuspended in 50 – 100µL 1 x LDS sample buffer (NuPAGE LDS sample buffer, Invitrogen).

2.4 Biochemical Protein Analysis

Protein quantitation by Bradford Assay

Quantification of total protein concentration of purified samples was determined by Bradford protein assay (Bradford, 1976), using Coomassie dye (Sigma). A test curve of protein concentration was generated using dilutions of a 0.5mg/mL BSA (NEB) solution in dye and, following an incubation at room temperature for 5 min, by measuring their absorbance at 595nm, using dye alone as the reference. Absorbance of each BSA standard was plotted against the standard sample's concentration. Next, a set of dilutions of the unknown protein sample in dye were generated and their absorbance at 595nm was measured, again following a 5 min incubation at room temperature. Using a dilution of the unknown protein sample reading in the linear range of the standard curve generated, the concentration of the unknown protein sample could be determined.

SDS-PAGE gel electrophoresis

Proteins were resolved on SDS-PAGE gels. The resolving component of the gel contained an appropriate percentage (usually 12 – 15%) of acrylamide (Severn Biotech, 30% (w/v) acrylamide, 37.5:1 acrylamide: bisacrylamide) and 0.38M Tris Cl, pH 8.8, 0.1% (w/v) SDS, whereas the stacking component contained acrylamide at 6%, as well as 25mM Tris Cl, pH 6.8, 0.2% (w/v) SDS. Polymerisation of the gels was achieved by addition of 10µL 10% APS/mL gel and 1µL TEMED/mL gel. Protein samples were prepared for gel electrophoresis by the addition of one sample volume 1 x LDS sample buffer and were boiled for 5 min at 95°C prior to loading. Sample resolution on the gel occurred by electrophoresis for 1h at 180V in SDS-running buffer (25mM Tris, 192mM glycine, 0.5% (w/v) SDS).

Protein gel staining by Coomassie

Protein gels were stained in Coomassie Blue (0.2% Coomassie Blue, 50% methanol, 10% glacial acetic acid) prior to destaining in destain (25% methanol, 10% glacial acetic acid). Destaining was repeated with fresh destaining solution for as long as necessary and protein gels were subsequently washed in water.

Protein gel staining by silver

Protein gels for silver staining were washed for 2 x 10min in 50mL 50% methanol subsequent to electrophoresis as above. The gels were then washed for 2 x 10 min with 50mL 5% methanol, before soaking in 250mL sterile water containing 8µL 1M DTT for 10min. The gels were rinsed prior to soaking in 250mL sterile water containing 0.25g silver nitrate for 15 min. Next, the gels were rinsed and then soaked in developing solution (7.5g sodium carbonate and 125µL 40% formaldehyde in 250mL sterile water) until bands were visible. The reaction was stopped by the addition of citric acid. The gels were then washed in and transferred to water.

Western blot analysis

Samples for analysis by Western blot were prepared for and electrophoresed on an SDS-Page gel as above. The proteins were then transferred onto a nitrocellulose membrane (Hybond ECL Nitrocellulose Membrane, GE Healthcare) in transfer buffer (2 x SDS-running buffer, 20% methanol). Protein transfer occurred for at 4°C for 1h in transfer buffer at 240mA. Following transfer, the membrane was incubated in blocking solution (5% (w/v) dried milk (Marvel) in 1 x TBS-T buffer (20mM Tris base, pH 7.6, 137mM NaCl, 0.1% (v/v) Tween 20) at room temperature for 1h with gentle agitation or at 4°C overnight. Next, the membrane was

incubated with the appropriate primary antibody in 10mL 1 x TBS-T either for 1-2h at room temperature or overnight at 4°C according to manufacturer's instructions or optimised protocols for individual antibodies. The membrane was washed four times at room temperature in 1 x TBS-T, before incubation with the relevant, HRP-conjugated, secondary antibody in 1 x TBS-T, for 1h at room temperature with gentle agitation. Prior to visualisation, the membrane was again washed four times in 1 x TBS-T. Visualisation of protein bands occurred by enhanced chemiluminescence (ECL) using Western Lightning ECL (Perkin Elmer) according to manufacturer's instructions. Membranes were exposed to X-ray film (Konica Milota X-ray film AX, Data Services) for, typically 1s – 15 min, depending on protein sample and antibody used for signal detection.

On occasion membranes were probed with multiple antibodies. In these cases, the membrane was stripped of the previous primary antibody by incubation in stripping buffer (62.5mM TrisCl pH 6.7, 100mM β-mercaptoethanol, 2% (w/v) SDS) at 55 – 70°C for 30 min. Subsequent to stripping, the membrane was repeatedly washed in 1 x TBS-T at room temperature. The membrane was then blocked and incubated with primary and secondary antibodies for visualisation by ECL as described previously.

Antibodies used in this study

Antibody	Conditions used	Source
α-FLAG	1:2500 dilution, 1h at room temperature	Sigma
α-H2A	1:4000 dilution, overnight at 4°C	Abcam
α-HIS	1:3000 dilution, 1h at room temperature	Sigma
α-Mouse	1:4000 dilution, 1h at room temperature	Sigma
α-Rabbit	1:10000 dilution, 1h at room temperature	Sigma

Expression and solubility trials

Following successful cloning of *IES6* into bacterial expression vectors, small-scale expression and solubility trials were performed. Small bacterial cultures were grown to an absorbance at 600nm of 0.5. Protein expression was then induced with 1mM IPTG. Cultures were subsequently grown at different temperatures (37, 30, 25 or 18°C) and samples were taken at time-points post-induction, typically 0, 1, 2, 3 and 4h, apart from cultures growing at 18°C post-induction, as these were grown overnight at the lower temperature. Bacterial cells were harvested by centrifugation at 5K for 20 min at 4°C. The cells were then resuspended in 1mL/50mL original culture volume resuspension buffer 1(50mM TrisCl, pH7.5, 100mM NaCl, 0.5 mM EDTA, pH8.0, 0.1mM DTT, 1% (w/v) sucrose) with triton-X 100 added to 0.1% (v/v) and lysosyme to 0.5mg/mL and shaken at room temperature for 30 min to lyse the bacteria. Next, the samples were centrifuged at 14.5K for 8 min. The resulting supernatant contained the “soluble” protein fraction; the pellet containing the “insoluble” fraction was resuspended in 0.1% (w/v) SDS. Prior to analysis by SDS-Page samples were heated at 95°C for 5 min.

Purification of MBP-tagged recombinant Ies6

Ies6 was PCR-amplified from yeast genomic DNA (W303a) and cloned into the *E.coli* expression vector pMAT10 (Kelly Littlefield and Darerca Owen, unpublished). 1L Luria Broth (LB) containing 100µg/mL ampicillin (LBamp) was inoculated using an overnight starter culture of Rosetta cells harbouring the pMAT10-ies6 plasmid in LBamp. The culture was grown to an absorbance at 600nm of 0.5 at 37°C with shaking. Next the culture was placed at 18°C with shaking and protein expression was induced overnight following the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) to 1mM. Bacteria were harvested by centrifugation at 5K for 20min at 4°C (SL-1500, Sorvall RC6) and cells were resuspended in 1mL Buffer A (20mM TrisCl, pH7.5, 200mM NaCl, 10mM β-mercaptoethanol, 1mM EDTA, 0.5% Triton-X-100, 1µg/mL

pepstatin A, 0.5µg/mL aprotinin, 1µM leupeptin, 100µg/mL PMSF)/50mL original culture. Following sonication (4 x 30s using the microtip at 35%), the sample was centrifuged at 14.5K for 8min at 4°C (SS34, Sorvall RC6). Ammonium sulphate was added to 42.5% (w/v) to the soluble fraction over a period of 30 min during which the sample remained on ice. The sample was centrifuged at 10K for 10 min at 4°C (SS34, Sorvall RC6) and the pellet resuspended in 1mL Buffer B (as Buffer A, but no EDTA)/50mL original culture. This sample (=input) was loaded onto a talon resin (equivalent to 1mL talon resin slurry, washed with dH_2O and equilibrated with Buffer B) and the flow-through collected. The column was then washed with 1.5mL Buffer B/50mL original culture and the washes collected. The protein was finally eluted in 2 x 1mL fractions of Buffer C (Buffer B + 250mM imidazole, no protease inhibitors). Glycerol was added to 10% to each elution fraction and the protein samples were stored at -80°C.

DNA binding assay

Radioactively-labelled DNA duplex, Y-fork and X-structure DNA were prepared essentially as in (Rass and West, 2006). Each DNA binding assay contained ~ 12.5fmol radiolabelled DNA and varying concentrations of purified, recombinant MBP-tagged Ies6 in a final volume of 10µL. Some reactions also contained 10ng poly [dIdC] (Sigma). The binding buffer contained 20mM TrisCl pH7.5, 10mM MgOAc, 10mM β-mercaptoethanol and 20% glycerol. The reactions were incubated at room temperature for 30 min. The samples were then electrophoresed on a 4% polyacrylamide gel in 1xTBE at 25mA, for ~2.5h. Gels were dried under vacuum before exposure to a phosphoscreen.

Analysis of DNA binding data

The amount of free probe and total DNA was quantified using ImageQuant® and both were corrected for background. The percentage of free probe was then obtained using:

%free probe = ((freeprobe-background$_{freeprobe}$)*100)/(total DNA-background$_{totalDNA}$).

The percentage of free probe in the "no protein" lane was then set as 100% free probe and the percentage free probe in all other lanes were corrected accordingly using:

%corr free probe = ((%free probe)*100)/%free probe$_{"no\ protein"}$.

The data was then plotted in Excel®. The bar charts show standard error of the mean, with n=2 for the duplex and Y-fork and n=3 for the X-structure, as well as all binding assays with the DNA-binding mutants.

3.1 Introduction

The Ino80 complex belongs to the Ino80-family of chromatin remodellors: large, multi-subunit protein complexes involved in the alteration of protein-DNA contacts, thus modulating the chromatin environment for enzymes involved in DNA metabolism, such as transcription. Initially identified via its role in inositol metabolism (Ebbert et al., 1999), it was further shown likely to be involved in the DNA damage response due to the sensitivity of the *ino80* deletion strain to DNA damaging agents (Shen et al., 2000). In 2004, both the Shen and Gasser groups ChIPped the Ino80 complex to sites of HO-endonuclease-induced DSBs, thus presenting evidence for the direct involvement of the complex at sites of DSBs (Morrison et al., 2004; van Attikum et al., 2004). The recruitment of the Ino80 complex was seen to be dependent on the phosphorylation of H2A at the DSB, one of the earliest events in the DNA damage response (Downs et al., 2004; Shroff et al., 2004). Furthermore, its requirement for the efficient repair of DSBs, as seen by sensitivity to DNA-damaging agents, was not dependent on a possible transcriptional role of the complex (Morrison et al., 2004; van Attikum et al., 2004). More recently, the Ino80 complex has also been shown to play a critical role at stalled replication forks (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008). Indeed, in the absence of Ino80, yeast strains displayed impaired S-phase progression (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008). Moreover, upon exposure to HU and the ensuing replication stress leading to replication forks stalling, strains lacking the Ino80 remodelling complex are unable to restart stalled replication forks and display a high rate of replication fork collapse (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008). The involvement of the Ino80 complex both in the DNA damage response, both at sites of DSBs and at replication forks, thus attributes an important role in the maintenance of genomic stability to this chromatin remodelling complex.

Most chromatin remodellors so far identified are large protein complexes composed of numerous subunits. In the light of this modular build, it is reasonable to assume that some of the subunits may play selective roles in the different aspects of Ino80's many functions. Indeed, varying degrees of importance have been ascribed to certain subunits: Ino80 complex function *in vitro* is abolished in the absence of Arp8, which does not seem to affect complex integrity (Shen et al., 2003). Deletion of *ARP5* leads to a significant decrease in the complex's *in vitro* acitivity, whereas the decrease observed in *nhp10* deletion mutants is far less severe (Shen et al., 2003). Accordingly, the DNA damage sensitivities of the three strains reflect their *in vitro* activity (Hogan et al., 2010; Shen et al., 2003) although it remains unclear which events may be mediated by these subunits. Specialised subunits are likely to mediate the recruitment of the complex to sites of DNA damage and indeed both Nhp10 and Arp4 have been implicated in the recruitment of the Ino80 complex to DSBs via an interaction with phosphorylated H2A (Downs et al., 2004; Morrison et al., 2004).

The Ino80 complex also contains a set of subunits, named Ies1-6, of which Ies1 and Ies3-5 would seem to be unique to fungal Ino80 and all of which remain largely uncharacterised. Ies2 and Ies6 however are conserved across species and have been identified in fission yeast, as well as humans (Hogan et al., 2010; Jin et al., 2005).

In light of this evolutionary conservation, it is reasonable to assume that Ies2 and Ies6 may each play a conserved role in at least one of the multiple functions fulfilled by the Ino80 chromatin remodelling complex. To determine a possible involvement of one of these subunits in the maintenance of genomic stability mediated by the Ino80 complex, budding yeast strains harbouring deletions of these genes were characterised.

3.2 Absence of Ies6 renders cells hypersensitive to Hyroxyurea compared to absence of other Ies subunits

Investigation of the possible involvement of one of the Ies subunits of the Ino80 complex in DNA damage responses was performed by DNA damage response assays, which were performed at 30°C, the optimal growth temperature for budding yeast. Variations in the phenotype observed for different mutant yeast strains thus provide information regarding the cellular pathways in which the protein is involved. Moreover, these spot tests offer a qualitative approach to analyse yeast strains for survival in the presence of DNA damaging agents. The difference in survival under such conditions can be compared between both the wild-type and the mutant strains, as well as between the mutant strains. The DNA damaging agent employed in these DNA damage response assays is hydroxyurea (HU). Hydroxyurea inhibits ribonucleotide reductase, which in turn leads to a decrease in cellular dNTP pools, causing replication fork stalling and the generation of S-phase specific DNA DSBs following replication fork collapse (Bilsland and Downs, 2005).

The *ies6* deletion strain, compared to the parent background strain (BY4741) already displays hypersensitivity to HU, as seen by a decrease in survival, at very low doses of HU (Figure 3.1). Indeed a decrease in survival for this strain can already be observed at doses of 20-40mM HU. This marked sensitivity to HU increases dramatically as the amount of HU is increased and at 100mM HU the *ies6* yeast deletion strain is no longer viable.

The *ies2* deletion strain also displays hypersensitivity to HU compared to both the parent strain and the *ies1* and *ies3-5* deletion strains. However the *ies2* deletion strain is less sensitive to HU than the *ies6* deletion strain; this is particularly apparent at 80-100mM HU. Of the remaining deletion strains, *ies1* and *ies5* deletion strains display slight sensitivity to HU at very high doses.

The hypersensitivity of the *ies6* deletion strain suggests Ies6 is indeed likely to be involved in the Ino80 complex's role in the DNA damage response.

3.3 The HU-hypersensitivity in the absence of Ies6 is comparable to the HU-hypersensitivity in the absence of Ino80

Further to comparing the HU-sensitivity of the *ies6* deletion strain to the other *ies* deletion strains, which is striking, it is of interest to investigate the *ies6* deletion strain's HU sensitivity compared to the deletion strain of the catalytic subunit of the Ino80 complex. The *ino80* deletion strain is hypersensitive to a variety of DNA damaging agents, especially HU (Papamichos-Chronakis and Peterson, 2008; Shen et al., 2000; Shen et al., 2003; van Attikum et al., 2004; Vincent et al., 2008). For the purpose of comparing the *ino80* and *ies6* budding yeast deletion strains, the DNA damage response assay to HU was repeated with these strains, as well as the wild-type control (Figure 3.2)

In these spot tests, the *ies6* deletion strain was again seen to be hypersensitive to HU compared to the BY4741 wild-type control strain and complete loss of survival is observed at around 60-80mM HU.

The *ino80* deletion strain was also hypersensitive to increasing concentrations of HU when compared to the wild-type control strain. The hypersensitivity of the *ino80* deletion strain to increasing HU concentration is comparable to results previously published in the literature (Papamichos-Chronakis and Peterson, 2008; Shen et al., 2003; van Attikum et al., 2004). When comparing both deletion strains, they show similar profiles of hypersensitivity to HU, suggesting that strains lacking *IES6* thus phenocopy strains lacking *INO80*, the catalytic subunit of the Ino80 chromatin remodelling complex.

3.4 Absence of Ies6 and absence of Arp8 lead to a similar HU-hypersensitivity phenotype

The deletion of the Arp8 subunit of the Ino80 chromatin remodelling complex abolishes the *in vitro* Ino80 complex DNA binding and nucleosome mobilisation activity without affecting the complex's integrity (Shen et al., 2003) and the *arp8* deletion strain is also hypersensitive to DNA damaging agents, such as HU (Shen et al., 2003; van Attikum et al., 2004). Its use as a phenotypic mimic of the *ino80* deletion strain is wide-spread in the literature (Falbo et al., 2009; Shen et al., 2003; Shimada et al., 2008). In light of the extensive use of the *arp8* deletion strain the DNA damage response assay with HU was repeated, this time comparing the *ies6* and *arp8* deletion strains to each other and to the BY4741 wild-type control (Figure 3.3).

The *arp8* deletion strain is also observed to be very sensitive to increasing HU concentration in comparison to the wild-type parent strain, although not as strikingly sensitive as the *ies6* deletion strain. When compared to the ino80 deletion strain (Figure 3.2), we find that the *arp8* deletion strain does in fact phenocopy the *ino80* deletion strain very well, as previously reported in the literature (Falbo et al., 2009; Shen et al., 2003; Shimada et al., 2008).

3.5 In the absence of Ies6 replication forks collapse following acute exposure to HU

HU causes replication fork stalling by depleting the cellular nucleotide pool, which eventually, upon chronic exposure as in the above DNA damage response assays, leads to the generation of DSBs (Bilsland and Downs, 2005). As the *arp8* and *ies6* deletion strains both display hypersensitivity to chronic HU exposure (Figures 3.1, 3.2, 3.3), it is of interest whether they also display sensitivity to acute exposure to HU, which is more reflective of the cells' ability to process stalled replication forks and resume replication (Shimada et al., 2008). In this assay, cells are treated with 200mM HU for increasing lengths of time before being plated onto rich media, allowing for recovery. The yeast strain's ability to resume replication can thus be

quantitatively assessed by analysing the strain's ability to form colonies following removal from HU (Shimada et al., 2008). Inviability and inability to form colonies in this assay are a read-out of an inability to process and stabilise stalled replication forks and thus of replication fork collapse (Shimada et al., 2008). Replication resumption in the absence of Ies6 was thus investigated by the means of acute exposure to HU as described above, using the BY4741 parent background and the arp8 deletion strain as controls (Figure 3.4).

After 6h exposure to 200mM HU prior to recovery on rich media, ability to form viable colonies was determined from three independent experiments to be 47.6%±8.5, 40.5%±8.5 and 20.4%±2.4 (error is standard deviation of the three independent experiments) in the parent, *arp8* deletion and *ies6* deletion strains, respectively. Viability in the *arp8* deletion strain is decreased by 15% compared to the wild-type, which whilst reflecting increased difficulty with regards to replication resumption and increased fork collapse, is less striking than in the *ies6* mutant. Thus the colony formation score in the *ies6* deletion strain is decreased by 57% with regards to the wild-type control. This decrease in ability to form colonies is comparable to that of a *rad51* deletion strain investigated in a previous study (Shimada et al., 2008), but not as severe as the *mec1* deletion strain (Cobb et al., 2005; Paciotti et al., 2001). Furthermore, this decrease in survival is similar to that observed for both *arp5* and *ino80* deletion strains in the same assay performed by the Gasser group (Shimada et al., 2008). While another study also found *ino80* mutant cells hypersensitive to acute HU exposure (Papamichos-Chronakis and Peterson, 2008), the hypersensitivity was more striking. The reason for the different degree of lethality is not clear. Taken together these data are consistent with the failure of replication resumption and likely fork collapse in the *ies6* deletion strain after HU exposure, comparable to that reported in the literature for *arp5* and *ino80* deletion strains.

3.6 Summary

Ies2 and Ies6 belong to a group of the Ies 1-6 subunits found in the Ino80 complex and are currently the only members of this group that are conserved across species. In light of this, it is reasonable to hypothesise that they might play an important and conserved role in the complex's function. Investigation into the DNA damage sensitivity to HU of the Ies subunits, reveals that whilst both *ies2* and *ies6* deletion strains are hypersensitive to HU compared to all other Ies subunits, the *ies6* deletion strain displays the most striking HU-sensitivity. Further experiments evaluating the HU sensitivity of the *ies6* deletion strain reveal that the observed hypersensitivity is comparable to that of both the *ino80* and *arp8* deletion strains. It is therefore likely that the Ies6 subunit is involved in the Ino80 complex's role in the DNA damage response. As HU causes replication fork stalling and the Ino80 complex plays a critical role in S-phase progression and at stalled replication forks (Papamichos-Chronakis and Peterson, 2008; Shimada et al., 2008), the ability of the *ies6* deletion strain to resume replication following acute HU-exposure was also investigated. Indeed, the *ies6* deletion strain displayed reduced viability after acute HU exposure, indicating a possible role for this subunit in the Ino80 complex's role in maintaining replication fork stability.

4.1 Introduction

The large, multi-subunit nature of chromatin remodellers such as the Ino80 complex makes it possible for numerous protein activities present in the different subunits to be grouped together. This grouping of protein activities allows them to act both in concert and complimentarily. It also adds the possibility of a further layer of complexity in protein function and regulation, by allowing interacting subunits to modulate each other's activities. A number of Ino80 complex subunits have been identified as possessing certain biochemical activities, however many more remain uncharacterised. Both Nhp10 and Arp4 have been implicated in the recognition and binding of phosphorylated H2A (Downs et al., 2004; Morrison et al., 2004). The catalytic subunit, Ino80, is to date perhaps the most extensively studied and has been shown to possess helicase and DNA- or nucleosome dependent ATPase activity (Shen et al., 2000). Furthermore, the Nhp10 subunit, a high mobility group protein has been shown to bind DNA (Ray and Grove, 2009). However many of the complex's subunits and their activities and functions remain unknown.

The Ino-eighty-subunit 6 (Ies6), a small, highly basic protein remains uncharacterised to date, however the protein contains a C-terminal YL1 domain. Although largely uncharacterised, human YL1 has been mapped to chromosome 1q21 and the protein has been shown to be nuclear (Horikawa et al., 1995). The protein is highly conserved between humans and mice and is thought to be a transcription factor (Horikawa et al., 1995). Interestingly, the protein was shown to bind DNA, although the protein does not contain a characterised DNA binding domain (Horikawa et al., 1995). The authors of this study propose the protein's DNA binding ability may be mediated by a putative α-helix structure (Horikawa et al., 1995). More recently the mammalian YL1 protein was identified as a subunit of both the TRRAP/TIP60 and the SRCAP (similar to budding yeast SWR1) complexes where it interacts with an uncharacterised zinc-finger protein (Cai et al., 2005). Although a number of subunits have been implicated in

DNA or chromatin interactions, thus contributing to the recruitment of the Ino80 complex to DNA, given the size and the multiple roles of the complex it is highly likely that further subunits will be identified to fulfil further DNA/chromatin binding roles.

Additionally, the human, mouse and fission yeast Ino80 complexes contain the YY1 protein (Cai et al., 2007; Hogan et al., 2010; Wu et al., 2007), which not only binds DNA, but is also able to recognise structured DNAs (Wu et al., 2007). This appears to be absent from the budding yeast Ino80 complex, making it likely that one of the budding yeast subunits fulfils this role.

Taking the above evidence into account it was hypothesised that Ies6 might be a putative DNA binding protein. To determine whether Ies6 exhibits DNA binding activity, recombinant protein was generated and its activity in DNA binding assays was investigated.

4.2 Generation of a recombinant, N-terminally MBP-tagged Ies6

In order to investigate a possible DNA binding of the Ies6 protein, recombinant protein was generated. Initially, an N-terminally HIS-tagged protein was generated by cloning budding yeast *IES6* into pET-15b. The protein was tagged at its N-terminus, as use of a C-terminal tag had been found to abolish function *in vivo* (Ben Wardleworth, Jessica Downs, personal communication). However the N-terminally HIS-tagged, recombinant Ies6 was largely insoluble (data not shown). In order to increase solubility, a variety of induction and lysis conditions were tested, but a significant increase in protein solubility was not achieved (data not shown). Additionally, an N-terminal truncation of *ies6* was cloned into pET-15b, as the N-terminal region of the protein is predicted to be highly disordered, possibly explaining the difficulty in obtaining soluble protein. Unfortunately, the HIS-tagged $\Delta_{1\text{-}43}$Ies6 was found to be equally insoluble as the full-length protein (data not shown). Finally, full-length *IES6* was cloned into pMAT10 (from Luca Pellegrini), thus generating an N-terminally MBP-HIS-tagged Ies6. The MBP

(Maltose binding protein) tag was chosen as this tag has been shown to increase the solubility of proteins from overexpression in bacterial systems ((Lee et al., 2006; Sachdev and Chirgwin, 1999; Terpe, 2003) and Luca Pellegrini, personal communication). Addition of this tag did increase protein solubility, which, with changes to induction, as well as lysis conditions resulted in a solubility: insolubility ratio of around 40:60% (Figure 4.1 a, lanes 1 & 2).

Purification of the tagged protein was trialled using a Nickel affinity resin, however high proteolytic degradation of the protein was observed (data not shown), which may result from activation of proteases by the Nickel ions. This proteolytic degradation was not significantly reduced by the addition of numerous protease inhibitors (see Materials & Methods 2.4) (data not shown). In order to increase the amount of full-length protein purified, an ammonium sulphate precipitation step was included (Figure 4.1 a, lane 3) and Talon affinity resin was used in place of the Nickel affinity resin (see Materials and Methods, 2.4).

The incorporation of an ammonium sulphate precipitation, as well as the exchange of the Nickel for the talon affinity resin improved the obtained elution both in terms of overall purity and in terms of decreased, though not abolished, proteolysis, and permitted the generation of recombinant Ies6 for use in *in vitro* biochemical assays (Figure 4.1 a, lane 6, Figure 4.1 b).

4.3 Recombinant Ies6 binds DNA

In order to perform DNA binding assays, radio-labelled DNA structures (linear duplex DNA, as well as Y-fork and Holliday Junction structures) were generated, following a published protocol developed by ((Rass and West, 2006), Figure 4.2 a)). The purified, recombinant Ies6 protein was then employed to investigate the protein's putative DNA binding activity. The MBP-HIS-tag alone was used in "mock" DNA binding assays to assess whether this protein might exhibit any DNA binding activity and contribute to any possible activity displayed by the MBP-HIS-Ies6

fusion protein. However, the purified MBP-HIS tag (kind gift from Anna Chambers) did not display any DNA binding activity (Figure 4.2 b). To begin with, the protein's binding activity was assessed in an *in vitro* gelshift assay, in which increasing amounts of purified protein were incubated at room temperature with a radioactively labelled 49bp DNA duplex (see Materials and Methods 2.4) prior to resolution by native PAGE gel electrophoresis. These assays were performed in the presence of dIdC, a nonspecific, noncompetitive inhibitor which sequesters unwanted DNA-protein interactions (Figure 4.3). All DNA binding assays were analysed using ImageQuant® and the amounts of free and bound protein were quantified (see Materials and Methods, 2.4, Figure 4.4)

Ies6 was indeed found to bind the duplex in a protein concentration-dependent manner, as observed by the shift seen on the gels (Figure 4.3, Figure 4.4). Initially, at low protein concentrations (lanes 1-3), no significant shift is observed and the amount of free probe present is similar to the "no protein" control. At a protein concentration of 402nM however (lane 4), a more significant shift can be seen on the gel, indicating the formation of protein:DNA complexes, accounting for 50% of the radioactivity in this lane. In lane 7, at a protein concentration of 644nM, the amount of free probe has decreased to ~10%. In the remaining lanes on the gel, the amount of free probe decreases slightly further and the appearance of higher order structures at the top of the gel, which may indicate the presence of complex aggregates, can be observed.

4.4 Recombinant Ies6 binds to structured DNAs

In humans and mice, the Ino80 complex contains the YY1 protein (Cai et al., 2007; Wu et al., 2007), of which no clear homologue exists in budding yeast. Aside from its role in transcriptional activation (Cai et al., 2007), it was also seen to be involved in DNA repair by homologous recombination, thus contributing to the maintenance of genomic stability (Wu et

al., 2007). In this study, the authors postulate a role for YY1 analogous to RuvA, although the proteins sequences are not related (Wu et al., 2007). In the course of their investigations, the authors show that YY1 preferentially binds both Holliday Junctions and Y-structures, which are thought to be similar to intermediates arising during the repair of stalled replication forks and suggest that YY1 may potentially play a role in recruiting the Ino80 complex to recombination intermediates in mammalian cells (Wu et al., 2007). To determine whether Ies6 may play a similar role, the DNA binding assays were repeated with radiolabelled Holliday Junction and Y-structure DNA (see Materials & Methods 2.4).

Ies6 is able to bind both the Y-fork and the Holliday Junction (Figures 4.5, 4.6, 4.7 & 4.8). Again, slight binding activity can be observed at low protein concentrations for both the Y-fork and Holliday Junction (Figures 4.5 & 4.7, lanes 1-3). Furthermore, at a protein concentration of 322nM, ~50% of the DNA is bound by protein, as seen in the shift up through the gel (Figures 4.5 & 4.7, lanes 3) and analysed quantitatively using ImageQuant® (Figures 4.6 & 4.8). In lanes 6 of Figure 4.5 and Figure 4.7, (564nM protein) the level of free probe has decreased to ~10% and ~15% in presence of the Y-fork and Holliday Junction substrate, respectively, confirming that recombinant Ies6 can bind to both of these structured DNAs well. Furthermore, the intense, higher order structures seen in presence of the duplex can also be observed for the structured DNA substrates, forming at protein concentrations of 644nM (Figures 4.5 & 4.7, lanes 7). The protein's ability to bind to DNA was further analysed by a rough estimation of the K_d for each of the three structures tested, which indeed confirmed that the protein has higher affinity for the branch-structured DNAs:

DNA structure:	Duplex	Y-fork	Holliday Junction
K_d (nM):	407	300	294

4.5 Recombinant Ies6 displays a small, but reproducible preference for binding to branch-structured DNAs

Ies6 analogously to YY1, is not only able to bind linear, duplex DNA, but can also bind structured DNAs *in vitro*. Although YY1 was seen to bind linear, as well as Holliday Junction and Y-fork DNA, the protein displayed a statistically significant preference for the structured DNAs, as it bound the linear duplex with significantly reduced efficiency and unlabelled, linear duplex was a poor competitor against radio-labelled Holliday Junction DNA (Wu et al., 2007). To investigate whether this binding preference also applies to the Ino80 subunit Ies6, the *in vitro* gel shift assays and their analyses were compared (Figures 4.9 & 4.10). A slight increase in binding activity can be observed at low protein concentrations in the case of the Y-fork and the Holliday Junction over the linear, duplex DNA (Figure 4.9, lanes 1-3). Additionally, 50% of the DNA is bound by 322nM for both the Y-fork and the Holliday Junction structures (Figure 4.9, lane 3). In contrast, 402nM Ies6 was required to shift 50% of the duplex DNA (Figure 4.9, lane 4). By the time the level of free probe has decreased to ~10-15% for the Y-fork and Holliday Junction DNA, respectively, the level of free probe in the presence of the linear duplex DNA is 30%. Notably, the higher-order structures observed at higher protein concentrations start forming at slightly lower protein concentrations and reach greater intensities in the Y-fork and Holliday Junction DNA binding experiments compared to the linear duplex.

In comparison to YY1, the preference of the Ino80 complex subunit Ies6 for branched DNAs is perhaps less striking, but it is nonetheless reproducible. Whilst YY1 displays a clear preference for the Holliday structure over the Y-fork structure over the linear duplex (Wu et al., 2007), Ies6 displays a similar slight preference for both branched-structured DNAs, possibly even favouring the Y-fork over the Holliday Junction (Figures 4.9 & 4.10).

4.6 Recombinant Ies6 binds to ssDNA

Single-stranded DNA is an intermediate of many DNA repair processes, including DSBs, but especially at stalled replication forks where ssDNA is thought to be a major component of the checkpoint, recruiting Mec1 and the 9-1-1 complex (see Introduction, 1.3). Not only is ssDNA energetically unfavourable when compared to dsDNA, but it also poses a threat to the cell in the form of unwanted re-annealing events and chemical modification (Bochkarev and Bochkareva, 2004; Wold, 1997). The cell is protected from such unwanted events by a number of proteins which bind ssDNA. Most ssDNA binding proteins display no sequence specificity, but show high affinity for ssDNA (Bochkarev and Bochkareva, 2004). Many ssDNA binding processes play a role in mediating DNA repair. The most well-known is RPA, which coats both the ssDNA generated from resection at DSBs and the ssDNA tracts generated at stalled replication forks and is involved in the recruitment of further repair factors, downstream checkpoint signalling and the removal of secondary structure, such as hairpins from ssDNA tracts (see Introduction 1.3, (San Filippo et al., 2008)). Furthermore, many of the recombination mediators, such as *S.cerevisiae* Rad52 bind ssDNA, often in preference to dsDNA, thus according importance to both the generation and the recognition of ssDNA throughout DNA repair and recombination processes (San Filippo et al., 2008).

Due to the presence of the Ino80 complex at stalled forks and previous results, which indicated that replication forks collapse in the absence of the Ino80 subunit Ies6 under conditions of replication stress, it was hypothesised that Ies6 may also bind ssDNA. Therefore the DNA binding assay was repeated under the same conditions as previously, but using a radiolabelled, ssDNA 49mer as the DNA substrate. To minimise the formation of double-stranded secondary structure, the binding assay was also performed at 30°C instead of room-temperature (lane 3). These data show that Ies6 does not only bind dsDNA, but is also capable of binding ssDNA (Figure 4.11). Indeed at a protein concentration of 1.29 μM (1000-fold molar excess), almost all

the ssDNA is bound by protein, as seen by the shift of the band up the gel (lane 2). Furthermore, the protein is seen to bind the ssDNA when the binding reaction was performed at 30°C (Figure 4.11), confirming that Ies6 is capable of binding ssDNA (lane 3).

4.7 Summary

The Ino80 remodelling complex contains 15 subunits which are all likely to play specific roles within the complex's multiple functions and a number of subunits have been implicated in DNA and chromatin binding and thus the potential recruitment of the complex to sites of DNA damage as opposed to recruitment for transcriptional activation. The Ies6 subunit remains fairly uncharacterised, but is thought to contain a C-terminal YL1 domain, which is believed to bind DNA. Although other Ino80 subunits are able to bind DNA it was hypothesised that Ies6 might also display DNA binding and it is reasonable to assume that subunits may act in concert or in a complimentary fashion in binding and complex recruitment. Indeed, the protein is not only able to bind linear DNA duplex, but was also seen to bind branch-structured DNAs. Thus Ies6 may perform an analogous function to YY1 although YY1 displays a greater preference for structured DNAs *in vitro* (Wu et al., 2007). However even if Ies6 performs an analogous function to human YY1, it is also likely to play a further, important role given the conservation of this protein demonstrated by its presence in other organisms, especially humans (Jin et al., 2005). Furthermore, the protein also binds ssDNA, an important intermediate of many DNA repair and checkpoint processes. Thus the Ies6 protein's ability to bind DNA, structured DNA and ssDNA, possibly via its YL1 domain, may mediate the probable involvement of the protein within the Ino80 complex's function at sites of damage and at stalled replication forks, as suggested by previous data showing the HU-hypersensitivity and replication fork collapse in the absence of Ies6 (see Chapter 3).

5.1 Introduction

The Ino80 chromatin remodelling complex encompasses a large number of subunits, of which a number, such as Ino80, the catalytic subunit, as well as Arp4 and Nhp10, have been implicated in DNA and chromatin interactions (Downs et al., 2004; Morrison et al., 2004; Ray and Grove, 2009; Shen et al., 2000). Ino-eight subunit 6 is a small and relatively uncharacterised subunit of the Ino80 complex. However, together with Ies2, but in contrast to Ies1 and Ies3-5, Ies6 is highly conserved across species (Fritsch et al., 2004; Hogan et al., 2010; Jin et al., 2005; Klymenko et al., 2006). Ies6 is thought to contain a C-terminal YL1 domain. Again, the YL1 domain remains relatively uncharacterised, however human YL1 protein is believed to be a transcription factor and possesses the ability to bind DNA ((Horikawa et al., 1995), see section 4.1 for a more detailed discussion).

Ies6 was hypothesised to be a putative DNA binding protein and *in vitro* gelshift assays employing radiolabelled DNA, as well as recombinant, MBP-HIS-tagged Ies6 confirmed that the Ies6 protein does indeed bind DNA. Furthermore, the protein not only bound double-stranded, linear DNA duplex, but also bound Holliday Junction and Y-fork DNA structures. Ies6 displayed a small, but reproducible preference for such structured DNAs, which is similar to human YY1, a protein subunit of human Ino80, with no clear homologue in budding yeast, which binds structured DNAs with a marked preference over linear DNA.

To determine whether the C-terminal YL1 domain of the Ies6 protein is involved in the protein's DNA binding activity, recombinant, MBP-HIS-tagged proteins were generated harbouring mutations in the YL1 domain and their DNA binding ability was investigated in *in vitro* DNA binding assays (see Chapter 4 & Materials & Methods 2.4). Furthermore, the importance of any mutations to the YL1 domain was also investigated in an *in vivo* system.

5.2 Site-directed mutagenesis of the Ies6 protein's C-terminal YL1 domain

To investigate the putative functional involvement in the protein's DNA binding ability of the Ies6 protein's C-terminal YL1 domain, a number of residues, as well as combinations of residues were targeted by site-directed mutagenesis (Figure 5.1). Not only the Ies6 protein, but also the human YL1 protein and YL1 domains in general remain largely uncharacterised. However, the YL1 domain is a putative DNA binding domain, although no established DNA binding domains were identified in the human YL1 protein (Horikawa et al., 1995). Residues for mutagenesis were therefore chosen largely by the presence of polar side chains, such as serines, as these often mediate DNA recognition and binding (Harrison and Aggarwal, 1990; Hatt and Youngman, 2000), whilst residues such as prolines were not targeted for mutation, as these were more likely to play a structural role within the protein (Figure 5.1). The residues targeted for site-directed mutagenesis were altered to alanines, so as to abolish a possible role in DNA binding for these residues whilst concomitantly attempting to avoid significant structural changes to the protein. Evolutionary sequence conservation was also taken into account (Figure 5.1) and indeed, T119 is highly conserved amongst the yeasts and present as a serine, a very similar residue in both mouse and humans (Figure 5.1). Although not fully conserved, homologous residues of T129 in budding yeast, are all polar residues often involved in binding DNA (Figure 5.1, (Harrison and Aggarwal, 1990; Hatt and Youngman, 2000)). Similarly, although K126 is not fully conserved, the lysine is present in most of the yeasts represented in Figure 5.1 and present as a polar residue in mouse and humans (Figure 5.1). A number of recombinant, MBP-HIS-tagged Ies6 mutants were generated successfully and subsequently screened in the established *in vitro* DNA binding assay to investigate their ability to bind DNA.

5.3 The Ies6-S127A/T129A mutant displays wild-type DNA binding activity *in vitro*

The first mutant generated for investigation of its DNA binding activity was Ies6-S127A/T129A (Figure 5.2). The mutant and the wild-type proteins were used in the *in vitro* gel shift assay side-by-side to evaluate any binding differences. In all binding assays, increasing amounts of either wild-type or mutant protein were incubated with radiolabelled linear duplex, Holliday Junction or Y-fork DNA before resolution by native PAGE gel electrophoresis (see Materials & Methods 2.4). All DNA binding assays were analysed using Image Quant® and the amounts of free and bound protein were quantified (see Materials and Methods, 2.4).

The mutations of serine 127 and threonine 129 to alanines do not cause any apparent decrease in the amount of DNA binding activity observed compared to the wild-type protein on the linear duplex DNA (Figure 5.3 a). A more detailed analysis of the DNA binding activity of the Ies6-S127A/T129A mutant, revealed only very slight differences between the two proteins, which were not statistically significant. Indeed, at a protein concentration of 402nM (lanes 2, respectively), the amount of free probe present in the wild-type binding assay had decreased to 25%, whereas in the mutant the amount of free probe present was 28%. Similarly, at a protein concentration of 564nM (lanes 4, respectively), the free probe remaining was quantified at 12% and 17% for the wild-type and S127A T129A Ies6 proteins, respectively.

Comparable results were observed in an analogous DNA binding assay in which radiolabelled Holliday Junction DNA was used as a substrate. Again, no remarkable difference in the binding activity of the mutant protein was observed when compared to wild-type Ies6 (Figure 5.3 b). Indeed, the results obtained by analysis in ImageQuant® were very similar: at a low protein concentration of 242nM, for example, the amount of free probe present in the wild-type Ies6 binding experiment was 24%, whereas 21% of the DNA substrate was present as free probe in the Ies6-S127A/T129A mutant DNA binding experiment. Similarly, though reversed for the wild-type and mutant, at a slightly higher protein concentration of 483nM, the amount of free

probe was seen to be 8% and 10% in the wild-type and mutant DNA binding assays, respectively. Analogous results were obtained employing radiolabelled Y-fork as the DNA substrate (data not shown), thereby demonstrating that mutation of S127 and T129 of Ies6 to alanines displayed little effect on the protein's ability to bind DNA.

5.4 An Ies6 quadruple mutant (T119A K122A S127A T129A) displays significantly decreased DNA binding *in vitro*

Since the mutation of both serine 127 and threonine 129 to alanines does not impinge on the ability of the Ies6 protein to bind DNA as observed in *in vitro* gelshift assays, two additional mutations were created in the Ies6 protein, thus generating the Ies6-T119A K122A S127A T129A mutant (Figure 5.4). Subsequently, the behaviour of this quadruple mutant was investigated in the DNA binding assays performed as previously. In contrast to the double mutant, the quadruple mutant displays reduced DNA binding activity compared to the wild-type Ies6 protein's ability to bind DNA in initial binding assays.

As indicated by the initial binding assays, the quadruple mutant displayed decreased binding activity. When the radio-labelled, linear duplex was used as the DNA substrate (Figure 5.5), the amount of unbound DNA present at a protein concentration of 402nM (lane4) was 42% for the wild-type protein and 56% for the quadruple mutant. Similarly, at the increased protein of 564nM (lane 6), the unbound DNA made up 18% and 41% of the DNA present in the wild-type and mutant binding reactions, respectively. This confirms that the quadruple mutant does indeed display reduced DNA binding activity.

Similarly, the reduced, though not abolished DNA binding activity of the quadruple mutant was also seen when either the Holliday Junction or Y-fork DNA was used as the DNA substrate (Figure 5.6 and Figure 5.7). In the case of the Holliday Junction, the amount of free probe at

protein concentrations of 402nM and 564nM (lanes 4 and 6) was 23% and 13% for the wild-type protein, whereas it was seen to be increased to 55% and 35% for the Ies6-T119A/K122A/S127A/T129A mutant. Likewise, a similar result was obtained using the Y-fork, branched structure as the binding substrate: at protein concentrations of 322nM and 564nM (lanes 3 and 6), the unbound DNA made up 45% and 10% of the wild-type Ies6 binding reactions, while in the DNA binding reactions containing the quadruple mutant, it constituted 65% and 30% at the same protein concentrations, respectively. Indeed, comparison of the protein's affinity for each of the DNA structures as compared to the wild-type (see Chapter 4) by a rough estimation of the K_d (nM) confirmed that the mutant possess decreased affinity for DNA:

	Duplex	Y-fork	Holliday Junction
Ies6	407	300	294
Ies6-T119A/K122A/ S127A/T129A	444	546	562

Mutation of T119, K122, S127 and T129 to alanines thus significantly decreased the ability of the protein to bind all three DNA structures tested in the *in vitro* gel shift assays, although it did not completely abolish the protein's DNA binding activity.

5.5 Decreased DNA binding activity *in vivo* correlates with yeast strain HU-hypersensitivity

The above data confirm that the Ino80 chromatin remodellor subunit Ies6 does indeed bind DNA *in vitro*. However, given the fact that multiple subunits of the Ino80 complex have been implicated in DNA binding and chromatin interactions (Downs et al., 2004; Morrison et al., 2004; Ray and Grove, 2009; Shen et al., 2000) and that all the above data characterise the

protein's DNA binding *in vitro*, it was important to determine the relevance of these findings *in vivo*.

In order to determine the physiological relevance of the Ies6 protein's ability to bind DNA, a set of complementation plasmids was generated. The plasmid backbone (pRS416) contains both a bacterial and a yeast (CEN/ARS) origin and the multiple cloning site was utilised for the insertion of the *IES*6 gene together with its promoter and terminator. The clone containing the wild-type *IES*6 gene was then used as the template for site-directed mutagenesis, as previously, to generate plasmids harbouring various mutant *ies6* genes, including *ies6*-T119A/K122A, *ies6*-S127A/T129A or *ies6*-T119A/K122A/S127A/T129A.

To assess the *in vivo* effect of the mutant proteins, it was first necessary to establish whether the protein was being expressed from the complementation plasmid constructs. In order to investigate protein expression, a FLAG-tag was inserted 5' of the ATG start codon (GenScript). These constructs were then transformed into the deletion strain and following protein extraction from yeast cultures grown to mid-log phase, protein expression levels were analysed by Western blot (Figure 5.8). Indeed, both the wild-type Ies6 and the mutants are expressed (Figure 5.8 a) in similar amounts relative to the H2A loading control (Figure 5.8 b) and there was no signal in the empty plasmid control using the anti-FLAG-tag antibody (Figure 5.8 a).

Loss of *IES6* has a striking effect on the yeast strain's ability to survive in the presence of HU, as shown previously by DNA damage response assay (see Chapter 3). Thus, in order to investigate the *in vivo* relevance of the Ies6 protein's DNA binding activity, the complementation plasmids were transformed into the *ies6* deletion strain and the strains' ability to survive in the presence of HU was evaluated.

The DNA damage response assays performed again demonstrate the hypersensitivity of the *ies6* deletion strain compared to the BY4741 parent strain (Figure 5.9, both strains contain the

empty pRS416 plasmid backbone to allow for selection on –URA). Indeed at an HU concentration of 40mM, the decrease in survival seen for the *ies6* deletion strain compared to the parent strain is already highly substantial.

Introduction of the wild-type *IES6* gene under the control of its endogenous promoter in the pRS416 backbone is seen to restore survival of the *ies6* deletion strain to almost wild-type levels (Figure 5.9). In fact, at low concentrations of HU, such as 40mM or 60mM, the phenotypes of the parent strains and the *ies6* deletion strain harbouring wild-type *IES6* in the HU DNA damage response assay are almost indistinguishable. At the highest concentration of HU used in this assay (150mM HU), introduction of the wild-type *IES*6 plasmid fails to fully complement the absence of ies6 to the level of viability seen in the parent strain. Survival is still however markedly increased when compared to the *ies6* deletion strain.

Both of the *ies6* deletion yeast strains containing the complementation mutants carrying the double mutations of *ies6* (T119A/K122A or S127A/T129A) display a very similar phenotype to the deletion strain harbouring the wild-type complementation plasmid. Data presented above (see section 5.3 and Figure 5.3) show that mutation of residues serine 127 and threonine 129 to alanines did not impact on the protein's ability to bind DNA. Thus the strain containing the S127A T129A mutant still able to bind DNA displayed a wild-type phenotype, as did the strain harbouring the other double mutant (T119A K122A), although this mutant combination was not evaluated for DNA binding activity.

The quadruple mutant (*ies6*-T119A/K122A/S127A/T129A), which displays reduced DNA binding ability *in vitro*, was also investigated in this HU DNA damage response assay. At low concentrations of HU (10-40mM HU), the presence of the quadruple mutant seemingly rescues the HU sensitivity of the *ies6* deletion strain. However with increasing concentrations of HU, the presence of the quadruple mutant increasingly fails to complement for the absence of *ies6*, seen by the decrease in survival of this strain compared with the parent BY4741 strain, as well

as the wild-type complementation strain. Thus, interestingly, the presence of the quadruple mutant only partially rescues the HU-hypersensitivity observed in the absence of *ies6*. This partial rescue may possibly correlate with the fact that whilst the quadruple mutant displays decreased DNA binding activity *in vitro*, its DNA binding activity is not all together abolished.

5.6 Summary

The Ino80 chromatin remodelling complex subunit Ies6 has been shown to bind DNA in *in vitro* DNA gelshift assays, displaying a slight preference for branch-structured DNAs (see Chapter 4). The hypothesis that the Ies6 protein's C-terminal YL1 domain mediated DNA binding was investigated by site-directed mutagenesis and *in vitro* DNA binding assays. Mutagenesis of two residues, S127 and T129, to alanines did not impair the protein's ability to bind DNA compared to the wild-type, both with regards to DNA binding activity on linear and branch-structured DNAs. A quadruple (T119A K122A S127A T129A) Ies6 mutant however, displayed significantly reduced DNA binding activity compared to the wild-type Ies6 protein. The reduction in DNA binding activity was also found to be similar for the linear, Holliday Junction and Y-fork DNAs employed in the DNA binding assay. These assays demonstrate a role for the Ies6 YL1 domain in the protein's DNA binding activity. Complementation plasmids harbouring the wild-type, the double or the quadruple mutant were generated to assess the relevance of these findings. Protein expression from these vectors was confirmed. DNA damage response assays were performed with the complementation plasmids and whilst transformation of the wild-type or the double mutant rescued the *ies6* deletion strain's HU-hypersensitivity, the presence of the quadruple mutant failed to fully rescue the deletion strain's HU-hypersensitivity. Thus loss of DNA binding activity of the Ies6 protein correlates with the *in vivo* HU-hypersensitivity similar to the sensitivity seen in the absence of *IES6*.

6.1 Introduction

In S-phase, the cell's entire genome must be faithfully replicated once and once only and over-replication of DNA by processes such as re-replication or endoreduplication, leads to an increase in cellular ploidy. Although increased ploidy may confer an increased ability to survive to some cells, polyploidy usually alters cellular physiology in a detrimental fashion. For example, the increase in cellular size seen in polyploidy cells leads to altered ratios of cellular components (Galitski et al., 1999). Furthermore, polyploid cells have altered gene expression and display problems passaging through both mitosis and meiosis (Galitski et al., 1999). Interestingly, the mitotic difficulties of polyploid cells have been observed to cause mitotic chromosome loss, thus generating aneuploid daughter cells, a sign of genomic instability (Mayer and Aguilera, 1990).

A screen for ploidy-specific lethality identified genes belonging to three distinct groups: sister chromatid cohesion, kinetochore function and homologous recombination (Storchova et al., 2006). All three groups of genes identified also play a role in maintaining genome stability (Galitski et al., 1999; Mayer and Aguilera, 1990) and not surprisingly, wide-spread genomic instability was proposed as a defining characteristic of polyploid yeast (Storchova et al., 2006; Storchova and Pellman, 2004; Thorpe et al., 2007). The dependence on homologous recombination displayed by yeast with increased cellular ploidy is thought to be due to increased DNA damage arising either spontaneously or during S-phase and due to the challenge of replicating the increased cellular DNA content (Storchova et al., 2006).

Although the above screen investigated the existence of ploidy-lethal genes, it is likely that there exists a further subset of genes, which constitute barriers to an increase in cellular ploidy. Moreover, it might be expected that such genes would also be involved in the maintenance of genome stability. Loss of such a gene would not only lead to polyploidy, but would also contribute to the genomic instability and DNA-damage sensitive phenotype

displayed by polyploid cells. Given the dependence on recombination and the DNA damage sensitivity, as well as the S-phase specific problems displayed by polyploid cells, it is plausible that chromatin remodellors, such as the Ino80 complex might be involved in maintaining correct cellular ploidy. The Ino80 complex is a particularly good candidate for such a function, given its role not only in the DNA damage response, but also in replication (see Chapter 3 & Introduction, section 1.5). To investigate a possible link between the Ino80 complex and the Ies6 subunit and cellular ploidy, budding yeast strains were analysed for their DNA content by Fluorescence Activated Cell Sorting (FACS).

6.2 Loss of *IES6*, but not *ARP8* leads to an increase in cellular ploidy

In order to obtain more information about the role of both the Ino80 chromatin remodelling complex and the Ies6 subunit in S-phase progression and during replication, cells from mid-log cultures of a number of yeast strains were analysed by FACS. Strikingly, the haploid *ies6* deletion strain employed showed a "diploid"-like FACS profile compared to haploid and diploid parent strain controls, as well as the *arp8* deletion strain (data not shown and Figure 6.1). However the strain was not a true diploid, as sporulation assays failed to produce any tetrads (data not shown). Furthermore, the strain could still be arrested using α-factor (data not shown). It was thus hypothesised, that the FACS profile observed for the *ies6* deletion strain may reflect an increase in cellular ploidy. To determine whether this was the case, independently-derived haploid *ies6* strains (both strains from Euroscarf, see Materials and Methods 2.3) were analysed by FACS alongside both the haploid and diploid parent strains BY4741 and BY4743, respectively, as well as an *arp8* deletion strain (Figure 6.1). The haploid and diploid parent both display profiles consistent with normal ploidy. The *arp8* haploid deletion strain shows a similar profile to the haploid BY4741 wild-type control (Figure 6.1). A "diploid" FACS profile is however observed for both *ies6* deletion strains (Figure 6.1). It is

noteworthy that the increase in cellular ploidy appears to have occurred in a discrete manner (Figure 6.1), advocating a discrete doubling of the cellular genome in these mutant strains. Together, these data suggest that loss of *IES6* leads to an increase in cellular ploidy and thus that Ies6 may function as a barrier to aberrant increases in cellular ploidy.

6.3 Loss of either the catalytic subunit *INO80* or the *IES6* subunit leads to polyploidy

With regards to the Ino80 chromatin remodelling complex's hyper-sensitivity to HU, both *arp8* and *ies6* deletion strains have been seen to phenocopy the *ino80* deletion strain (kind gift from Xuetong Shen) and indeed the *arp8* deletion strain is often employed as the strain of reference for loss of Ino80 complex function in lieu of the *ino80* deletion strain (Falbo et al., 2009; Shen et al., 2003; Shimada et al., 2008). Therefore, as loss of *IES6*, but not *ARP8* was observed to lead to increased cellular ploidy, it is of interest to investigate whether this effect is specific to the loss of *IES6* or is also seen in the case of loss of the catalytic subunit. To this effect, FACS analysis was performed on asynchronous yeast cultures in mid-log growth phase (Figure 6.2 a & b). Compared to the wild-type haploid and diploid parent strains, both the *ino80* and *ies6* deletion strains were observed to have a "diploid" profile in the FACS analysis (Figure 6.2 a & b), whereas the *arp8* deletion strain is haploid as previously demonstrated (Figure 6.2 b). The polyploidy seen in both the *ies6* and *ino80* deletion strains is seemingly of a similar nature, with a discrete increase in the observed cellular ploidy. Thus loss of *IES6* not only phenocopies loss of *INO80* with regards to yeast strain HU hyper-sensitivity, but also with regards to the observed polyploidy, which is not the case for the *arp8* deletion strain. Increased ploidy in the same *ino80* deletion strain as used in the above experiments was also observed in the Peterson lab (Craig Peterson, personal communication).

6.4 In contrast to loss of *IES6*, loss of the other *IES* genes does not lead to an increase in cellular ploidy

Ies1 and Ies3-5 are specific to budding yeast, whereas Ies2 and Ies6 are found to be highly conserved across species. In DNA damage response assays, only deletion of *IES6* results in the same, striking, HU hyper-sensitivity as seen for the deletion strain of the catalytic subunit of the Ino80 complex (see Chapter 3), with deletion of any of the other *IES* subunits resulting in little, or no HU hyper-sensitivity (see Chapter 3). In light of these previous findings, it was of interest to investigate whether deletion of any of the other *IES* genes also affected cellular ploidy, as seen with the *ies6* or *ino80* deletion strains.

Similarly to the data obtained from the DNA damage response assays, it was observed that deletion of any of the *IES1-5* subunits did not affect cellular ploidy (Figure 6.3). Although there is possibly a very small population with higher ploidy in the *ies2* and *ies4* deletion strains, it is not nearly as striking as in the *ies6* deletion strain, which displays a "diploid" profile by FACS (Figure 6.3). These data show that both the catalytic subunit of the INO80 complex, as well as the Ies6 subunit, impinge on the regulation of cellular ploidy. Of the Ino80 subunits tested, this property is unique to these two complex subunits.

6.5 Tetrad analysis of an *IES6/ies6* heterozygous diploid shows that loss of *IES6* is concomitant with cellular development towards higher polyploidy

Loss of *IES6* may be a largely lethal event for budding yeast, with only a small population of cells containing higher ploidy being able to survive. Alternatively, *IES6* may constitute a barrier to polyploidy, thus explaining the "diploid" FACS profile so far observed for all *ies6* deletion strains. To distinguish between these two possibilities, an *IES6/ies6* heterozygote yeast strain was generated. The heterozygous strain was generated in the BY4743 budding yeast

background as described previously (see Materials & Methods 2.3 & Figure 6.4 a). The FACS profile of the *IES6/ies6* heterozygous yeast strain generated is normal for a diploid strain (Figure 6.4 a), suggesting that a single copy of *IES6* is sufficient to maintain normal ploidy in a diploid. The heterozygous yeast strain was sporulated and the tetrads obtained were dissected (see Materials & Methods 2.3). In all cases, the four spores were viable, suggesting that loss of *IES6* per se is not a lethal event. Loss of *IES6* was seen to segregate 2:2 (Figure 6.4 & Figure 6.5) and correlated with a slow-growing phenotype, characteristic of *ies6* deletion strains (Figure 6.5). The genotype of the spores was confirmed both by the spores ability to grow on rich media supplemented with G418 (Figure 6.4 b) and by PCR for the inserted KanMX deletion cassette (data not shown).

Immediately upon growth of the dissected spores, spores were streaked onto rich media and grown for 48 hours before liquid cultures were inoculated. Following overnight growth to stationary phase, the yeast cultures were subcultured and grown to mid-log phase prior to analysis by FACS. In all cases, the spores containing wild-type *IES6* displayed wild-type-like, haploid, FACS profiles. In contrast, all spores deleted for *IES6* displayed a subpopulation with higher cellular ploidy (Figure 6.6). Notably, the majority of cells at this stage show a normal haploid FACS profile. This demonstrates that loss of *IES6* can occur in cells with a normal haploid content, contradicting the hypothesis that loss of *IES6* is lethal in haploids and survival only occurs in a small, polyploid, population. Furthermore, these data show that the slow-growth phenotype associated with loss of *IES6* is not due to the higher state of ploidy, as slow-growth was observed immediately after tetrad dissection when a large population of cells still display a haploid FACS profile. Finally, these findings suggest that the switch to higher ploidy is an early event following loss of *IES6*, as a subpopulation of polyploid cells is detectable at the earliest time-point it was possible to investigate.

6.6 Summary

Analysis of *ies6* and *ino80* deletion strains by FACS revealed that, in contrast to *arp8* or any of the *ies1-5* deletion strains, these strains possess a "diploid"-like FACS profile. Thus loss of *INO80*, the catalytic subunit or *IES6* leads to increased cellular ploidy. In order to obtain further information as to the lethality of deletion of *IES6* as well as to gain further information regarding the temporal development of polyploidy, a heterozygote *IES6/ies6* yeast strain was generated. Loss of *IES6* was not a lethal event in this budding yeast parent strain and loss of *IES6* was seen to segregate 2:2. Analysis of the spores by FACS confirmed a haploid FACS profile for the wild-type spores, whereas in the *ies6* deletion spores, a subpopulation with increased cellular ploidy was observed, even at an early time-point after tetrad dissection. These data suggest that the Ino80 chromatin remodelling complex plays a role in maintenance of correct cellular ploidy and are consistent with the hypothesis that the Ino80 complex acts as a barrier to polyploidy. Finally, these data show that the Ies6 subunit plays a role in the complex's activity in ploidy maintenance.

7.1 Introduction

Polyploidy can occur stably and relatively commonly in plants and some lower eukaryotes, such as fish and amphibians and is thought to contribute to evolutionary diversity (Comai, 2005; Doyle et al., 2008; Ohno et al., 1968; Thorpe et al., 2007). Although polyploidy is well tolerated in some lower eukaryotes, it is rare in higher eukaryotes, apart from a few somatic cells of highly proliferative tissue, such as the megakaryocyte of the bone marrow (Ravid et al., 2002). In general, the occurrence of polyploidy in higher eukaryotes is associated with a variety of pathological processes (Storchova and Pellman, 2004).

Polyploid cells are characterised by a number of deleterious physiological changes. Arguably, the most interesting hallmark of these cells is a marked genomic instability (Andalis et al., 2004; Huang and Koshland, 2003; Mayer and Aguilera, 1990; Storchova et al., 2006; Thorpe et al., 2007). Indeed, increased cellular ploidy renders cells hypersensitive to DNA damaging agents, as well as microtubule poisons (Andalis et al., 2004; Storchova et al., 2006) and also correlates with increased chromosomal loss and gross chromosomal rearrangements (Huang and Koshland, 2003; Mayer and Aguilera, 1990). Moreover, a more recent study on the genetic constraints of polyploidy in budding yeast identified three groups of genes which were ploidy-lethal (Storchova et al., 2006). Interestingly, all three groups (homologous recombination, sister chromatid cohesion and mitotic spindle maintenance) are involved in maintaining genome stability, once again highlighting the genomic instability of polyploid cells (Storchova et al., 2006). In contrast to the above identified ploidy-lethal genes, there is also likely to exist a subset of genes which constitute a barrier to changes in cellular ploidy and to which *IES6* and the Ino80 chromatin remodelling complex likely belong (see Chapter 6).

In contrast to polyploidy, aneuploidy is characterised by a variation in chromosome number distinct from the full haploid content, usually chromosome loss (Williams and Amon, 2009). At an organismal level, aneuploidy exists rarely and is often associated with embryonic lethality

or developmental defects and death (Williams and Amon, 2009). Furthermore, aneuploidy, that is loss of chromosomes, is the most common change in ploidy observed in cancerous cells (Rajagopalan and Lengauer, 2004) and at this cellular level is associated with cancerous hyper-proliferation in mammals. Since its first observation as a trait of cancerous cells (Boveri, 1914), the exact role of aneuploidy in carcinogenesis has been both unclear and a matter of debate (Duesberg and Rasnick, 2000; Matzke et al., 2003; Nigg, 2002; Rajagopalan and Lengauer, 2004; Storchova and Pellman, 2004). Indeed its observation as merely a characteristic of cancer cells somehow arising during carcinogenesis is advocated by some, whereas others ascribe it an early, fundamental and active role contributing to the cancerous development of the cell (Duesberg and Rasnick, 2000; Matzke et al., 2003; Nigg, 2002; Rajagopalan and Lengauer, 2004; Storchova and Pellman, 2004). To add to the debate, the generation of aneuploid cells also remains unclear. Interestingly, there is evidence from both yeast and human cancer predispositions, such as Barrett's oesophagus to suggest that aneuploidy may result from a preceding switch to polyploidy followed by progressive chromosome loss (King, 2008; Mayer and Aguilera, 1990; Reid et al., 1996).

The DNA-damage-hypersensitive nature of polyploid cells and the possible involvement of polyploidy as a precursor of aneuploidy accord the budding yeast ploidy-lethal genes a role in the prevention of progressive genomic instability. To determine whether *IES6*, previously shown to act as a barrier to increased cellular ploidy (see Chapter 6) may also play a role in the prevention of aneuploidy and progressive genomic instability, the DNA content of budding yeast *ies6* deletion strains was investigated by FACS over time.

7.2 Loss of *IES6* leads to progressive changes in cellular ploidy and genomic instability

In order to investigate the consequences on cellular ploidy of strains lacking *IES6*, the *ies6* deletion strain harbouring an empty pRS416 vector was regularly subcultured and cell samples in asynchronous growth phase were analysed by FACS. Unfortunately, due to technical difficulties, the initial time-points taken could not be analysed. In the samples analysed, the changes in cellular ploidy observed over time in the *ies6* deletion strain are striking (Figure 7.1). Over the course of this experiment the *ies6* deletion strain was seen to display a strong tendency towards even higher cellular ploidy than its characteristic "diploid-like" FACS profile. Indeed the cellular ploidy observed throughout the experiment is increased, less well defined and variable over time (Figure 7.1) compared with the ploidy of the restreaked *ies6* deletion strain previously observed (Chapter 6). It is likely, that the general broadening of the FACS profiles which is seen to occur over time also shows that the progression towards genome instability in these samples is a relatively stochastic process, in which some cells are developing towards higher ploidy, whereas others are experiencing chromosome loss and yet others still have likely lost viability. Additionally, the small, sub-G1 peak observed may be indicative of cells having undergone mitotic catastrophe. Furthermore , a tendency towards chromosome loss over time, as well as sub-G1 cells were observed over the time course of this experiment, primarily at later stages, such as days 37 and 40 (Figure 7.1), possibly indicating that the increase in ploidy may precede or at the least facilitate such chromosome loss. *IES6* has previously been shown to constitute a barrier to aberrant, higher cellular ploidy (see Chapter 6), however these data also show that loss of *IES6* leads to progressive changes in cellular ploidy over time and it is possible to conclude from these FACS profiles that *IES6* consequently also plays a role in maintaining genome stability.

7.3 Re-introduction of *IES6* halts progression towards further genomic instability

To determine whether re-introduction of the wild-type *IES6* into the *ies6* deletion strain had an effect on the changes in ploidy and genome stability observed in the *ies6* deletion strain harbouring an empty plasmid only, a culture of the *ies6* deletion strain transformed with the vector containing wild-type *IES6* was also analysed over time by FACS (Figure 7.2). Interestingly, in the case of the *ies6* deletion strain harbouring wild-type *IES6* on a plasmid, the "diploid-like" FACS profile characteristic of the *ies6* deletion strain was now essentially maintained (Figure 7.2). Whilst minor shifts in the FACS profile may be observed (Figure 7.2), they are relatively subtle, especially when compared to the dramatically aberrant FACS profiles seen in the *ies6* deletion strain (Figure 7.1). It is also noteworthy that whilst re-introduction of *IES6* seemingly prevents further changes in cellular ploidy and progressive genome instability, it does not lead to a change back to a normal "haploid" FACS profile (Figure 7.2). These data thus confirm that *IES6* plays a role in maintaining cellular ploidy and thus genome stability.

7.4 The Ies6 protein's ability to bind DNA correlates with the protein's ability to maintain genomic stability

A significant decrease in the ability of the Ies6 protein to bind DNA results in HU-hypersensitivity similar to that observed in the *ies6* deletion strain, thus according the protein's DNA binding activity an important function in the protein and the Ino80 complex's role in response to DNA damage (see Chapter 5). In light of the role *IES6* is now also seen to play in the maintenance of correct cellular ploidy and prevention of genomic instability (Chapter 6 and 7.2, 7.3), it is of interest to investigate whether the Ies6 protein's ability to bind DNA is also important for these processes. To determine the relevance of the protein's ability to bind DNA with regards to its role in maintaining both cellular ploidy and genome stability, cultures of the *ies6* deletion strain transformed with either the vector containing *ies6*-

S127A/T129A, which still binds DNA *in vitro*, or *ies6*-T119A/K122A/S127A/T129A, which displays significantly reduced *in vitro* DNA binding activity, and were also analysed over time by FACS. Presence of the *ies6*-S127A/T129A mutant, which retains DNA binding activity (see Chapter 5), is initially seen to maintain the "diploid" profile of the ies6 deletion strain (Figure 7.3), analogously to re-introduction of the wild-type protein (see 7.1). However over the course of the experiment, from about days 25-30, a general tendency of increased cellular ploidy and genomic instability is observed (Figure 7.3). These features become more distinct with each subsequent time-point analysed, until at ~day 37, the FACS profile is very similar to that observed in an *ies6* deletion strain (Figure 7.3), likely to be indicative of a number of cell populations displaying increased ploidy, chromosome loss, mitotic catastrophe, cell death and wide-spread genome instability.

Strikingly, in the case of the *ies6*-T119A/K122A/S127A/T129A, which previously was shown to display significantly decreased DNA binding activity *in vitro*, as well as HU-hypersensitivity *in vivo* (see Chapter 6), dramatic changes in ploidy are seen, which are very similar to those of the *ies6* deletion strain (Figure 7.3). Indeed, from the first time point analysed here, the samples display altered, largely increased, cellular ploidy and a general broadening of the FACS profile, suggestive of a non-uniform development of genome instability in sample subpopulations (Figure 7.3). Indeed, the hypothesis of a stochastic nature for this process is further corroborated by repetition of the time course (see Appendix A.2).

7.5 Analysis of *ies6* versus *IES6* spores suggests progression towards general genome instability may occur in a timely fashion

In order to gain further insight into the development of such drastic changes in cellular ploidy observed in the absence of *IES6*, cultures of the spores previously analysed were also investigated by FACS analysis over time (Figure 7.4). These cultures were prepared from

glycerol stocks that were made immediately following the analysis of the spores by FACS in Chapter 6. It is noteworthy that although the first time-point analysed here was taken as soon as possible after the cells were retrieved, the profile now observed for both spores harbouring a deletion of *IES6* are now completely "diploid-like" (Figure 7.4 a), providing further evidence for the hypothesis that this increase in cellular ploidy in the absence of *IES6* is a very early event. No changes are observed from Day 1 to Day 3 (Figure 7.4 b), however, strikingly, the profile observed on Day 5, shows a dramatic change in the FACS profile of cultures from both spores harbouring a deletion of *IES6* (Figure 7.4 c). Both spores 2a and 2c display broadened profiles, displaying both chromosome loss and an increase in cellular ploidy, again demonstrating the stochastic nature of these events within the population of cells (Figure 7.4). Interestingly, both spores are seen to undergo dramatic alterations in cellular ploidy in a timely fashion, between Day 3 and Day 5 (Figure 7.4), which was also seen to be the case for the other spores investigated (see Appendix A.3 and A.4) suggesting there may exist a relatively well-defined time frame for the progression to the aberrant cellular ploidy and genome instability that is the hallmark of loss of *IES6*.

7.6 Summary I

Analysis over time by FACS of the cellular ploidy of the *ies6* deletion strain harbouring an empty vector revealed that in the absence of *IES6*, cells develop towards higher ploidy, chromosome loss, mitotic catastrophe, cell death and significant genome instability in what is likely to be a stochastic process. This process could be prevented by re-introduction of wild-type *IES6*, however this did not lead to a reversal of the "diploid-like" FACS profile characteristic of the *ies6* deletion strain, but rather maintenance of the profile that existed at the time of re-introduction of *IES6*. Re-introduction of the *ies6*-S127A/T129A or the *ies6*-T119A/K122A/S127A/T129A mutants showed that the double mutant slowed, but did not

prevent progression towards aberrant cellular ploidy and genome instability, whereas strains harbouring the quadruple mutant displayed the same profile as the *ies6* deletion strain. This demonstrates that the DNA binding activity of the Ies6 protein is not only important with regards to the protein's role in HU-hypersensitivity , but also plays a critical role in the protein's function in maintaining correct cellular ploidy and, crucially, genome stability. Finally, analysis of *IES6* and *ies6* spores further confirmed the development to aberrant ploidy and genome instability in the absence of IES6 and also suggests there may possibly exist a relatively defined time-frame for this process.

7.7 Hypotheses regarding possible genetic insight into the role of the Ino80 complex

Loss of *IES6* not only leads to HU-hypersensitivity and failure to stabilise replication forks in the presence of DNA damaging agents, such as HU, but also causes a "diploid-like" FACS profile, which, over time and in a stochastic process leads to further increases in cellular ploidy, as well as chromosome loss and sub-G1 populations (see Chapters 3, 6 and data presented above). *IES6* thus plays a role in maintaining genome stability, for which its ability to bind DNA, including branch-structured Y-fork and Holliday Junction DNA intermediates, is critical (see Chapters 4 and 5). In order to gain further insight into the function of *IES6* and the Ino80 complex in these events, a number of hypothesises regarding the role of the Ino80 complex in a variety of relevant cellular processes, such as decatenation and the G2/M checkpoint were constructed. These hypotheses were tested by overexpression of proteins involved in these pathways in the *ies6* deletion strain and investigation of the resulting strains' behaviour in HU DNA damage response assays.

Yeast strains lacking, or expressing catalytic-dead versions of either topoisomerases II (*TOP2*) or III (*TOP3*) display similar phenotypic characteristics of the *ies6* deletion strain with regards to slow growth, DNA damage hypersensitivity, chromosome loss and genomic instability

(Baxter and Diffley, 2008; Chakraverty et al., 2001; Gangloff et al., 1994; Wallis et al., 1989). The type II topoisomerase, Top2, is responsible for the decatenation of catenated, double-stranded DNA molecules resulting from replication completion. Indeed, down-regulation of Top2 results in decreased decatenation leading to chromosomal mis-segregation, chromosome breakage and cell death (Baxter and Diffley, 2008). Top3 on the other hand, whilst also required for decatenation, decatenates hemicatenane DNA intermediates arising during the repair of DSBs or of stalled replication forks in such a manner as to prevent cross-overs and thus loss or aberrant alteration of the cell's genetic information (Ira et al., 2003; Mankouri and Hickson, 2006; Wu and Hickson, 2003).

Other proteins of which the effects of overexpression on the *ies6* deletion strain were investigated were Clb2 and Cdk1, which play important roles in the regulation of the *S.cerevisiae* cell cycle: Clb2 belongs to the B-type family of cyclins, which bind Cdk1 and target it to its relevant substrates (Bloom and Cross, 2007). Clb2 is required for mitotic events, such as the onset of anaphase, as well as the prevention of mitotic exit and cytokinesis (Bloom and Cross, 2007). Cdk1 is the only cyclin-dependent kinase required in *S.cerevisiae* for cell cycle progression and impinges on a variety of cellular processes in each phase of the cell cycle (Bloom and Cross, 2007; Enserink et al., 2009). Importantly, Cdk1 is responsible for determining the cell's preference for repair by HR over NHEJ in S/G2 (Caspari et al., 2002; Ira et al., 2004). Interestingly, Cdk1 impinges on numerous pathways involved in maintaining genome stability, such as DNA repair by HR and the prevention of mitotic catastrophe (Enserink et al., 2009; Ira et al., 2004; Kitazono and Kron, 2002; Myung et al., 2004). In this capacity, Cdk1 is seen to influence repair by HR both at an early and at a late stage and in the case of the latter is thought to facilitate recombination intermediate processing by Sgs1 and Top3, the activity of which is maintained by both Cdk1 and Rad9 (Caspari et al., 2002).

In light of the phenotype observed in the *ies6* deletion strain, it is reasonable to hypothesise that these proteins might act in common or related pathways. Furthermore, given the nature of these events, their effects on DNA topology and the constraints placed upon them by the chromatin environment they most take place in, it is reasonable to hypothesise a role for a chromatin remodelling complex in these processes. It has also been suggested, that the chromatin context may influence the choice of repair pathway (Caspari et al., 2002).

To investigate these pathways and determine possible effects of overexpression of any of these four proteins (Top2, Top3, Clb2 and Cdk1), overexpression constructs were transformed into the BY4741 parent and the *ies6* deletion strain and DNA damage response assays were performed in order to obtain some preliminary information.

7.8 Overexpression of *TOP2*, *TOP3*, *CDK1* or *CLB2* has a deleterious effect on the BY4741 parent strain

To determine whether overexpression of any of these four proteins had an effect on the BY4741 parent strain, overexpression plasmids were transformed into the strain. Overexpression of these proteins from the plasmid was under the regulation of the *GAL1-10* promoter and thus the yeast was initially grown in synthetic drop-out media containing glucose to both select for the plasmid and maintain the *GAL1-10* promoter switched off, thus causing minimal protein expression from the plasmid. The yeast strains were then used for DNA damage response assays on synthetic drop-out media containing galactose and supplemented with increasing amounts of HU (Figure 7.6).

The BY4741 parent strain and the *ies6* deletion strain harbouring an empty vector, as well as the deletion strain harbouring wild-type *IES6* on a plasmid, were used as controls. As expected, BY4741 grows robustly at all concentrations of HU employed, whereas the *ies6* deletion strain

displays a marked hyper-sensitivity the presence of this DNA damaging agent (Figure 7.6). Expression of wild-type *IES6* from a plasmid largely rescues the HU-hypersensitivity, as previously shown (Figure 7.6, Chapter 5, Figure 5.9).

Strikingly, overexpression of any of the four proteins investigated in this experiment in the BY4741 yeast strain lead to severely stunted growth even on the synthetic drop-out control plate, which did not contain HU (Figure 7.6). Indeed, even on this control plate, growth of strains overexpressing Top3, Top2 or Clb2 was hardly significant, whereas growth of the strain overexpressing Cdk1 was strongly reduced (Figure 7.6). Additionally, the small amount of growth observed in this strain was further reduced at low concentrations of HU, such as 20mM and abolished at higher HU-concentrations (Figure 7.6). There was no significant growth in the presence of overexpressed Top3, Top2 or Clb2 with increasing amounts of HU, however these strains did seem to develop suppressing mutations at a low frequency (Figure 7.6).

7.9 Overexpression of *TOP3* or *CDK1*, but not *TOP2* or *CLB2* largely rescues the slow-growth and HU-hypersensitive phenotype of the *ies6* deletion strain

The overexpression plasmid were next transformed into the *ies6* deletion strain and the same DNA damage response assay was performed in order to assess the effect of overexpression of these proteins in the absence of *IES6*. Again, growth of the wild-type background strain, BY4741, was largely unaffected by increasing amounts of HU, whereas the *ies6* deletion strain displays HU-hypersensitivity, which is mostly rescued by re-introduction of wild-type *IES6* on a plasmid (Figure 7.7).

Interestingly, with regards to the overexpression strains, there already exists a visible difference on the galactose-supplemented synthetic drop-out media. Indeed, overexpression of both Cdk1 and Top3 is viable in the *ies6* deletion background, as compared to the BY4741

parent strain and may even lead to a slight rescue of the slow-growing phenotype characteristic of the deletion strain (Figures 7.6 and 7.7).

Overexpression of Top2 or Clb2 in the *ies6* deletion strain lead to decreased growth compared to deletion of *IES6* alone (Figure 7.7). However overexpression of Top2 in the deletion strain compared to the parent strain is possibly less deleterious for yeast growth (Figures 7.6 and 7.7).

At increasing concentrations of HU, overexpression of Clb2 does not rescue the HU-hypersensitive phenotype of the *ies6* deletion strain (Figure 7.7). The *ies6* deletion strain overexpressing Top2 displays very slight growth on media supplemented with increasing amounts of HU, however this slight growth persists even at high concentration of HU (Figure 7.7, 200mM HU) and the appearance of suppressors seen frequently in the BY4741-Top2 overexpression strain is absent (Figures 7.6 and 7.7) indicating that Top2 may play some role in survival of the deletion strain.

Overexpression of either Cdk1 or Top3 in the *ies6* deletion strain background, not only possibly rescues the slow-growth phenotype of the ies6 deletion strain on synthetic drop-out media, but was also seen to rescue the HU-hypersensitivity of the deletion strain (Figure 7.7). Indeed this rescue becomes both more apparent and more striking with increasing amounts of HU and is very slightly greater in the case of overexpression of Cdk1 compared with that of Top3 (Figure 7.7). The level of rescue seen in the presence of overexpressed Cdk1 or Top3 is probably slightly less than that achieved by re-introduction of *IES6* under the *IES6*-promoter but nonetheless attains a level of rescue which is highly significant.

7.10 Summary II

Ies6 and the Ino80 complex have previously been shown to play a role in the stabilisation of stalled replication forks in S-phase and further investigations have also demonstrated a role for the complex in the maintenance of ploidy and genome stability. In the absence of Ies6, cells thus experience problems during replication and probably also prior to cytokinesis, possibly at the G2/M checkpoint. In light of these results, four proteins, Top2, Top3, Cdk1 and Clb2, which all impinge on aspects of these processes, were chosen for overexpression in the presence and absence of *IES6* for evaluation of their effects on cell survival in the presence of HU. Interestingly, overexpression of any of these proteins in the presence of *IES6* was highly deleterious to the cells, not only in the presence of increasing amounts of HU, but even on synthetic drop-out media. In the absence of *IES6*, however, a number of interesting effects were observed. Overexpression of Clb2, providing cells with a delay prior to cytokinesis had a detrimental effect in the absence of *IES6*, even in the absence of HU. Cells overexpressing Top2, which resolves catenated, dsDNA molecules following replication, also showed decreased growth on synthetic drop-out media, compared to the ies6 deletion strain, but the small amount of growth observed persisted even at high concentrations of HU. Strikingly, overexpression of either Cdk1, the only S.cerevisiae CDK which plays a role in numerous cellular processes, or Top3, which decatenates hemicatenanes arising during repair of DSBs and replication forks, resulted in a near wild-type rescue of both the slow growth and the HU-hypersensitive phenotype of the *ies6* deletion strain, suggesting the possibility of interplay between Top3 activity, certain aspects of Cdk1 activity and the role of Ies6 within the Ino80 chromatin remodelling complex.

8.1 Summary

Ino-eighty-subunit 6 is a small subunit which associates with the Ino80 chromatin remodelling complex, involved in the DNA damage response. In this study we have shown that Ies6 possesses DNA binding activity *in vitro*. The protein's DNA binding activity is mediated by its C-terminal YL1 domain and displays a slight, but reproducible preference for branch-structured DNAs, such as Y-forks and Holliday Junctions. Yeast strains harbouring a deletion of *IES6* phenocopy yeast strains lacking the catalytic subunit *INO80*. Both strains are not only strikingly hypersensitive to the DNA damaging agent HU, but are also defective in the stabilisation of stalled replication forks arising from acute exposure to HU. In the case of Ies6 the HU-hypersensitivity can be rescued by re-introduction of wild-type IES6 expressed from a plasmid under the control of its own promoter, but expression of a DNA binding mutant with significantly reduced DNA binding activity fails to rescue the strain's HU-hypersensitive phenotype. Interestingly, loss of *IES6* also leads to increased cellular ploidy, initially giving rise to a "diploid-like" FACS profile for this haploid deletion strain. Moreover the development to "diploid-like" ploidy was shown to be an early event following loss of *IES6*. This study also shows that whilst deletion of *IES6* initially leads to a "diploid-like" state of ploidy, over time and via a stochastic process, these cells undergo further changes in ploidy, which are again seen to be dependent on the protein's ability to bind DNA. This process resulted in further less well defined increases in cellular ploidy, as well as aneuploidy, chromosome loss and sub-G1 populations, indicative of mitotic catastrophe and cell death, all of which are hallmarks of cancer. Interestingly, it was demonstrated that overexpression of *TOP3* or *CDK1*, but not *TOP2* or *CLB2* leads to a rescue of the *ies6* deletion strain's HU-hypersensitivity to near wild-type levels.

8.2 Ies6, a DNA-binding protein

Ies6 contains a C-terminal YL1 (YL1_C) domain. Human YL1 is a relatively uncharacterised protein, which binds DNA, is believed to be a transcription factor, and was recently identified as a subunit of human TRRAP/TIP60 and the SRCAP (similar to yeast SWR1) complexes, where it interacts with an uncharacterised zinc-finger protein (Cai et al., 2005; Horikawa et al., 1995). Human YL1 was originally shown to be a DNA binding protein, possibly via a putative a-helical structure, though it does not contain a characterised DNA binding domain (Horikawa et al., 1995). Proteins containing YL1 domains are thus speculated to possess DNA binding activity. The finding that Ies6 does indeed possess *in vitro* DNA binding activity, which could be significantly decreased by site-directed mutagenesis of residues within the protein's YL1_C domain, thus suggests the YL1_C domain is indeed a novel DNA binding domain. Future work will therefore be aimed at further investigating this novel DNA binding domain and obtaining structural information to shed light on its ability to mediate DNA binding.

Ies6 also binds branch-structured DNAs, such as Y-forks and Holliday Junctions, with a slight, but reproducible preference. This is somewhat analogous to the DNA-binding YY1 protein, a polycomb group transcription factor which contains a zinc-finger, which is a subunit of the human Ino80 complex and displays a significant preference for branch-structured DNAs (Cai et al., 2007; Wu et al., 2007). No YY1 homologue has yet been identified in budding yeast, leading to speculation that Ies6 may function analogously to human YY1 and possibly function by participating in the recruitment of the Ino80 complex to sites of DNA damage. However the preference of Ies6 for branch-structured DNAs is less marked than that of YY1 and the estimation of its Kd, although similar to that of YY1 (Wu et al., 2007), shows significantly less affinity for these structures than RuvA from *E.coli* and *M.pneumoniae*, which have a Kd of 10-200nM (Ingleston et al., 2002; Iwasaki et al., 1992). However it is also possible that *in vivo* the Ies6 protein's affinity for DNA and branch-structured DNAs may be modulated and possibly

increased. Indeed, the protein may be subject to post-translational modifications, such as phosphorylation, which may allow its DNA binding to be regulated, possibly in response to DNA damage. Moreover, Ies6 must have at least one protein interaction partner within the Ino80 complex, binding to which may cause a conformational change in the protein, thereby affecting its DNA binding domain. Ies6 may also interact with proteins which are not subunits of the Ino80 complex, again allowing for conformational changes within the protein. Furthermore, the Ino80 complex contains a number of proteins thought to bind DNA and it is possible that this locates Ies6 to the DNA first, increasing its ability to recognise branch-structured DNA by proximity.

Although the Ino80 complex contains a number of DNA binding activities, this does not necessarily make their DNA binding functions redundant. The multiple DNA binding subunits are likely to work cooperatively and may well each possess a specialised function within the many aspects of chromatin remodelling by the Ino80 complex.

No YY1 homologue has yet been identified in budding yeast, however homologues exist in mice and flies (Cai et al., 2007; Jin et al., 2005; Klymenko et al., 2006; Wu et al., 2007). More recently, a YY1 homologue, Iec1, was also identified in *S.pombe* (Hogan et al., 2010). Given the high degree of conservation of the Ino80 complex across organisms, it is therefore possible that a YY1 homologue is yet to be discovered in *S.cerevisiae*. Furthermore, Ies6 and Ies2 are highly conserved across species (Hogan et al., 2010; Jin et al., 2005). Thus, although Ies6 displays some functional similarities to YY1, it is likely that the protein also fulfils a unique and important function of its own.

Interestingly, Ies6 displays some homology to the zinc-finger HIT domain (Jessica Downs, Tony Oliver, personal communication). This is a novel sequence motif which has been identified in a number of proteins and whilst its exact function is unknown, it has been proposed to be involved in gene regulation and chromatin remodelling (He et al., 2007). The putative zinc-

finger HIT domain in Ies6 would however not be able to coordinate zinc, as the coordinating residues in other family members are hydrophobic residues in Ies6 (Tony Oliver, personal communication). Interestingly, a Pfam database search performed by Bateman et al. revealed, that 12.4% of zinc-finger HIT domain containing proteins contain an additional domain, homologous to PAPA-1 (Bateman et al., 2004; He et al., 2007). Although Ies6 does not contain homology to PAPA-1, Ies2, the remaining Ies subunit of the budding yeast Ino80 complex to be highly conserved across species is the budding yeast homologue of human PAPA-1 (hIes2). It is therefore tempting to speculate that Ies2 and Ies6 may be interaction partners and further work will be aimed at investigating this intriguing possibility.

The DNA binding activity of the Ies6 protein was further shown to be highly significant *in vivo*, as the Ies6 mutant which possesses significantly reduced DNA binding ability was unable to rescue the HU-hypersensitivity of the *ies6* deletion strain. This highlights the need for further work to elucidate the physiological role of the Ies6 protein and its DNA binding activity, which may possibly function in conjunction with Ies2 and potentially revolve around the recognition of DNA structures such as stalled replication forks and recruitment of the Ino80 complex.

8.3 There exists a subset of genes that are barriers to increased cellular ploidy

Loss of *IES6* correlates with an increase in ploidy, giving the *ies6* deletion strain a “diploid-like” FACS profile. The development to a “diploid-like” ploidy status was further shown to be an early event following loss of *IES6*. However, this study also showed that loss of *IES6* is not a lethal event per se and can occur in haploids. Furthermore, it was also demonstrated that the loss of *IES6*, rather than the increase in cellular ploidy, is responsible for the slow-growth phenotype observed. Moreover, the same increased ploidy was also observed in a budding yeast strain with a deletion of INO80 (this study and Craig Peterson, personal communication), thus demonstrating again that the *ies6* deletion strain phenocopies the strains lacking *INO80*.

Interestingly, polyploidy was also observed to be a consequence of loss of *YY1* in MEFs (Wu et al., 2007).

Multiple processes lead to increases in cellular ploidy, which may or may not give rise to partial or perfect genome duplications. The well-defined, diploid-like profile displayed by the *ies6* deletion strain may indicate that the increase in cellular ploidy observed is due to endoreduplication, that is a discrete doubling of the genetic content by a second, complete S-phase, rather than re-replication, that is stochastic re-firing of origins throughout the genome, leading to a less well-defined increase in genome size, however the exact process by which cells lacking *IES6* might undergo a second, complete round of replication remains unclear. It is also noteworthy, that the cells seemingly undergo exactly one round of endoreduplication, suggesting that a "diploid-like" state of ploidy is a consequence of loss of *IES6* and may confer an advantage, however higher states of ploidy do not. The possible mechanism by which budding yeast cells lacking *IES6* arrive at a "diploid-like" ploidy status will be investigated in future experiments.

These data also highlight the importance of investigating the ploidy of budding yeast strains employed for experiments as changes in ploidy lead to significant changes in budding yeast cell physiology (Storchova et al., 2006; Storchova and Pellman, 2004; Thorpe et al., 2007), which are likely to impact on experimental data obtained which cannot be neglected .

Finally, it has been demonstrated that there exists a group of "ploidy-lethal" genes, which are essential for the viability of budding yeast cells with higher ploidy (Storchova et al., 2006). Interestingly all "ploidy-specific lethal" genes identified by the above study are involved in the maintenance of genomic stability (Storchova et al., 2006) and the authors further conclude that genomic instability might be the most significant change in cellular physiology displayed by budding yeast with increased cellular ploidy (Storchova et al., 2006). These conclusions, together with the data presented from this study demonstrating the increase in cellular ploidy

as a consequence of the loss of *IES6*, show that it is likely there exists a further group of "ploidy-barrier" genes, to which *IES6* and *INO80* belong. It can thus be concluded that Ies6 and the Ino80 complex play an important role in preventing a switch to increased cellular ploidy in budding yeast, though the exact mechanism remains unclear.

8.4 The role of Ies6 in preventing progressive genomic instability is reminiscent of tumour cells

Although budding yeast lacking *IES6* were initially observed to display a well-defined, "diploid-like" FACS profile, when monitored over time, the DNA content of this strain was seen to drift. Consequently, increased ploidy, as well as aneuploidy and sub-G1 populations, indicative of mitotic catastrophe and cell death were observed in an apparently stochastic process. Crucially, this was not only seen to be the case in the absence of *IES6*, but was also demonstrated to be a consequence of an inability of the Ies6 protein to bind DNA. Moreover, data presented here suggest there may be a distinct time-frame over which this stochastic process takes place. Furthermore, the small sub-G1 population of cells observed, which are likely to have undergone mitotic catastrophe, are possibly an indicator that cells lacking *IES6* have indeed undergone endoreduplication by a process involving abortive cytokinesis and a full repetition of the cell's S-phase.

These observations are in agreement with observations from MEFs lacking *YY1*, which display both increased ploidy, aneuploidy and chromosomal abnormalities, similar to those seen in cells with defects in DNA repair, such as Bloom's, Fanconi anaemia and BRCA1 and BRCA2-breast or ovarian cancers (Wu et al., 2007). Moreover, they are also in accordance with results from a recent study using siRNA to human *INO80* in HeLa cells, which led to both polyploidy and aneuploidy (Hur et al., 2010).

The characteristics of cells lacking *IES6* are also hallmarks of cancer cells, which are often found to harbour altered ploidy, especially aneuploidy (Storchova and Pellman, 2004). Indeed, some cancers characteristically display a triploid-like content (Storchova and Pellman, 2004), which may be similar to a subset of the FACS profiles observed for the *ies6* deletion strains' profiles here. Furthermore, the stochastic process observed is also reminiscent of tumour cells, which are believed each to be aneuploid in a distinct way (Storchova and Pellman, 2004). Interestingly, the fact that the gross genomic instability observed for cells lacking *IES6* or containing Ies6 with a significantly reduced DNA binding activity is preceded by a phase of seemingly stable higher ploidy, would argue that increased ploidy may indeed be a possible cause of cancer rather than necessarily a by-product. These data thus support the idea that increased ploidy in itself may be a driving force along the road to carcinogenesis. Furthermore, these observations suggest a likely role for Ies6 and the Ino80 chromatin remodelling complex in protecting genome integrity and preventing some of the hallmark changes frequently observed in tumour cells.

8.5 Speculation on possible mechanisms of action of the Ino80 complex

This study has shown that loss of *IES6* leads to HU-hypersensitivity and failure to stabilise stalled replication forks following acute exposure to HU. Furthermore, deletion of *IES6* or a significant reduction in the protein's ability to bind DNA, quickly leads to an increase in cellular ploidy, which appears to be perfectly "diploid-like" at early time points, develops towards progressively higher ploidy and aneuploidy over time, further displaying FACS profiles consistent with mitotic catastrophe and cell death. Interestingly, overexpression of budding yeast Top3 or Cdk1, but not Top2 or Clb2 rescued the *ies6* deletion strain's characteristic phenotype of slow growth and hypersensitivity to chronic HU exposure.

While the effect of overexpression of these genes has not yet been tested on the changes in ploidy in the *ies6* deletion strain, so far the effects of IES6 mutations on DNA binding correlated with all of the phenotypes of the *ies6* deletion strain, such as HU-hypersensitivity, maintenance of genome stability, and slow growth. Therefore, it is not unreasonable to speculate that overexpression of Top3 or Cdk1 will also suppress the genome instability of the *ies6* mutant cells and this is currently being tested.

The detrimental effect of Clb2 overexpression seen here, which leads to a delay at G2/M in the *ies6* deletion strain, may indicate that the increase in time spent in G2/M prior to cytokinesis does not aid with the recovery of the problems experienced by the loss of *IES6* in replication and potentially maintenance of normal ploidy. The fact that Top2 overexpression does not seem to rescue the HU-hypersensitivity of the *ies6* deletion strain may possibly indicate that the hypothesis for a role of the Ino80 complex in setting up a correct chromatin environment for decatenation to occur does not hold true and that in fact increased Top2 activity leads to further chromosome missegregation and problems following cytokinesis. However endogenous budding yeast Top2 is subject to tight regulation, often relying on correct sister chromatid cohesion, which is likely to be affected in the *ies6* deletion strain and thus this result remains to be verified with the use of overexpression of a viral Top2, which is not subject to such tight control (D'Ambrosio et al., 2008).

There are a number of possible hypotheses that may explain the rescue by Top3 or Cdk1 seen in the data presented above. Ies6 may be involved in the processing and repair of DNA structures generated at stalled replication forks, explaining the *ies6* deletion strain's HU-hypersensitivity and its failure to stabilise stalled replication forks. In this role, its ability to bind branch-structured DNA might be crucial to the activity of the Ino80 complex in processing these structures. This would also open up the possibility for a role for the complex's Rvb proteins, which as yet have no known role ascribed to them within the Ino80 complex. Should

the Ino80 complex be able to process these structures independently of the Sgs1-Rmi1-Top3 pathway, overexpression of Top3 may increase the number of collapsed replication forks processed by the latter pathway in the absence of Ies6, thereby leading to an increase in cellular survival. However it is also possible that the Ies6 and thus the Ino80 complex may act in conjunction with Top3, either via the Sgs1-Rmi1-Top3 complex or with Top3 alone, as there is the possibility Top3 may process a subset of replication fork collapse induced recombination structures in an Sgs1-Rmi1-independent pathway (Bachrati and Hickson, 2003). Furthermore, a role for the Ino80 complex in the processing of these intermediates would protect from genomic instability arising from deleterious cross-overs between sister chromatids (Wu and Hickson, 2003). The precise role of the Ino80 complex remains unclear, but may involve the modulation of the chromatin environment thus allowing certain repair pathways over others and facilitating or inhibiting the generation and processing of certain recombination intermediates.

A further intriguing possibility is that Ies6 and therefore the Ino80 complex is required for the establishment of sister chromatin cohesion during S-phase and replication. Failure to correctly establish sister chromatid cohesion in the absence of Ies6 might explain the changes in ploidy observed in this strain, as this leads to missegregation of sister chromatids, which may both cause polyploidy, by causing a delay prior to cytokinesis, which the cells finally escape from by a second round of replication and which is an important contributing factor to aneuploidy (Ganem et al., 2007). The role of Ino80 as a chromatin remodelling complex and its localisation to the replication fork place it in an ideal position to potentially modulate the chromatin environment for the establishment of correct sister chromatin cohesion. Furthermore there have been reports that Ino80 may impact on the association of factors important for the establishment of sister chromatid cohesion with the replication fork, such as Ctf18 (Ogiwara et al., 2007). Additionally, it has been suggested that Top3 may play a role in sister chromatid cohesion, which would possibly explain the level of rescue seen in this experiment (Lai et al.,

2007). Interestingly, the possible defect in sister chromatin cohesion observed in the *ies6* deletion strain here would have to differ from that observed in the sister chromatid cohesion-defective strains identified in a previous budding yeast screen for ploidy-lethal genes, as those strains were inviable following an increase in ploidy (Storchova et al., 2006).

The rescue of the *ies6* deletion strain's HU-hypersensitivity by overexpression by Cdk1 is made difficult to interpret by the numerous cellular processes Cdk1 impinges upon. In budding yeast, but in contrast to other organisms, Cdk1 levels increase following DNA damage, as the protein plays a role in the DNA damage response and is further responsible for the cell's preference for repair by homologous recombination in S/G2 (Enserink et al., 2009; Ira et al., 2004). Cdk1 has also been implicated in the prevention of mitotic catastrophe (Myung et al., 2004). Although Cdk1 alone does not seem to aid recovery from stalled replication forks, it may do so in conjunction with Mre11 (Enserink et al., 2009) and it further influences late stages of repair by HR by maintaining Top3 activity (Caspari et al., 2002). Interestingly, a relatively recent study shows that Cdk1 may paradoxically increase viability of cells faced with repairing broken chromosome ends in a manner prone to gross genomic rearrangements (Enserink et al., 2009).

8.6 Perspectives on Ies6, the Ino80 complex and cancer

Although a very small protein, which only co-purifies with the Ino80 complex under low salt conditions, Ies6 nonetheless seems to play a critical role within the complex. In this study loss of *IES6* was identified as most closely phenocopying loss of *INO80* of the subunits tested, suggesting it is likely to play an important part. This is further highlighted by its conservation across species, especially humans. Ies6 plays a crucial role in the maintenance of genomic stability and cells lacking this protein go on to develop many of the phenotypic characteristics which are hallmarks of cancer cells. These data suggest Ies6 and the Ino80 complex are likely to act in a tumour-suppressing capacity. Further investigations are needed to identify the

precise mechanism of action of this chromatin remodelling complex in these processes and whether the observations presented here hold true in human cells. Chromatin remodelling complexes impinge on numerous processes central to cancer biology, however there is a lack of information on both their precise mechanism of action, as well as on individual subunit activity and thus potential targets. Nonetheless, it is intriguing to speculate that the Ino80 complex and more specifically, Ies6 and its DNA binding activity may in the future be of relevance in the development of novel anti-cancer agents.

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	Saccharomyces cerevisiae	*Schizosaccharomyces pombe*	*Drosophila melanogaster*	*Homo sapiens*
ATPase	Ino80	Ino80	INO80	INO80
RuvB-like	Rvb1 Rvb2	Rvb1 Rvb2	Reptin Pontin	RUVB1/TIP49a RUVB2/TIP49b
Actin	Act1	Act1	Actin	b-Actin
Actin-related proteins	Arp4 Arp5 Arp8	Alp5 Arp5 Arp8	 ARP5 ARP8	BAF53A ARP5 ARP8
YEATS protein	Taf14	Not known	Not known	Not known
GLI-Krueppel zinc-finger transcription factor-like	Not known	Iec1	Pleiohomeotic	YY-1
Other subunits, conserved	Nhp10 Ies2 Ies6	HMG-box, Nhp10-like Ies2 Ies6	Not known	 IES2 (PAPA-1) IES6
Other subunits, Non-conserved	Ies1 Ies3-5	SPCC1259.04 SPCC16C4.20	Not known	Amida CCDC95 FLJ20309 MCRS1 NFRKB UCH37

Table 1.5.1 Evolutionary conservation of Ino80 complex subunits

No.	Name	Sequence
1	gelshift-01	5'- GAC GCT GCC GAA TTC TGG CTT GCT AGG ACA TCT TTG CCC ACG TTG ACC C - 3'
2	gelshift-02	5' - TGG GTC AAC TGT GGC AAA GAT GTC CTA GCA ATG TAA TCG TCT ATG ACG TT - 3'
3	gelshift-03	5' - CAA CGT CAT AGA CGA TTA CAT TGC TAG GAC ATG CTG TCT AGA GAC TAT CGA - 3'
4	gelshift-04	5' - ATC GAT AGT CTC TAG ACA GCA TGT CCT AGC AAG CCA GAA TTC GGC AGC GT - 3'
5	gelshift-05	5' - GGG TCA ACG TGG GCA AAG ATG TCC TAG CAA GCC AGA ATT CGG CAG CGT C - 3'
6	gelshift-06	5' - TGG GTC AAC GTG GGC AAA GAT GTC CGG ACA TGC TGT CTA GAG ACT ATC GA - 3'
7	Recles6-01	5' - GTA CAT ATG AGC GGT AGT AGG GGC AA - 3'
8	Recles6-02	5' - ATG CTC GAG CTA TTT TAG AAC GAA GTT GGC - 3'
9	Recles6 N-term trunc F44	5' - GTA CCC CAT ATG TTT CCC TCT AGA TTC AAG TC - 3'
10	Y2HIes6-03	5' - GTA CCC ATG GGC GGT AGT AGG GGC AA - 3'
11	S127AT129AIes6-01	5' - GGG TTG AAG GGC TTC TAC AAG GCG CCT GCG AAC AAC ATT CGG - 3'
12	S127AT129AIes6-02	5' - CCG AAT GTT GTT CGC AGG CGC CTT GTA GAA GCC CTT CAA CCC - 3'
13	T119AK122AIes6-01	5' - GCC AAG AAG TAC TGC GAT GTT GCT GGG TTG GCG GGC TTC TAC - 3'
14	T119AK122AIes6-02	5' - GTA GAA GCC CGC CAA CCC AGC AAC ATC GCA GTA CTT CTT GGC - 3'

15	*IES6* -350	5’ - GTA GAA TTC GCA TAG TTT ATT AGT CTG TG - 3’
16	*IES6* +200	5’ - ATG AAG CTT ATG CTG GAC ATA GGT AGG AG - 3’
17	Ies6-del-01	5’ - AAG GCC CTG TCG CCG CAC AT - 3’
18	Ies6-del-02	5’ - AGA CGA TGC TGG ACA CAG GA - 3’

Table A.1 Primers used in this study

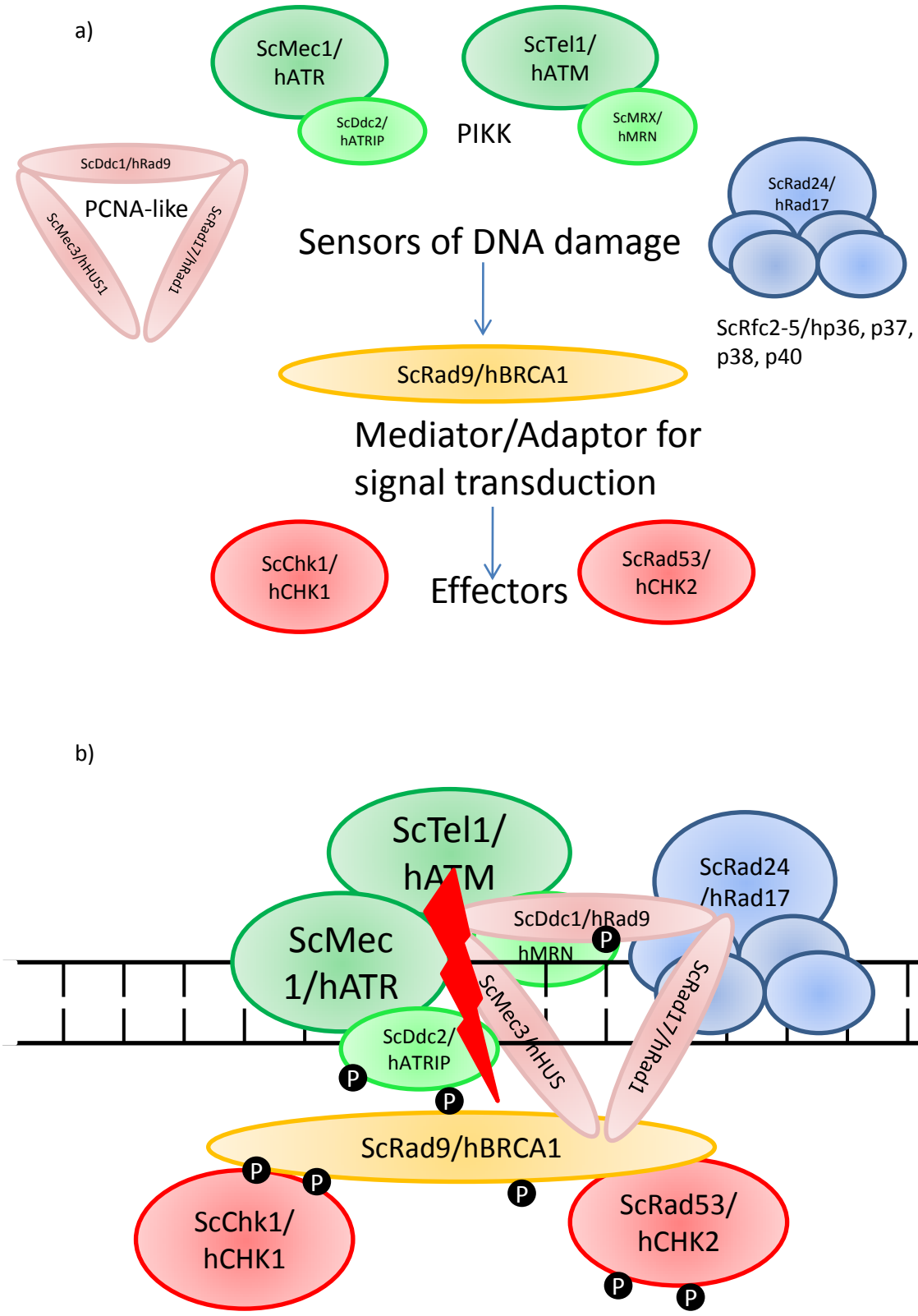


Figure 1.1.1 The DNA damage response is composed of a multitude of protein components and signalling activities a) Presentation of some key proteins involved in the DNA damage response according to their role in the signalling pathway b) The players in the DNA damage response work in concert to effect downstream cellular signalling leading to initiation of the DNA damage checkpoint and cell cycle arrest, allowing for timely DNA repair

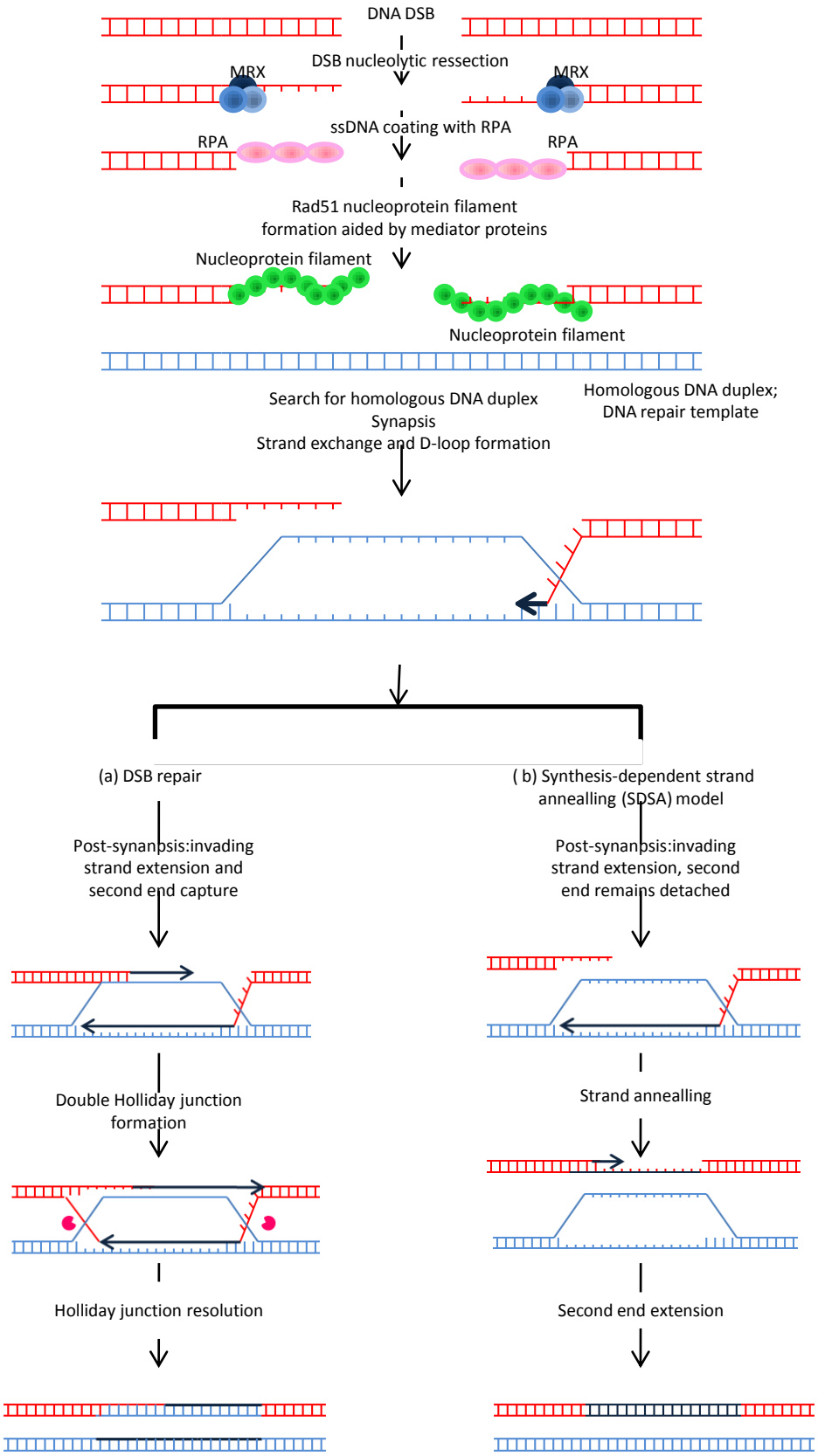


Figure 1.1.2 Homologous recombination is one of the key mechanisms for repair of DSBs Diagram of some of the stages of HR, including pre-synapsis, synapsis and postsynapsis; the latter may be accomplished by a) the DSB repair or the b) SDSA model. The damaged and undamaged sister chromatid/homologous chromosome template DNA are shown in red and light blue, respectively, the newly synthesised DNA is shown in dark blue.

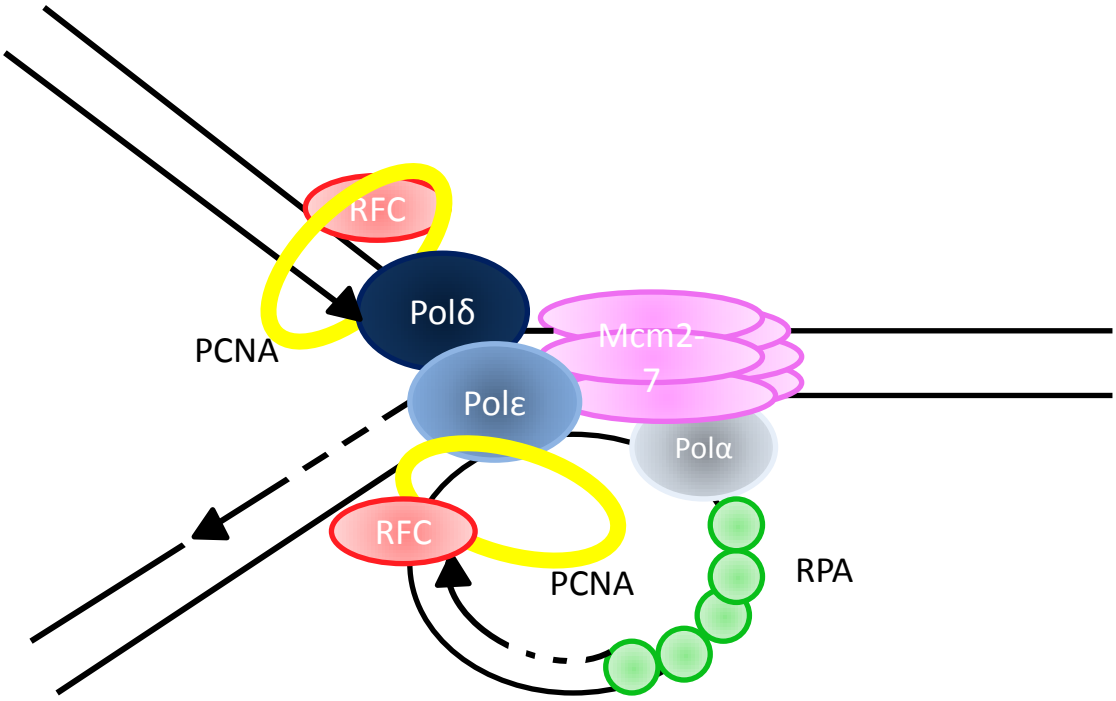


Figure 1.2.1 Simplified schematic molecular anatomy of a replication fork Presentation of some of the key players of replication fork progression

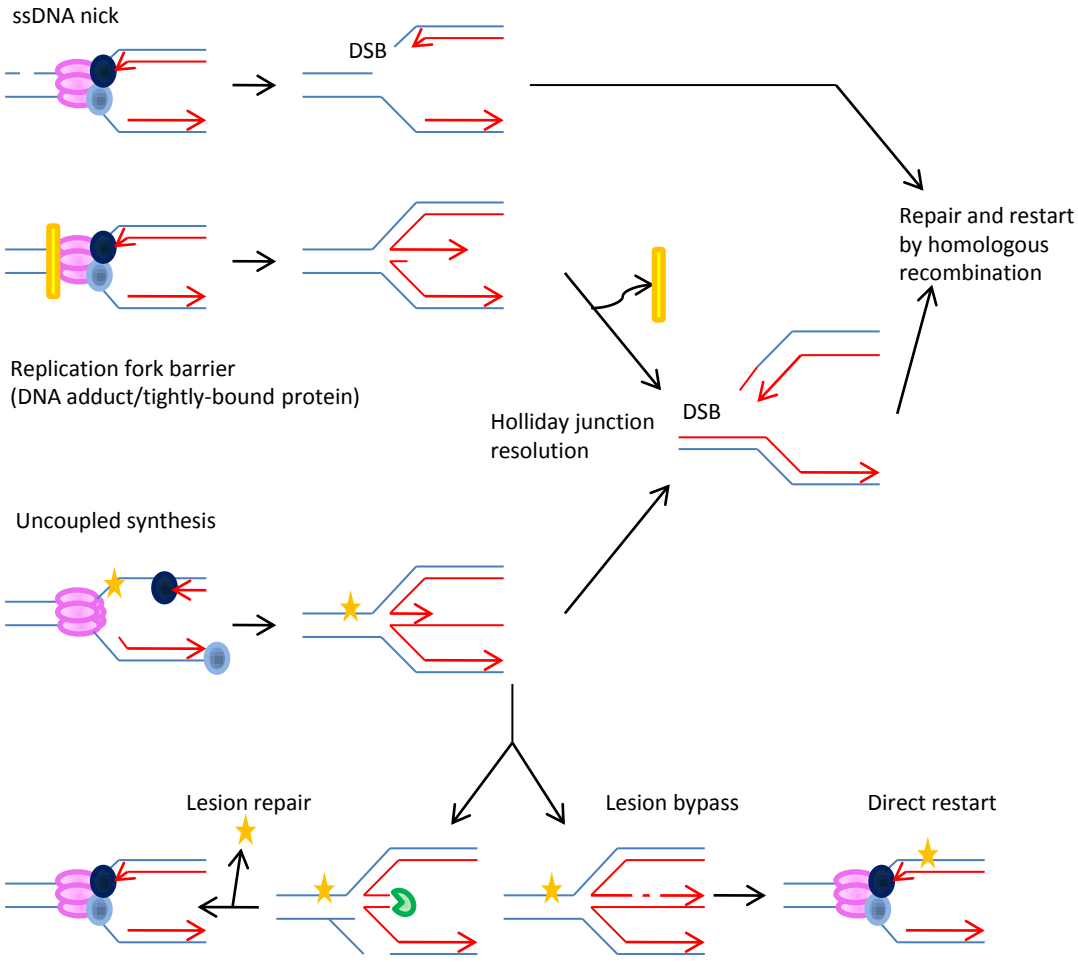


Figure 1.2.2 There exist a multitude of possible outcomes following replication fork stress, many of which give rise to DSBs Replication forks encountering ssDNA nicks collapse generating a DSB, whereas DNA adducts or tightly bound proteins lead to inhibition of the replication fork, which may be processed by the formation of a DSB or which may cause uncoupling of the replicative polymerases. Other lesions may affect the synthesis on one strand but not necessarily impede replication fork progression (Figure adapted from Aguilera and Gomez-Gonzalez, 2008)

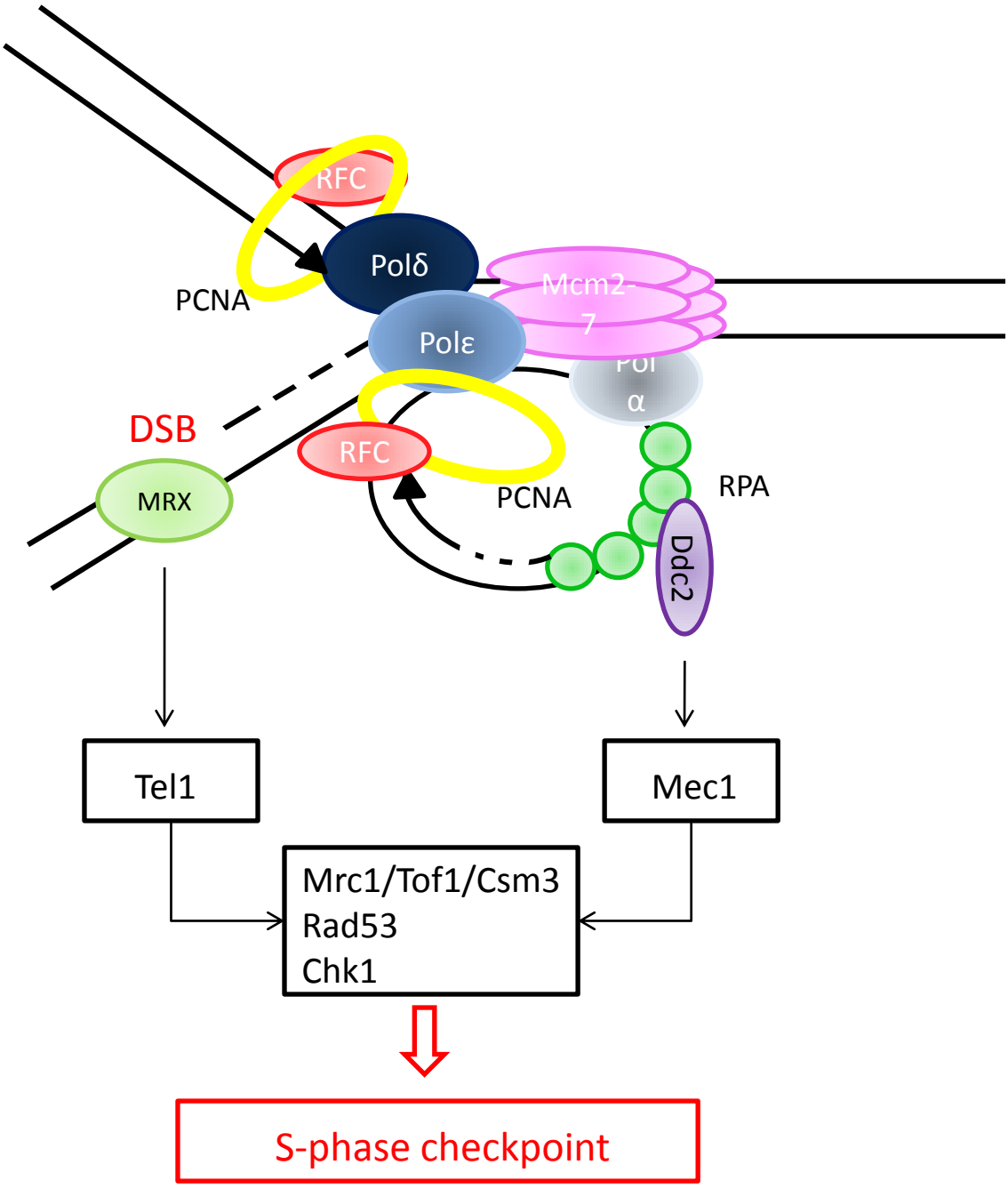
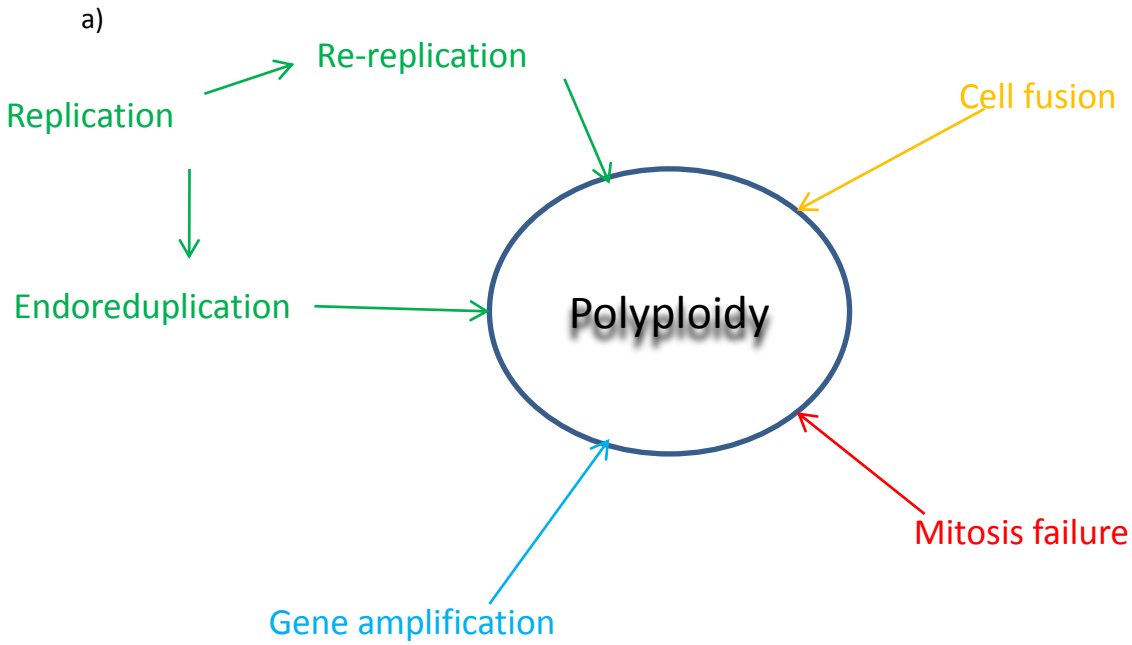


Figure 1.2.3 Signalling at a stalled replication fork leads to the activation of the S-phase checkpoint Diagram of some of the key factors involved in S-phase checkpoint activation at stalled replication forks, analogously to the DNA damage checkpoint activation at DSBs (discussed in section 1). Correct checkpoint activation and downstream signalling allow the cell to process aberrant structures and ensure faithful replication of the cellular genome.



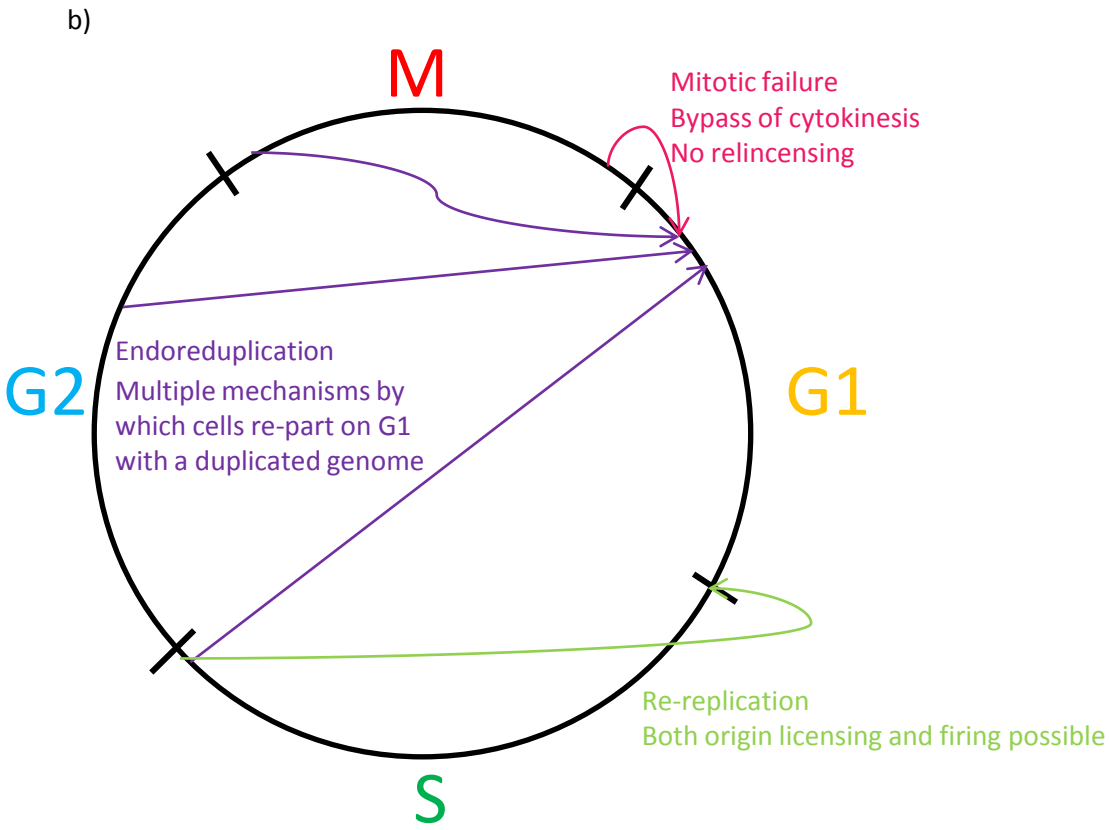


Figure 1.3.1 Many roads lead to polyploidy a) A multitude of cell-cycle events may lead to polyploidy, characterised by an increase in genome size b) endoreduplication, characterised by an exact doubling of genetic material may result from problems at varying stages of the cell cycle

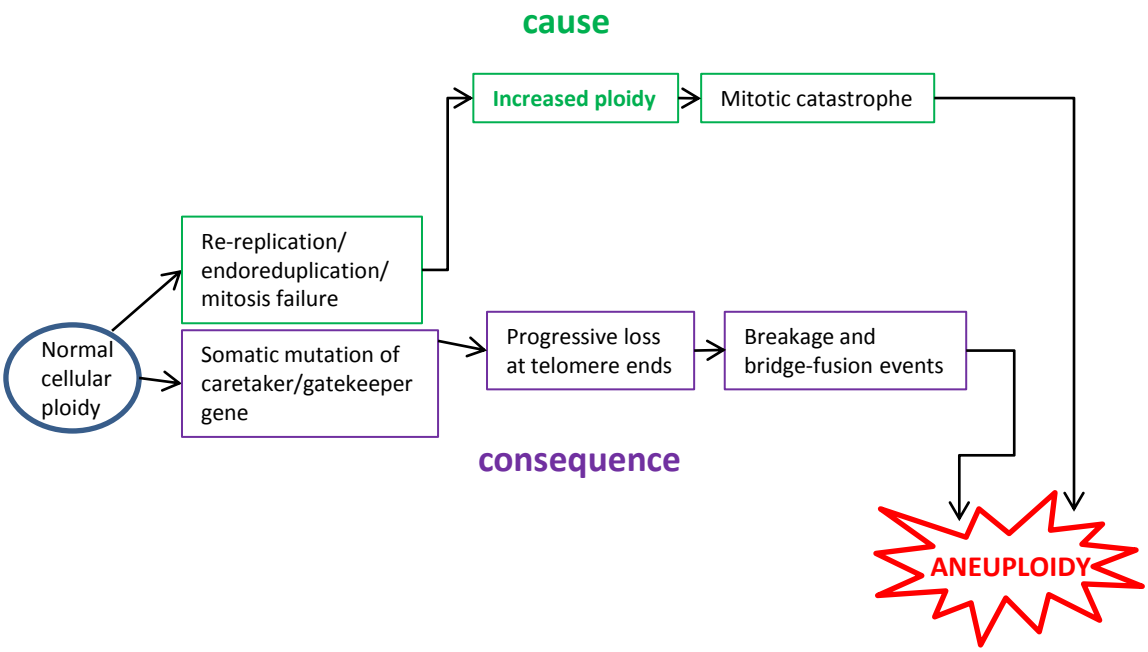


Figure 1.3.2 Aneuploidy, a hallmark of cancerous cells – cause or effect? Whilst aneuploidy is a well-established hallmark of cancer cells, its precise role in the evolution of a cancer remains controversial

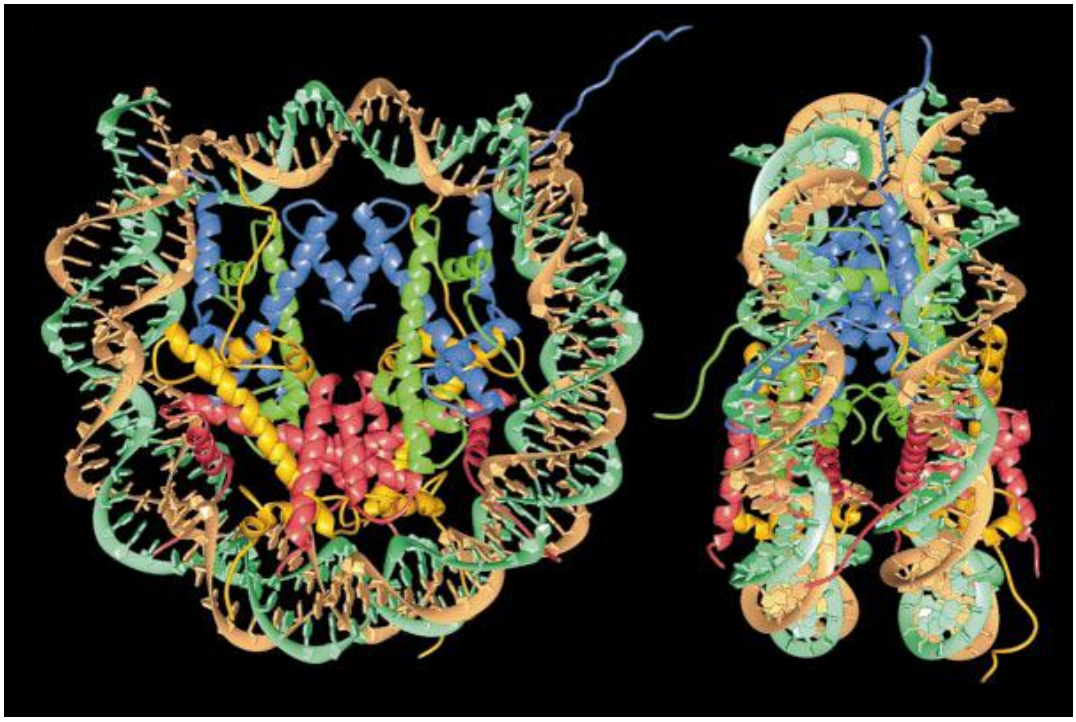


Figure 1.4.1 X-ray crystal structure of the nucleosome core particle (Luger at al., 1997). The nucleosome consists of eight histone proteins (2 each of H2A, H2B, H3 and H4, shown in yellow, red, blue and green, respectively) around which the DNA is wrapped. The nucleosome is viewed down the DNA superhelix axis on the left and perpendicular to it on the right. Figure taken from (Luger at al., 1997).

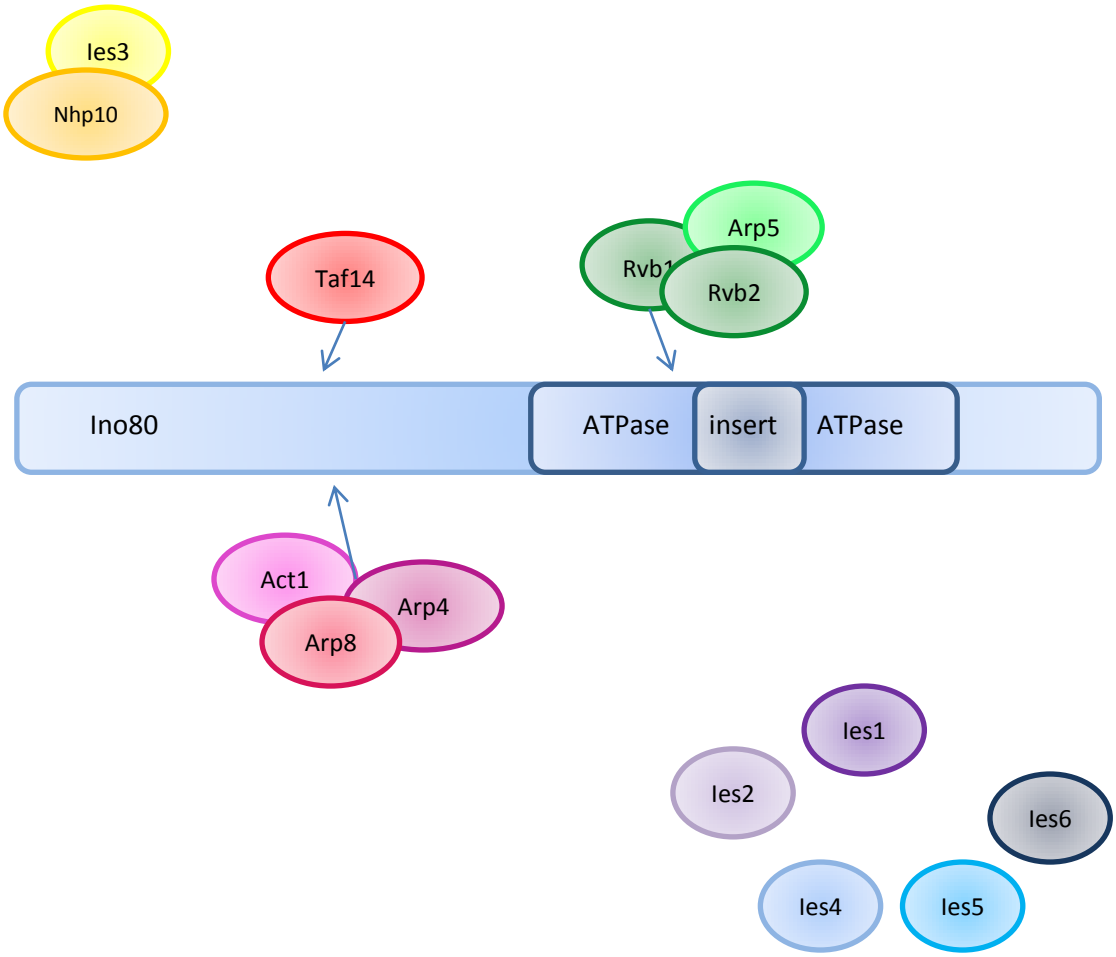


Figure 1.5.1 The Ino80 complex is a large, multi-protein-subunit chromatin remodelling complex Schematic representation of the composition of the *Saccharomyces cerevisiae* Ino80 complex, subunits shown overlapping have been identified as likely interacting subunits, arrows mark the likely interaction of these sub-complexes with the catalytic subunit where these have been established

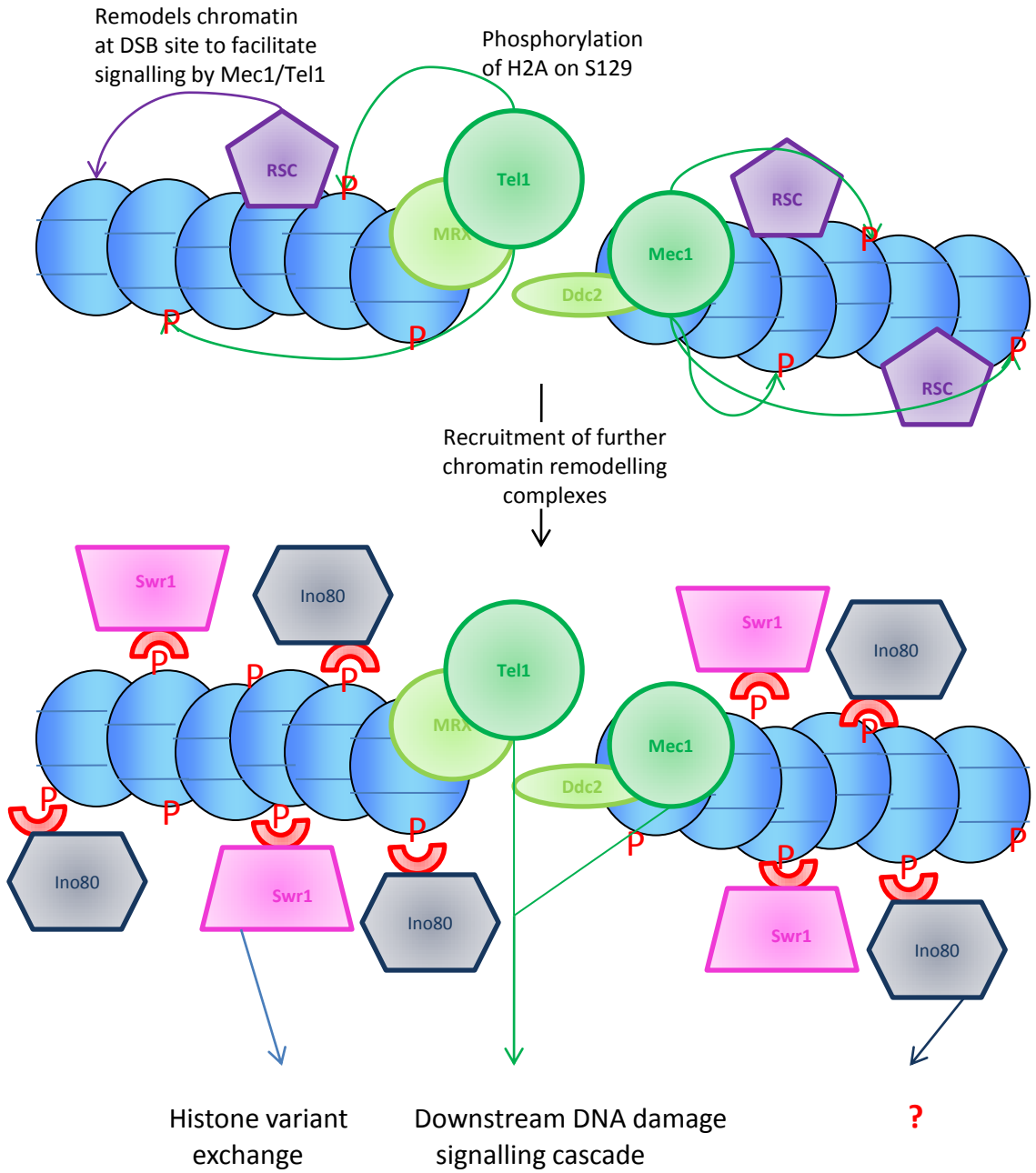


Figure 1.5.2 Chromatin remodellors at DSBs The processing of cellular DSBs involves concerted action from multiple chromatin remodellors, although their exact mechanisms of action often remain unclear

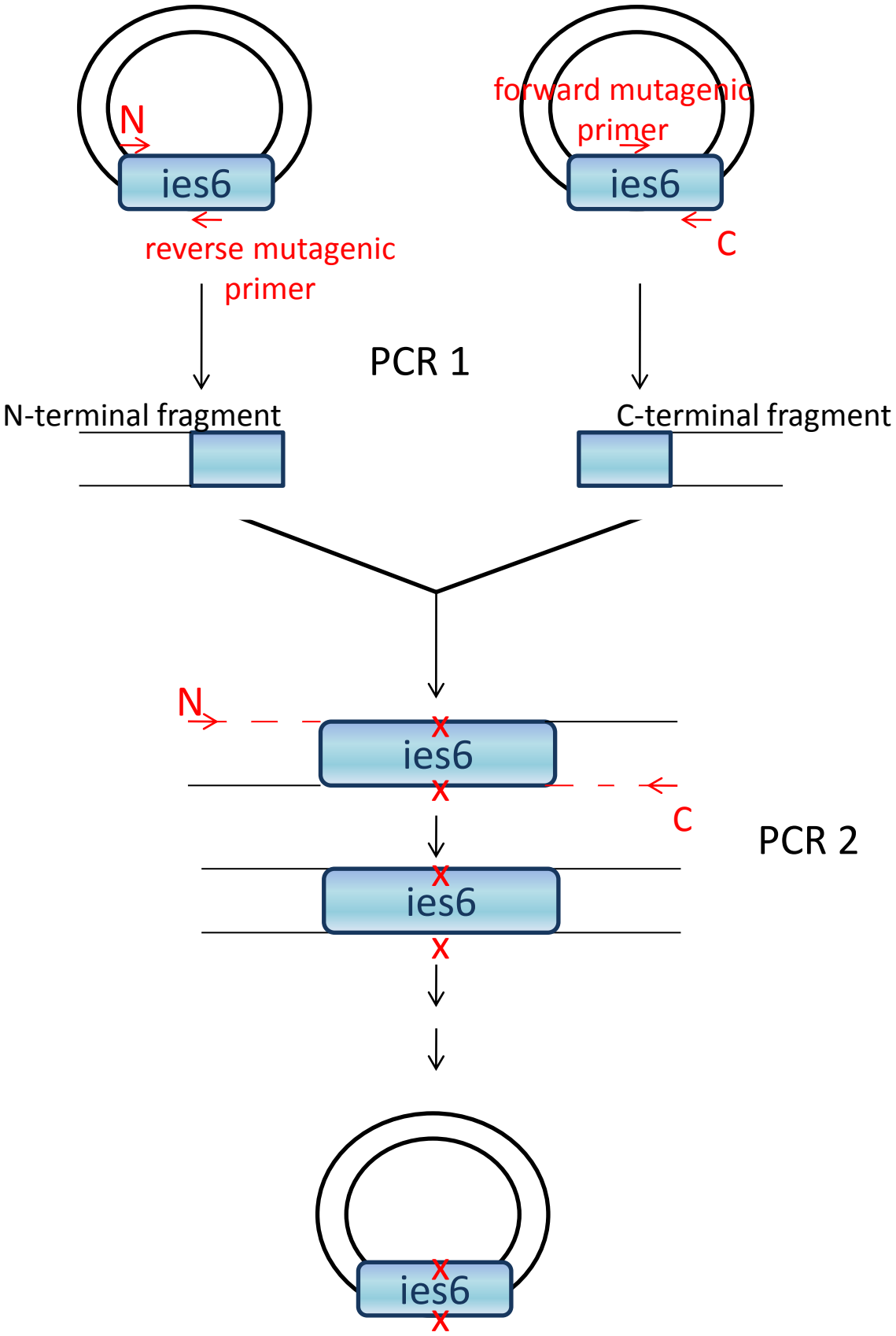


Figure 2.1.1 Cloning strategy for the generation of plasmids containing *ies6* point mutants by site-directed mutagenesis

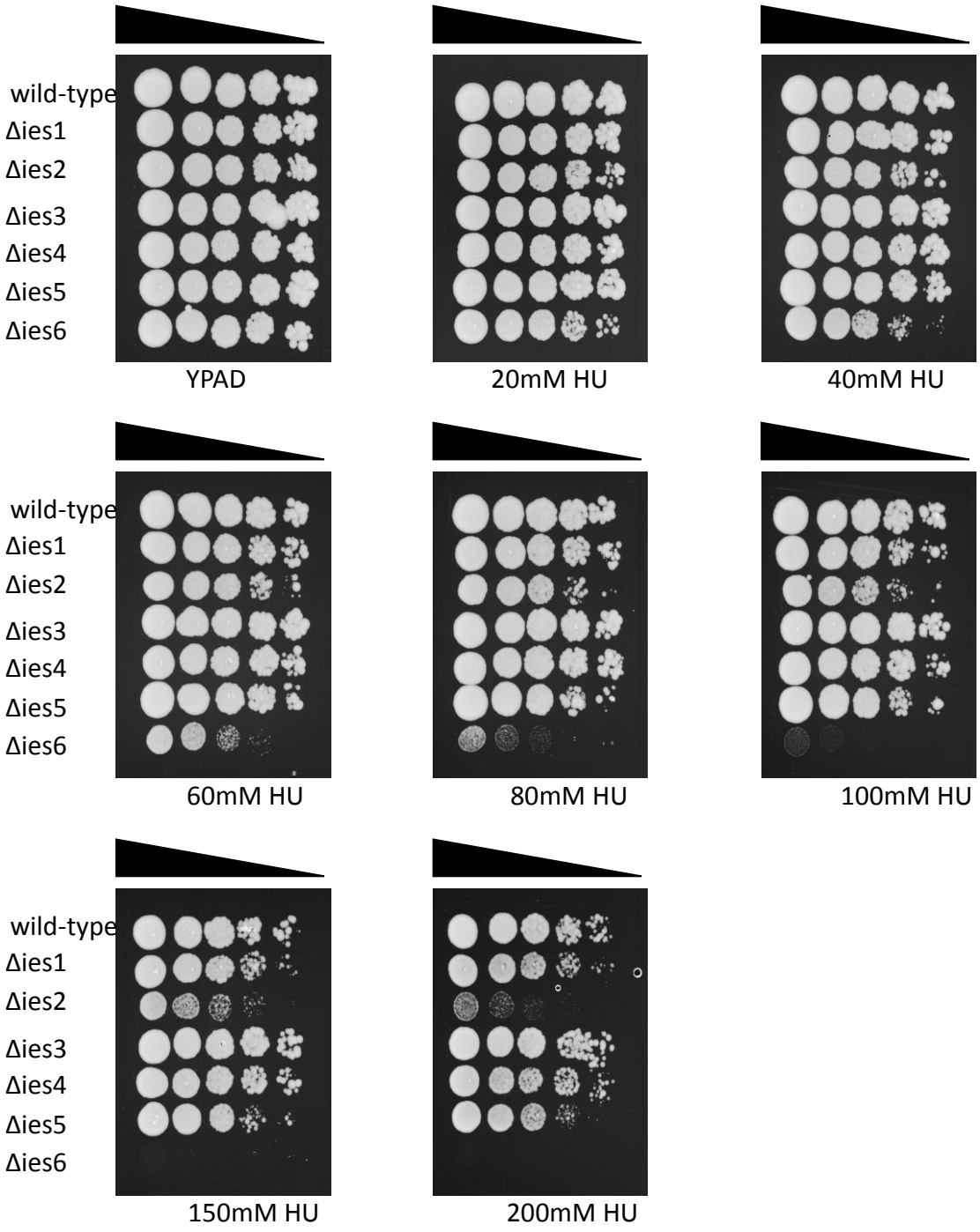


Figure 3.1 Absence of Ies6 renders cells hypersensitive to hyroxyurea compared to absence of other Ies subunits The wild-type yeast strain, or isogenic yeast strains harbouring a deletion of one of the genes encoding the ino-eighty-subunits 1-6 were analysed for their level of sensitivity to hydroxyurea (HU) by plating serial five-fold dilutions of mid-log yeast cultures onto rich media containing amounts of HU as indicated

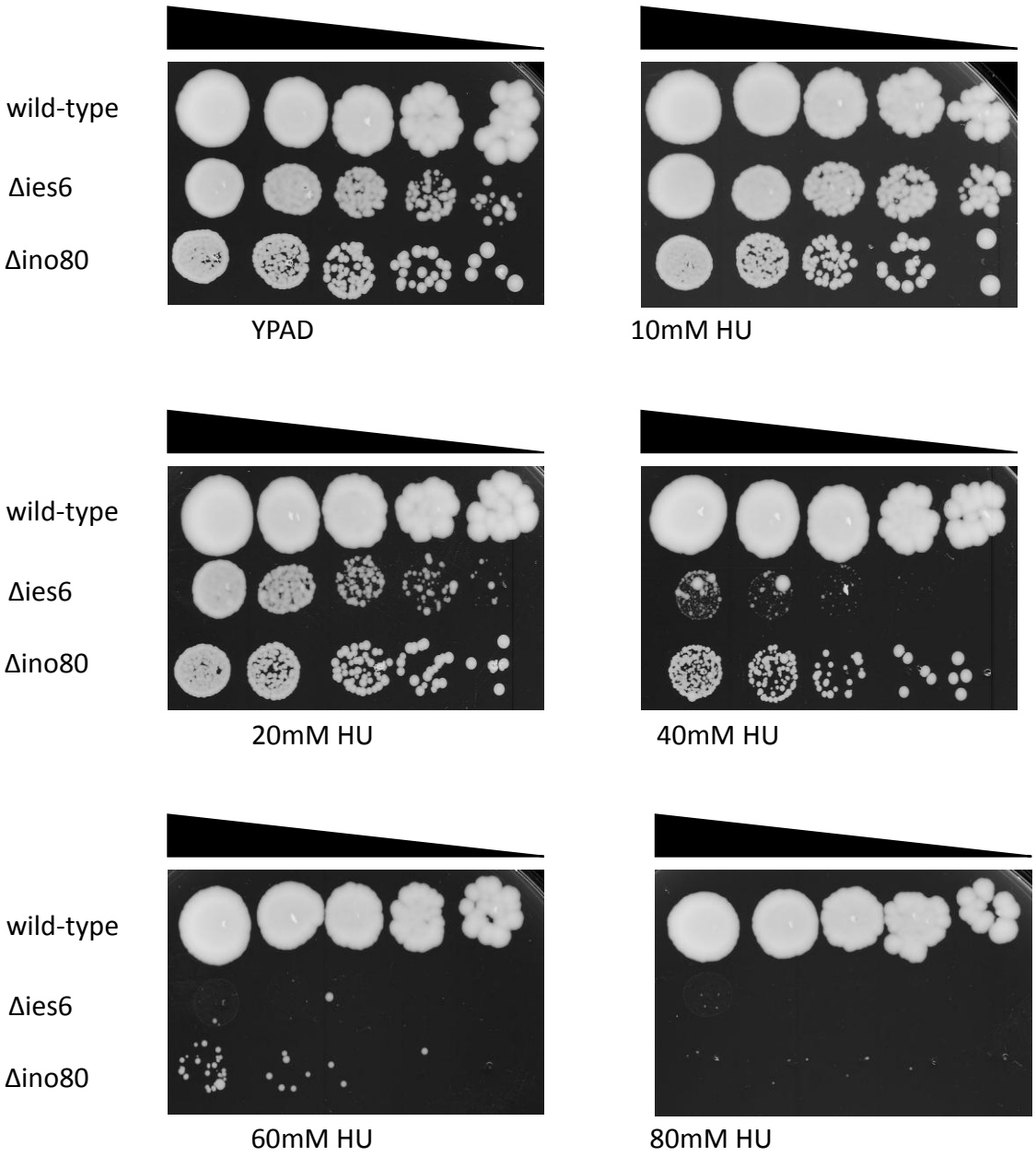


Figure 3.2 The HU-hypersensitivity in the absence of Ies6 is comparable to the HU-hypersensitivity in the absence of Ino80 The wild-type yeast strain, or isogenic yeast strains harbouring a deletion of *ies6* or *ino80* were analysed for their sensitivity to Hydroxyurea (HU) by plating serial five-fold dilutions of mid-log yeast cultures onto rich media containing of HU as indicated.

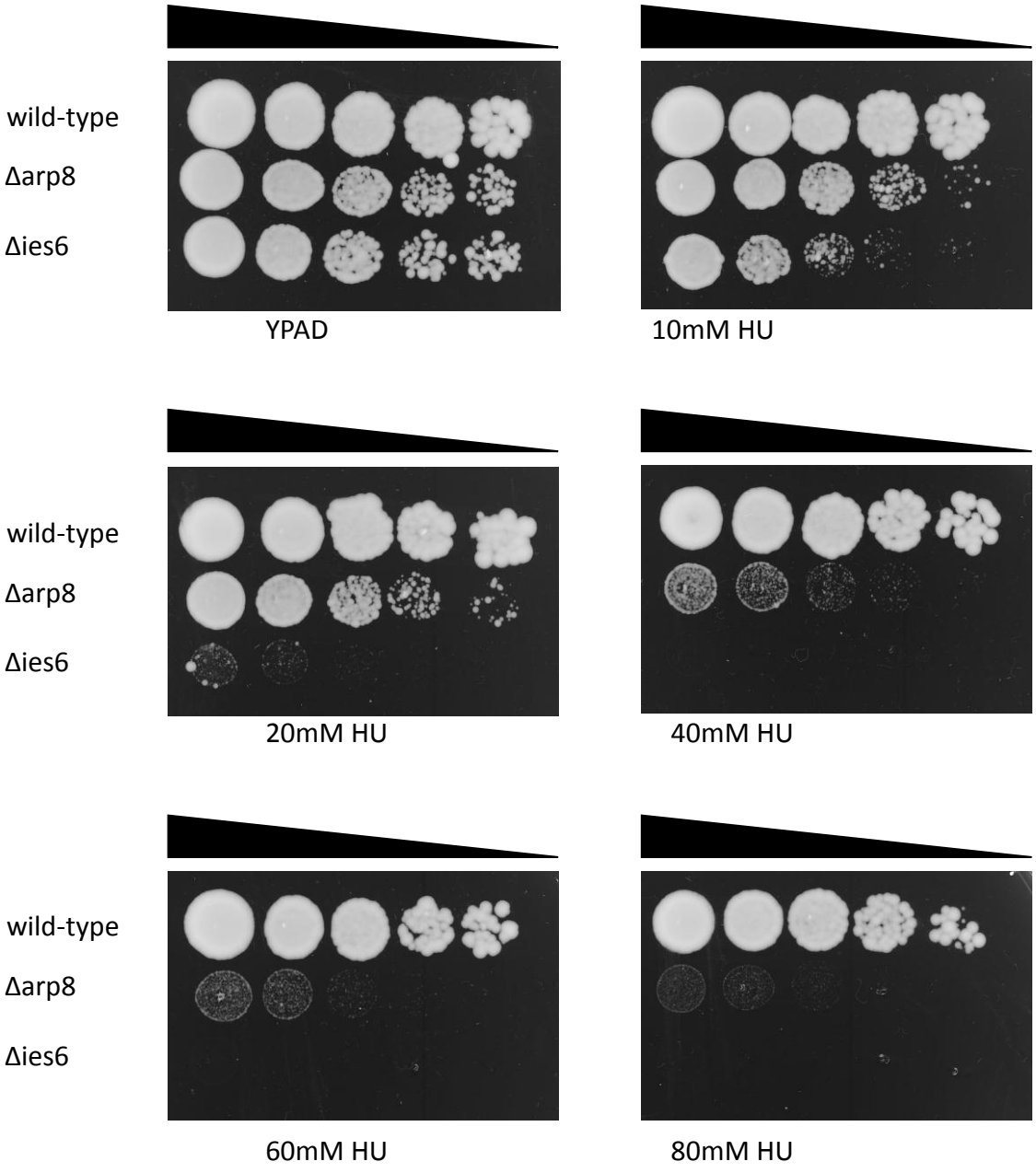


Figure 3.3 Absence of Ies6 and absence of Arp8 lead to a similar HU-hypersensitivity phenotype The wild-type yeast strain, or isogenic yeast strains harbouring a deletion of *ies6* or *arp8* were analysed for their sensitivity to Hydroxyurea (HU) by plating serial five-fold dilutions of mid-log yeast cultures onto rich media containing HU as indicated

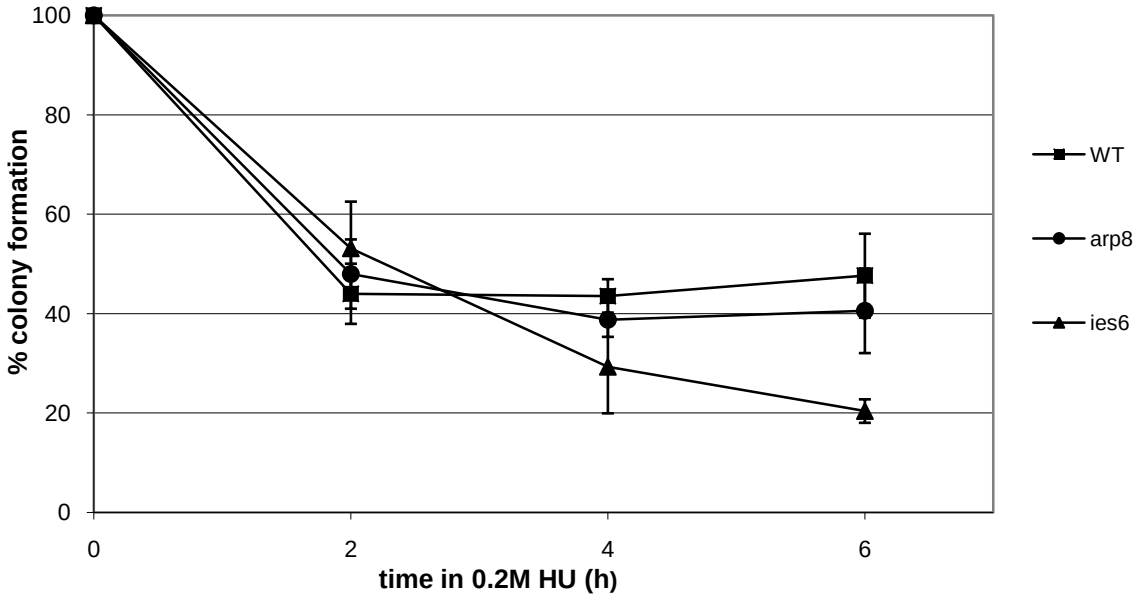
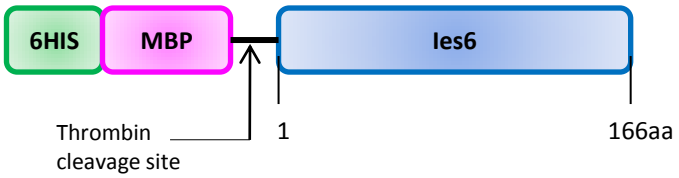
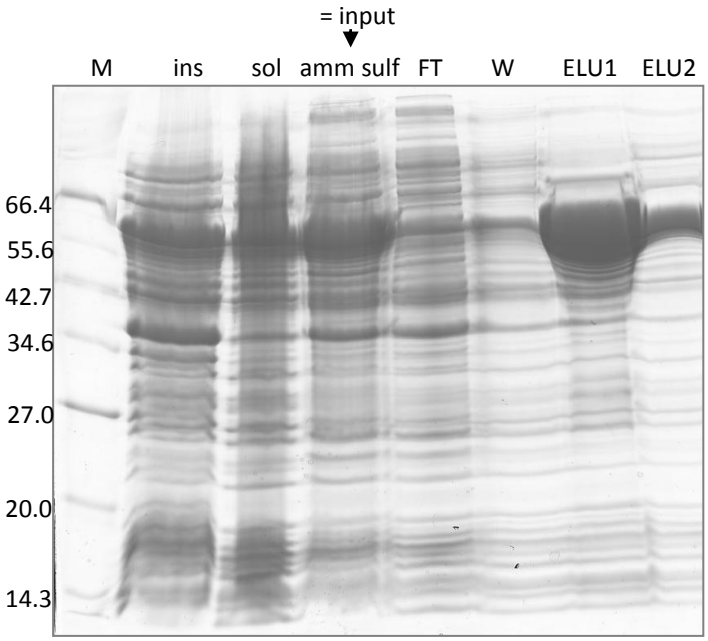


Figure 3.4 In the absence of Ies6 replication forks collapse following acute exposure to HU Wild-type or yeast strains harbouring a deletion of *IES6* or *ARP8* were arrested in G1 using α-factor. Following release into rich media or rich media containing 0.2M HU the strains were plated onto rich media at the time-points indicated above. The strains ability to recover was quantified by scoring their ability to form viable colonies as indicated.

a)



b)



c)

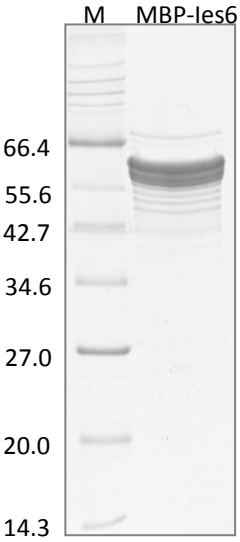


Figure 4.1 Purification of recombinant Ies6 a) Schematic of 6HIS-MBP-tagged Ies6 fusion protein (tag not to scale) b) Example purification of recombinant Ies6: 1/200th of each fraction (insoluble (ins), soluble (sol) ammonium sulfate (amm sulf), flow-through (FT), wash (W), elution 1 (ELU1) and elution 2 (ELU2)) was electrophoresed on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. c) 1/1000th elution 1 fraction (resolution as in b))

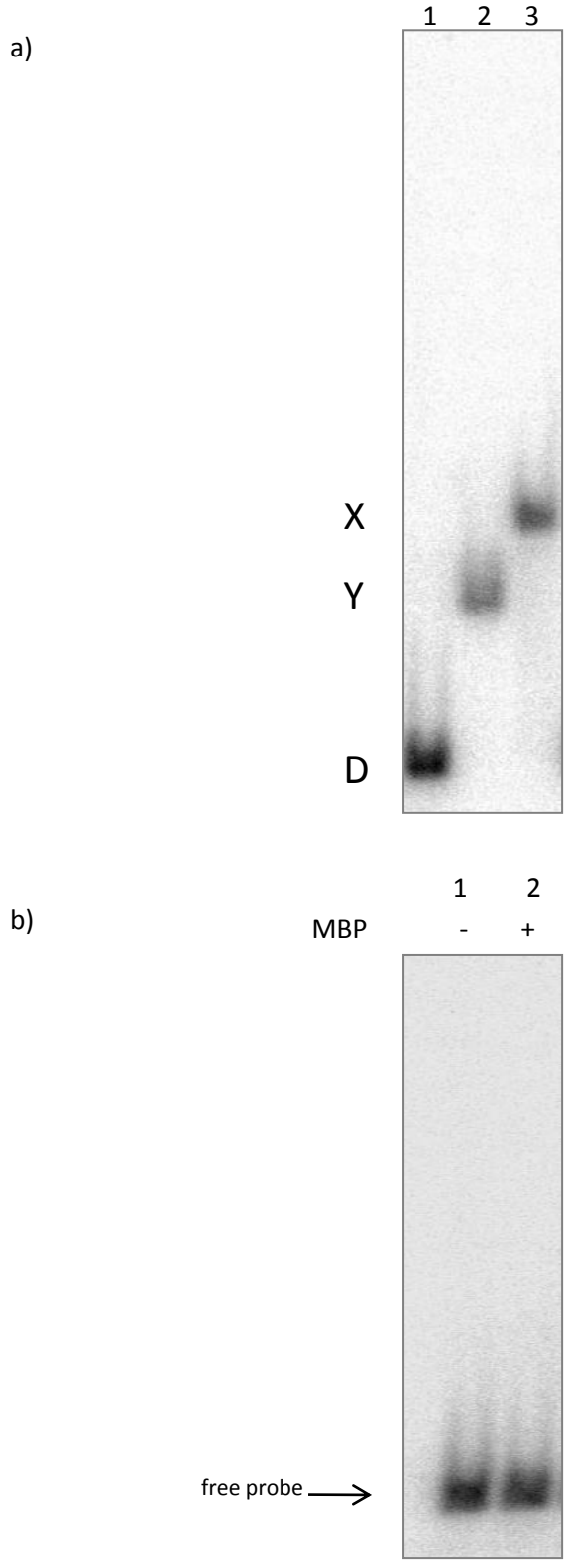


Figure 4.2 Preparation of *in vitro* DNA binding substrates to investigate the putative DNA binding activity of Ies6 a) Resolution of 12.5 fmol of each of the three radio-labelled DNA structures (linear duplex (D), Y-fork (Y) and Holliday Junction (X)) generated following a published protocol (Rass and West, 2006). b) Recombinant MBP does not possess intrinsic DNA binding activity: 12.5 fmol radio-labelled, linear duplex DNA was incubated at room temperature for 30 min with recombinant MBP added to a concentration of 2.33µM prior to resolution on a 4% non-denaturing TBE-polyacrylamide gel. Position of the free probe is indicated.

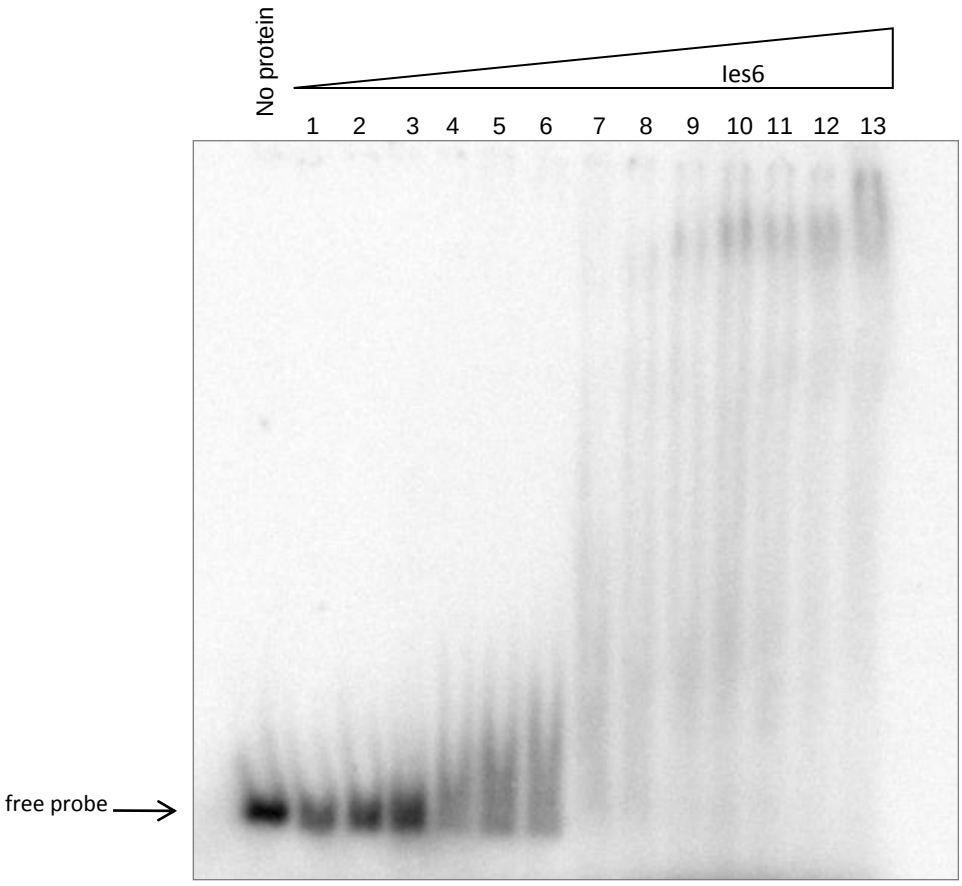


Figure 4.3 Recombinant Ies6 binds linear duplex DNA *in vitro* Recombinant, MBP-HIS-tagged Ies6 was incubated with 12.5 fmol DNA probe at room temperature for 30 min prior to resolution on a 4% non-denaturing TBE-polyacrylamide gel. Reactions 1-13 contained 160nM, 242nM, 322nM, 402nM, 483nM, 564nM, 644nM, 806nM, 967nM, 1.12µM, 1.29µM, 1.45µM, 1.61µM protein, respectively. The position of the free probe is indicated.

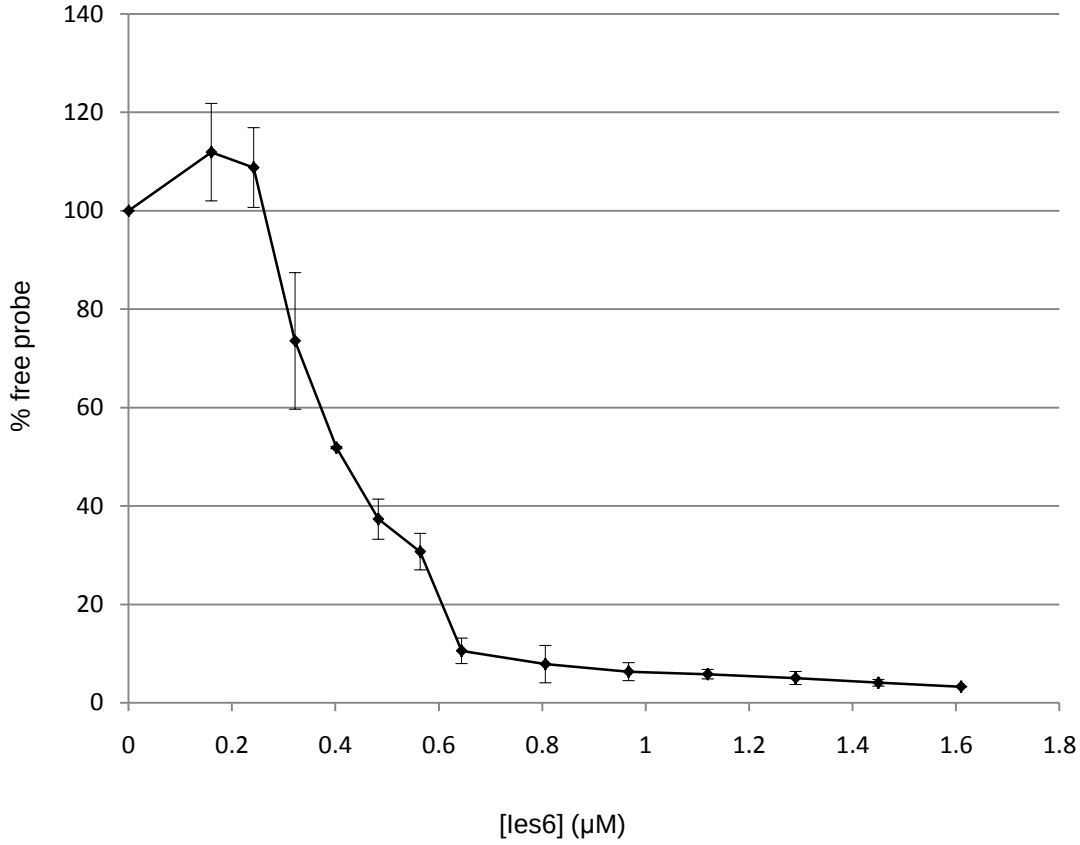


Figure 4.4 Quantification of the recombinant Ies6 protein's DNA binding activity to linear duplex DNA *in vitro* Quantification of the amount of unbound probe as a percentage of the total amount of radioactivity present in each lane, using the amount of unbound probe in the "no protein" control lane as 100% (see Figure 4.3 and Materials and Methods, 2.4) Error bars shown are standard error of the mean, with n = 2.

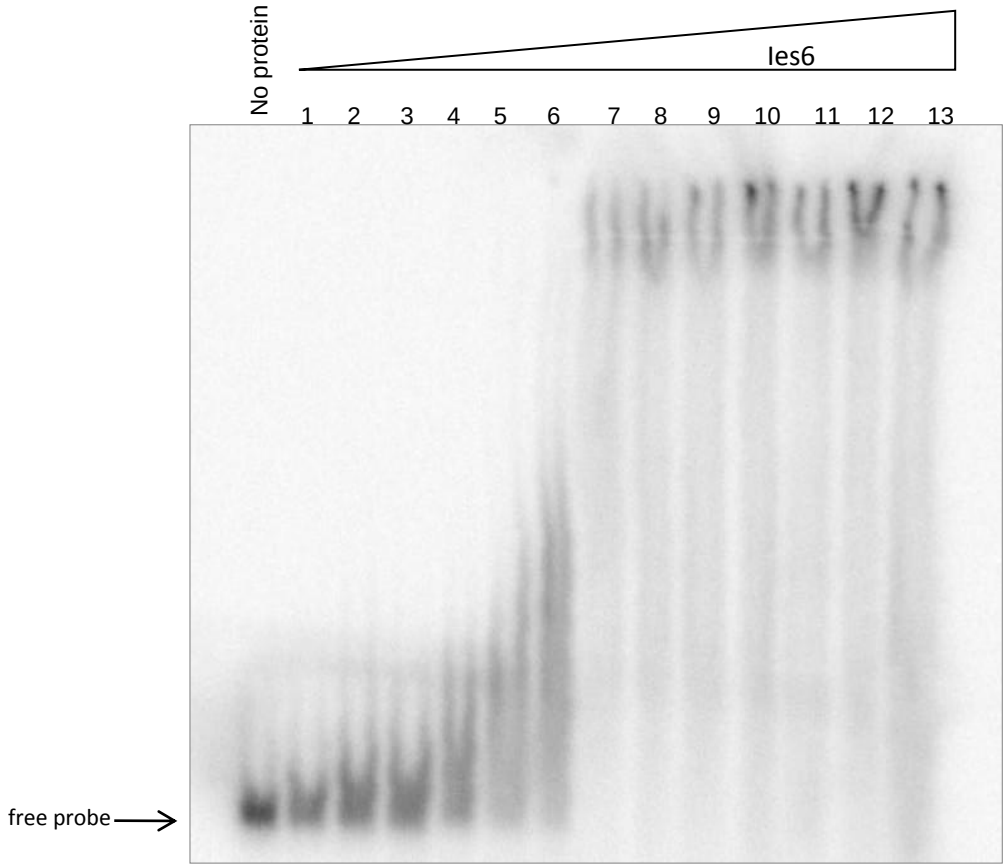


Figure 4.7 Recombinant Ies6 is able to bind Y-fork DNA *in vitro* Recombinant, MBP-HIS-tagged Ies6 was incubated with 12.5 fmol DNA probe at room temperature for 30 min prior to resolution on a 4% non-denaturing TBE-polyacrylamide gel. Reactions 1-13 contained 160nM, 242nM, 322nM, 402nM, 483nM, 564nM, 644nM, 806nM, 967nM, 1.12µM, 1.29µM, 1.45µM, 1.61µM protein, respectively. The position of the free probe is indicated.

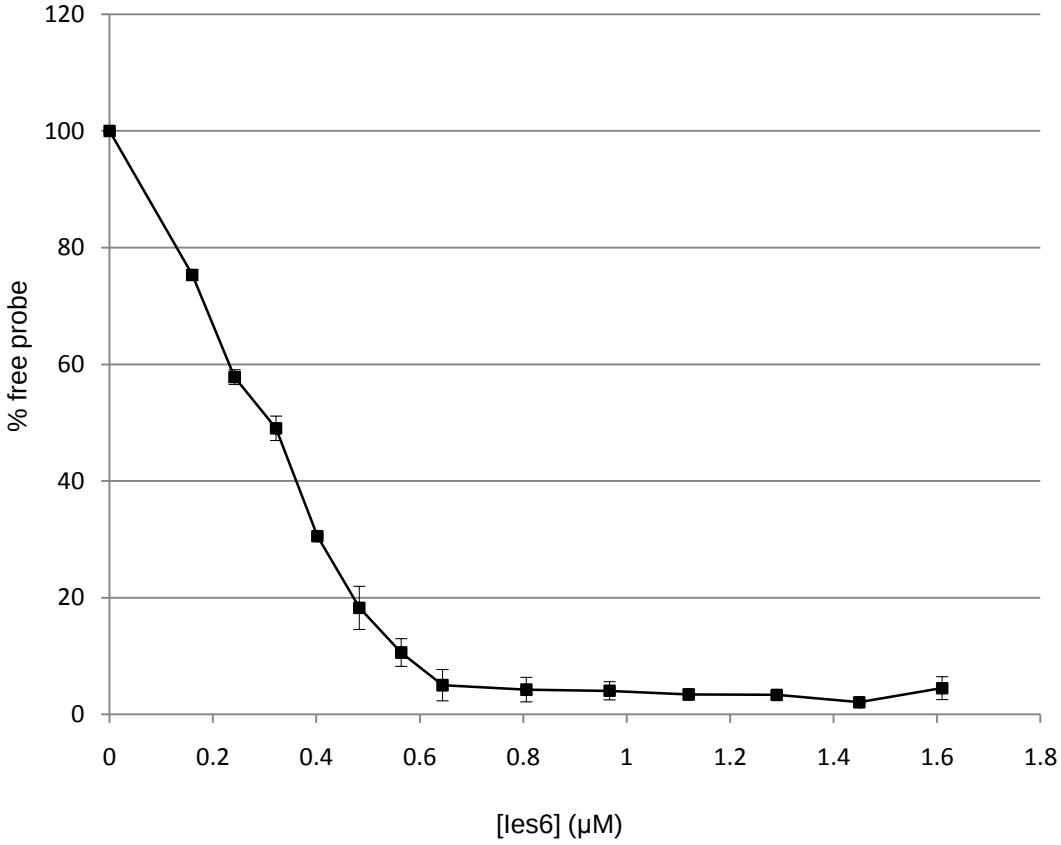


Figure 4.8 Quantification of the recombinant Ies6 protein's DNA binding activity to Y-fork DNA *in vitro* Quantification of the amount of unbound probe as a percentage of the total amount of radioactivity present in each lane, using the amount of unbound probe in the "no protein" control lane as 100% (see Figure 4.7 and Materials and Methods, 2.4) Error bars shown are standard error of the mean, with n = 2.

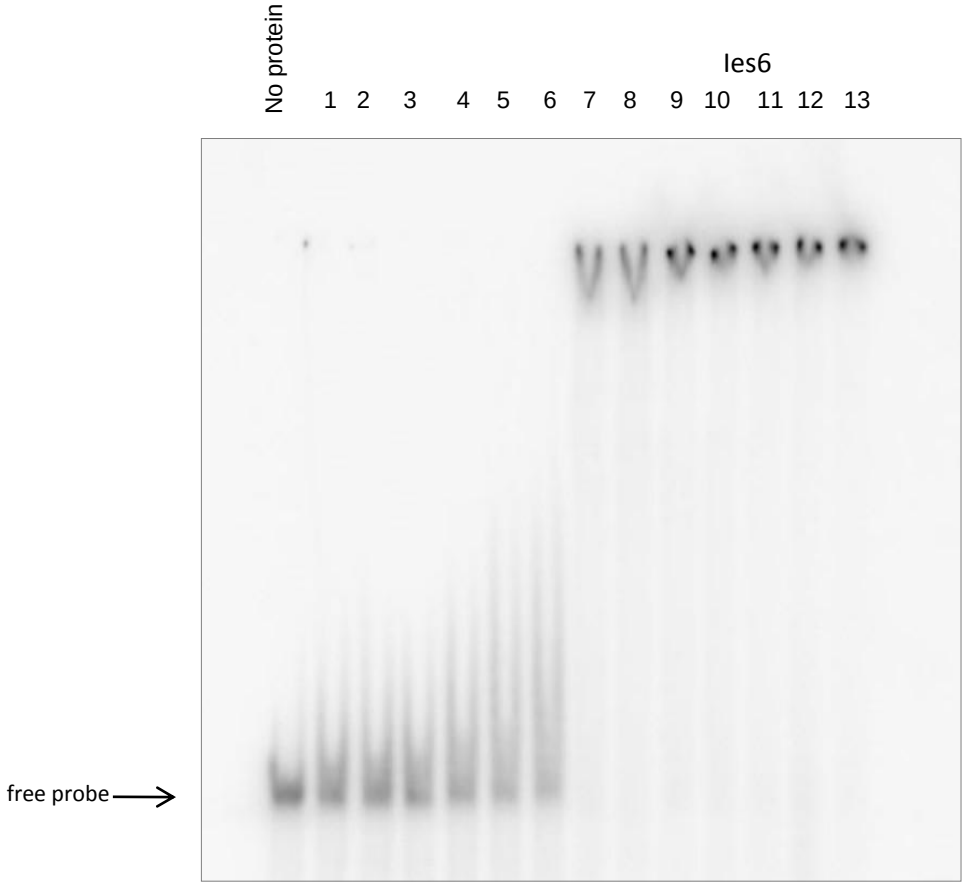


Figure 4.5 Recombinant Ies6 is capable of binding Holliday Junction DNA *in vitro* Ies6 was incubated with 12.5 fmol DNA probe at room temperature for 30 min prior to resolution on a 4% non-denaturing TBE-polyacrylamide gel. Reactions 1-13 contained 160nM, 242nM, 322nM, 402nM, 483nM, 564nM, 644nM, 806nM, 967nM, 1.12µM, 1.29µM, 1.45µM, 1.61µM protein, respectively. The position of the free probe is indicated.

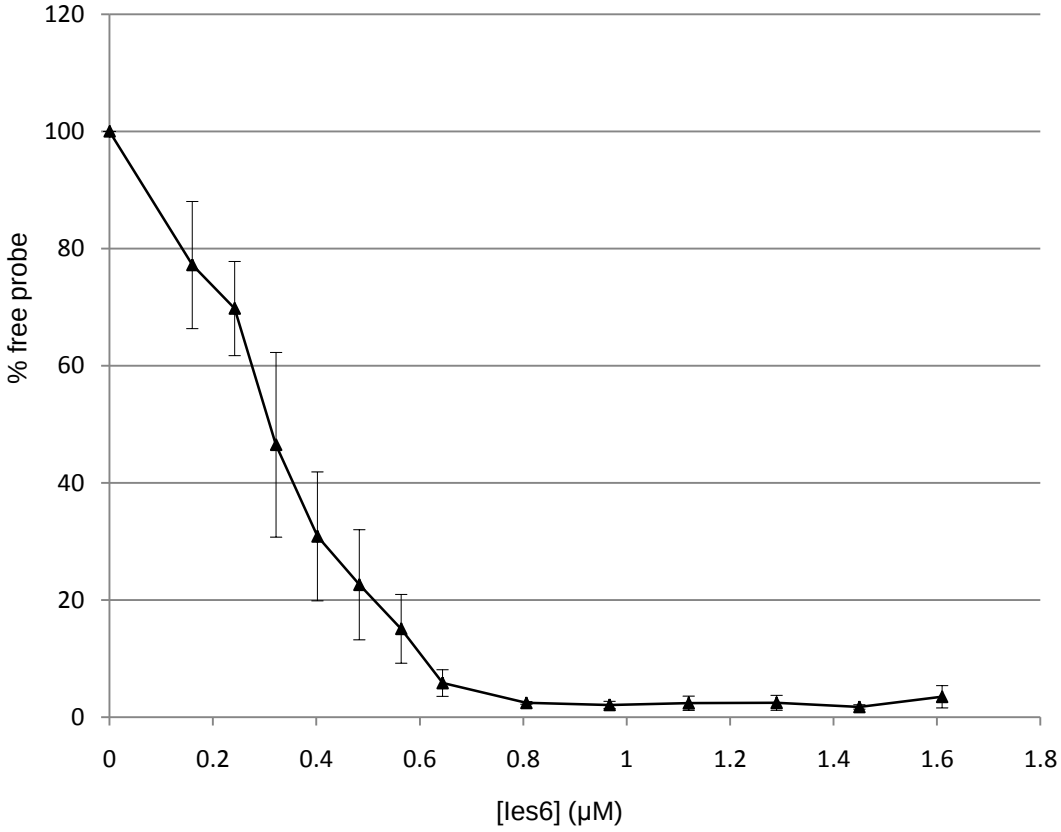


Figure 4.6 Quantification of the recombinant Ies6 protein's DNA binding activity to Holliday Junction DNA *in vitro* Quantification of the amount of unbound probe as a percentage of the total amount of radioactivity present in each lane, using the amount of unbound probe in the "no protein" control lane as 100% (see Figure 4.7 and Materials and Methods, 2.4) Error bars shown are standard error of the mean, with n = 3.

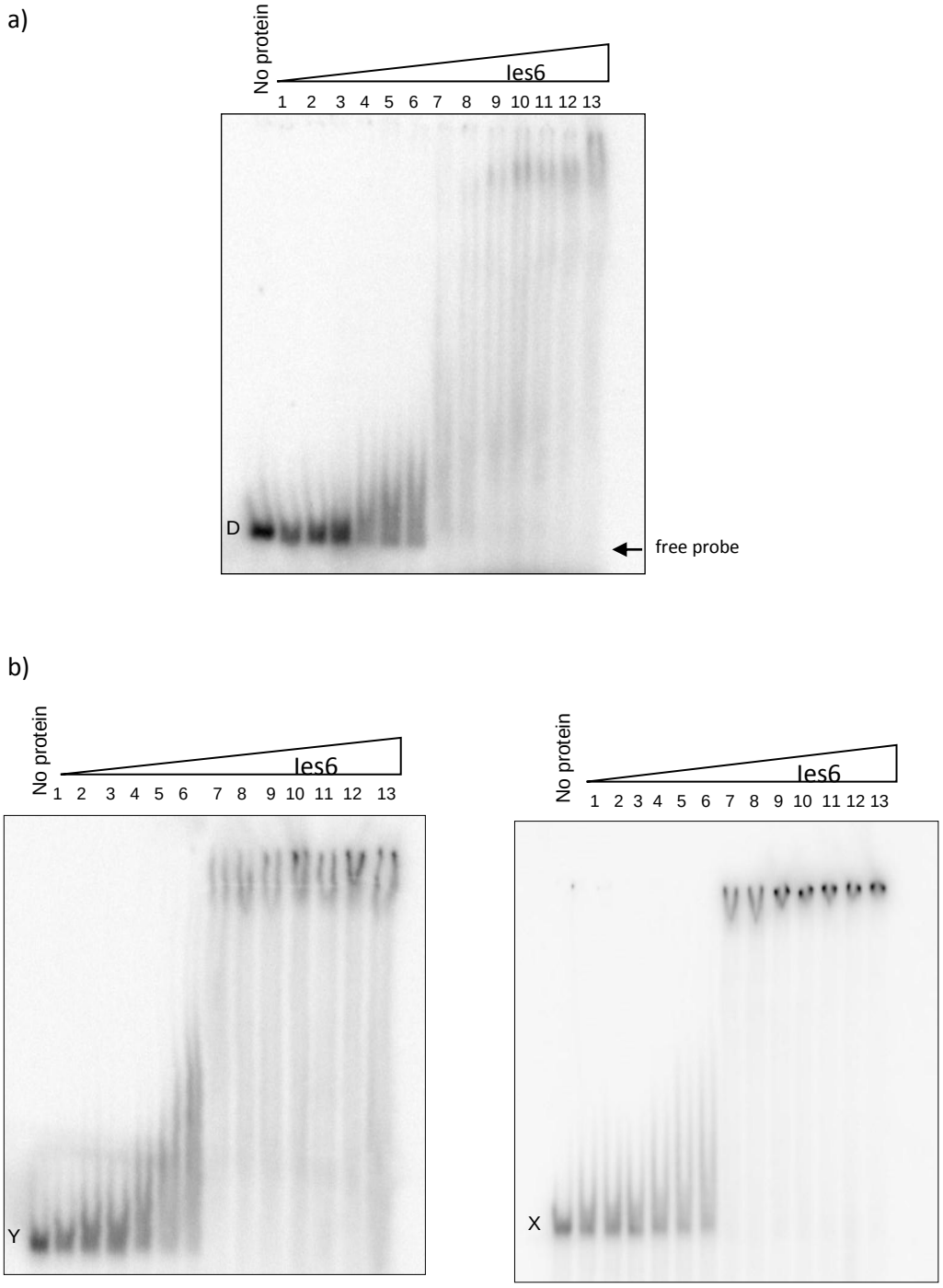


Figure 4.9 Comparison of the Ies6 protein's ability to bind linear DNA duplex, as well as branch-structured DNAs *in vitro* All DNA assays were performed as described above and protein concentrations are the same in the numbered lanes on all gels, respectively. The DNA substrates are radio-labelled linear DNA duplex (a, D), as well as branch-structured DNAs (b, the left panel shows Y-fork DNA (Y), the right panel shows Holliday Junction DNA (X)).

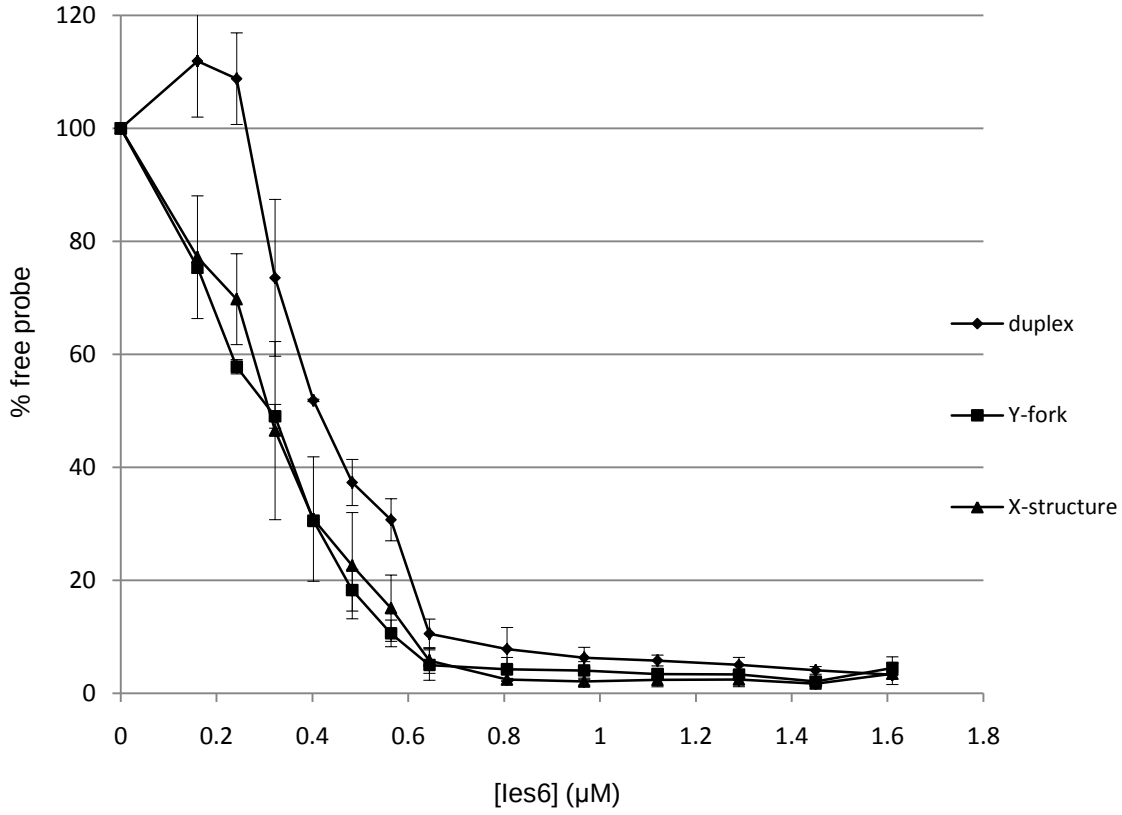


Figure 4.10 Comparison of the Ies6 protein's ability to bind linear DNA duplex, compared with branch-structured DNAs *in vitro* Quantification of the amount of unbound probe as a percentage of the total amount of radioactivity present in each lane, using the amount of unbound probe in the "no protein" control lane as 100% (see Figure 4.9 and Materials and Methods, 2.4). Error bars shown are standard error of the mean, with n = 2 for the linear duplex and Y-fork and n = 3 for the Holliday Junction

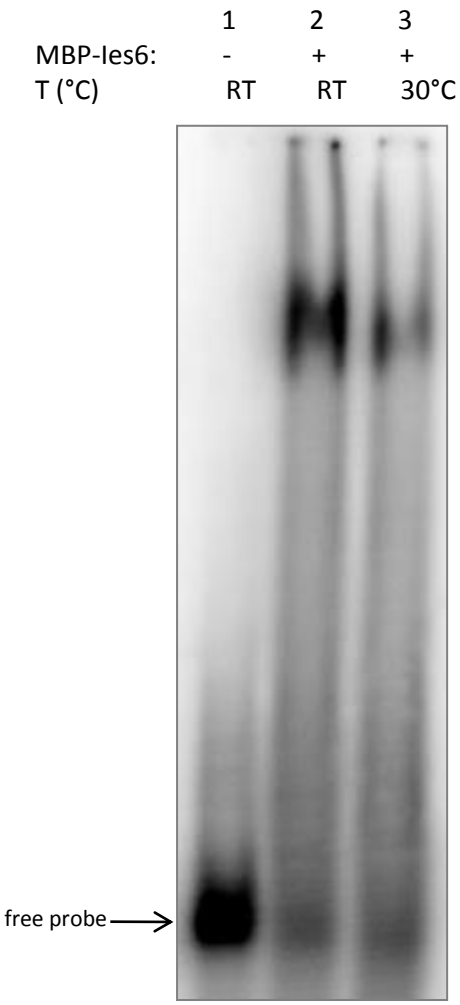


Figure 4.11 Recombinant Ies6 also displays single-stranded DNA binding activity 12.5 fmol ssDNA was incubated with 1.29µM protein for 30 min either at room temperature (RT, lane 2) or 30°C, as indicated prior to resolution on a 6% non-denaturing TBE-polyacrylamide gel. The position of the free, single-stranded probe is indicated.

a)

MSGSRGNSSNSSVSNNSNNNNNNDGGDERLLFLRSVGERNEIGFPSRFKSAHYKKPTRRHKSARQLI

SDENKRINALLTKANKAAESSTAARRLVPKATYFSVEAPPSIRPAKKYCDVTGLKGFYKSPTNNIRYHN

AEIYQLIVKPMAPGVDQEYLKLRGANFVLK

b)

```
C.albicans      ------------------------------MSNTPGRSMSSTPITTTTTTQVN------- 23
P.pastoris      -------------------------------------MSAP-----EKQPVD------- 10
S.cerevisiae    ------------------------------MSGSRGNSSNSSVSNNSNNNNNNDGGDERL 30
S.pombe         ------------------------------------------------MEKN------- 4
H.sapiens       MAAQIPIVATTSTPGIVRNSKKRPASPSHNGSSGGGYGASKKKKASASSFAQGISMEAMS 60
M.musculus      MAAQIPIVAATSTPAVARNSKKRPASPSHN-SSGGGYGASKKKKLSASGFAQGVSIEAMN 59
                                                                      .

C.albicans      --LHELSEITTKPHSFKQNPNRKQQSNRRYKPSRQLISDELK---YLQSKQ--------- 69
P.pastoris      --LYKLSEIAEGPHSFKQNPNRLRPPARRYKPARQVIGDEQK---YLQTK---------- 55
S.cerevisiae    LFLRSVGERNEIGFPSRFKSAHYKKPTRRHKSARQLISDENKRINALLTKANKAAESSTA 90
S.pombe         ---SSVDSLDISLLARPFRNPNYKAQPRRNRNLRQIIQNDPVQ----------------- 44
H.sapiens       ENKMVPSEFSTGPVEKAAKPLPFKDPNFVHSGHGGAVAGKKNRTWKNLKQILASER---A 117
M.musculus      ESKMASSELSSGPVEKAAKPLPFKDPNFVHSGHGGAVAGKKNRTWKNLKQILAAER---A 116
                      ..          .    :            : ..

C.albicans      SNLKFDTPTYNSIMSPPSLKPTMKYCDITGLPTNYKCPSNQLRFYNSEIYQEVIKNMPAG 129
P.pastoris      ENIKFDTPTWFSVAAPPSLVPQKHYCDITGLRGKYKNPANSLRFHNVEIYQEIIKNMPPG 115
S.cerevisiae    ARRLVPKATYFSVEAPPSIRPAKKYCDVTGLKGFYKSPTNNIRYHNAEIYQLIVKPMAPG 150
S.pombe         --NEPSKFSYSSIEAPPSVLPQPKYCDVTGLLAIYTDPKTRLRYHNKEIYG-LIRELPSG 101
H.sapiens       LPWQLNDPNYFSIDAPPSFKPAKKYSDVSGLLANYTDPQSKLRFSTIEEFS-YIRRLPSD 176
M.musculus      LPWQLNDPNYFSIDAPPSFKPAKKYSDISGLLANYTDPQSKLRFSTVEEFS-YIRRLPSD 175
                       .: *: :***. *  :*.*::**   *. * . :*: . * :   :: :...
                                          ↑  ↑    ↑↑ ↑

C.albicans      VDQEYLQLRGANVILK 145
P.pastoris      VDQEYLELRGANVILK 131
S.cerevisiae    VDQEYLKLRGANFVLK 166
S.pombe         ADQEYLKLRSSDVVLK 117
H.sapiens       VVTGYLALRKATSIVP 192
M.Musculus      VVTGYLALRKATSIVP 191
                .   ** ** :  ::
```

Figure 5.1 Sequence, mutagenesis and alignment of the budding yeast Ies6 protein a)Sequence of budding yeast Ies6 protein (from Saccharomyces Genome Database), the protein's YL1 domain is highlighted in blue, residues for site-directed mutagenesis are shown in red b) CLUSTAL 2.0.12 multiple protein alignment (UniProt) of the Ies6 protein from funghi (P32617(S.c.), Q9UTE8 (S.p.), Q5A1D0 (C.a.), A3GF12 (P.p.)), mouse (Q8BHA0) and human (Q6PI98)

a)

MSGSRGNSSNSSVSNNSNNNNNNDGGDERL
LFLRSVGERNEIGFPSRFKSAHYKKPTRRHKSAR
QLISDENKRINALLTKANKAAESSTAARRLVPKA
TYFSVEAPPSIRPAKKYCDVTGLKGFYKSPTNNIR
YHNAEIYQLIVKPMAPGVDQEYLKLRGANFVLK

b)

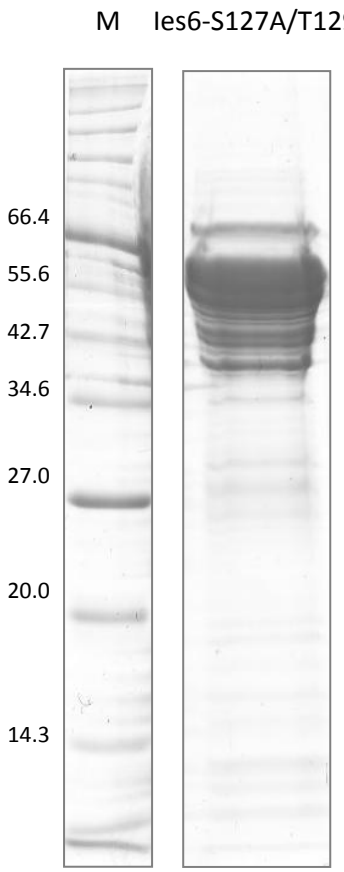


Figure 5.2 Generation of a recombinant, Ies6-S127A/T129A a)Ies6-S127A/T129A mutant sequence, the protein's YL1 domain is shown in blue, mutated residues are highlighted in red b) purified recombinant Ies6-S127A/T129A was resolved in a 12% SDS-polyacrylamide gel and stained with Coomassie blue.

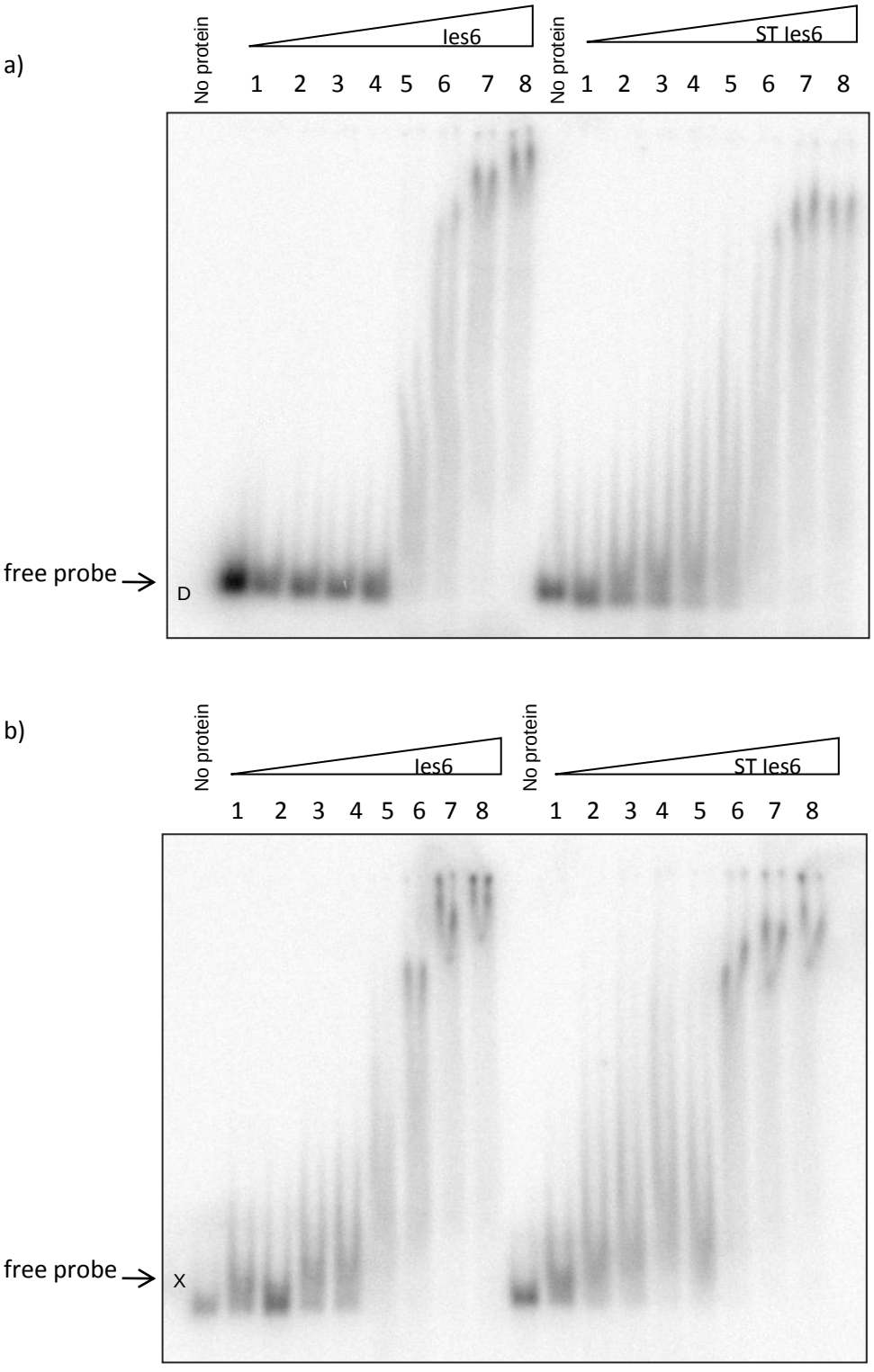


Figure 5.3 The recombinant, Ies6-S127A/T129A mutant displays normal DNA binding activity The above DNA binding assays were performed as previously, using 12.5 fmol radio-labelled linear duplex DNA (a) or Holliday Junction DNA (b) Recombinant wild-type or S127A T129 Ies6 were used at concentrations of242nM, 402nM, 483nM, 564nM, 644nM, 967nM, 1.29µM & 1.61µM of each protein as indicated in lanes 1-8, respectively. Following incubation at room temperature for 30 min, complexes were resolved on a non-denaturing 4% TBE-polyacrylamide gel.

a)

MSGSRGNSSNSSVSNNSNNNNNNDGGDERLLF
LRSVGERNEIGFPSRFKSAHYKKPTRRHKSARQLI
SDENKRINALLTKANKAAESSTAARRLVPKATYFS
VEAPPSIRPAKKYCDVTGLKGFYKSPTNNIRYHNA
EIYQLIVKPMAPGVDQEYLKLRGANFVLK

b)

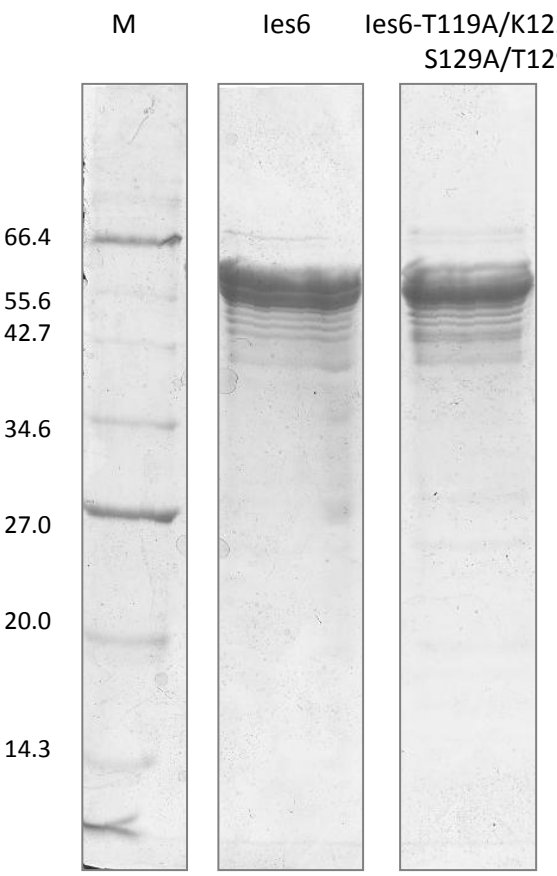
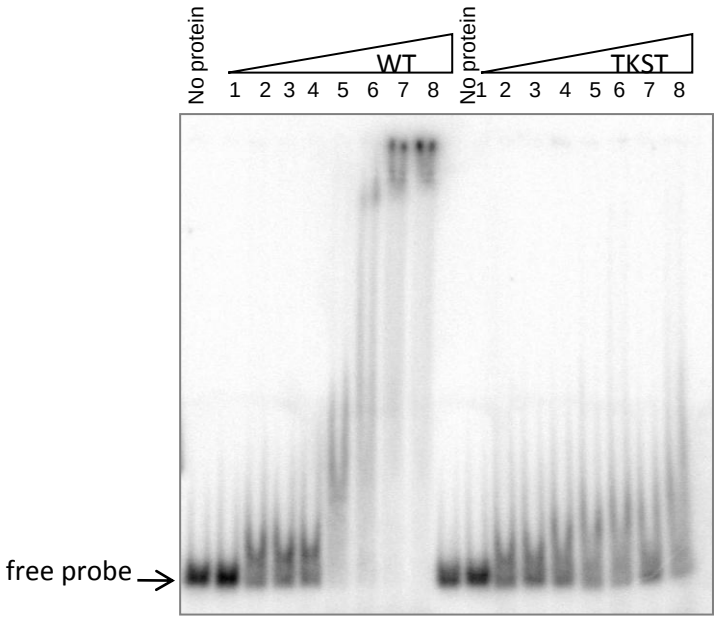


Figure 5.4 Generation of a recombinant Ies6-T119A/K122A/S127A/T129A a)Ies6-T119A/K122A/S127A/T129A mutant sequence, the protein's YL1 domain is shown in blue, mutated residues are highlighted in red b) purified recombinant Ies6-T119A/K122A/S127A/T129A was resolved in a 12% SDS-polyacrylamide gel and stained with Coomassie blue.

a)



b)

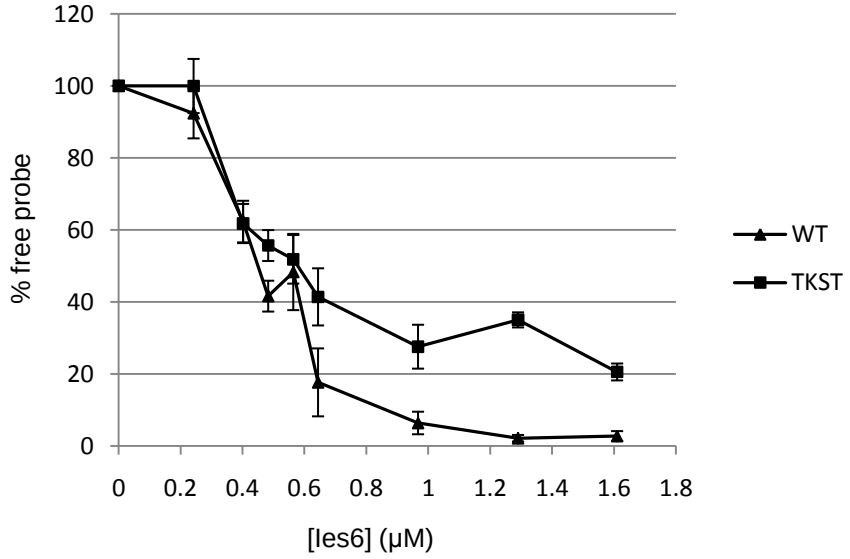


Figure 5.5 The Ies6-T119A/K122A/S127A/T129A mutant displays reduced DNA binding activity to the wild-type protein on linear, duplex DNA DNA binding assays were performed as previously. Protein concentrations are 242nM, 402nM, 483nM, 564nM, 644nM, 967nM, 1.29μM & 1.61μM for each protein in lanes 1-8, respectively. Quantification was performed as in Chapter 4. Error bars are standard error of the mean, with n = 3.

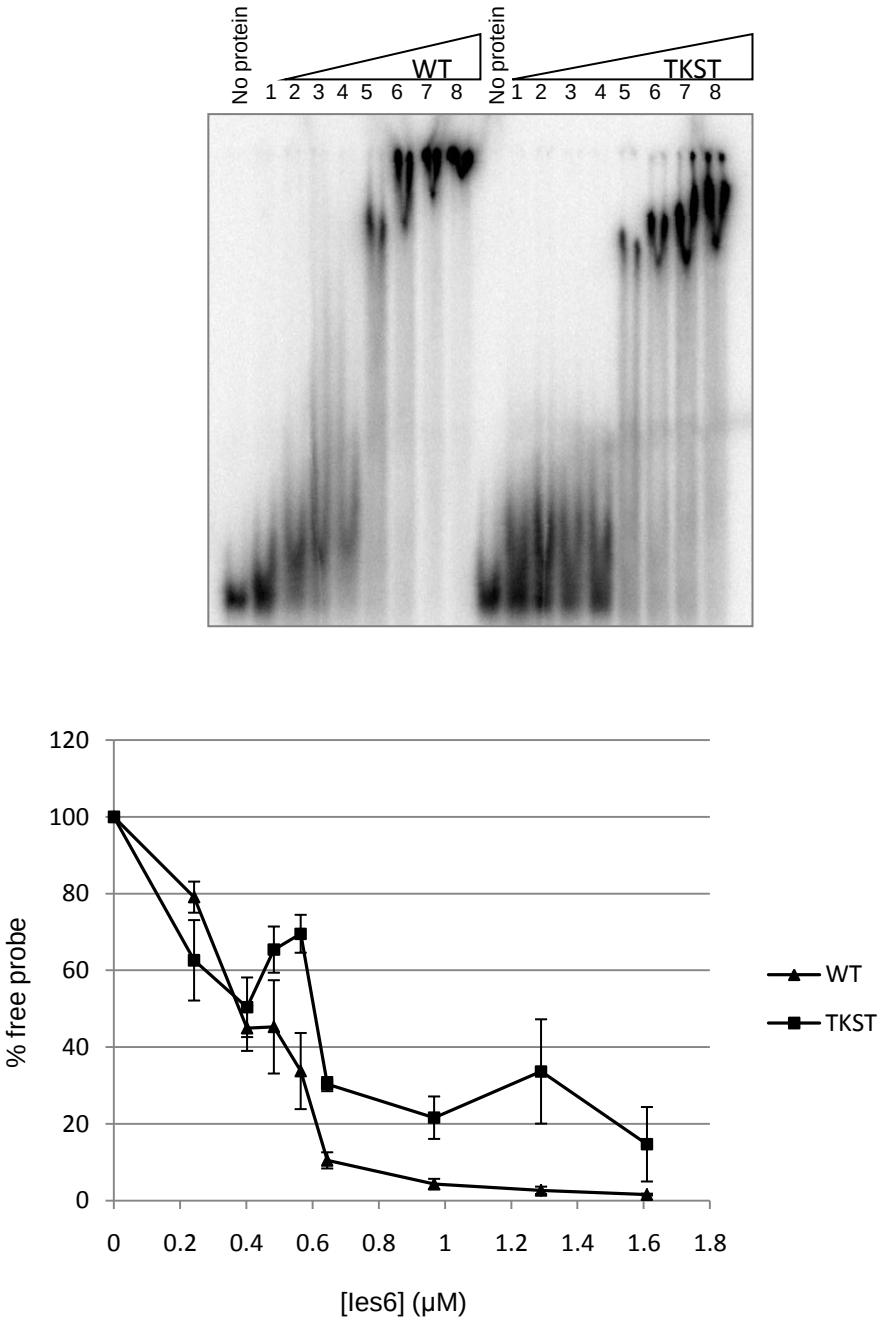


Figure 5.6 The Ies6-T119A/K122A/S127A/T129A mutant displays reduced DNA binding activity compared to the wild-type protein on Holliday Junction DNA DNA binding assays were performed as previously. Protein concentrations are 242nM, 402nM, 483nM, 564nM, 644nM, 967nM, 1.29µM & 1.61µM for each protein in lanes 1-8, respectively. Quantification was performed as in Chapter 4. Error bars are standard error of the mean, with n = 3.

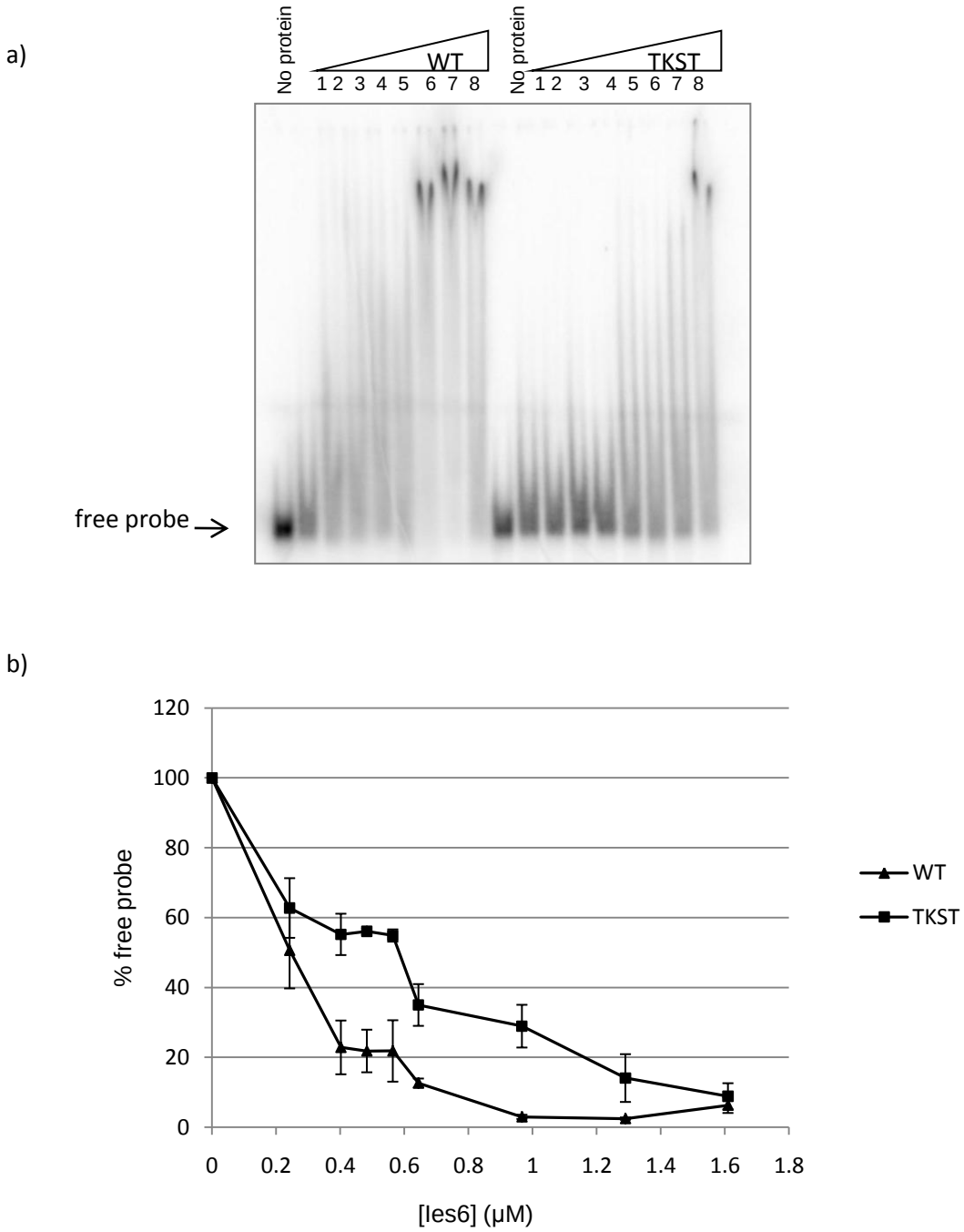
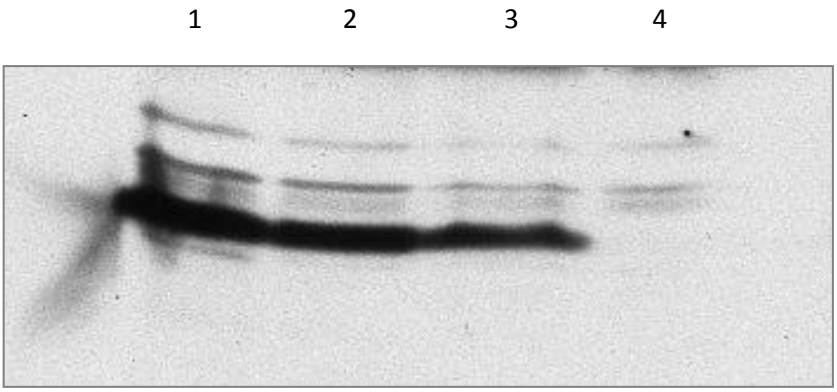


Figure 5.7 The Ies6-T119A/K122A/S127A/T129A mutant displays reduced DNA binding activity compared to the wild-type protein on Y-fork DNA DNA binding assays were performed as previously. Protein concentrations are 242nM, 402nM, 483nM, 564nM, 644nM, 967nM, 1.29μM & 1.61μM for each protein in lanes 1-8, respectively. Quantification was performed as in Chapter 4. Error bars are standard error of the mean, with n = 3.

a)



b)

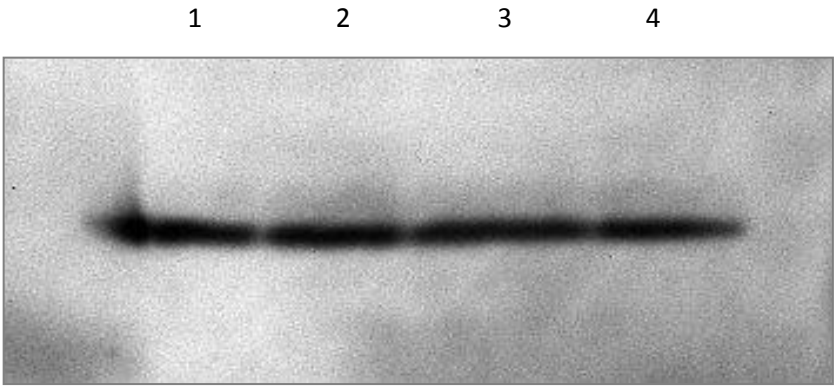


Figure 5.8 Analysis of protein expression levels of the Flag-tagged complementation plasmids for wild-type, S127A T129A and the quadruple mutant T119A K122A S127A T129A Yeast cultures of the *ies6* deletion strain harbouring the FLAG-tagged wild-type (lane 1), *ies6*-S127A/T129A (lane 2) , *ies6*-T119A/K122A/S127A/T129A (lane 3) or the empty complementation vector (lane 4) were grown to mid-log phase in selective media to maintain plasmid selection. Protein extraction was performed prior to resolution on a 15% SDS-PAGE polyacrylamide gel and Western blot analysis using an antibody to the FLAG-tag (a), as well as H2A as a loading control (b) was performed.

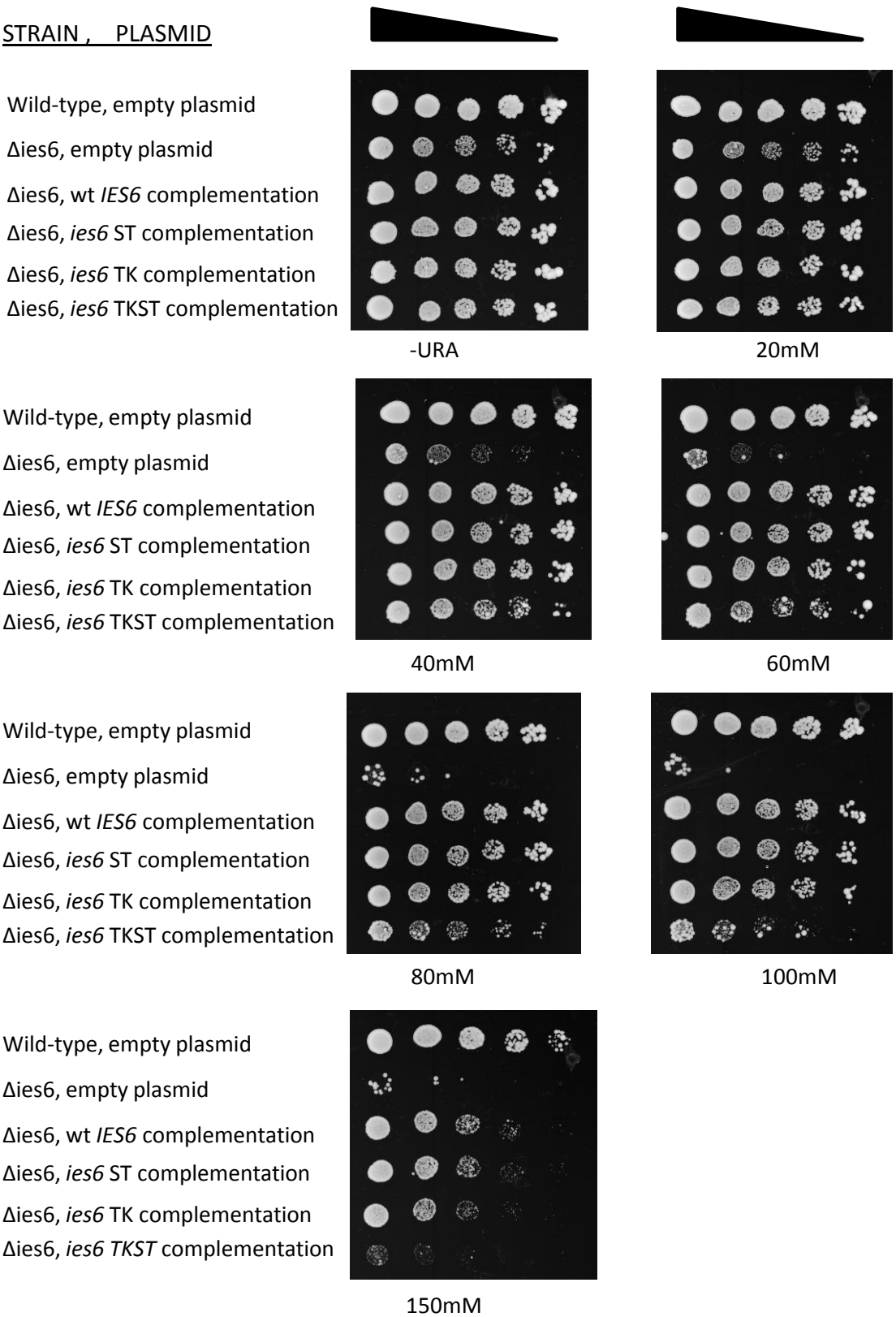


Figure 5.9 The Ies6-T119A/K122A/S127A/T129A DNA binding mutant fails to rescue the HU-hypersensitivity of the ies6-deletion strain The wild-type yeast strain, or yeast strains harbouring a deletion of *IES6*, containing either empty vector (pRS416) or a complementation vector (pRS416 containing either wild-type (wt), *ies6*-S127A/T129A (ST), *ies6*-T119A/K122A (TK) or *ies6*-T119A/K122A/S127A/T129A (TKST) under the control of its endogenous promoter and terminator) were analysed for their level of sensitivity to hydroxyurea (HU) by plating serial five-fold dilutions of yeast cultures onto media lacking uracil (-URA) for maintenance of plasmid selection, containing increasing amounts of HU as indicated.

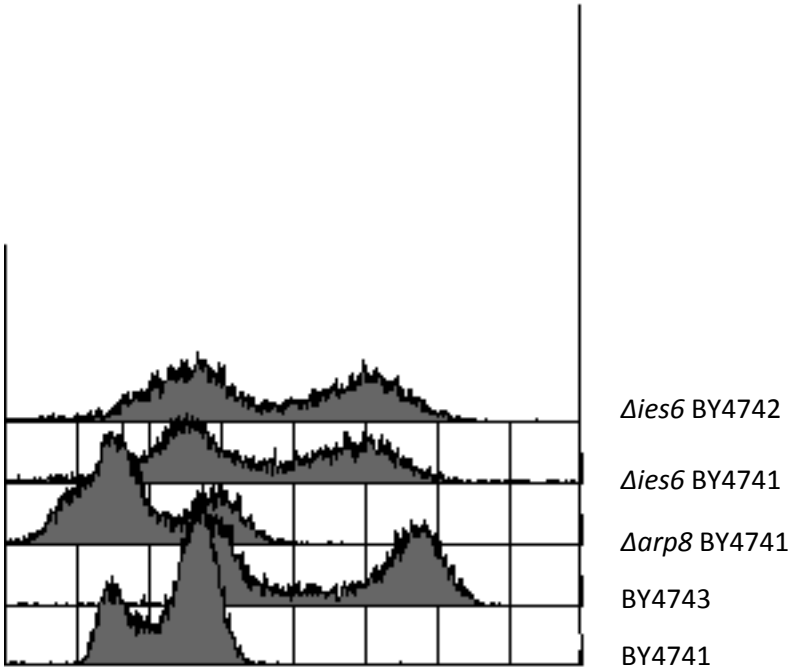
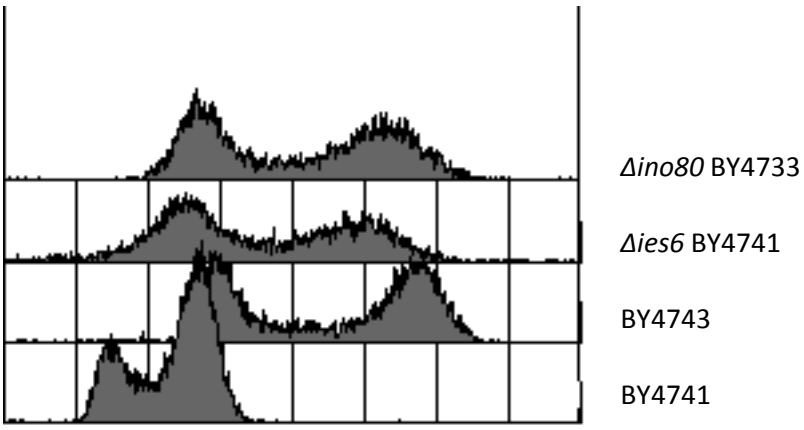


Figure 6.1 Loss of the Ino80 chromatin remodelling complex subunit Ies6, but not Arp8 leads to an increase in cellular ploidy FACS analysis of cell samples from asynchronous haploid or diploid parent strain or mutant (as indicated) yeast cultures in mid-log growth phase

a)



b)

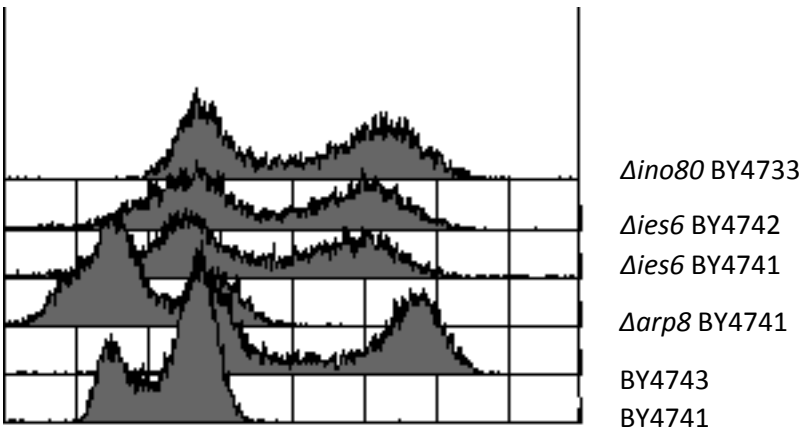


Figure 6.2 Loss of the Ino80 chromatin remodelling complex catalytic subunit Ino80, or subunit Ies6, but not Arp8 leads to an increase in cellular ploidy a & b) FACS analysis of cell samples from asynchronous haploid or diploid parent strain or mutant (as indicated)yeast cultures in mid-log growth phase

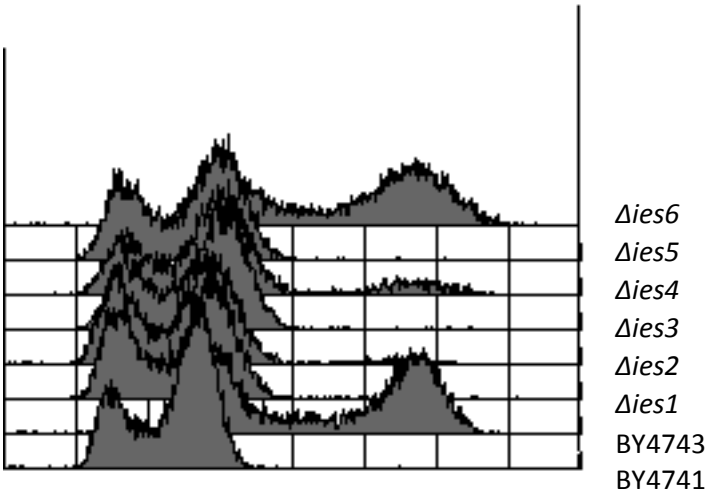
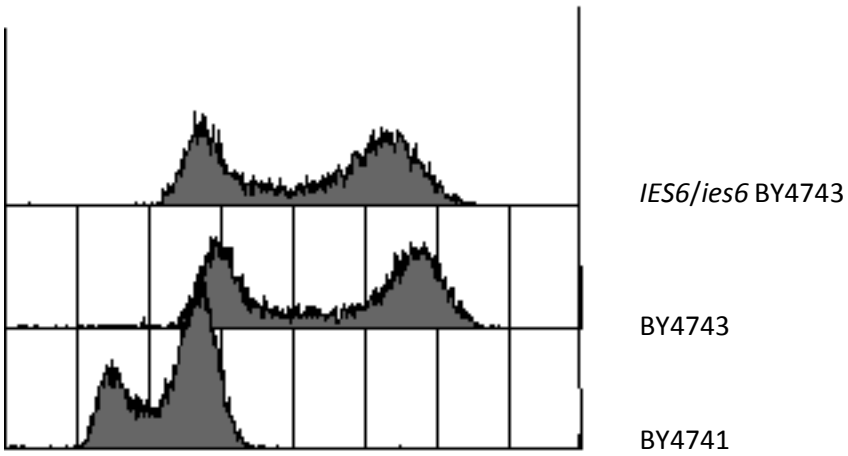


Figure 6.3 Loss of Ies6, but not the other Ies subunits leads to an increase in cellular ploidy FACS analysis of cell samples from asynchronous haploid or diploid parent strain or mutant (as indicated) yeast cultures in mid-log growth phase

a)



b)

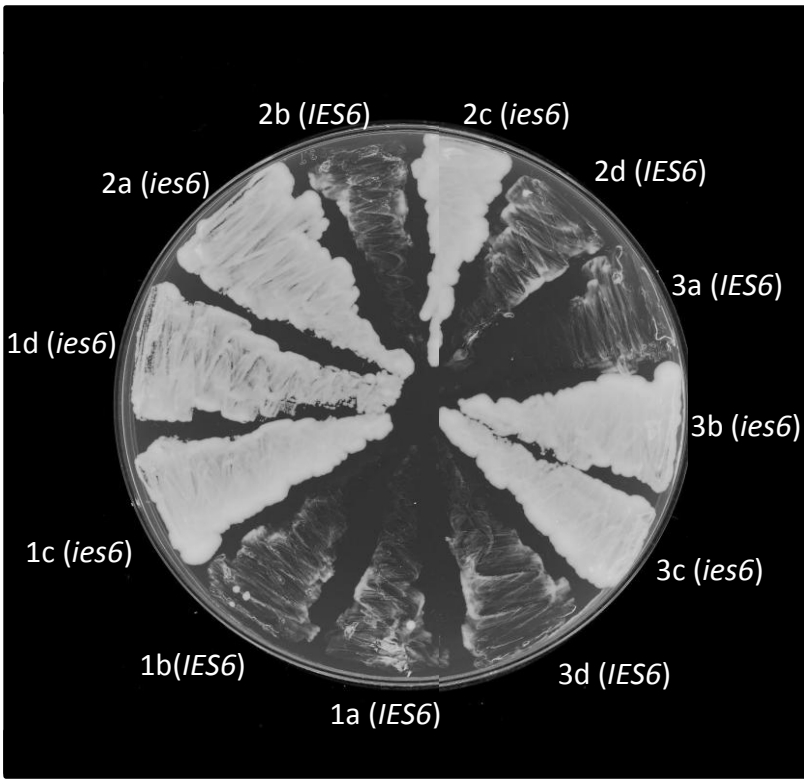


Figure 6.4 Analysis of the *IES6/ies6* heterozygote budding yeast strain a) Analysis of the *IES6/ies6* heteroygote by FACS of asynchronous yeast cultures grown to mid-log phase b) Confirmation of the spores' genotype by analysis of spore growth on rich media supplemented with G418, only the spores harbouring a deletion of *IES6* are able to survive on the presence of G418 as the deletion cassette contains a KanMX marker.

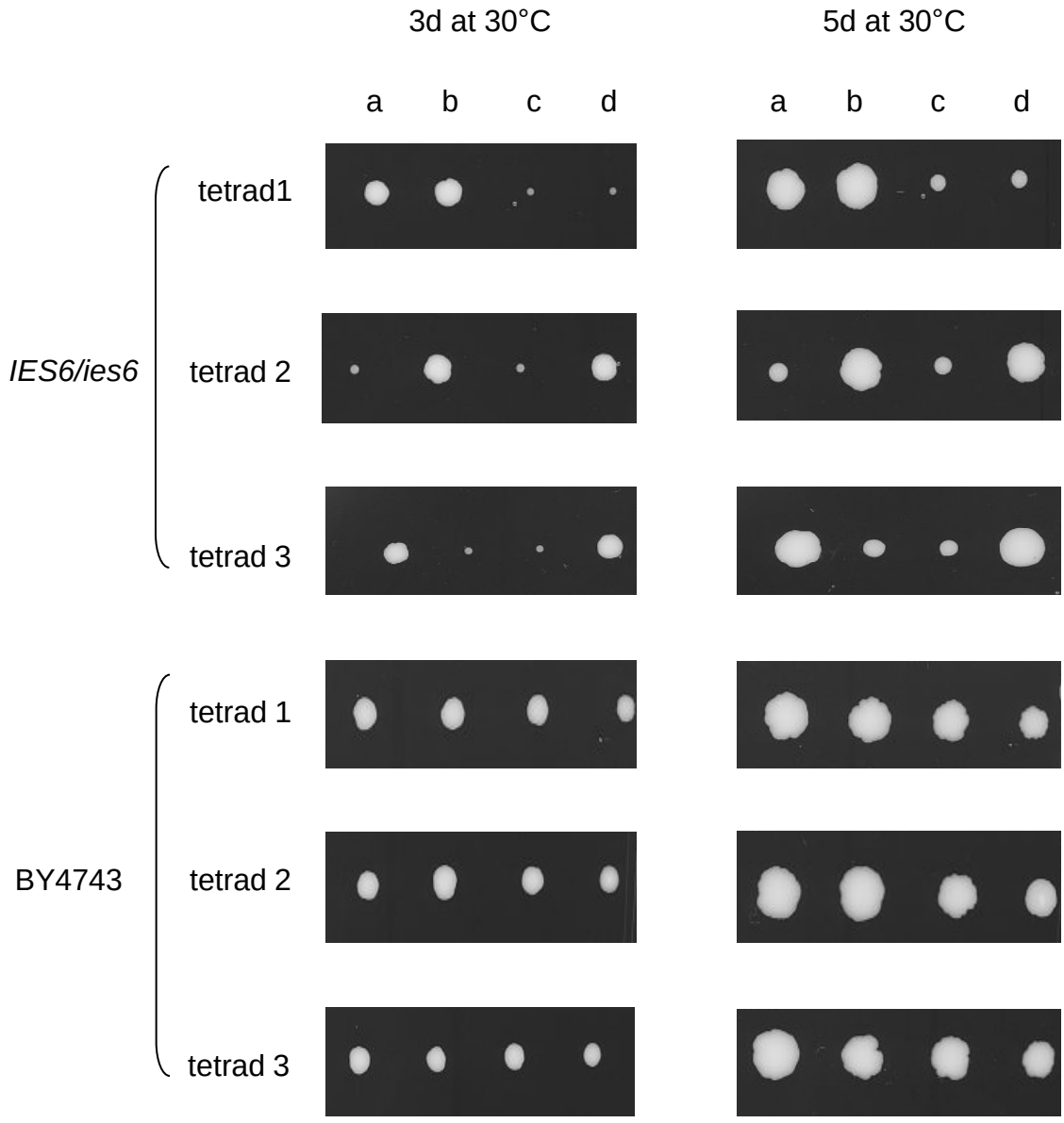


Figure 6.5 Tetrad analysis of the *IES6/ies6* BY4743 heterozygous yeast strain Deletion of *IES6* segregates in a 2:2 manner as seen by the slow-growth phenotype on rich media of the *ies6*-deletion spores

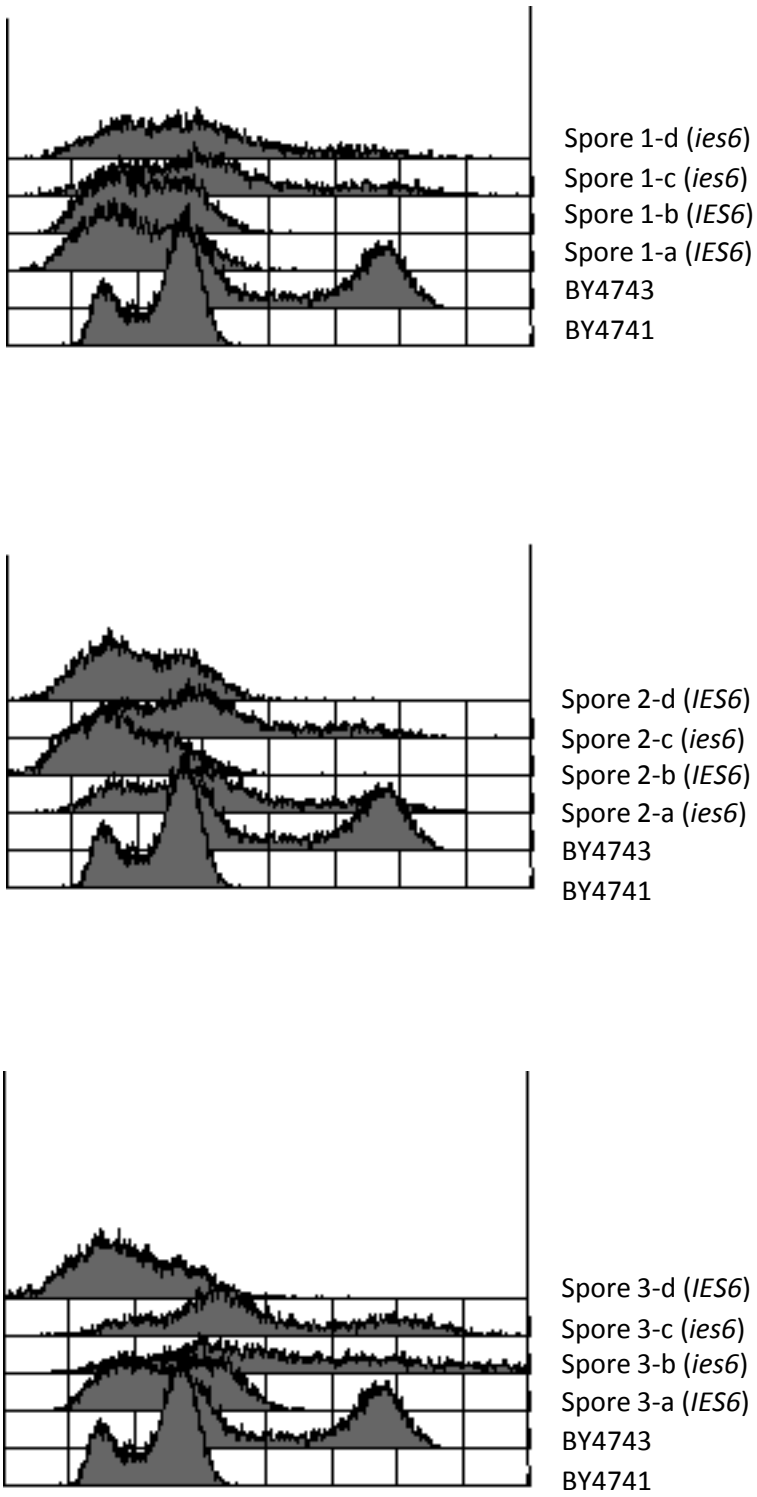


Figure 6.6 Spores harbouring a deletion of *IES6* display a tendency towards higher ploidy analysis of cell samples from asynchronous haploid or diploid parent strain or mutant (as indicated) yeast cultures in mid-log growth phase

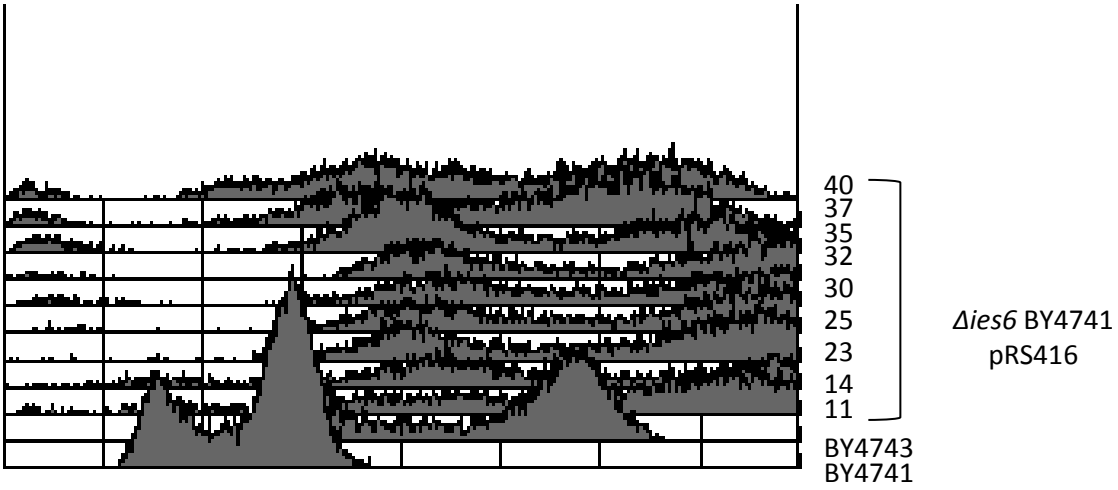


Figure 7.1 Loss of *IES6* leads to progressive genomic instability FACS analysis of cell samples from asynchronous yeast cultures in mid-log growth phase of haploid (BY4741) or diploid (BY4743) controls (as indicated) or from a regularly subcultured *Δies6* BY4741, empty pRS416 vector, asynchronous yeast culture over time as indicated, in days following transformation into *Δies6* BY4741

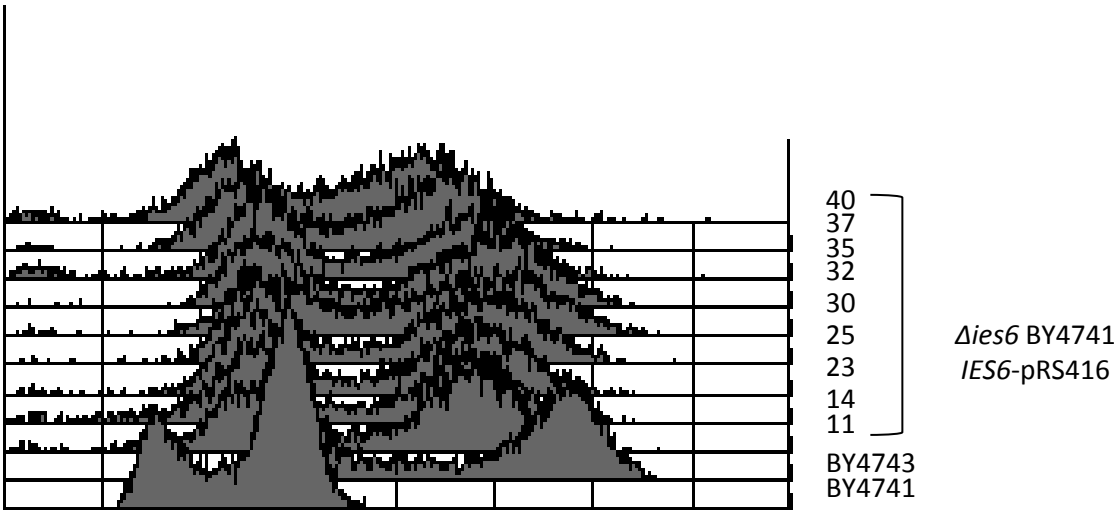
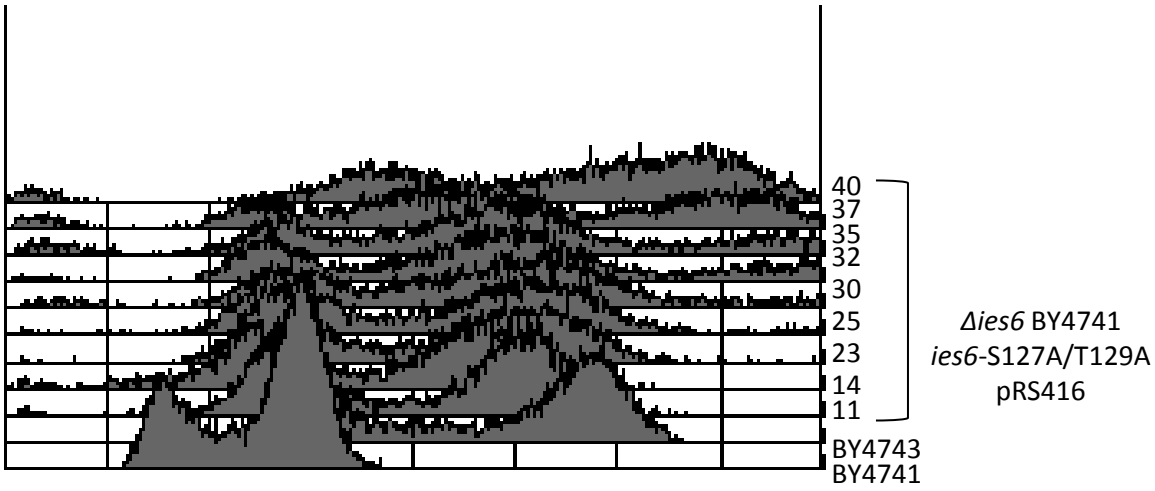


Figure 7.2 Re-introduction of wild-type *IES6* halts progression towards genomic instability FACS analysis of cell samples from asynchronous yeast cultures in mid-log growth phase of haploid (BY4741) or diploid (BY4743) controls (as indicated) or from a regularly subcultured *Δies6* BY4741, wild-type *IES6* on the pRS416 vector, asynchronous yeast culture over time as indicated, in days following transformation into *Δies6* BY4741

a)



b)

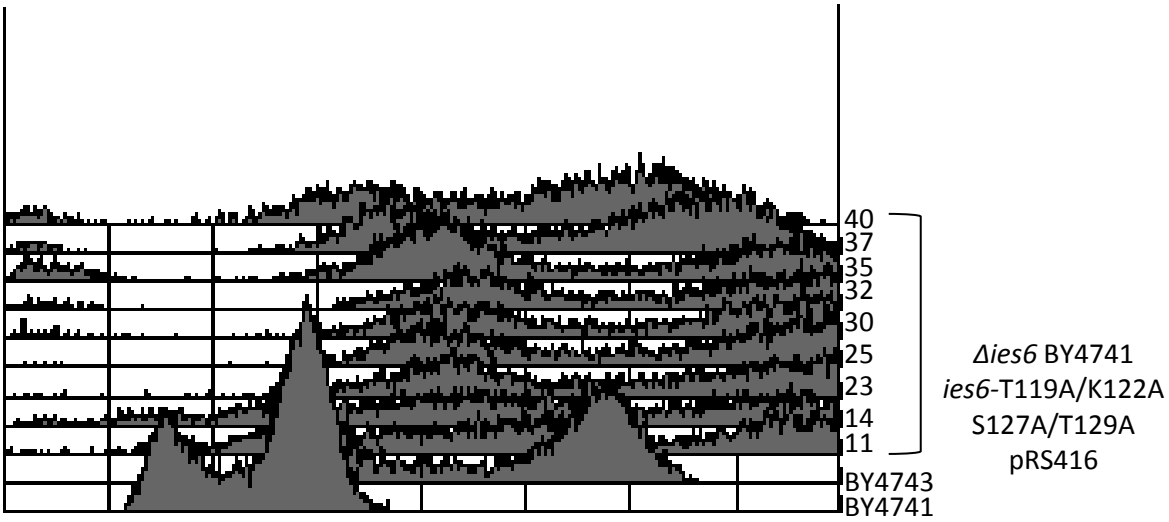


Figure 7.3 The Ies6 protein's ability to bind DNA correlates with its ability to maintain genome stability FACS analysis of cell samples from asynchronous yeast cultures in mid-log growth phase of haploid (BY4741) or diploid (BY4743) controls (as indicated) or from a regularly subcultured *Δies6* BY4741, with either mutant *ies6*-S127A/T129A (a) or ies6-T119A/K122A/S127A/T129A (b) on the pRS416 vector, asynchronous yeast culture over time as indicated, in days following transformation into *Δies6* BY4741

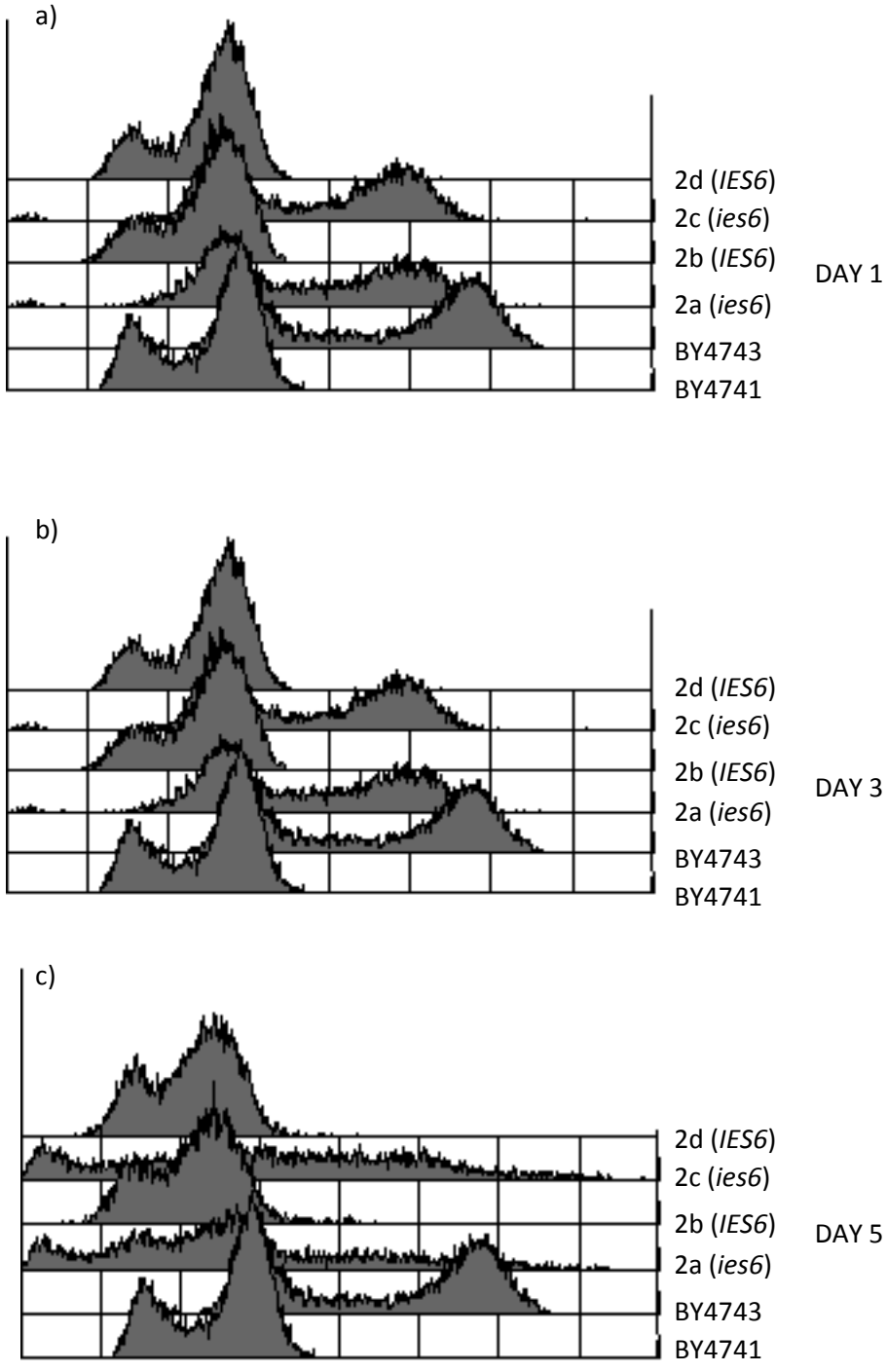


Figure 7.4 Spores lacking *IES6* all develop towards significant genome instability in an apparently timely manner FACS analysis of cell samples from asynchronous yeast cultures in mid-log growth phase of haploid (BY4741) or diploid (BY4743) controls (as indicated) or from regularly subcultured, asynchronous yeast samples of spores a-d of tetrad 2 over time at days 1(a), 3(b) and 5(c) following initial subculture

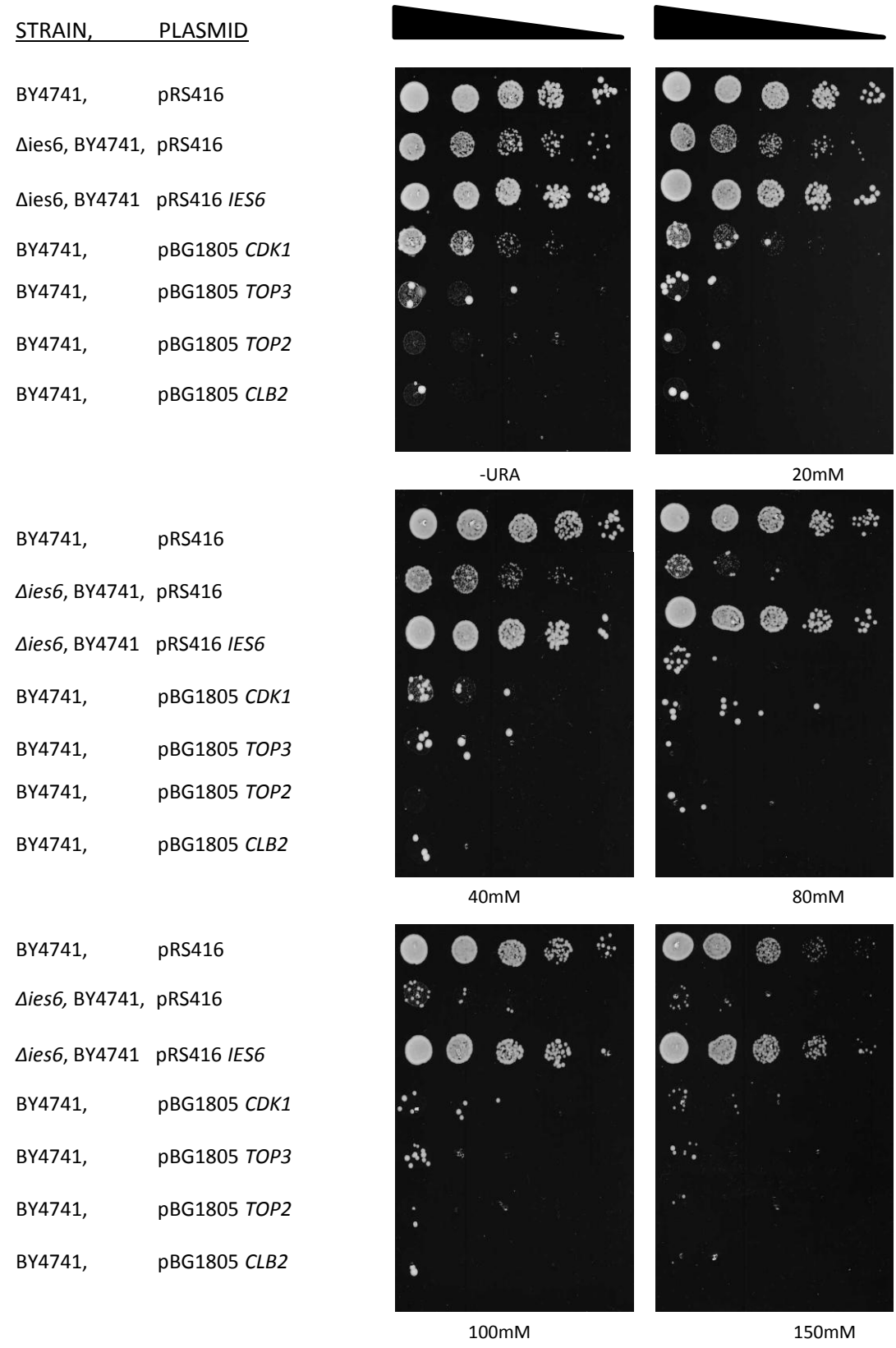


Figure 7.5 Overexpression of any of *CDK1*, *TOP3*, *TOP2* or *CLB2* negatively impacts on the survival of the wild-type BY4741 yeast strain The BY4741 parent strain harbouring either an empty vector or overexpression constructs of *TOP2*, *CDK1*, *TOP3* or *CLB2* (as indicated), as well as the *ies6* deletion strain and the *ies6* deletion strain harbouirng *IES6* on a plasmid under control of its own promoter, were analysed for their level of sensitivity to hydroxyurea (HU) by plating serial five-fold dilutions of mid-log yeast cultures onto media lacking uracil (-URA) for maintenance of plasmid selection, containing increasing amounts of HU as indicated.

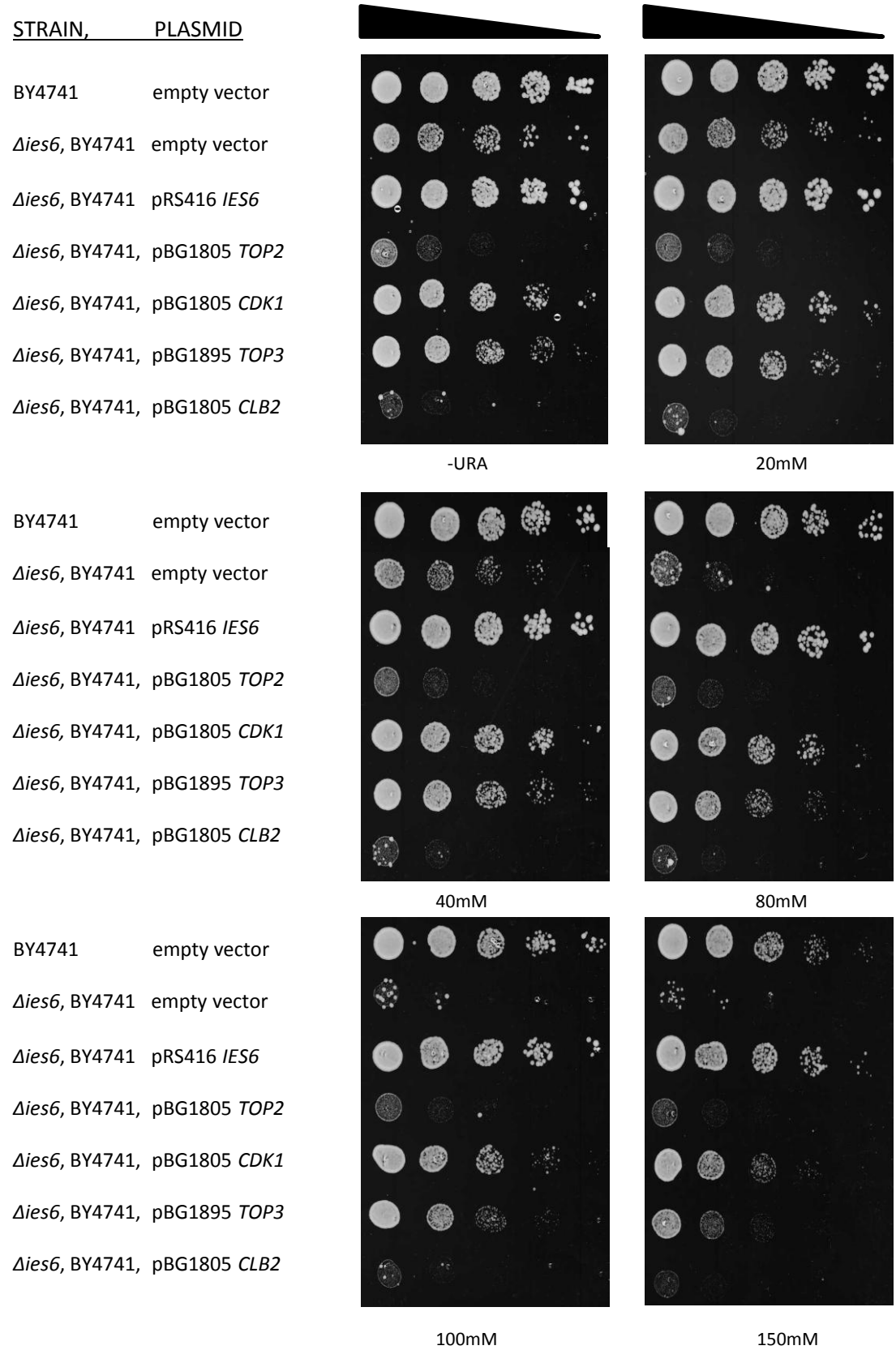


Figure 7.6 Overexpression of *TOP3* or *CDK1*, but not *TOP2* or *CLB2* leads to near wild-type rescue of the slow-growth and HU-hypersensitive phenotype of the *ies6* deletion strain The *ies6* deletion strain harbouring either an empty vector, *IES6* under control of the *IES6*-promoter or overexpression constructs of *TOP2*, *CDK1*, *TOP3* or *CLB2* (as indicated), as well as the parent strain were analysed for their level of sensitivity to hydroxyurea (HU) by plating serial five-fold dilutions of mid-log yeast cultures onto media lacking uracil (-URA) for maintenance of plasmid selection, containing increasing amounts of HU as indicated.

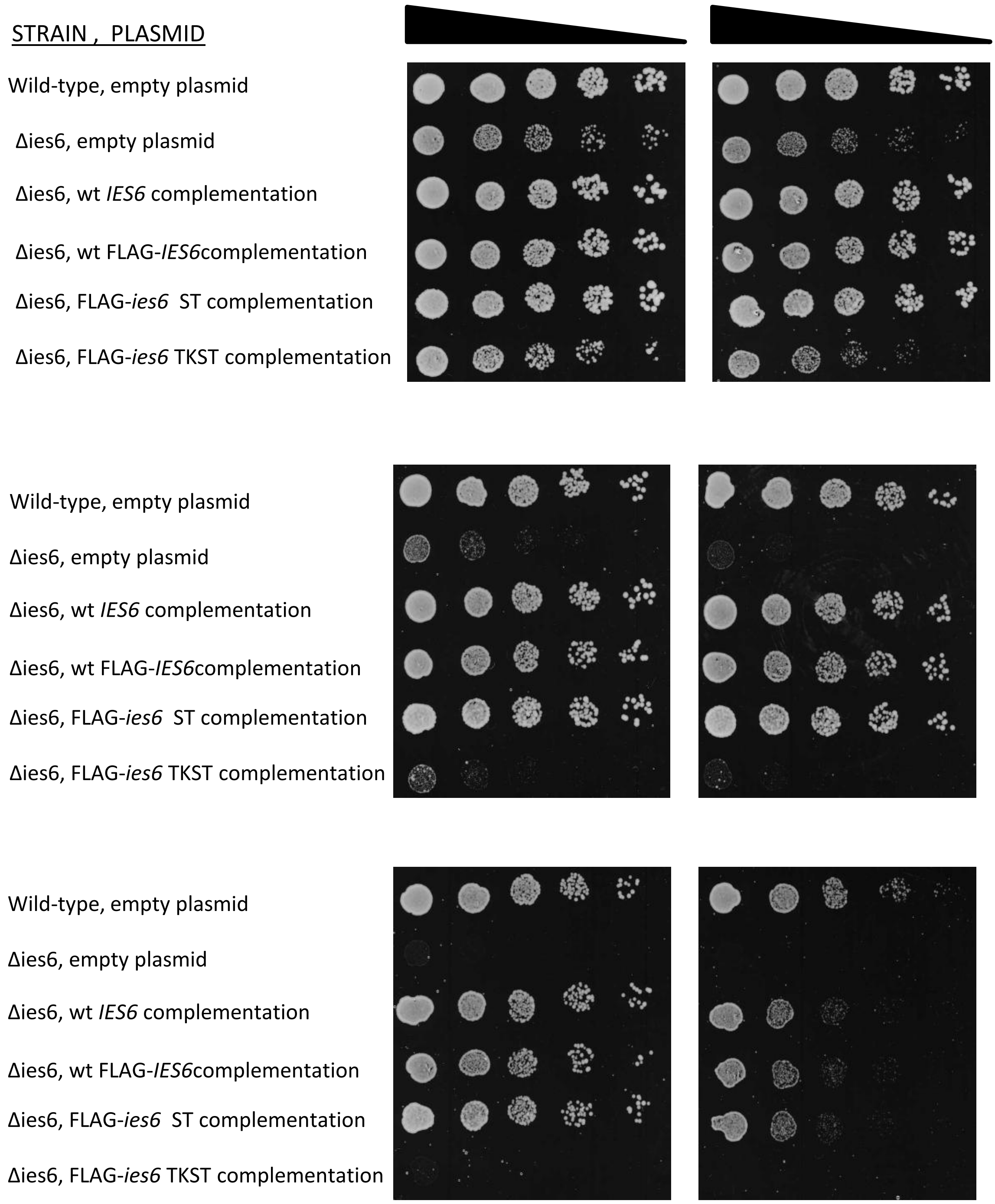


Figure A.1 The FLAG-tagged wt-Ies6 and Ies6-S127A/T129A, but not the FLAG-tagged Ies6-T119A/K122A/S127A/T129A rescue the HU-hypersensitivity of the *ies6*-deletion strain The wild-type yeast strain, or yeast strains harbouring a deletion of *IES6*, containing either empty vector (pRS416) or a complementation vector (pRS416 containing either wild-type (wt), FLAG-tagged wild-type (wt FLAG-I*ES6*), *ies6*-S127A/T129A (FLAG ST), or *ies6*-T119A/K122A/S127A/T129A (FLAG TKST) under the control of its endogenous promoter and terminator) were analysed for their level of sensitivity to hydroxy-urea (HU) by plating serial five-fold dilutions of yeast cultures onto media lacking uracil (-URA) for maintenance of plasmid selection, containing increasing amounts of HU as indicated.

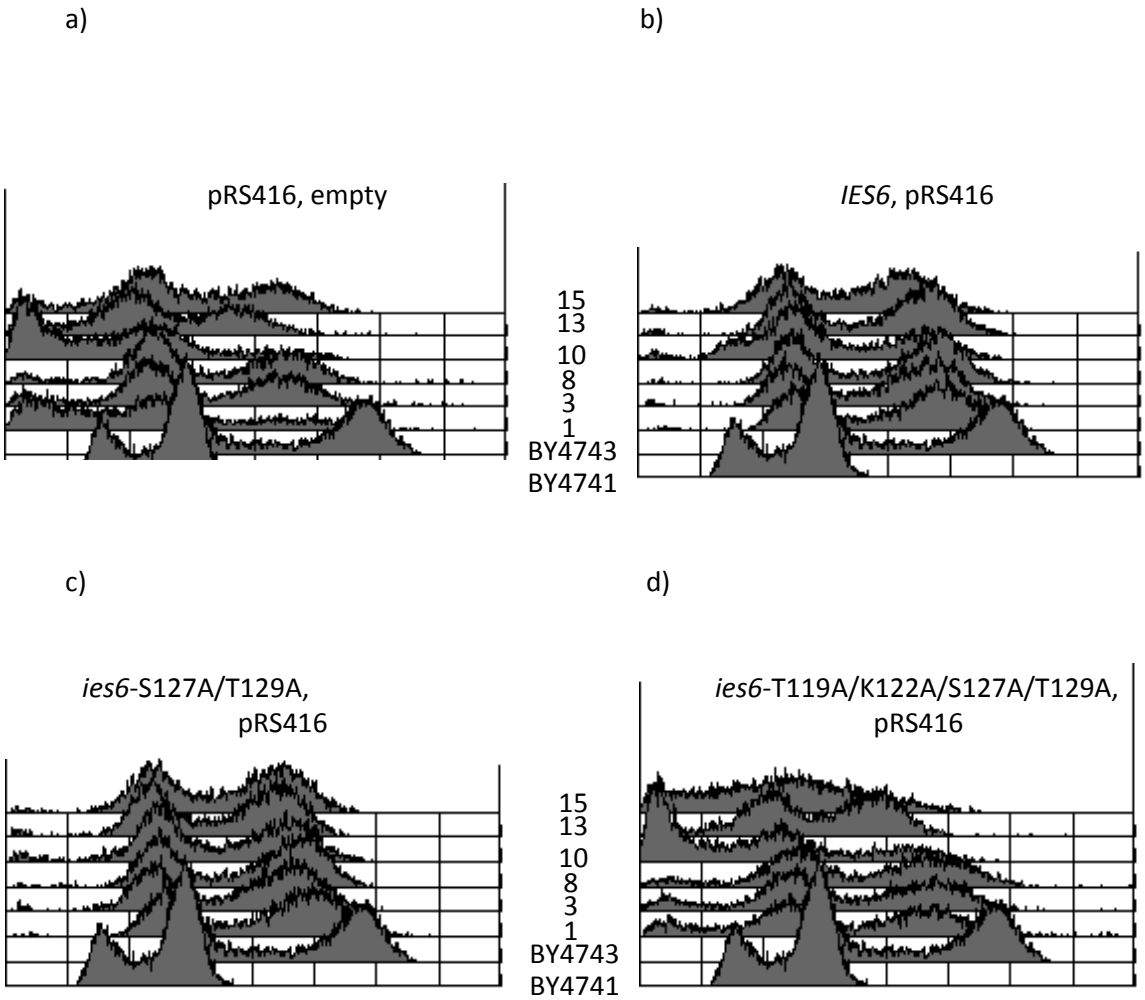


Figure A.2 Genome instability as seen by alteration in ploidy following loss of *IES6* is a stochastic process FACS analysis of cell samples from asynchronous yeast cultures in mid-log growth phase of haploid (BY4741) or diploid (BY4743) controls (as indicated) or from a regularly subcultured *Δies6* BY4741, with empty vector (a), wild-type (b) or mutant *ies6* (c & d) on the pRS416 vector, asynchronous yeast culture over time as indicated, in days following transformation into *Δies6* BY4741

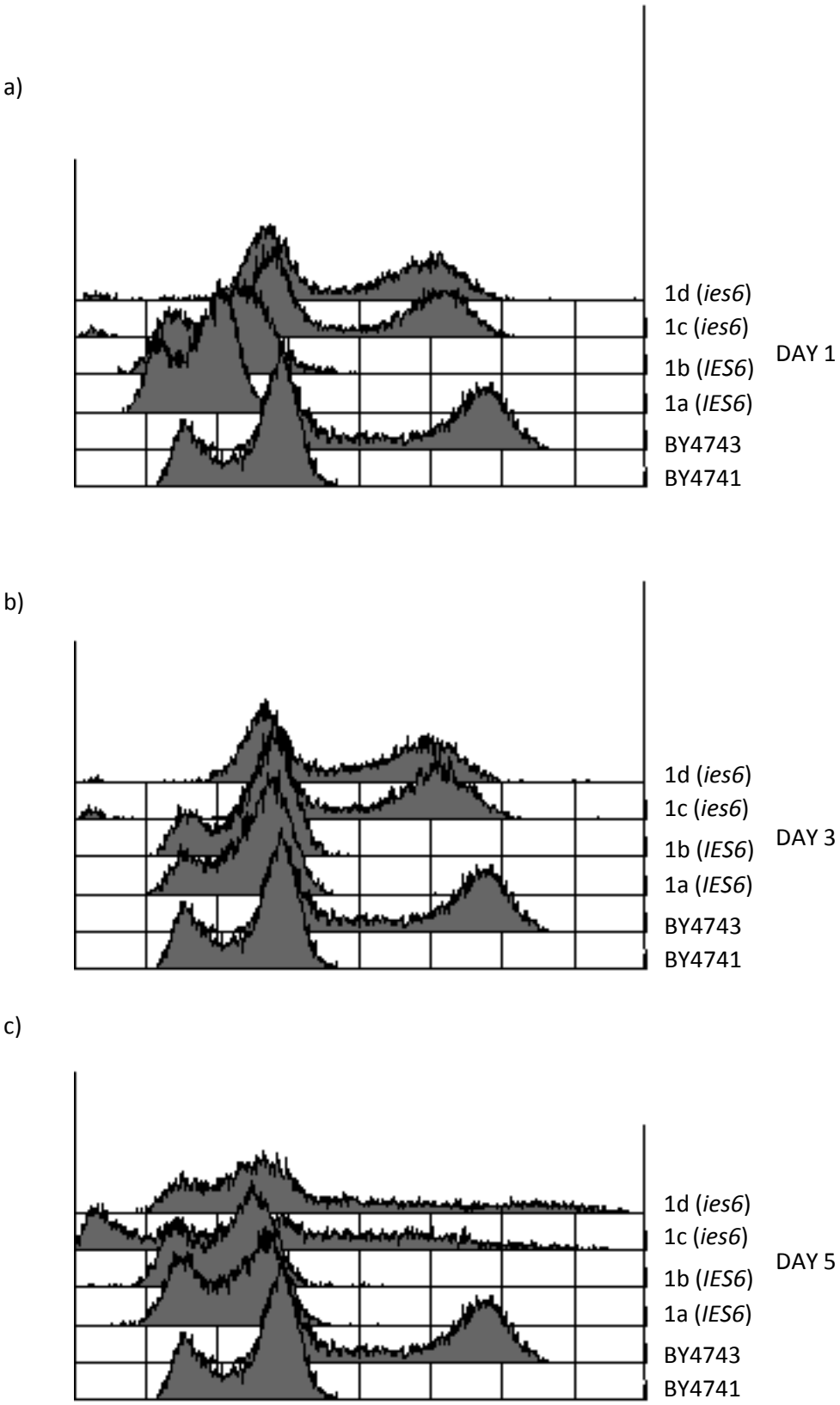


Figure A.3 Spores lacking *IES6* all develop towards significant genome instability in an apparently timely manner FACS analysis of cell samples from asynchronous yeast cultures in mid-log growth phase of haploid (BY4741) or diploid (BY4743) controls (as indicated) or from regularly subcultured, asynchronous yeast samples of spores a-d of tetrad 1 over time at days 1(a), 3(b) and 5(c) following initial subculture

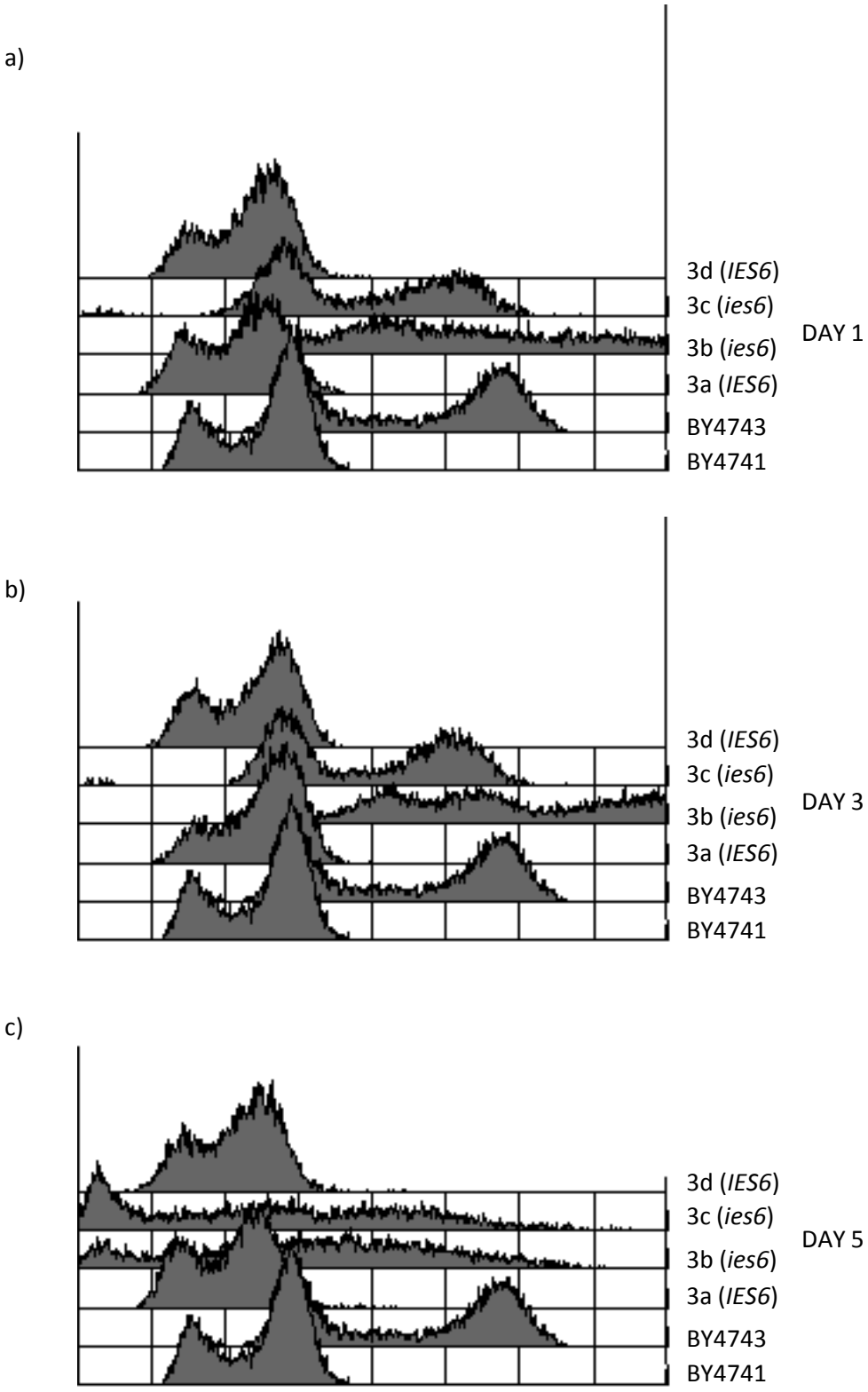


Figure A.4 Spores lacking *IES6* all develop towards significant genome instability in an apparently timely manner FACS analysis of cell samples from asynchronous yeast cultures in mid-log growth phase of haploid (BY4741) or diploid (BY4743) controls (as indicated) or from regularly subcultured, asynchronous yeast samples of spores a-d of tetrad 3 over time at days 1(a), 3(b) and 5(c) following initial subculture