FUS, RNA and the nucleolus

A thesis submitted to the University of Sussex for the degree of Doctor of Philosophy

By

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I declare that this thesis has not been submitted and will not be submitted to another university for the award of any other degree, and that the work described in the text is my own unless otherwise stated.

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Abstract

Fused-in-sarcoma (FUS) is an RNA binding protein, thought to be involved in a wide variety of cellular processes, and mutations in FUS are known to be causative for amyotrophic lateral sclerosis (ALS). The mechanism of pathogenesis for ALS has not been established but it has been proposed that dysfunction in cellular functions involving RNA could be responsible. Investigations into a FUS-ALS patient cell line showed sensitivity to the transcriptional inhibitor camptothecin (CPT) and demonstrated constitutively fragmented nucleoli, a phenotype that has been associated with rRNA dysfunction, as well as a possible defect in ribosomal RNA (rRNA) maturation. In addition a reversible relocalisation of FUS to the nucleolus in response to inhibition of RNA polymerase II was observed in all cell lines examined. This relocalisation appeared to be dependent on the activity of phosphodiesterase 8 (PDE8) and on the presence of rRNA, as pre-inhibition of RNAP I (which produces rRNA) prevented relocalisation of FUS. However treatment of both RNAP I and RNAP II at the same time resulted in FUS relocalisation and the protein remaining in the nucleolus for hours if inhibition was maintained - long after RNA would be depleted at the site were RNAP I inhibited in isolation. These findings suggest that FUS may have a role in protecting pre-rRNA transcripts from degradation during transcriptional stress.

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4E-BP1 - eukaryotic translation initiation factor 4E-binding protein 1

53BP1 - p53-binding protein 1

ActD - actinomycin D

ADP/ATP - adenosine di/triphosphate

ANOVA - analysis of variance

APE - apurinic-apyrimidinic endonuclease 1

APLF - aprataxin-and-PNKP-like factor

ATM - ataxia-telangiectasia mutated

ATR - ataxia- and Rad3-related

ATRIP - ATR interacting protein

B23 - nucleolar phosphoprotein B23/nucleophosmin

BRCA1/2 - breast cancer 1/2

BSA - bovine serum albumin

cAMP - cyclic 3',5'-adenosine monophosphate

CB - coiled body

cGMP - cyclic 3',5'-guanosine monophosphate

CENPF - centromere protein F

Chk1 - checkpoint kinase 1

Chk2 - checkpoint kinase 2

CK2 - casein kinase 2

CPD - cyclobutane pyrimidine dimers

CPSF - cleavage and polyadenylation specificity factor

CPT - camptothecin

DAPI - 4',6-diamidino-2-phenylindole

DEPC - diethylpyrocarbonate

DFC - dense fibrillar component

DMEM - Dulbecco's modified Eagle medium

DMSO - dimethyl sulphoxide

DNA - deoxyribonucleic acid

DNA-PKcs - DNA-dependent protein kinase, catalytic subunit

DRB - 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

DSB - double strand break

DSIF - DRB-sensitivity inducing factor

EDTA - ethylenediaminetetraacetic acid

eIF4A/B/E/G - eukaryotic initiation factor 4A/B/E/G

ETS - external transcribed spacer

EU - ethynyl uridine

FAT - FRAP, ATM, TRRAP

FATC - FAT C-terminus

FC - fibrillar centre

FcS - foetal calf serum

FEN1 - flap endonuclease 1

FKBP12 - FK506-binding protein

FTLD - frontotemporal lobar degeneration

FUS/TLS - fused-in-sarcoma/translocated-in-liposarcoma

GABA - γ-aminobutyric acid

GC - granular component

GDP/GTP - guanosine di/triphosphate

GFP - green fluorescent protein

Gy – Gray

Hdm2 - human double minute 2

HEAT - huntingtin, elongation factor 3, A subunit of protein phosphatase 2A and TOR1

hTERT - human telomerase reverse transcriptase

HU - hydroxyurea

IBMX - 3-isobutyl-1-methylxanthine

IgG - immunoglobulin G

INR - Initiator

IR - ionising radiation

kDa - kilodalton

LAP - localisation-affinity-purification

LB - Laemmli buffer

MEFs - mouse embryonic fibroblasts

MEM - minimum essential medium

miRNA - micro RNA

mRNA - messenger RNA

MOPS - 3-(N-morpholino)propanesulfonic acid

mTOR - mammalian target of rapamycin

mTORC1/2 - mTOR complex 1/2

ncRNA - non-coding RNA

NLS - nuclear localisation sequence

NOR - nucleolar organising region

NELF - negative transcription elongation factor

PAR - poly ADP ribose

PARG - poly ADP ribose glycohydrolase

PARP - poly ADP ribose polymerase

PBS - phosphate buffered saline

PBSTS - phosphate buffered saline with TWEEN and SDS

PCNA - proliferating cell nuclear antigen

PDE - phosphodiesterase

PFA - paraformaldehyde

PKA - cAMP dependent protein kinase

PKG - cAMP dependent protein kinase

PIC - pre-initiation complex

PI3K - phosphatidylinositol 3-kinase

PIKK - phosphatidylinositol 3-kinase-related kinase

PNKP - polynucleotide kinase 3'-phosphatase

PRD - PIKK-regulatory domain

P-TEF - positive transcription elongation factor

Rb - retinoblastoma protein

RGG - arginine-glycine-glycine box

RNA - ribonucleic acid

RNAP - ribonucleic acid polymerase

RPA(32) - replication protein A (32 kDa subunit)

RQ1 - RNA qualified

RRM - RNA recognition motif

rRNA - ribosomal RNA

S6K1 - S6 kinase 1

SDS - sodium dodecyl sulphate

SDS-PAGE - SDS polyacrylamide gel electrophoresis

shRNA - small hairpin RNA

siRNA - small interfering RNA

SL1 - selectivity factor 1

SMC1 - structural maintenance of chromosomes 1

SMG1 - suppressor of morphogenesis in genitalia 1

snRNA/snRNP - small nuclear RNA/ribonucleoprotein

snoRNA/snoRNP - small nucleolar RNA/ribonucleoprotein

SSB - single strand break

SSC - saline-sodium citrate

TAE - tris-acetate-EDTA

TAF - TBP-associated factor

TBP - TATA binding protein

TBS - tris-buffered saline

TBST - tris-buffered saline with TWEEN

TDP1/2 - Tyrosyl-DNA phosphodiesterase 1/2

TDP43/TARDBP - trans-activating response region DNA-binding protein with a molecular mass of 43kDa

TDP1/2 - tyrosyl-DNA phosphodiesterase 1/2

TOP - terminal oligopyrimidine track in the 5' untranslated region

TOP1/2 - topoisomerase 1/2

TOP1/2cc - TOP1/2 cleavage complex

TopBP1 - DNA topoisomerase II binding protein

TRRAP - transformation/transcription domain-associated protein

TSC1/2 - tuberous sclerosis 1/2

UBF - upstream binding factor

UPF - up frameshift

UV(A) - ultraviolet (A)

VP16 - etoposide

XLF - DNA ligase 4 and XRCC4-like factor

XRCC1 - X-ray repair cross-complementing protein 1

WT - wild-type

ZnF - zinc finger domain

1. Introduction

1.1 Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD)

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease - patient records at the Eleanor and Lou Gehrig Research Centre indicate ALS accounted for 76.3% of motor neuron disease diagnoses between 2000 and 2007 at the centre (Kim et al. 2009). ALS has an incidence of 2 to 3 new cases per 100,000 people per year in western Europe - incidence peaks between the ages of 50 and 75 and affects up to 1.5 male patients for every female patient (Zufiría et al. 2016). The disease affects both upper motor neurons (in the cerebral cortex) and lower motor neurons (in the brainstem and spinal cord) (Ludolph et al. 2015). Degeneration in the latter category of motor neurons leads to the muscle weakness and wasting that leads to death (via respiratory failure) and this death typically occurs two to five years after onset (Worms 2001; Wijesekera & Leigh 2009). ALS can express as both sporadic and familial forms with 5-10% of ALS cases being familial (Shaw 2005).

Frontotemporal lobar degeneration (FTLD) is a relatively common dementia - estimated to comprise 12% of dementia diagnoses in patients under the age of 65 (Harvey et al. 2003). This is the third most common diagnosis after Alzheimer's disease and vascular dementia. Clinically there are a few variants of FTLD and the behavioural FTLD variant; characterised by disinhibition, apathetic behaviour and stereotypic behaviour; is sometimes found associated with motor neuron diseases (Ferrari et al. 2011). As the name implies FTLD is associated with progressive neurodegeneration in the frontal and temporal lobes (Brun et al. 1994). FTLD variants can also be classified pathologically on the basis of molecular characteristics of protein inclusions found in affected neurons (MacKenzie et al. 2010) - these inclusions are also morphologically diverse. Like ALS cases FTLD cases can be sporadic or familial although, unlike with ALS, familial cases are relatively common, constituting approximately 40% of cases (Ferrari et al. 2011).

Intermediate diseases between ALS and FTLD have been reported since the 1980s, with patients diagnosed as ALS patients demonstrating symptoms of FTLD with the associated degeneration in the forebrain and vice versa. Intermediate ALS-FTLD cases are typically associated with a shorter time to death (Ferrari et al. 2011), and some symptoms

uncommon in pure ALS or FTLD, such as hallucinations, can occur in these intermediate cases (van Langenhove et al. 2012). Up to half of ALS patients may demonstrate a degree of impairment in behaviour or cognition and that potentially over 15% may meet the clinical criteria of FTLD (van Langenhove et al. 2012). Cases of FTLD with ALS like symptoms are comparatively rare, with only up to 10% of patients showing ALS like symptoms, though some reports claim much higher rates of FTLD cases with subclinical motor neuron degeneration (Ferrari et al. 2011). Notably both diseases demonstrate often demonstrate ubiquitin and 43-kDa transactive response DNA-binding protein (TDP43/TARDBP) positive inclusions in affected neurons (Arai et al. 2006; Hasegawa et al. 2008a) though this is near universal in ALS, seen in most ALS cases that are not caused by mutations in superoxide dismutase 1 (SOD1) (Mackenzie et al. 2007), but present merely in the majority of FTLD cases (Mancuso & Navarro 2015) where there are varieties which are TDP43 negative but positive for tau or fused-in-sarcoma (FUS) (MacKenzie et al. 2010).

Currently there approximately two dozen genes where mutations have been linked to familial ALS. These include genes encoding SOD1, c9orf72 (hexanucleotide expansion of GGGCCC repeats), TDP43, FUS, angiogenin, senataxin, ubiquilin-2 and valosin-containing protein (Zufiría et al. 2016). Of these the c9orf72 expansions are known to be the most common cause of familial ALS (DeJesus-Hernandez et al. 2011; Renton et al. 2011). Prior to the discovery of c9orf72 expansions SOD1, FUS and TDP43 were the three gene products most commonly implicated in familial ALS - with angiogenin being the fourth most common (Li & Hu 2010).

FTLD can be caused by mutations in many of the same genes (Ferrari et al. 2011) but not all ALS mutations cause FTLD or vice-versa: for instance mutations in tau are a common cause for familial FTLD but are not causative of familial ALS (van Swieten & Spillantini 2007) and FUS mutations are a relatively common cause of familial ALS but are very rare in familial FTLD (Dormann & Haass 2011).

There are several proposed mechanisms by which ALS pathology may develop. Two popular hypotheses are that protein aggregation or dysfunction in cellular processes involving RNA are responsible for ALS pathogenicity - proposals centring around oxidative stress have also

been proposed but oxidative stress-based mechanisms are no longer considered likely to be the primary cause of pathogenesis (Morgan & Orrell 2016).

The protein aggregation hypothesis implies a gain of function mechanism in ALS: SOD1 aggregates are near universally found in both sporadic and familial ALS patient cells (Shibata et al. 1994) and have been shown to activate microglia *in vitro* (Roberts et al. 2013); aggregates of TDP43 (Neumann et al. 2006) and of FUS (Deng et al. 2010) have been found in most non-SOD1 patient cells while aggregates of ubiquilin 2 have been found in patient cells with or without mutations in the gene (Deng et al. 2011).

Furthermore some of the genes in which mutations can cause familial ALS, such as valosin containing protein or ubiquilin 2, are thought to be involved in protein degradation or ubiquitination (Verma & Tandan 2013) and also signs of chronic endoplasmic reticulum (an organelle implicated in protein quality control) stress have been found in mouse models and ALS patients (Zufiría et al. 2016). It could be that defects in protein degradation and quality control can increase the likelihood of pathogenic aggregate formation. Prion-like mechanisms of toxicity have been proposed for neurotoxicity of ALS - for instance SOD1 cellular and mouse models have shown aggregate toxicity to be transferrable from mutant to wild type cells (Nagai et al. 2007; Chia et al. 2010; Haidet-Phillips et al. 2011) and it has been proposed that such mechanisms can account for this (Verma & Tandan 2013). Prion-based mechanisms of pathogenesis have also been proposed for FUS and TDP43. These two proteins have also both been found in cytoplasmic stress granules and it is thought that these may be precursors to pathogenic aggregates - this is discussed further in the individual chapters for these proteins.

RNA dysfunction is another compelling mechanism as many of the major familial ALS proteins are involved in RNA processing and a proposed mechanism of pathogenicity of c9orf72 expansion is that RNA transcripts of the GGGGCC repeats result in formation of secondary RNA structures, sequestration of RNA binding proteins and defects in processes such as transcription (Haeusler et al. 2014). FUS and TDP43, their functions described in depth later, are known to be involved in RNA processing, as are senataxin, key for resolving DNA:RNA hybrids (Skourti-Stathaki et al. 2011), and angiogenin, which may be a transcription factor for RNA polymerase I (RNAP I) (Tsuji et al. 2005; Li & Hu 2010) though it

is also an inhibitor of apoptosis (Li et al. 2012). Angiogenin is known to be a ribonuclease and most characterised angiogenin ALS mutations have been found to be deficient in ribonucleolytic activity (Wu et al. 2007; Crabtree et al. 2007). Curiously overexpression of the gene was also found to be associated with neuronal survival in a SOD1 animal model of the disease (Kieran et al. 2008).

Some models of ALS pathogenesis also propose a mutual link between RNA dysfunction and oxidative stress (Bozzo et al. 2016) and there is some evidence that the two may affect each other. For instance mitochondrial abnormalities are associated with ALS mutants of FUS (Huang et al. 2010; Huang et al. 2012; Tradewell et al. 2012) and TDP43 (Xu et al. 2010; Duan et al. 2010; Hong et al. 2012) despite neither having any direct antioxidant function (as SOD1 does). Conversely changes in transcription and splicing of neuron-specific genes has been reported in cells subjected to oxidative stress but also in mice harbouring ALS-associated SOD1 mutations (Lenzken et al. 2011).

1.2 Fused-in-sarcoma (FUS)

FUS, also known as translocated-in-liposarcoma (TLS), was identified as a component of a fusion oncogene (Rabbitts et al. 1993; Crozat et al. 1993) but has more recently been the focus of study due to its involvement in neurodegenerative disease, such as ALS. FUS is associated with up to 4% of familial ALS cases as well as in in rare sporadic cases (Verma & Tandan 2013), familial FUS ALS cases also typically have a lower age of onset than sporadic ALS cases - with an estimated 61% of FUS ALS cases having an onset before the age of 40, as opposed to only 9.7% of sporadic ALS cases (Shang & Huang 2016).

FUS is a member of the FET family (FUS, EWSR1 and TAF15) of RNA binding proteins (Crozat et al. 1993; Dormann & Haass 2013) and consists of an N terminal transcriptional activation domain, a central DNA/RNA binding domain and an atypical proline-tyrosine (PY) nuclear localisation sequence (NLS), which allows nuclear import via transportin (Niu et al. 2012; Dormann & Haass 2013). The transcriptional activation domain contains a glutamine, glycine, serine and tyrosine rich region (SYGQ-rich) up to residue 165, proposed to have prion-like properties (Cushman et al. 2010), and a glycine rich region in residues 165-267. The nucleic acid binding domains consist of three arginine-glycine-glycine boxes (RGG) between which are located an RNA recognition motif (RRM), which may contain a nuclear

export sequence (Sun et al. 2015), and a zinc finger domain (ZnF) (Dormann & Haass 2013). Some sources, such as Shang & Huang 2016, describe FUS domains slightly differently - typically combining the N-terminal side of the SYSQ-rich domain and the most C-terminal RGG domain into a single glycine-rich domain. The RGG boxes are major targets of arginine methylation, which can affect the cellular localisation (Bedford & Clarke 2009), FUS being a protein that shuttles between the nucleus and cytoplasm (Zinszner, Sok, et al. 1997). For instance knockdown of protein N-arginine methyltransferase 1, responsible for the majority of arginine dimethylation, restores wild-type localisation to the P525L mutant which is typically subject to a severe nuclear import block. It is thought that the unmethylated RGG3 domain stabilises the PY-NLS/transportin interaction (Dormann et al. 2012; Sproviero et al. 2012). It has also been recently reported that phosphorylation of tyrosine 526 negatively regulates nuclear import of FUS (Darovic et al. 2015).

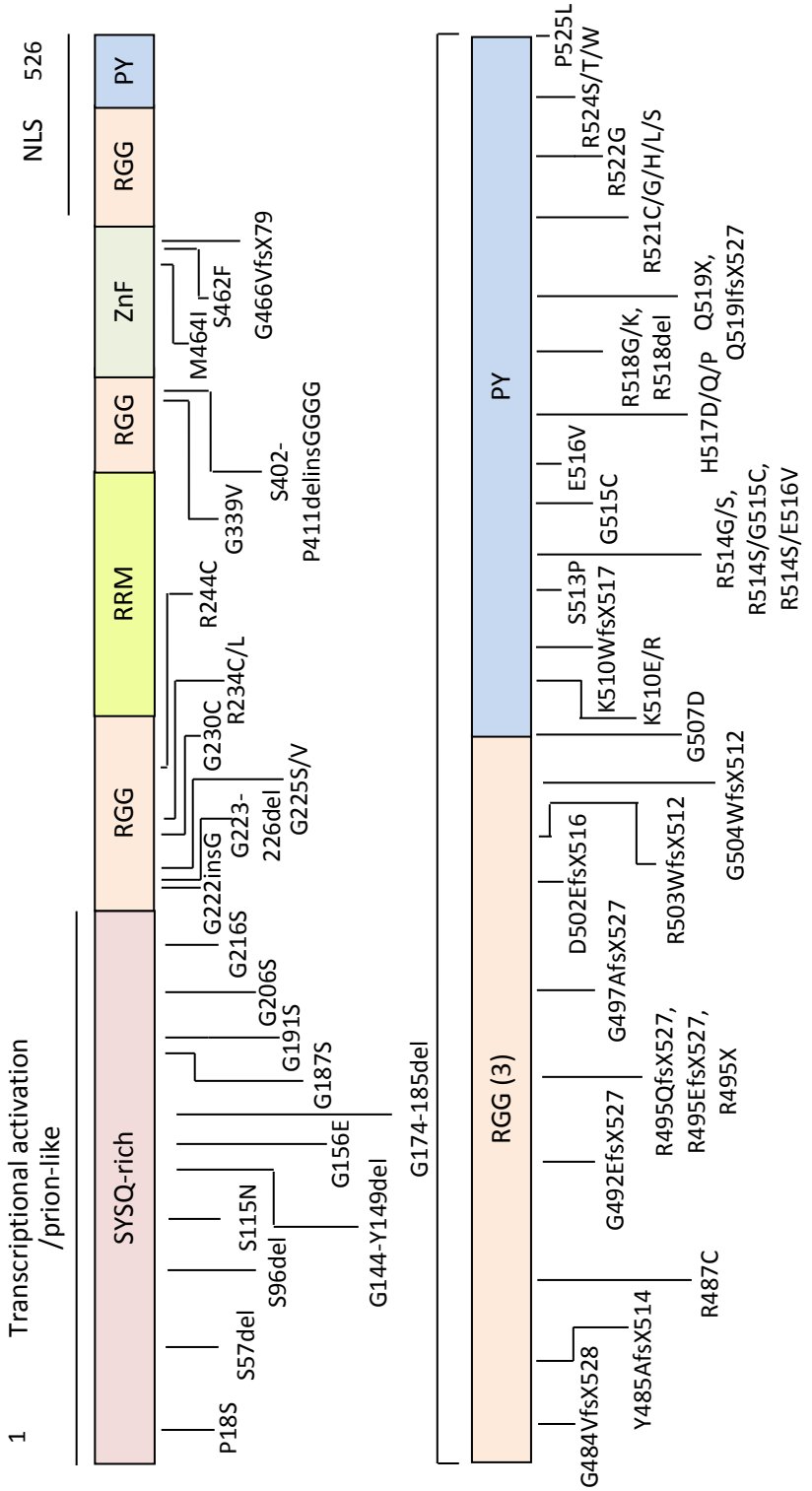


Figure 1.1 | **Diagram of FUS primary structure and location of ALS-causative mutations.** Most known mutations are clustered near the C-terminal NLS region or the SYGQ-rich prion-like domain, the number of mutations in each domain is listed here. List of mutations sourced from Dormann & Haass 2013 and Shang & Huang 2016. In addition, although FUS mutation in FTLD is not commonly observed, there have been single cases of FUS-FTLD patients with the following mutations: P106L, G206S and M254V - all in the SYSQ-rich or RGG domains (Dormann & Haass 2013). fs = frameshift, X = termination, del = deletion, ins = insertion

FUS has been implicated in DNA damage processes with FUS knockout mice demonstrating radiosensitivity (Kuroda et al. 2000) and chromosomal instability (Hicks et al. 2000), though these mice lack ALS-FUS symptoms. Curiously overexpression of human FUS in mice results in FUS-ALS like symptoms but no reported DNA damage repair defects (Mitchell et al. 2013) and expression of R521C human mutant FUS resulted in both these symptoms and evidence of increased DNA damage in the brain (Qiu et al. 2014). The first role proposed for FUS in DNA damage repair is its role in the homologous recombination (HR) pathway of double strand break (DSB) repair. In HR DSBs are repaired in a processing involving using an intact sister chromatid as a template, and this requires the formation of a D-loop structure where the two strands of DNA are separated by a third - formation of this loop is promoted by FUS *in vitro* (Baechtold et al. 1999). A later study implicated FUS in non-homologous end joining (NHEJ), another major path of DSB repair, using plasmid based assays (Mastrocola et al. 2013). FUS has also been proposed to act with histone deacetylase 1 at the earliest stage of the DSB repair process (Wang et al. 2013).

FUS has been also been implicated in the DNA damage response by way of papers indicating it is as a target of one or more of ataxia-telangiectasia mutated (ATM) (Gardiner et al. 2008) or the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Deng et al. 2014), signalling molecules involved in the DNA damage response of the phosphatidylinositol 3-kinase-related kinases (PIKKs) family. The exact role of these signalling molecules in the activities of FUS remains unclear.

Additionally three studies have used laser microirradiation to show the recruitment of FUS to sites of DNA damage and the requirement of this on a class of DNA damage signalling molecules, the poly ADP ribose polymerase (PARP) family (Mastrocola et al. 2013; Rulten et al. 2014; Britton et al. 2014) - with the Britton paper stating it was recruited in a complex containing two other proteins. The Britton paper also demonstrated that removal of FUS from sites of damage was dependent on the concerted action of ATM, DNA-PK and a third PIKK called ataxia- and Rad3-related (ATR), while the other two papers demonstrated a lack of involvement of ATM in recruitment.

FUS is heavily involved in the process of transcription as well, being found to directly bind RNA polymerase II (RNAP II) and to interact with RNAP III. FUS binds RNA polymerase II

(RNAP II), preventing inappropriate phosphorylation at serine 2 of the C-terminal domain repeats of RNAP II near the start of genes. Typically RNAP II, transcribing messenger RNA (mRNA), is phosphorylated at serine 5 near the start of sites of transcription with phosphorylation at serine 2 accumulating as it travels downstream, but loss of FUS has been shown to result in abnormal accumulation of serine 2 hyperphosphorylated RNAP II at transcriptional start sites. This results in favouring of formation of mRNAs utilising early polyadenylation sites (Schwartz et al. 2012). FUS is also found in some RNAP II pre-initiation complexes, as part of the transcription factor IID (TFIID) complex (Bertolotti et al. 1996), and has been shown to be a repressor of RNAP III transcription (Tan & Manley 2010).

FUS has been shown to interact with PGC-1α, a transcriptional co-activator and is thought to encourage its promotion of expression of genes that are protective against oxidative stress (Sama et al. 2014). It has also been implicated in transcriptional control of the cell cycle - reducing transcription of the cyclin D1 gene and encouraging cell cycle arrest, in a mechanism involving non-coding RNA and inhibition of transcriptional co-activators (X. Wang et al. 2008). Inappropriate re-entry of postmitotic neurons into the cell cycle has been linked to neurodegeneration (Herrup & Yang 2007; Bonda et al. 2010), complexes including cyclin D are implicated in reinitiation of the cell cycle (Sherr 1994) and cyclin D has been found elevated in ALS patient tissue (Nguyen et al. 2003; Ranganathan & Bowser 2003). FUS is known to bind and decrease the activity of the Spi-1 transcription factor (Hallier et al. 1998) as well as increasing that of nuclear factor κB (Uranishi et al. 2001). However, these specific modulations have not yet been shown to occur in neuronal tissue. FUS (and TDP43) have also been implicate in the expression of histone deacetylase 6 mRNA through an unclear mechanism involving direct interaction of the two proteins (Kim et al. 2010) and methylated FUS has also been reported to regulate survivin (an inhibitor of apoptosis) expression through an unclear mechanism (Du et al. 2011).

It has also been proposed that FUS may be a general transcriptional regulator, directly binding promoters with a preference for single stranded motifs and regions of G-quadruplex DNA (Tan et al. 2012). Surprisingly, however, some studies indicate knockdown of FUS appears to have only modest effects on levels of mRNA expression, albeit over hundreds of genes (Lagier-Tourenne et al. 2012) - though others show more dramatic results on broad

gene expression with overexpression of wild type or mutant FUS resulting in substantial changes in expression of ribosomal and spliceosomal genes (van Blitterswijk et al. 2013).

FUS can bind a wide variety of RNA sequences - some reports suggest it preferentially binds GGUG-rich sequences (Lagier-Tourenne et al. 2012; Liu et al. 2013), but others suggest it has far greater affinity for different structures such as stem loops (Hoell et al. 2011) or that FUS has very limited sequence specificity with the protein binding in a saw-tooth pattern across long stretches of mRNA (Rogelj et al. 2012; Wang et al. 2015), though sources agree that there is a preference for binding to the 5' end of long introns (Hoell et al. 2011; Lagier-Tourenne et al. 2012; Rogelj et al. 2012). Despite disagreements on sequence specificity RNA crosslinking and deep sequencing studies have led to identification of many species of RNA that FUS binds. Some notable species are RNAs encoding actin and the actin stabilising protein Nd1-L (Fujii & Takumi 2005), tau (associated with microtubules) (Ishigaki et al. 2012; Lagier-Tourenne et al. 2012; Orozco et al. 2012; Rogelj et al. 2012), SOD1 and FUS itself (Lagier-Tourenne et al. 2012). Broader categories of RNAs that FUS has been found to bind include those involved in the endoplasmic reticulum, mitochondria, neuronal function, the unfolded protein response, transcriptional regulation, cell cycle regulation, DNA repair, ribosome biogenesis and spliceosome assembly (Hoell et al. 2011; Colombrita et al. 2012; Lagier-Tourenne et al. 2012).

FUS has been identified in a proteomic screen of the spliceosome (Zhou et al. 2002), specifically as part of the U1 component (Sun et al. 2015), and is involved in splicing of RNA species (Hallier et al. 1998; Yang et al. 1998; Meissner et al. 2003; Kameoka et al. 2004), including its own mRNA (Zhou et al. 2013), though details remain unclear. Exon array analyses on FUS knockout mouse brains have demonstrated changes in inclusion of thousands of exons including many involved in neuronal function, development or degeneration (Rogelj et al. 2012; Ishigaki et al. 2012). Notably, FUS promotes exclusion of exon 10 of tau - inclusion of which is associated with FTLD (Orozco & Edbauer 2013) and mice expressing human R521C mutant FUS in their brains demonstrated defects in their dendrites and synapses as a consequence of defects of splicing in mRNAs encoding proteins such as brain-derived neurotrophic factor. This led to defective signalling between cells (Qiu et al. 2014). In non-neuronal cells FUS mutant overexpression has been shown to induce exon skipping and intron retention (van Blitterswijk et al. 2013), and it has been proposed

that Wnt signalling can affect splicing via FUS (Sato et al. 2005) and that FUS can autoregulate by favouring alternative splicing into an isoform favoured for processing by nonsense mediated decay (Zhou et al. 2013). FUS may also regulate micro RNA (miRNA) biogenesis (many are encoded in introns of protein-encoding genes) (Gregory et al. 2004; Morlando et al. 2012; Ibrahim et al. 2012) and may autoregulate via this mechanism (Modigliani et al. 2014).

FUS is also implicated in telomere maintenance as it has been shown to specifically bind G-quadruplex RNA in telomeric repeat-containing RNA - an important structural component of the telomere (Dejardin & Kingston 2009; Takahama et al. 2009; Takahama & Oyoshi 2013). The last of these cited studies proposed that FUS regulates telomere length through modulating histone methylation as well.

The shuttling of FUS between compartments is known to be linked to mRNA transport between the nucleus and cytoplasm (Zinszner, Sok, et al. 1997) and a loss of FUS in some neurons has been shown to cause abnormal morphology in neuronal spines. It is thought that this is due to FUS shuttling mRNA to dendritic spines in response to synaptic activation in order to enhance local translation (Fujii & Takumi 2005; Fujii et al. 2005). This is consistent with the association of FUS with both actin and microtubule associated molecular motors (Kanai et al. 2004; Yoshimura et al. 2006; Takarada et al. 2009), its localisation in translationally active RNA granules associated with cell migration, and its requirement for some translational activity of the granules (Yasuda et al. 2013). This function relates to mRNAs encoding neuronal receptors and may be a method of regulating their activity (Fujii et al. 2005; Udagawa et al. 2015). Finally it has also been reported that FUS is involved in sumoylation of proteins (Oh et al. 2010).

The nuclear localisation of the protein may be of important to FUS-ALS pathogenesis as in cases of both familial and sporadic ALS dominant FUS mutations are regularly found and a small minority of these mutations are located in or at the PY NLS, with severe blockage of nuclear import correlating with severe ALS symptoms (Sproviero et al. 2012). This is often considered evidence for FUS-ALS pathogenesis being due to a loss of function associated toxicity but it is also often proposed that FUS-ALS is mediated by a toxic (possibly prion-like) gain of function.

However FUS inclusions are also a significant feature of FUS-ALS: arginine-methylated FUS can be found in inclusions within the cytoplasm (Dormann et al. 2012) and some reports have shown inclusions containing FUS, TDP43, p62 and ubiquitin in neurons of all examined non-SOD1 ALS patients (Deng et al. 2010). Similar inclusions of other proteins tend to be hyperphosphorylated and ubiqutinylated, though this is not thought to be the case with FUS inclusions (Blokhuis et al. 2013). Cytoplasmic inclusions of FUS notably include components of stress granules such as poly(A)-binding protein 1 (Dormann et al. 2010).

Stress granules are transient cytoplasmic aggregates of mRNAs and translation pre-initiation complexes - possibly involved in selectively marking different mRNA species for translation or degradation (Anderson & Kedersha 2009), sequestering signalling proteins to promote cell survival during stress (Takahara & Maeda 2012) and reducing energy intensive activities such as ribosome biogenesis (Bentmann et al. 2013). Mutant FUS has been observed being recruited to these structures (Bosco et al. 2010; Dormann et al. 2010), it is thought that only the cytoplasmic pool of FUS is recruited for this purpose and that the RNA-binding activity at the C-terminal end (near the ZnF) is vital for this (Bentmann et al. 2012). Although this is associated with mutant FUS it was shown that upon application of oxidative stress that the mutated FUS recruited wild-type FUS into the stress granules as well (Vance et al. 2013) and that hyperosmolar stress can recruit wild-type FUS even in the absence of mutant protein (Sama et al. 2013). Protein aggregates in other diseases also often contain stress granule related proteins, and other proteins associated with ALS (such as SOD1, TDP43 and angiogenin) are associated with the structures (Bentmann et al. 2013).

It is worth noting that FUS inclusions are not unique to ALS with FUS-positive intranuclear inclusions appearing in a variety of disorders such as Huntingdon's disease and some varieties of FTLD (Woulfe et al. 2010). Indeed FUS-FTLD is one of the three major subtypes of FTLD (albeit by far the least common of them), with the others being associated with inclusions of tau or TDP43 (MacKenzie et al. 2010). FTLD-FUS inclusions differ from those in FUS-ALS as the protein within usually lacks mutations, is unmethylated (Dormann et al. 2012) and co-localises with other FET proteins and transportin 1 in inclusions (Brelstaff et al. 2011; Neumann et al. 2012). FUS-FTLD and FUS-ALS are therefore often thought to have distinct pathogenic mechanisms (Neumann et al. 2012; Dormann & Haass 2013), although it is worth noting that even though the FUS pathology of the two diseases seem distinct that

there are cases of neurodegeneration intermediate between ALS and FTLD in phenotype (Lillo et al. 2012). The role of FUS in FUS-FTLD pathogenesis is currently unknown in any case (Kurz et al. 2014).

Some mechanisms integrate elements of both loss of function in nuclear import of FUS and of inclusion toxicity - for instance one proposed ("two-hit") mechanism of ALS-FUS inclusion formation is that dysfunctional nuclear import of FUS combined with formation of stress granules (due to stressors such as oxidative damage or hypoxia) leads to accumulation of FUS in these stress granules. These then convert into insoluble FUS inclusions through an unknown mechanism (Dormann et al. 2010). As FUS is very prone to aggregation in cell-free systems and most mutations that are not in the NLS are in the SYGQ prion-like region (Polymenidou & Cleveland 2011) a prion-like mechanism has been suggested. A defect in autophagy was also suggested (Dormann & Haass 2013) as p62, a protein involved in degrading protein aggregates (Pankiv et al. 2007), is a common component of protein aggregates in a range of neurodegenerative diseases (Zatloukal et al. 2002). Finally a study demonstrated that FUS can form metastable liquid compartments at sites, including stress granules, that can then convert into a more thermodynamically stable solid fibrillar state in a process accelerated by FUS mutations or increased local FUS concentration (Patel et al. 2015).

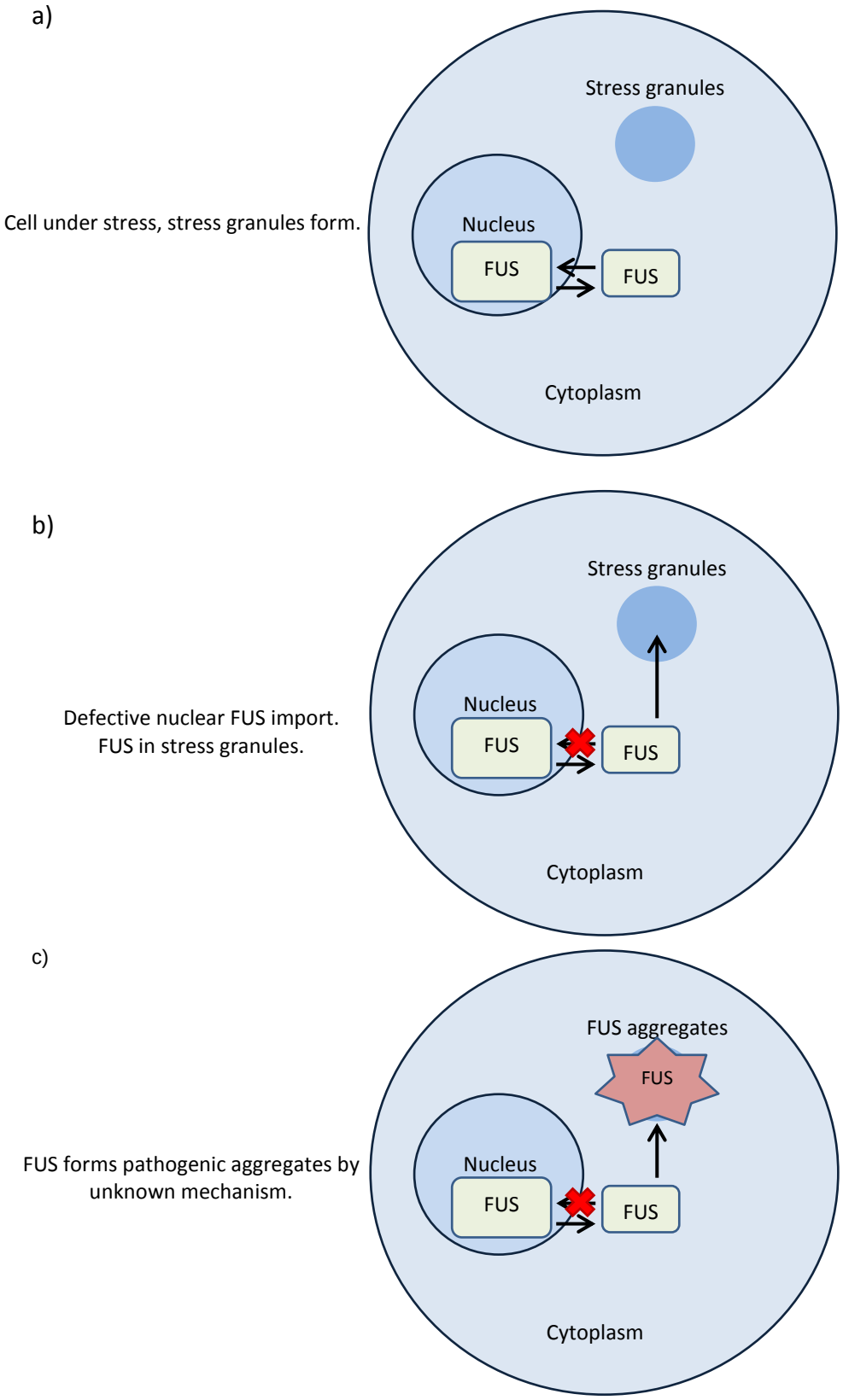


Figure 1.2 | **A two-hit hypothesis of FUS aggregate formation.** a) Cellular stress leads to formation of stress granules in the cytoplasm. FUS shuttles between the nucleus and cytoplasm, at a higher concentration in the nucleus. b) Impaired nuclear import leads to accumulation of FUS in these granules. c) FUS-containing stress granules form into pathogenic aggregates.This may involve a liquid to solid transition, accelerated by increased FUS concentration or pathogenic mutations.

1.3 Trans-activating response region DNA-binding protein with a molecular mass of 43kDa (TDP43)

Unlike FUS TDP43 was not identified in the context of cancer biology but rather in a virology paper - where it was found that it bound, and repressed gene expression from, the trans-activating response element of the HIV-1 virus (Ou et al. 1995). However the protein was later found to be a component of protein aggregates in many case of ALS and FTLD (Neumann et al. 2006) and furthermore mutations in the gene were found to be causative for familial ALS (Lagier-Tourenne & Cleveland 2009) - with the mean age of onset of TDP43-ALS almost ten years later than that of FUS-ALS (Zufiría et al. 2016). Therefore, like FUS, the protein is heavily studied in the context of neurodegenerative disease.

TDP43 consists of a NLS, two RRM domains (the latter of which also contains a nuclear export sequence) and a C-terminal glycine-rich domain. The various RNA binding functions of TDP43, discussed later, are largely related to RNA binding via its RRM1 domain (Buratti & Baralle 2001; Wang et al. 2004; Ayala et al. 2005) and a structure of TDP43's RRM2 domain bound to DNA exists (Kuo et al. 2009). The glycine-rich domain of TDP43 is associated with binding of the protein to heterogenous nuclear ribonucleoproteins (Buratti et al. 2005; D'Ambrogio et al. 2009; Ling et al. 2010; Freibaum et al. 2010) and is thought to be a prion-like domain (Cushman et al. 2010). Similarly to FUS TDP43 is found primarily in the nucleus but is not absent from the cytoplasm, and has been shown to shuttle between these compartments (Winton et al. 2008; Youhna M. Ayala et al. 2008).

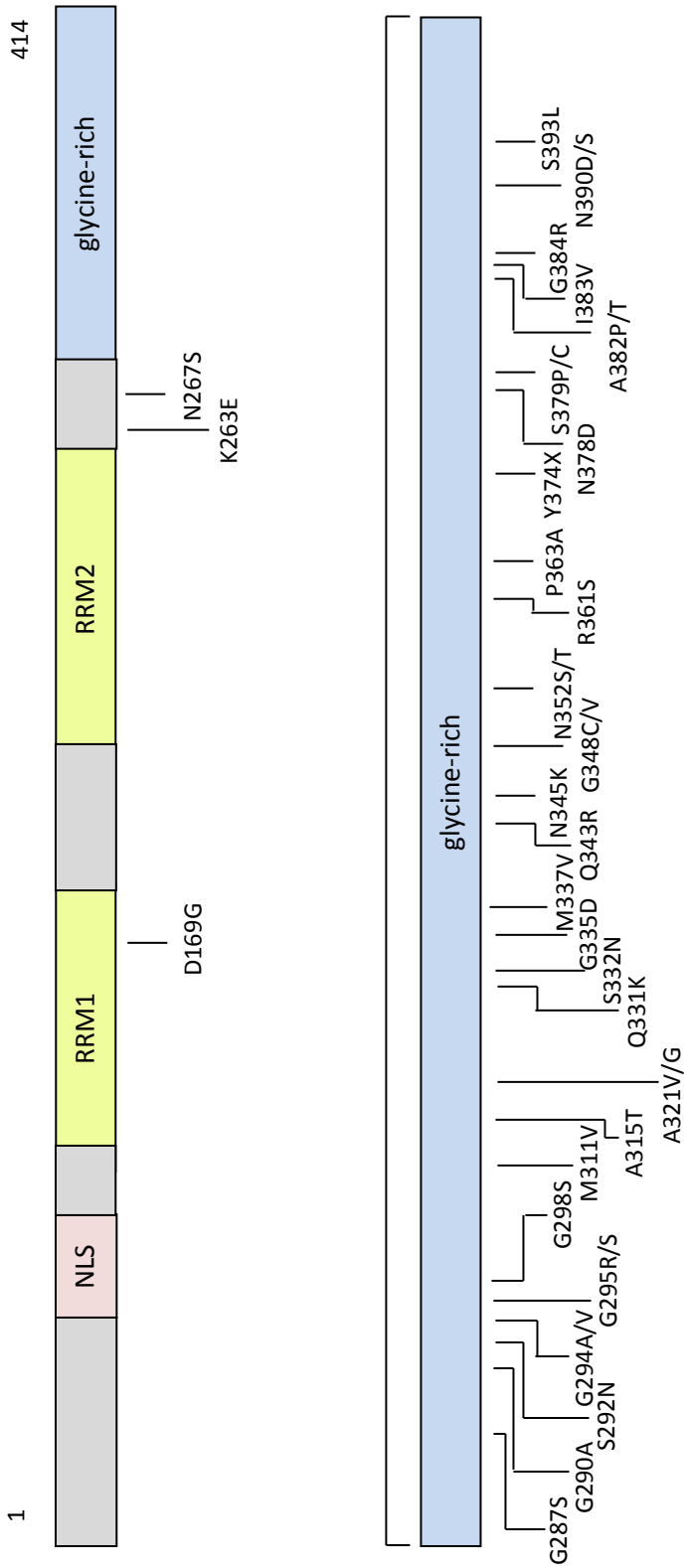


Figure 1.3 | **Diagram of TDP-43 primary structure and location of ALS-causative mutations.** The vast majority of known mutations are clustered near the C-terminal glycine rich domain, the number of mutations in each domain is listed here. List of mutations sourced from Lee et al. 2012. Unlike with FUS, there are many documented mutations of TDP43 in sporadic ALS cases (Lagier-Tourenne & Cleveland 2009). X = termination

Like FUS TDP43 is a nucleic acid binding protein and like FUS a preference for specific RNA sequences has been observed with TDP43 (for UG repeats) but the protein has also been found bound to unrelated sequences (Buratti & Baralle 2001; Buratti et al. 2004; Ayala et al. 2005; Polymenidou et al. 2011; Tollervey et al. 2011). High-throughput studies have indicated that it binds to a very large number of RNA species and has a preference for introns, 3' untranslated regions and non-coding sequences. TDP43 binds many transcripts associated with synaptic activity or neuronal development and has a preference for binding long transcripts (Polymenidou et al. 2011), another similarity to FUS. These studies also indicated that TDP43 mediates the splicing of many of these transcripts, a finding in agreement with other studies indicating a role for TDP43 in regulating splicing of various mRNAs (Buratti et al. 2001; Mercado et al. 2005; Bose et al. 2008; Dreumont et al. 2009; Costessi et al. 2014). It also regulates its own expression - the initial report showing this proposed that it bound its own 3' untranslated region, that its autoregulation worked partially through promoting mRNA instability and partially through exosome mediated protein degradation but not via alternative splicing (Ayala et al. 2011). However other studies have implicated splicing and choice of polyadenylation site as key factors in TDP43 autoregulation (Eréndira Avendaño-Vázquez et al. 2012). It is also known to affect expression of long noncoding RNAs (Tollervey et al. 2011; Liu et al. 2012; Guo et al. 2015; Li et al. 2015) though the mechanisms by which it does this remain unknown (Ratti & Buratti 2016).

TDP43 is also associated with mRNA turnover, as it been shown to regulate the levels of some transcripts (Strong et al. 2007; Y. M. Ayala et al. 2008; Fiesel et al. 2010; Godena et al. 2011) including histone deacetylase 6 and microtubule associated proteins and is known to have a role in miRNA biogenesis - affecting their levels and binding the Drosha complex which processes miRNAs (Gregory et al. 2004; Fukuda et al. 2007; Buratti et al. 2010). It has also been proposed to regulate RNA trafficking (Elvira 2005; I. F. Wang et al. 2008), particularly along axons (Alami et al. 2014). It is also a known component of stress granules (Volkening et al. 2009; Colombrita et al. 2009; Nishimoto et al. 2010; Freibaum et al. 2010; Liu-Yesucevitz et al. 2010; Meyerowitz et al. 2011) and has a role in regulating their dynamics (Dewey et al. 2011; McDonald et al. 2011). 35-kDa C terminal fragments of TDP43, also colocalise with stress granules (Freibaum et al. 2010; Nishimoto et al. 2010).

TDP43 also binds FUS (Ling et al. 2010; Freibaum et al. 2010), itself a protein associated with RNA, and also binds single stranded DNA (Buratti & Baralle 2001; Ayala et al. 2005; Acharya et al. 2006; Abhyankar et al. 2007; Kuo et al. 2009; Furukawa et al. 2011). The protein was identified for its binding to the trans-activating response region motif in HIV DNA (Ou et al. 1995) and is known to inhibit transcription of these sequences but the mechanism by which it does this remains unknown (Lee et al. 2012).

As with FUS mutations in TDP43 can cause neurodegenerative disease - in this case both ALS and FTLD (Geser et al. 2010), approximately 5% of familial ALS cases are related to TDP-43 mutation (Dormann & Haass 2011). Most of these mutations are dominant missense mutations (Lee et al. 2012). Mutations in the gene encoding TDP43 are known to promote FUS and TDP43 association (Ling et al. 2010) and *D. melangogaster* models of neurodegeneration have shown TDP43 and FUS acting in the same genetic pathway (Wang et al. 2011; Lanson et al. 2011) - so it is possible that pathogenesis between the two proteins is linked.

Furthermore TDP43 is regularly found in the cytoplasmic inclusions characteristic of many neurodegenerative diseases. As previously mentioned TDP43-FTLD is a major subtype of the disease, comprising 45% of cases (Lee et al. 2012), but TDP43-positive inclusions are also extremely common in ALS even in non TDP43-ALS cases (Neumann et al. 2006), except those associated with mutations in FUS or SOD1 (Mackenzie et al. 2007; Kwiatkowski et al. 2009; Lee et al. 2012), and have been observed in other neurodegenerative diseases too. Typically the morphologies of the inclusions are much more diverse in TDP43-FTLD than in TDP43-ALS, where they are fairly uniform (Lee et al. 2012). Mutations in the NLS of TDP43 have been shown to increase aggregate formation in the cytoplasm of cells and, similarly to FUS, recruit non-mutant TDP43 into these aggregates (Nonaka et al. 2009) and post-mortems of TDP43-FTLD and TDP43-ALS patients have found a deficiency in components of the classical nuclear import pathway through which TDP43 is shuttled (Nishimura et al. 2010). Overexpression of truncated TDP43 C-terminal fragments or seeding of pre-formed inclusions also encourages inclusion formation (Winton et al. 2008; Nonaka et al. 2009; Igaz et al. 2009; Furukawa et al. 2011) and oxidative stress has been reported to promote TDP43 aggregation by oxidising cysteine residues (Cohen et al. 2012).

However there is some evidence from rat neurons that the toxicity of TDP43 depends more on the amount of expression in the cytoplasm rather than formation of inclusions or mutations in the protein (Barmada et al. 2010), though this is not uncontroversial as, load of inclusions in neurons is inversely correlated with (though not necessarily causal of) longer survival times in TDP43-ALS patients (Nishihira et al. 2009). In addition some animal models of TDP43 have produced results that are in contrast with features of the disease in humans even while still recapitulating neurodegeneration - such as the TDP43-positive inclusions sometimes being rare (Wegorzewska et al. 2009; Stallings et al. 2010), the presence of mitochondrial inclusions (Shan et al. 2010; Xu et al. 2010) and even motor dysfunction being accompanied by only mild mortality of lower motor neurons (McGoldrick et al. 2013).

Most TDP43 inclusions include protein phosphorylated at serines 409 and 410 and truncated 20-25-kDa C-terminal fragments of the protein (Hasegawa et al. 2008b; Neumann et al. 2009) and many also contain ubiquitinated TDP43 (Neumann et al. 2006). The mechanisms of phosphorylation and its importance in pathogenesis remain unclear, though it is clear that it is associated with insolubility of the TDP43 protein (Lee et al. 2012) and that phosphorylated TDP43 has a longer half life than unmodified protein, suggesting it might have a degree of resistance to protein degradation (Zhang et al. 2010).

Granular, less dense inclusions thought to represent an earlier stage in inclusion development are often ubiquitin negative (Strong et al. 2007; Giordana et al. 2010) implying that ubiquitination of TDP43 is a later process in inclusion formation. This may suggest a defect in the turnover of the protein as part of pathogenesis, as TDP43 is degraded by the ubiquitin-proteasome system, albeit at a very slow rate (Ling et al. 2010; Pesiridis et al. 2011) and inhibition of the deubiqitinating enzyme USP14 inhibits aggregation (Lee et al. 2010). TDP43 is thought be degraded by autophagosomes due to mutations in some of these pathways resulting in TDP43 pathologies. For instance rare cases of familial ALS involve mutations in ubiquilin 2, associated with autophagic protein degradation, and central nervous system tissues samples from these patients demonstrated inclusions not only of this protein but also of TDP43 (Deng et al. 2011). Overexpression of the autophagy associated p62 also inhibits aggregation (Brady et al. 2011).

Finally C-terminal fragments of TDP43, possibly the product of proteolytic cleavage by caspase 3 (Zhang et al. 2007; Y.-J. Zhang et al. 2009; Dormann et al. 2009; Nishimoto et al. 2010), are often found in inclusions even though they are relatively quickly degraded in the cell under normal circumstances (Pesiridis et al. 2011). Curiously in both ALS and FTLD inclusions in the brain are immunoreactive only to C-terminal TDP43 antibodies (suggesting a lack of full length protein) but this was not true in inclusions found in the spinal cord (Igaz et al. 2008). As the C-terminal fragment of TDP43 still contains the domain required for aggregation but not the NLS it is easy to imagine this domain encouraging cytoplasmic inclusion formation. However the amount of C-terminal fragments of TDP43 present in cells has been reported to not correlate with disease status and studies with these fragments in various animal models have been inconsistent (Lee et al. 2012).

There are some, though few, TDP43 inclusions that are nuclear, these are more common in TDP43-FTLD than TDP43-ALS and are even fairly numerous in one TDP43-FTLD subtype (Neumann et al. 2006; Sampathu et al. 2006; Mackenzie et al. 2006; Mackenzie et al. 2011). However most inclusions are cytoplasmic and are also often associated with an increase of the levels of TDP43 in the cytoplasm and a decrease in the nucleus (called nuclear clearance) (Neumann et al. 2006; Lee et al. 2012) leading to similar debate about whether TDP43 pathology is mediated by a gain of function or loss of function mechanism (Lee et al. 2012).

It is thought that TDP43 nuclear clearance is an early event in TDP43 ALS and FTLD pathogenesis, as it is observed in even in cells with ubiquitin-negative TDP43 inclusions (Giordana et al. 2010). As TDP43 autoregulates its expression it can be observed that in mice overexpressing human TDP43 that endogenous expression decreases (Igaz et al. 2011). Moreover in these mice neurodegeneration resembling FTLD and ALS is observed, correlating with endogenous TDP43 expression but not with the number of inclusions found in cells. These data imply nuclear clearance of TDP43 is key for neurodegeneration, possibly simply just because TDP43 is not performing its nuclear activities, and may even suggest that TDP43 inclusions are not necessary for pathogenesis. However the caveats with TDP43 mouse models mentioned previously still apply.

A model integrating a role for TDP43 inclusions with nuclear clearance could be that if TDP43 autoregulation occurs within the nucleus, as has been suggested, that cellular

stresses could result in aggregation of TDP43 in the cytoplasm (possibly in stress granules) and that these may be hard to degrade. Meanwhile, as nuclear TDP43 is depleted expression of the protein may increase, more may get exported to the cytoplasm and formation of aggregates and inclusions would increase. This is the loss of autoregulation model (Polymenidou et al. 2011; Ayala et al. 2011; Lee et al. 2012).

The converse, gain of autoregulation model can also be imagined if TDP43 autoregulation occurs in the cytoplasm. Logically an export of TDP43 to the cytoplasm would increase autoregulation and therefore decrease expression of nuclear TDP43 (Lee et al. 2012) This would differ in that nuclear clearance would be both a cause and effect of excessive export of TDP43 to the cytoplasm and that pathogenicity would be mediated by loss of nuclear TDP43 functions rather than inclusion toxicity.

Finally TDP43, like FUS, has a prion-like domain associated with aggregation and protease resistance (Johnson et al. 2009; Fuentealba et al. 2010; Guo et al. 2011; Gitler & Shorter 2011) and localises to stress granules. It is not unreasonable to hypothesise that the "two-hit" model proposed for FUS may be applicable to TDP43 as well (Dormann & Haass 2011; Parker et al. 2012)

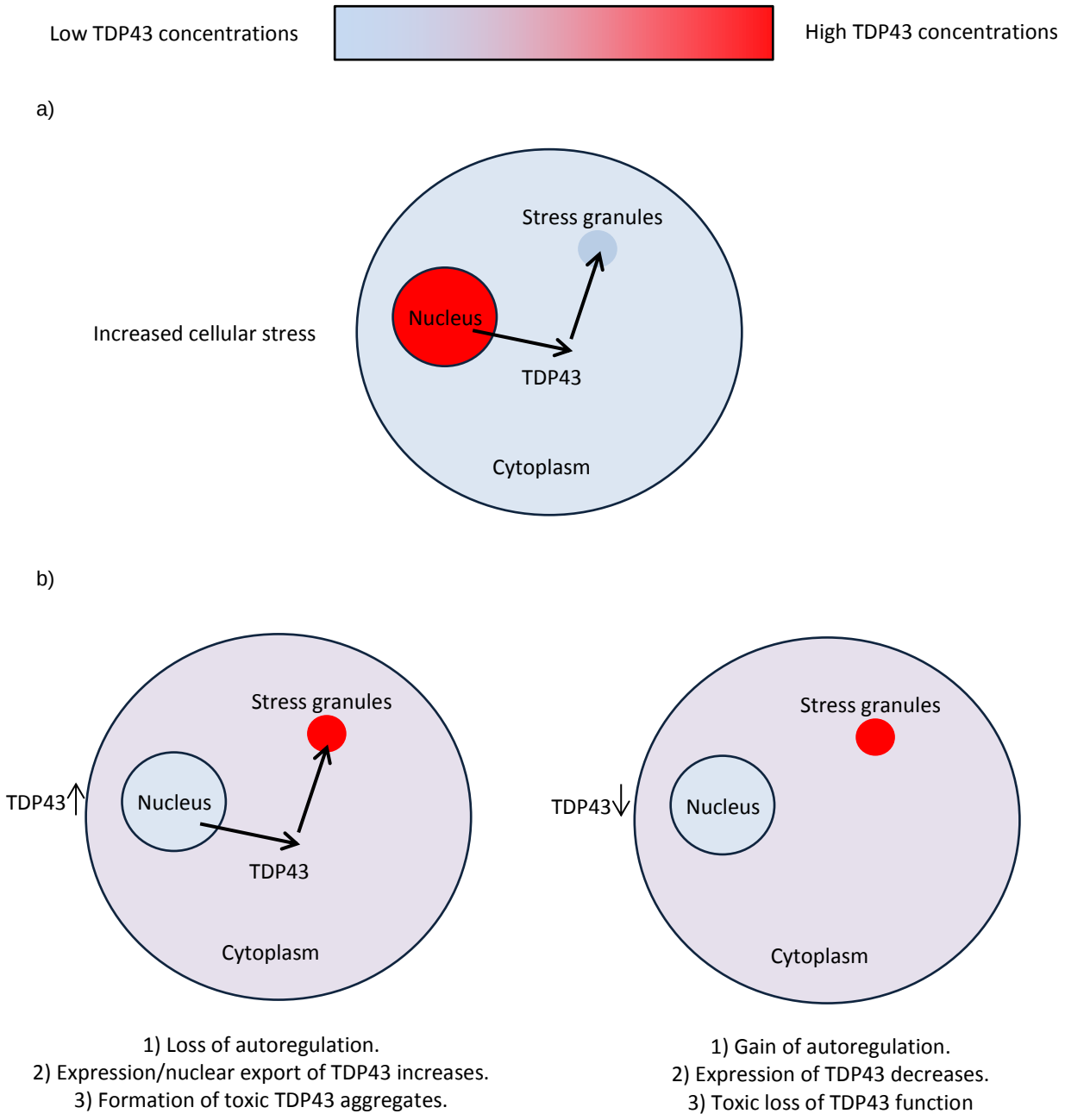


Figure 1.4 | **TDP43 nuclear clearance.** Simple models of the loss and gain of autoregulation models of TDP43 nuclear clearance - the former assumes TDP43 autoregulates its mRNA stability in the nucleus, the latter in the cytoplasm. a) Stressors result in increased cytoplasmic localisation of TDP43 and inclusion in stress granules. Nuclear TDP43 decreases as a result of this export. b) If the autoregulation of TDP43 (mRNA stability) occurs in the nucleus TDP43 expression increases and more is exported, resulting in toxic TDP43 aggregation, possibly in stress granules (loss of autoregulation). If autoregulation occurs in the cytoplasm TDP43 expression decreases, resulting in a toxic loss of TDP43 function being responsible for cell death (gain of autoregulation). In either event nuclear TDP43 concentration is decreased relative to the cytoplasmic concentration.

1.4 DNA damage repair

1.4.1 Single strand break repair

Single strand breaks (SSBs) in DNA are, as the name implies, breaks in a single strand of a DNA duplex - typically this break is not neat and is accompanied by nucleotide loss and damaged termini (Caldecott 2014). SSBs can be introduced from many sources including from ribonucleotide inclusion in DNA (Sparks et al. 2012; Reijns et al. 2012), oxidative attack of DNA (Bradley & Kohn 1979; Pogozelski & Tullius 1998) and the canonical activity of enzymes used in the base-excision and single strand break repair pathways (Pogozelski & Tullius 1998; Demple & DeMott 2002). Other repair enzymes can generate SSBs if their enzymatic activity is aborted mid-catalysis - topoisomerase I (TOP1) (El-Khamisy et al. 2005) is an example of this as the enzyme transiently cleaves and then religates a strand of DNA in order to relax supercoiling - but the religation step of the reaction can be inhibited by proximity to DNA lesions or collisions with transcription or replication machinery (El-Khamisy 2011).

SSBs are thought to be toxic to cells via three mechanisms: conversion to double strand breaks (DSBs) after collision of the SSB with DNA replication machinery (Kuzminov 2001), inhibition of transcription preventing normal cellular function (Kathe et al. 2004) and overactivation of the SSB repair enzyme poly ADP ribose polymerase 1 (PARP1). PARP1 modifies a range of proteins, including itself, with long branched chains of poly ADP ribose in a process dependent on nicotinamide adenine dinucleotide and adenosine triphosphate. Overactivation of the enzyme may be toxic due to depletion of these co-factors leading to necrosis (Andrabi et al. 2006; Heeres & Hergenrother 2007) though it is thought it may also induce caspase-independent apoptosis via release of mitochondrial apoptosis-inducing factor (Yu et al. 2006).

The process of SSB repair can be divided into four distinct steps: detection, DNA end processing, DNA gap filling and DNA ligation. DNA gap filling can be further subdivided into short patch and long patch pathways - where a single nucleotide is replaced or a stretch of nucleotides are synthesised with the newly synthesised nucleotides displacing those already present (Caldecott 2014).

The key protein for SSB detection is PARP1 - it transiently poly ADP-ribosylates a variety of target proteins, is the first line of SSB detection (Satoh & Lindahl 1992; Eustermann et al. 2015) and has been found in assays to increase rates of SSB repair (Fisher et al. 2007; Woodhouse et al. 2008). It is likely that specific levels of poly ADP-ribosylation are necessary as the enzyme reversing this modification, poly ADP ribose glycohydrolase (PARG), has also been shown to be important for maintaining high SSB repair rates (Davidovic et al. 2001; Fisher et al. 2007). The mechanisms by which PARP1 may increase rates of SSB are by modification of chromatin compaction and by recruitment of the scaffold protein X-ray repair cross-complementing protein 1 (XRCC1) (Caldecott 2014). Other members of the PARP family, such as PARP2, are thought to be involved at this step also (Schreiber et al. 2002; Fisher et al. 2007) and the role of PARP proteins in DNA damage repair is not limited to SSB repair (Beck, Robert, et al. 2014).

PARP1 can be located at the linker region between nucleosomes (Kim et al. 2004) and has been shown to relax chromatin (Poirier et al. 1982; Tulin et al. 2002), as discussed previously poly ADP-ribosylation is reversible so it follows that this relaxation would also be reversible. PARP1 targets many chromatin components including itself (Ogata et al. 1981), histones H1 and H2B (Poirier et al. 1982; Tanuma & Johnson 1983; Adamietz & Rudolph 1984; Huletskyl et al. 1989) and the proposed histone chaperone aprataxin-and-PNKP-like factor (APLF) (Iles et al. 2007; Bekker-Jensen et al. 2007; Mehrotra et al. 2011). It is thought that PARP1 reduces the affinity of histones for DNA (Poirier et al. 1982; Kim et al. 2004) by its production of poly ADP ribose chains, which are negatively charged and chemically resemble nucleic acids so may compete with them for binding (Mathis & Althaus 1987; Panzeter et al. 1992) and for which there are putative binding sites on histones (Pleschke et al. 2000; Karras et al. 2005). It is also possible that as PARP1 is found in nucleosome linker regions that it can open higher order chromatin simply by dissociating from the region (Caldecott 2007). It is also thought that PARP inhibitors increase the affinity for both PARP1 and PARP2 for DNA, and result in the enzymes being trapped on the DNA - effectively creating a cytotoxic lesion (Murai, Huang, Das, Renaud, et al. 2012).

XRCC1 (amongst other proteins) binds PARP1 and PARP2 via its poly ADP ribose bindng motif and PARP's BRCT domain and has a preference for the polyribosylated PARPs (Caldecott et al. 1996; Masson et al. 1998; Schreiber et al. 2002; El-Khamisy et al. 2003). This

allows recruitment of XRCC1 to sites of damage (El-Khamisy et al. 2003; Woodhouse et al. 2008). The protein also binds and stimulates a variety of SSB repair proteins such as PNKP, aprataxin, APLF and DNA polymerase β, as well as proliferating cell nuclear antigen (PCNA) (Loizou et al. 2004; Lan et al. 2004; Hirano et al. 2007, Caldecott 2014) and is necessary for the nuclear stability of DNA ligase 3α, forming a stable complex with it (Caldecott et al. 1995; Mortusewicz et al. 2006). In doing this it both concentrates SSB factors at breaks and stimulates their activity, having a key effect on SSB repair even without any known enzymatic activity. Although PARP1 recruitment of XRCC1 is vital, it does also recruit other proteins involved in SSB repair, such as tyrosyl-DNA phosphodiesterase 1 (TDP1), which will be discussed later (Das et al. 2014).

DNA end processing is a diverse process, reflecting the variety of damaged termini that occur in SSBs. Some common termini include the 3' phosphoglycolate terminus (arising from oxidative damage), the 5' deoxyribosephosphate terminus (arising from the activity of apurinic-apyrimidinic endonuclease 1 (APE1) on abasic sites (Izumi et al. 2000)) and the 3' phosphate terminus (which can arise from oxidative damage or the activity of DNA repair enzymes) (Caldecott 2014). These termini can be processed respectively by APE1 (Chen et al. 1991; Winters et al. 1992; Winters et al. 1994; Parsons et al. 2004), DNA polymerase β (Sobol et al. 2000) and polynucleotide kinase 3'-phosphatase (PNKP) (Karimi-Busheri et al. 1999; Jilani et al. 1999). APE1 and PNKP process other termini as well, such as a 3' αβ unsaturated aldehyde termini and a 5' hydroxyl termini respectively (Chen et al. 1991; Izumi et al. 2000; Wiederhold et al. 2004). The 2',3'-cyclic phosphate terminus, the 5' adenosine monophosphate terminus and the 3' TOP1 terminus are termini that occur which cannot be processed with these enzymes. The method of end processing for the former terminus remains unknown (Williams et al. 2013) but not for the latter two termini.

Aprataxin is thought to process the 5' adenosine monophosphate terminus, arising from abortive DNA ligase activity, though the evidence for this comes from *in vitro* work (Ahel et al. 2006; Rass et al. 2007). It is thought that accidental misincorporation of ribonucleotides into DNA increases the rate of abortive activity by DNA ligases (Rumbaugh et al. 1997; Pascal et al. 2004) and aprataxin may reverse adenylation at RNA-DNA junctions in order to allow activity of the RNAse H2-dependent pathway that removes misincorporated ribonucleotides (Tumbale et al. 2014).

TOP1-linked SSBs can be processed by long patch SSB repair, which will be discussed later, or by the actions of TDP1 (Zhang et al. 2011). 3' TOP1 termini arise from abortive TOP1 activity. TOP1 is one of six topoisomerases found in human cells and its role is to relax DNA that has been supercoiled by cellular processes such as replication and transcription. In order to do this it nicks one DNA strand and binds the 3' end of the nicked DNA with its catalytic tyrosine residue - forming a TOP1 cleavage complex (TOP1cc) which is effectively a 3' TOP1 terminus. The damaged strand then rotates at a high speed around the undamaged one until the supercoiling is relaxed, at which point the broken ends realign and a rapid relegation occurs. TOP1 requires no cofactors and also has a mitochondrial isoform that works in the same manner (Pommier et al. 2014). TOP1 can also generate a nick with a 2'-3' cyclophosphate and 5' hydroxyl ends when a ribonucleotide is misincorporated into DNA (Kim et al. 2011; Williams et al. 2013).

Camptothecin (CPT) is a drug that inhibits topoisomerase I (TOP1) at such a stage in catalysis that it generates TOP1ccs. It acts as an interfacial inhibitor - its binding traps a complex In such a conformation that it cannot achieve the topology required to finish the reaction. In this case it intercalates with and deforms downstream DNA and prevents the formation of the topology required for religation (Staker et al. 2002; Pommier & Marchand 2012). Collisions with replication machinery (Hisang et al. 1989; Furuta et al. 2003) or the transcription machinery (Wu & Liu 1997) can convert these into DSBs. CPT-induced TOP1cc formation also results in potent transcriptional inhibition (Bendixen et al. 1990) and it has been noted that nicks very close to TOP1 cleavage sites can directly induce DSBs (Pourquier, Pilon, et al. 1997).

TOP1ccs can be generated without the use of inhibitors and it is likely that physiological sources of TOP1ccs are biologically relevant, as many endogenous and exogenous lesions can trigger them. These include mismatched DNA (Pourquier, Ueng, et al. 1997), 8-oxoguanine bases (Lesher et al. 2002), ultraviolet (UV) induced damage (Lanza et al. 1996; Subramanian et al. 1998) and abasic sites (Pourquier, Ueng, et al. 1997), generated at an estimated rate of 10,000 lesions per cell per day (Lindahl & Nyberg 1972).

In order to process a TOP1cc/3'TOP terminus by short patch repair the majority of the TOP1 protein is degraded by proteolytic digestion or denaturation (Yang et al. 1996; Debethune et

al. 2002) and TDP1 hydrolyses the remaining 3' tyrosine terminus (Pouliot 1999). It can do this with up to approximately 100 residues of TOP1 remaining attached (Interthal & Champoux 2011) generating a 3' phosphate end that can then be processed by PNKP. At low efficiency TDP1 can also process 5' phosphotyrosyl bonds implying an ability to resolve TOP2ccs - the analogous structure to TOP1ccs formed by abortive TOP2 activity (Nitiss et al. 2006; Murai, Huang, Das, Dexheimer, et al. 2012). Although this is its most well known function TDP1 is also implicated in removing nucleotides (RNA or DNA) without 3' phosphorylation (Interthal et al. 2005; Dexheimer et al. 2010) - resolving oxidative DNA damage (Ben Hassine & Arcangioli 2009; El-Khamisy et al. 2009) and lesions resulting from alkylation at abasic sites, as well as in dealing with 3' phosphoglycolate and 3' dexoyribosephosphate ends (Inamdar et al. 2002; Zhou et al. 2005; Zhou et al. 2009; Lebedeva et al. 2011; Murai, Huang, Das, Dexheimer, et al. 2012). In fitting with the broad action of TDP1 at 3' termini it has also been observed to remove fluorophore tags at this site as well as chain-terminating nucleoside analogues (Interthal et al. 2005; Huang et al. 2013).

DNA gap filling via the short-patch method involves replacement of a single nucleotide at the SSB. DNA polymerase β is implicated in this role but DNA polymerases δ and ε are also capable and it has been hypothesised that DNA polymerases λ and ι (associated with DSB repair and translesion synthesis respectively) may also be usable (Fortini et al. 2000; Bebenek et al. 2001; García-Díaz et al. 2001; Braithwaite et al. 2005; Pascucci et al. 2005; Vermeulen et al. 2007). Gap filling by DNA polymerases δ and ε require PCNA (Hashiguchi et al. 2007).

However some termini, for instance oxidised 5' deoxyribosephosphate sites (Sung & Demple 2006), may be subject to the long patch pathway of SSB repair, though other factors such as adenosine triphosphate levels (Petermann et al. 2003) also influence pathway choice. In long patch repair multiple nucleotides are added to the end of the break by DNA polymerases δ and ε (Kim & Wilson III 2012) or β (Sung & Demple 2006), displacing the downstream strand. This forms a relatively long 5' flap adjacent to a 3' flap which is a substrate for flap endonuclease 1 (FEN1) which digests the displaced strand to create a ligatable substrate (Storici et al. 2002; Kim & Wilson III 2012). The RNAse H2-dependent ribonucleotide excision pathway feeds directly into long patch repair (Sparks et al. 2012).

DNA ligation is the final step of the process, with DNA ligase 3α and DNA ligase 1 being used in short and long patch repair respectively (Caldecott 2014).

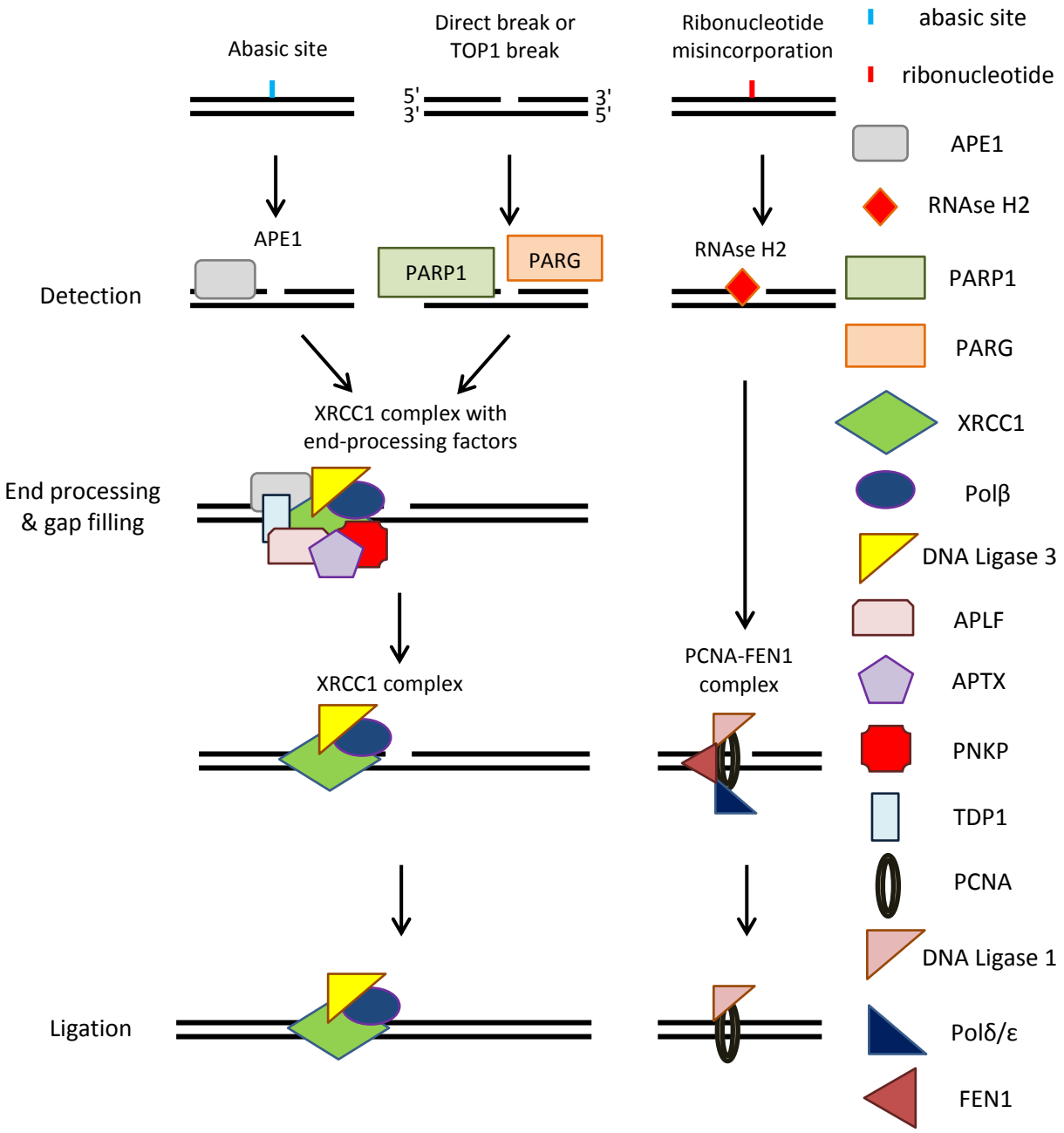


Figure 1.5 | **Single strand break repair.** It is likely that the interactions between XRCC1 and PNKP, APLF and aprataxin are mutually exclusive, but all three are portrayed in the same complex here for simplicity. i) Detection - abasic sites are recognised and cleaved by APE1, misincorporated ribonucleotides are recgonised and immediately processed by RNAse H2 and direct or TOP1 breaks are recognised by PARP1, which produces complex branched poly ADP-ribose chains. PARG is also required. ii) End processing & gap filling - XRCC1 recruits many proteins including end processing proteins to the site of damage, the ends are processed and Polβ fills the gap (short patch) or PCNA recruits FEN1, which resects from the gap - which is then filled in using Polδ/ε (long patch). iii) Ligation - DNA ligase 3 (short patch) or DNA ligase 1 (long patch) ligates the DNA, finishing the processing of the SSB.

1.4.2 Double strand break repair

DSBs are toxic lesions that can cause substantial genomic rearrangements and cell death, it is therefore imperative for the cell to resolve them. There are two major DSB repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ), with the former only operating in the S and G2 phases as it requires a sister chromatid for use as a template (Liu et al. 2014) - though other pathways such as the PARP1 driven and highly mutagenic alternative end joining/alternative NHEJ exist (Wang et al. 2006; Q. Cheng et al. 2011). HR and canonical NHEJ also have roles outside of DSB repair - for instance HR is used to resolve stalled replication forks and NHEJ in the maturation of cells in the adaptive immune system (Liu et al. 2014).

HR begins with recruitment of the MRN complex (named after the components Mre11, Rad50 and Nbs1) to DSB ends (Lamarche et al. 2010; Stracker & Petrini 2011). This carries out an initial resection at the break using endonuclease followed by exonuclease activity (the former cut promotes HR over NHEJ) (Shibata et al. 2014), promoted by the stimulation by CtIP (which is rapidly degraded outside of S and G2 phases) of Mre11's endonuclease activity (Jazayeri et al. 2006; Sartori et al. 2007). The presence of MRN recruits a complex of ataxia-telangiectasia mutated (ATM) and the histone acetyltransferase Tip60, although there are regulatory proteins such as ATMIN that can modulate ATM's interaction with MRN (Paull 2015). Tip60 acetylates ATM which then phosphorylates itself on serine 1981, converting itself from an inactive dimer/multimer to an active monomer (Bakkenist & Kastan 2003; Sun et al. 2005; Sun et al. 2009), and also phosphorylates a wide variety of other targets (Matsuoka et al. 2007; Bennetzen et al. 2010) including the mediator kinase Chk2 and p53 (Liu et al. 2014), key in the wider DNA damage response. Another key target of ATM is the histone H2AX, the phosphorylated form of which is known as γH2AX (van Attikum & Gasser 2009). Mediator of damage checkpoint 1 can bind the phosphorylated histone and allows a positive feedback loop, as it has a site that binds Nbs1 so it can effectively recruit more ATM (Spycher et al. 2008; Chapman & Jackson 2008), this leads to spreading of the γH2AX modification to large regions around DSBs - this is key for DSB repair processes though not critical for other signalling functions of ATM via p53 or checkpoint kinase 2 (Chk2) (Fernandez-Capetillo et al. 2002; Kang et al. 2005; Maréchal & Zou 2013).

Because of this detection by immunofluorescence of γH2AX foci can be used as a quantitative measure of DNA damage in cells (Rogakou et al. 1998; Löbrich et al. 2010).

ATM phosphorylates mediator of damage checkpoint 1 and the ubiquitin E3 ligase RNF8 binds the protein, RNF8 and (later) RNF168 ubiqutinates histone H2A (Mattiroli et al. 2012; Gatti et al. 2012; Bartocci & Denchi 2013; Zhao et al. 2014) possibly displacing any Ku heterodimers bound to DSBs (Feng & Chen 2012) and creating sites for recruitment of p53-binding protein 1 (53BP1) and breast cancer 1 (BRCA1) (Liu et al. 2014), the latter also being a direct phosphorylation target of ATM (Cortez et al. 1999). This allows cross-talk between HR and NHEJ, as the former protein is strongly associated with NHEJ (a process ATM is also associated with (Zha et al. 2011)) and the latter with HR - with 53BP1 thought to be a barrier for BRCA1-mediated HR (Bunting et al. 2010; Bouwman et al. 2010), and therefore essential for NHEJ. It also acts as a scaffold protein and as an amplifier of ATM signal (Panier & Boulton 2014).

A second longer-range resection is performed using DNA exonuclease 1 or a combination of DNA replication helicase 2 with BLM. It is unclear whether the BLM complex and DNA exonuclease 1 interact or work in parallel (Bolderson et al. 2010; Nimonkar et al. 2011; Tomimatsu et al. 2012). After resection a 3' overhang is produced which is bound by the single stranded DNA binding protein replication protein A (RPA), in turn displaced by Rad51 which forms a nucleoprotein filament which can invade a sister chromatid and form a transient D-loop in complex with a wide array of other proteins (Liu et al. 2014). Regulation of Rad51 displacement of RPA is complex, with BRCA2 as part of the BRCC complex (also containing BRCA1) being a major factor in the process (Sy et al. 2009; F. Zhang et al. 2009; Holloman 2011). The 3' end of the invading strand acts as a primer for elongation using the invaded chromatid as a template, requiring removal of Rad51 by Rad54 and Rad54B - the machinery used for DNA polymerisation is not well characterised though PCNA and DNA polymerases δ, κ and ν are implicated (Sebesta et al. 2013). In non-meiotic cells typically a short sequence is elongated from the invading strand (forming a Holliday junction), which then anneals with complementary DNA on the other side of the DSB, any gaps are filled and the DSB religated (Liu et al. 2014) - RTEL1 or BLM can promote disassembly of the D-loop in this manner (Bachrati et al. 2006; Barber et al. 2008). This subpathway is known as synthesis-dependent strand annealing and is the most common pathway in mitotic cells.

Alternatively the other end of the DSB is captured and a double Holliday junction formed, which can be cleaved to form crossover or non-crossover products. A variant on HR called single strand annealing also exists where there are two repeat sequences at either end of the DSB - the process repairs the break but deletes one copy of the repeat (Liu et al. 2014).

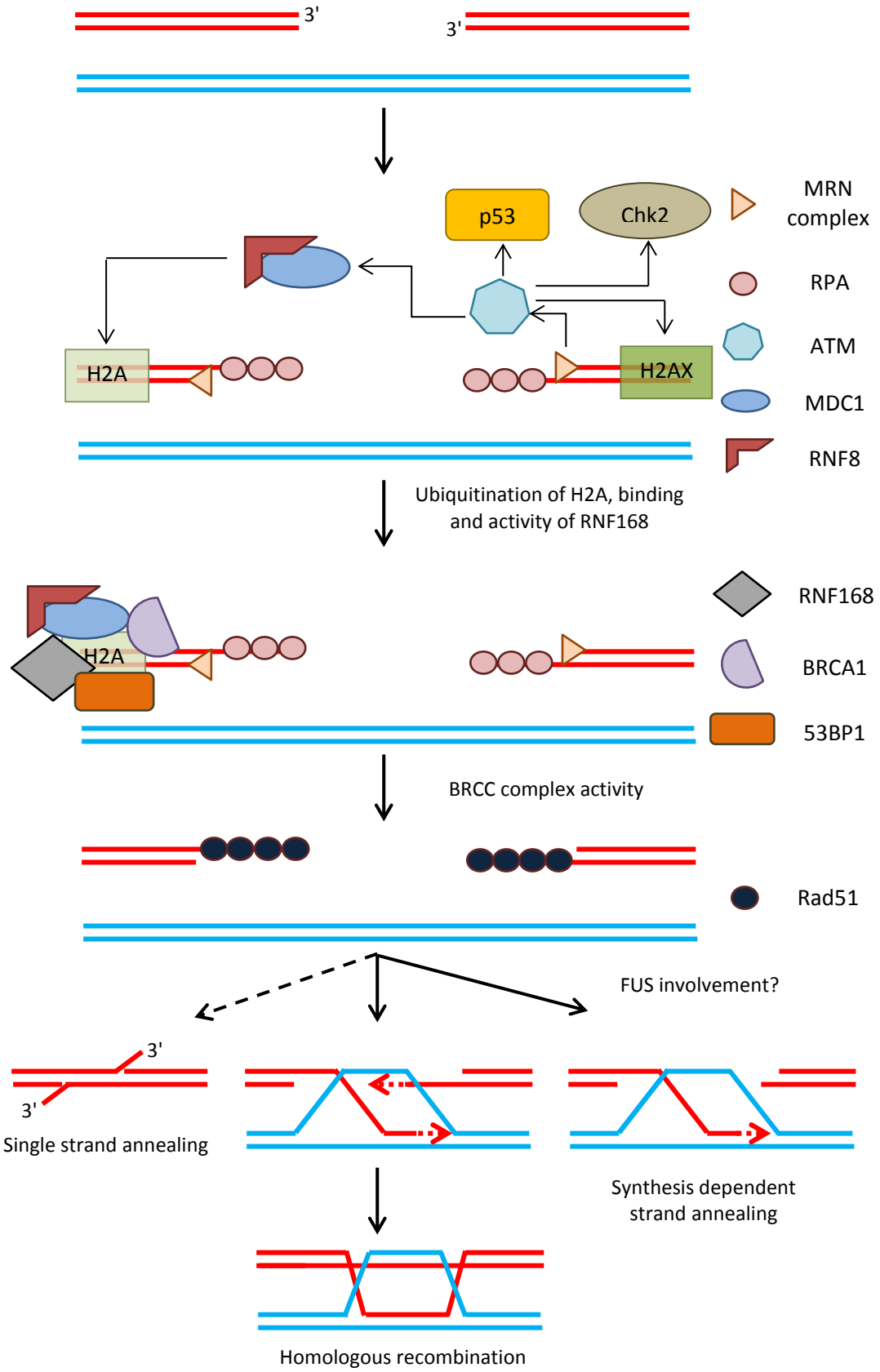


Figure 1.6 | **The homologous recombination pathway.** The second resection by DNA exonuclease 1 and/or BLM complex not pictured. The double Holliday junction in HR can be resolved to generate crossover or non-crossover products. Synthesis dependent strand annealing never generates crossover products.

In NHEJ the repair process is started by binding of a heterodimer of Ku70 and Ku80 to the ends of DSBs which stabilise the ends by forming a synaptic complex across the break and act as a scaffold for protein recruitment (Pang et al. 1997; Walker et al. 2001; Liu et al. 2014). Although associated with NHEJ there is also some evidence that Ku may stabilise the ends of DSBs initially even when HR is ultimately chosen as a repair pathway (Shao et al. 2012). A key protein recruited is the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) which is phosphorylates itself (Uematsu et al. 2007) and the nuclease Artemis (Soubeyrand et al. 2006), it is thought that the presence and activity of DNA-PKcs helps stabilise the NHEJ complex (Davis & Chen 2013) and help form a synaptic complex across the break (Pang et al. 1997; Cary et al. 1997). A great deal of other targets of DNA-PKcs have been identified *in vitro*, but not verified *in vivo* - these include the majority of NHEJ factors mentioned in the next two paragraphs, the Ku proteins, p53 and the histone H2AX (Davis & Chen 2013). Phosphorylation of itself is likely to allow its dissociation - two key clusters of phosphorylation sites for this have been identified, the serine 2056 site (B. P. C. Chen et al. 2005) and the threonine 2609 site, which is also a target for ATM and the related kinase ataxia- and Rad3-related (ATR) (Chan & Chen 2002; Yajima et al. 2006; Chen et al. 2007).

Independently of DNA-PKcs XRCC4 (a scaffold protein) is recruited along with DNA ligase 4 and XRCC4-like factor (XLF) (Nick McElhinny et al. 2000; Mari et al. 2006) - this complex can form a filament to bridge DSB ends (Liu et al. 2014), is stabilised and stimulated by APLF (Grundy et al. 2012) and is responsible for religation. Notably DNA ligase 4 can ligate across gaps and between DNA ends that are not compatible, an activity stimulated by XLF (Ahnesorg et al. 2006; Tsai et al. 2007; Lu et al. 2007; Gu et al. 2007).

Just like with SSBs the ends of DSBs can be damaged. The Ku complex itself resolves 5' deoxyribosephosphate termini (Roberts et al. 2010) and a range of end processing enzymes such as PNKP (Koch et al. 2004) and aprataxin (Clements et al. 2004) are recruited to deal with other damage. Also recruited are a range of DSB end resecting proteins such as APLF which acts as a nuclease generating 3' overhangs for the XRCC4 complex in *in vitro* end joining assays (S. Li et al. 2011). WRN is also recruited, with Ku and XRCC4 known to stimulate its 3' to 5' exonuclease activity only (Cooper et al. 2000; Kusomoto et al. 2008; Davis & Chen 2013). Artemis is implicated in many activities such as removal of 3' phosphoglycolate termini, 5' to 3' exonuclease activity on single stranded DNA and an

endonuclease activity which nicks 5' overhangs to generate blunt ends. The last of these activities requires DNA-PKcs phosphorylation (Ma et al. 2002; Povirk et al. 2007) and it is possible that it is also ATM-regulated (Davis & Chen 2013).

DNA polymerases μ and λ are also recruited for gap filling (Mahajan et al. 2002; Ma et al. 2004). μ is normally template dependent but can work with a discontinuous template in the presence of the Ku and XRCC4 complexes (McElhinny et al. 2005), whereas λ is template-independent and has lyase activity to remove damaged bases (Ramadan et al. 2004). This introduces the possibility of errors in DSB repair by NHEJ.

These steps have conventionally been thought to occur in a set order - Ku is recruited, followed by DNA-PKcs, followed by processing enzymes and finally the XRCC4-Ligase 4 complex (Davis & Chen 2013). However recruitment of the XRCC4 complex is not dependent on DNA-PKcs and the stepwise recruitment model has been called into question (Yano & Chen 2008; Reynolds et al. 2012) as XRCC4 is key for recruitment of many "earlier" NHEJ factors, for instance PNKP and aprataxin are known to bind XRCC4 in a casein kinase 2 dependent manner (Koch et al. 2004; Clements et al. 2004). Optimal PNKP binding to XRCC4 also require its phosphorylation by ATM and DNA-PKcs (Zolner et al. 2011) however. It may be the case that simple DSBs can be repaired using by NHEJ only the Ku and XRCC4 complexes, but that more complex breaks require DNA-PKcs and possibly ATM (Davis & Chen 2013). It is also worth noting that other processes, such as regulation of chromatin state, can be vital for NHEJ - as an example histone deacetylases 1 and 2 are heavily implicated in the process (Miller et al. 2010).

Topoisomerase activity can generate DSBs as, unlike TOP1, TOP2 operates on DNA duplexes and allows decatenation of DNA, though it also relaxes supercoiling. Two TOP2 proteins form a homodimer (of either TOP2α or TOP2β (Wu et al. 2011)) and each unit cleaves a strand, binding with their catalytic tyrosines to the 5' end of the nicked DNA on their respective strands. This generates a TOP2cc, a transient DSB, through which another DNA duplex is moved before relegation occurs. Unlike TOP1 TOP2 requires metal ion cofactors and ATP hydrolysis for function (Pommier et al. 2014), but many of the same lesions can trigger TOP2cc formation - such as abasic sites, 8-oxoguanine (Sabourin & Osheroff 2000) and UV damage (Corbett et al. 1991), though there are also lesions that are only known to

form TOP1ccs or TOP2ccs (Ledesma, El Khamisy, et al. 2009). The activity of the TOP2 inhibitor etoposide is also analogous to the activity of CPT on TOP1, trapping the enzyme in a cleavage complex by misaligning the ends of the nicks (Wu et al. 2011).

TOP2ccs have an enzyme that can process them analogous to TDP1 - TDP2. TDP2 was shown to process TOP2ccs after proteolytic degradation of TOP2 (Mao et al. 2001; Ledesma, El-Khamisy, et al. 2009) and has weak activity on 3' phosphotyrosyl bonds (Ledesma, El-Khamisy, et al. 2009; Zeng et al. 2012) and can therefore process TOP1ccs. Unlike TDP1 its activity is solely confined to hydrolysing phosphotyrosyl bonds (Gao et al. 2012) and has a preference for 5' tyrosyl bonds at the end of single-stranded DNA or termini with a 5' overhang. The protein generates a DSB with 4 base overhangs and undamaged termini, a substrate for Ku and DNA ligase IV. Although NHEJ is considered an error-prone process it has been proposed that NHEJ processes involving TDP2 are not and constitute an error-free subpathway of NHEJ (Gómez-Herreros et al. 2013), though repair of TOP2ccs involving resection can still be error-prone. TDP2 has also been proposed to act on TOP3ccs, analogous structures to TOP1ccs or TOP2ccs from the activity of the TOP3 proteins, which work on single stranded DNA or RNA (Pommier et al. 2014) and the protein has roles in signal transduction (C. Li et al. 2011; Do et al. 2012) distinct from its DNA repair functionality.

Finally ADP-ribosylation also has a significant role in DSB repair with PARP1 and PARP3 being stimulated by DSBs (Haince et al. 2008; Langelier et al. 2011), with PARP activation at DSB resulting in recruitment of chromatin remodelling complexes (Liu et al. 2014) and with many proteins involved in both branches DSB repair known to bind poly ADP ribose - these proteins include BRCA1, ATM, Ku70, Mre11, PARP9 and DNA-PKcs. It is likely that this results in a rapid recruitment of proteins to the site of damage (Beck, Robert, et al. 2014). PARP3 is known to promote NHEJ over HR and alternative end joining through two proposed pathways: acting in concert with aprataxin-and-PNKP-like factor to accelerate the action of XRCC4-DNA ligase 4 at DSBs (Rulten et al. 2011), thereby promoting NHEJ, and poly ADP-ribosylating the Ku70/Ku80 heterodimer to inhibit Mre11 driven end resection (Beck, Boehler, et al. 2014), thereby inhibiting HR and alternative end joining. PARP1's relevance in NHEJ remains much more unclear (Beck, Robert, et al. 2014) but it has a complex role regarding DSB repair pathway choice at stalled replication forks, having been shown to both

promote (Hochegger et al. 2006; Haince et al. 2008; Bryant et al. 2009) and inhibit (Ying et al. 2012) HR under different conditions - and to promote fork slowing and reversal over DNA repair in others (Ray Chaudhuri et al. 2012), presumably to discourage DSB formation in the first place. Both PARP1 and PARP2 are also implicated in reducing illegitimate recombination events (Beck, Robert, et al. 2014).

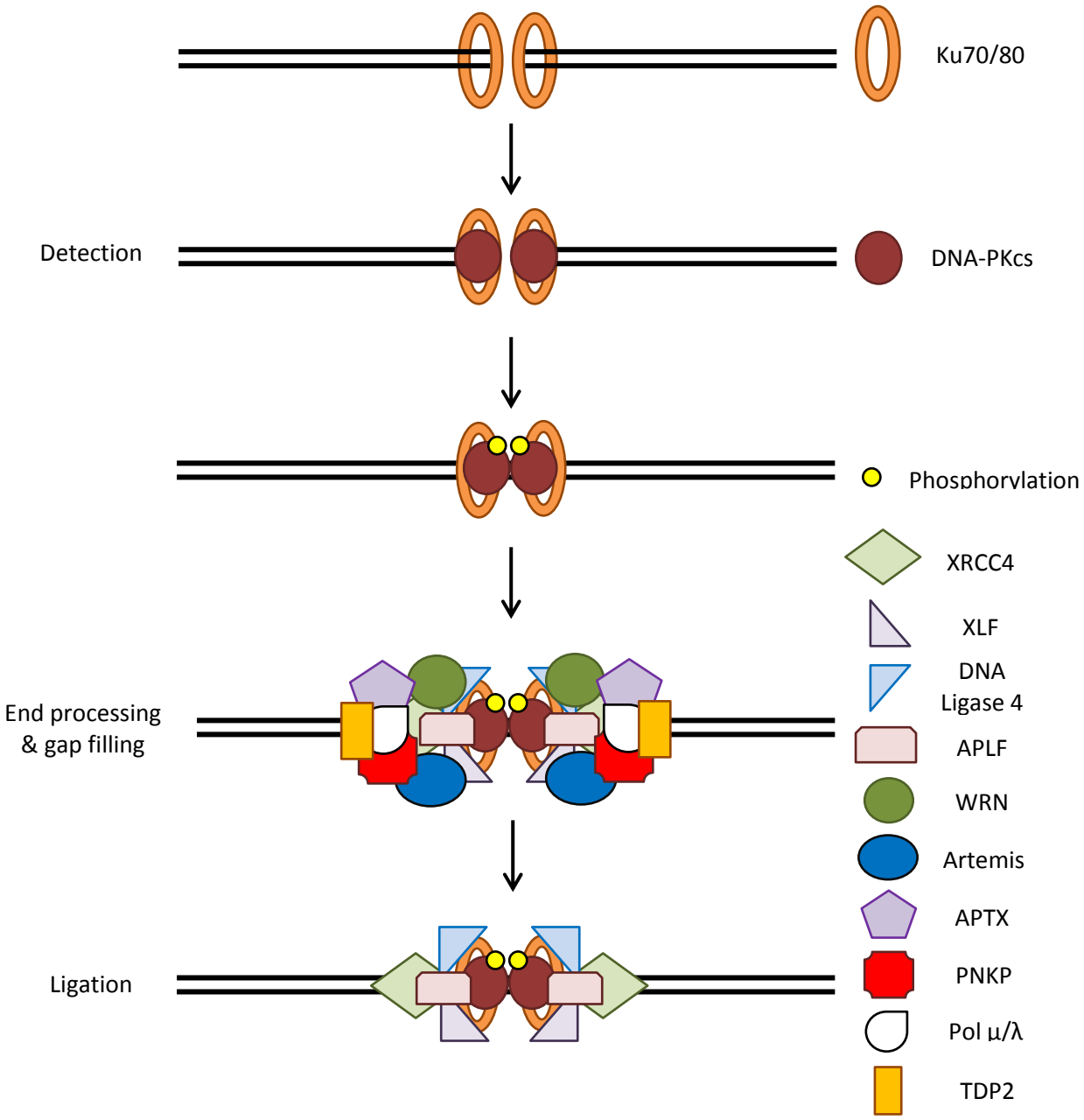


Figure 1.7 | **The classical non-homologous end-joining pathway.** The pathway is presented in the conventional step-wise model, though it is worth noting that there is some doubt about how step-wise the process is. XRCC4 complex may form filaments crossing the break, this is not depicted here. i) Detection - Ku70/80 binds the DSB, recruiting DNA-PKcs which then autophosphorylates and forms a synapse across the break. ii) End processing & gap filling - XRCC4 recruits a wide variety of proteins including APLF, polymerases, end processing factors, nucleases and DNA ligase 4. These process (or remove and replace) damaged termini and DNA polymerase μ or λ fills in the gap. iii) Ligation - a core XRCC4 complex consisting of XRCC4, APLF, XLF and DNA ligase 4 remains at the break. DNA ligase 4 performs the final religation.

1.5 Transcription

Nuclear transcription in humans is performed by three seperate RNA polymerases (RNAPs): RNA polymerase I, RNA polymerase II and RNA polymerase III. The first of these operates within the nucleolus and is responsible for production of pre-ribosomal RNA (pre-rRNA), which are processed into the catalytic RNA used in the ribosome. RNAP I is dedicated to synthesis of the precursor 47S transcript that is ultimately processed into every mature rRNA species but the 5S rRNA (Preti et al. 2013; Carron et al. 2011). The remaining two polymerases are nucleoplasmic, though 5S rRNA is transcribed close to the nucleolus (Fedoriw et al. 2012).

RNA polymerase II is involved in many transcriptional processes but is most well known for producing pre-messenger RNA (pre-mRNA) - RNA species that may be translated into proteins after processing. RNA polymerase III is similarly diverse in its functions but is most commonly noted for transcription of transfer RNAs (tRNAs), required for translation, and 5S rRNA (Schramm & Hernandez 2002). Other RNA species such as micro RNAs (miRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) may be transcribed by either RNA polymerase II or RNA polymerase III depending on the exact RNA species, with RNAP II transcription being more common (Egloff et al. 2008; Dieci et al. 2009; Schanen & Li 2011).

1.5.1 RNA polymerase I (RNAP I)

RNAP I transcribes around the nucleolar organiser regions (NORs) containing tandem arrays of ribosomal DNA (rDNA sequences) which encode the 47S sequence (Németh & Längst 2011) and a series of regulatory regions including promoters, enhancers and terminators, producing several million rRNA transcripts per cell per day (Goodfellow & Zomerdijk 2013). Humans have around 400 repeats of the rDNA per cell (Birch & Zomerdijk 2008), though only approximately half are transcribed at any given point (Goodfellow & Zomerdijk 2013) with the remainder found in a heterochromatic state. Furthermore there are variations between the coding sequences of the repeats which are known to be differentially expressed (Tseng et al. 2008; Santoro et al. 2010).

Active rRNA genes are found in a euchromatic state and are enriched in upstream binding factor (UBF) protein, which is key for organising DNA into NORs and decondensing the local chromatin (Chen et al. 2004; Mais et al. 2005; Sanij et al. 2008). The architecture around the

rDNA is complex and the maintenance of is not fully characterised (Goodfellow & Zomerdijk 2013).

The promoter regions for RNAP I transcribed genes include two notable regions, the core promoter and the upstream control element. The core promoter contains a TATA-like sequence and allows basal transcription in most species (Reeder 1984; Paule & White 2000) whereas the upstream control element stimulates the activity of RNAP I (Henderson & Sollner-Webb 1990; Paule & White 2000). Like the other RNA polymerases RNAP I must form a pre-initiation complex (PIC) in order to initiate transcription - however only the RNAP Iβ subpopulation of RNAP I (a minority of the population) can form PICs (Milkereit & Tschochner 1998; Miller et al. 2001).

An initial step of PIC formation is the binding of selectivity factor 1 (SL1) to the core promoter, essential for sequence specificity, and allows stable interactions between UBF and the rDNA promoter (Rudloff et al. 1994; Miller et al. 2001; Cavanaugh et al. 2002; Friedrich et al. 2005). SL1 consists of TATA-binding protein (TBP) and several TBP-associated factors (TAFs) (Goodfellow & Zomerdijk 2013) and SL1 binds the DNA directly (Rudloff et al. 1994); also binding TIF-IA/RRN3, TIF-1A in turn directly binds RNAP Iβ (Peyroche et al. 2000; Miller et al. 2001). In this way RNAP Iβ is brought to the core promoter along with other associated proteins such as TOP2α, which regulates supercoiling, and casein kinase 2 (CK2), known to promote PIC assembly by acting on UBF and to promote elongation by acting on TIF-1A (Lin et al. 2006; Panova et al. 2006). Other proteins can affect RNAP I transcription - angiogenin, mutations in which can cause familial ALS, is also thought to bind RNAP I promoters and to be an RNAP I transcription factor (Tsuji et al. 2005; Li & Hu 2010).

UBF, bound throughout the rDNA (O'Sullivan et al. 2002), binds to SL1 (Jantzen et al. 1992; Hempel et al. 1996; Kihm et al. 1998; Tuan et al. 1999; Goodfellow & Zomerdijk 2013) and directly to RNAP I (Hanada et al. 1996; Seither et al. 1997; Panov et al. 2006), promoting transcriptional activation (Goodfellow & Zomerdijk 2013). The RNAP I specific transcription inhibitor CX5461 is thought to work by preventing SL1 recruitment to the rDNA, therefore inhibiting initiation of transcription (Drygin et al. 2011; Haddach et al. 2012).

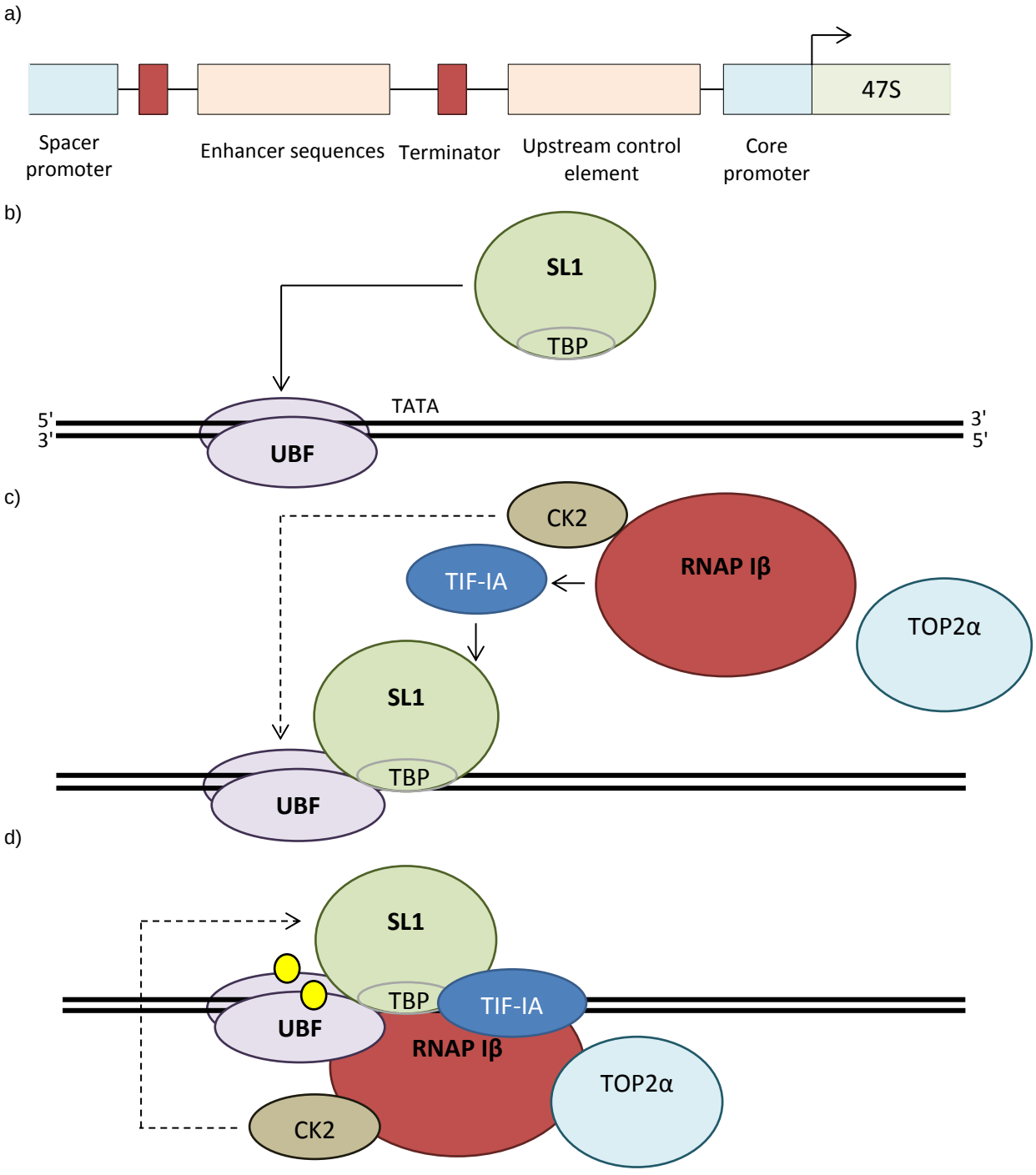


Figure 1.8 | **Process of formation of the RNA polymerase I pre-initiation complex.** SL1 and RNAP Iβ are large multi-protein complexes. TBP is shown in the SL1 complex but the complex also includes many TAF proteins. a) features of the DNA upstream of the sequence encoding 47S. Spacer regions may be used for silencing rRNA genes (Mayer et al. 2006). b) SL1 loads onto UBF, already present at the core promoter. c) RNAP Iβ binds TIF-IA in the SL1 complex and to UBF, TOP2α and CK2 are also recruited with it. CK2 phosphorylates UBF. d) The PIC is assembled, CK2 phosphorylates TIF-IA. The complex is initiated with UBF and SL1 left at the core promoter.

Once the PIC is assembled transcription begins and proceeds for a short distance but RNAP I must dissociate from promoter bound initiation factors in order to progress to elongation. This process is called promoter escape, and occurs at the point of TIF-1A dissociation from RNAP I (Milkereit & Tschochner 1998; Hirschler-Laszkiewicz et al. 2003; Goodfellow & Zomerdijk 2013) which is mediated by a phosphorylation event (Fath et al. 2001; Cavanaugh et al. 2002; Bierhoff et al. 2008). The exact details of how promoter escape occurs remain obscure, though UBF has been implicated.

SL1 and UBF remain at the promoter after escape (Panov et al. 2001; Lin et al. 2006), both proteins are implicated in regulating elongation by maintaining favourable topology for RNAP I transcription (O'Sullivan et al. 2002; Denissov et al. 2011; Goodfellow & Zomerdijk 2013). Many other factors promoting elongation have been identified, notably including proteins implicated in later rRNA processing such as nucleolin and nucleophosmin/B23 (Rickards et al. 2007; Murano et al. 2008). Actinomycin D inhibits RNAP I by intercalation of DNA and therefore at the elongation step and at higher concentations can inhibit other polymerases too (Fetherston et al. 1984; Drygin et al. 2011).

Termination of RNAP I transcription in *Homo sapiens* is mediated by binding of transcription termination factor 1 binding to terminator elements downstream of the coding sequence and pausing the RNAP I complex, leading to dissociation mediated by RNAP I itself and a release factor (Goodfellow & Zomerdijk 2013). Transcription termination factor 1 binding sites are also located upstream of the rDNA promoter and downstream of spacer promoters, it is thought that these sites are important for the topology of the rDNA allowing for rapid re-initiation after termination (Németh et al. 2008; Shiue et al. 2009; Németh & Längst 2011; Goodfellow & Zomerdijk 2013).

1.5.2 RNA polymerase II (RNAP II)

There are at least two distinct models of RNAP II PIC formation - one is for a human PIC and the other for yeast but they are highly structurally divergent models (Murakami et al. 2013; He et al. 2013). The model of human PIC formation will be described but a note of caution must be sounded for several reasons:

Firstly both presume initiation involving a TATA box approximately 30 nucleotides upstream of the start site. In reality mammalian RNAP II promoters are diverse and those containing a

TATA box are a distinct minority, mainly used in tissue specific genes, with the majority of RNAP II promoters being bidirectional and containing large stretches of CpG islands (Carninci et al. 2006).

Secondly there are other elements of DNA sequence and chromatin structure that affect PIC formation. Typically a transcriptional start site will be occupied by unstable nucleosomes (often histone variants such as H2AZ or H3.3) giving proteins more access to DNA (Jin & Felsenfeld 2007; Henikoff et al. 2009; Valen & Sandelin 2011) and some promoters are dependent on chromatin remodellers such as the SWI/SNF complex (Ramirez-Carrozzi et al. 2009). Transcription from any promoter is affected strongly by various histone modifications around the core of the promoter region (Karlić et al. 2010; C. Cheng et al. 2011) and other factors such as looping of DNA by complexes like Mediator (mechanism currently unknown) are likely to be important too (Allen & Taatjes 2015).

There are also both TATA-dependent and TATA-independent sequences which can affect PIC formation. Recognition elements for components of the PIC complex, such as TFIID (Lee et al. 2005), exist and can increase initiation efficiency. TATA-independent signals include the Initiator (INR) sequence located near the start site which can bind RNAP II, particularly when associated with other PIC components (Carcamo et al. 1990; Conaway et al. 1992) and can operate in conjunction with or independently of the TATA box, although it is dependent on additional activator sequences such as Sp1 (Yarden et al. 2009) in the latter case.

Thirdly the role of specific transcription factors, diverse proteins that can direct tissue or stimulus dependent transcription, is not touched upon in either of these models. There are potentially thousands of specific transcription factors, including important regulatory proteins such as p53, binding diverse enhancer or repressor sequences (Vaquerizas et al. 2009) and itself being subject to complex regulatory processes. PIC formation is the central event in transcriptional initiation but these specific transcription factors have large part in regulation of the process too. It is likely that the dynamic complex Mediator is key to much of this regulation, and is thought to have a role in recruitment of most members of the PIC (Allen & Taatjes 2015). Other specific transcription factors may affect transcription at later stages, for instance mechanisms by which NF-κB function operates during early elongation steps have been determined (Nowak et al. 2008; Huang et al. 2009; Fang et al. 2014).

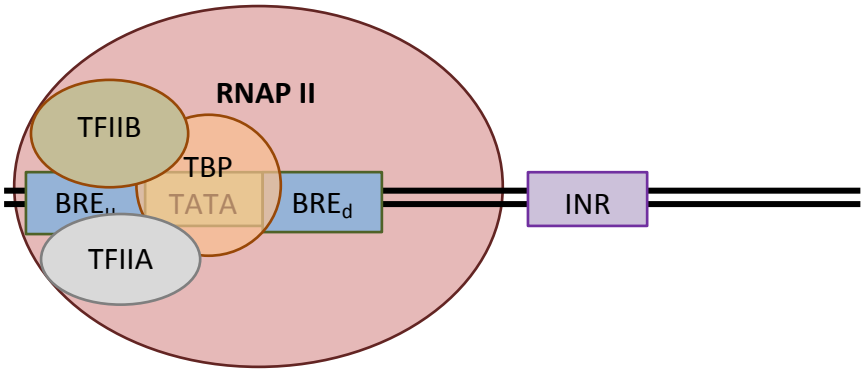
Finally some components of the core PIC being described in the following paragraphs have been observed to be replaceable by other factors. For example replacement of the core PIC protein TBP with TBP-like factor 3 during oogenesis has been observed (Gazdag et al. 2007).

With these caveats outlined it is clear that PIC formation requires a minimal set of proteins known as general transcription factors as well as RNAP II itself. These are TBP, TFIIB, TFIIF, TFIIE and TFIIH (He et al. 2013). TBP is typically found in the TFIID complex, associated with a variety of TAF proteins (Dynlacht et al. 1991), and binds the TATA box 30 nucleotides upstream of the transcriptional start site, bending the DNA (Luse 2013). TFIIB can bind specific promoter elements (BREs) up- or downstream of the TATA box (Lagrange et al. 1998; Deng & Roberts 2005) if present and can bind with the catalytic subunit of RNAP II (Sainsbury et al. 2013). TFIIF also binds RNAP II (Sopta et al. 1985) and may aid in loading of TFIIB or RNAP II itself (Luse 2013), CK2 phosphorylated TFIIF, although functional, is not retained within the PIC (Cabart et al. 2011) and so may be only transiently part of the complex.

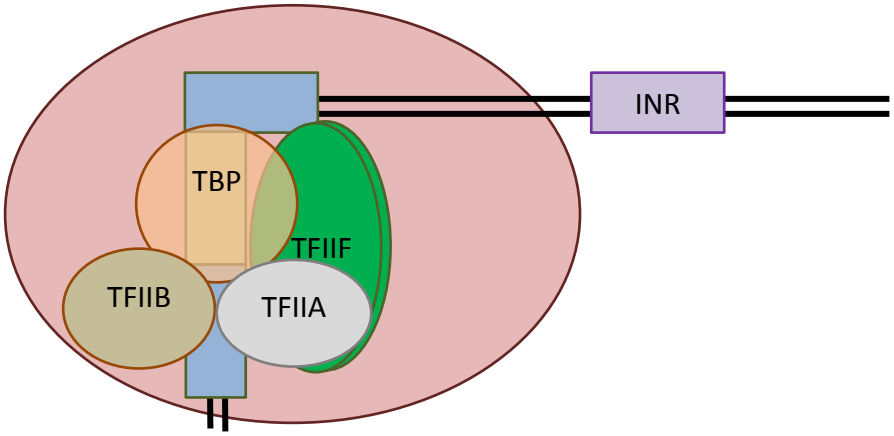
RNAP II also lacks intrinsic helicase activity and as such relies on the XPB helicase activity of TFIIH (which also contains cyclin dependent kinase 7 with cyclin H and XPD helicase) to unwind DNA (Tirode et al. 1999), pre-PICs consisting of PICs sans TFIIH have been found at mammalian promoters (Kouzine et al. 2013) implying TFIIH is loaded last in PIC formation in mammals. TFIIH, via cyclin dependent kinase 7, phosphorylates RNAP II at sets of 52 tandem YSPTSPS repeats at its C-terminus at serine 5 and serine 7. The serine 7 mark is associated with the start of splicing and serine 5 with processes such as capping and the start of elongation (Akhtar et al. 2009; Glover-Cutter et al. 2009). The TFIIH complex is thought to be loaded by TFIIE, which may also encourage melting at the promoter in its own right (Maxon et al. 1994; Ohkuma et al. 1995; Bushnell et al. 1996). Finally TFIIA is a non-essential component of the PIC thought to promote TBP-DNA interactions (Hieb et al. 2007).

A cryo-EM study of simplified human PIC assembly *in vitro* indicated a stepwise assembly with a TBP-TFIIA-TFIIB-RNAP II complex initially forming, before binding TFIIF and DNA and undergoing a major conformational change. TFIIE and then TFIIH were added after this (He et al. 2013). This would imply TFIIF was responsible for remodelling the PIC as it forms rather than for loading TFIIB.

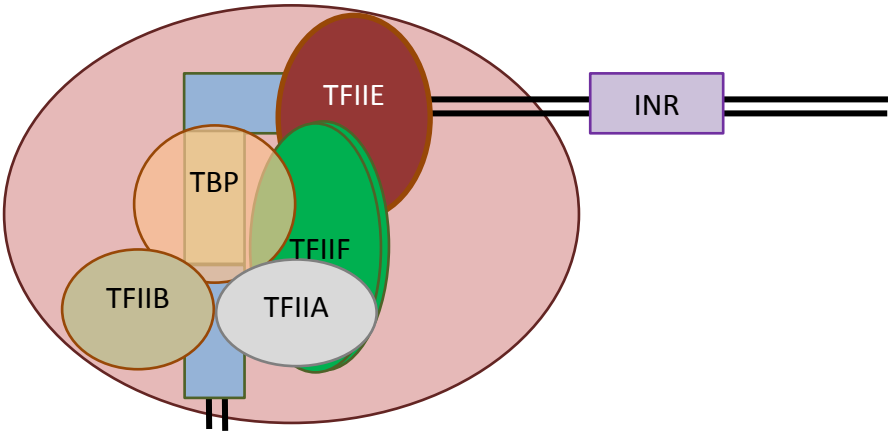
a)


RNAP II
TFIIB
TBP
BRE$_u$
TATA
BRE$_d$
INR
TFIIA

b)


INR
TBP
TFIIF
TFIIB
TFIIA

c)


TFIIE
INR
TBP
TFIIF
TFIIB
TFIIA

d)

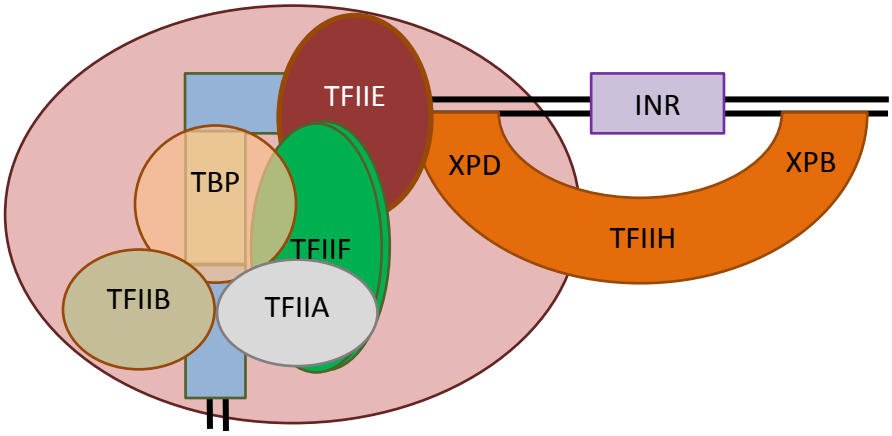

TFIIE
INR
XPD
XPB
TBP
TFIIH
TFIIF
TFIIB
TFIIA

Figure 1.9 | **Model of assembly of the human RNA polymerase II pre-initiation complex.** RNAP II is a large multi-protein complex and the TFIIH complex also contains cyclin dependent kinase 7 and cyclin H (not pictured). Due to the conditions of the experiments of He et al. some proteins likely present in vivo are excluded (for instance TBP is not shown as part of the TFIID complex). BREu refers to the upstream BRE and BREd to the downstream BRE. a) A complex of TBP (bound to the TATA box), RNAP II and TFIIA and TFIIB (bound to the upstream BRE). b) TFIIF binds, stabilising the promoter site. c) TFIIE binds. d) TFIIH binds last, with XPB downstream of the INR site.

When the PIC complex is assembled and the promoter DNA melted to form a transcription bubble (Goodrich & Tjian 1994; Holstege et al. 1996; Kim et al. 2000) initiation begins and the promoter clearance stage begins with partial disassembly of the PIC. Some components are left at the promoter for reinitiation as RNAP II starts to elongate (Zawel et al. 1995; Yudkovsky et al. 2000).

Elongation is a highly regulated process with RNAP II with an initial pausing of RNAP II tens of nucleotides downstream of the start site after promoter clearance (Marshall & Price 1992; Rahl et al. 2010). This pausing is primarily due to the actions of negative elongation factor (NELF) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)-sensitivity inducing factor (DSIF) (Wada et al. 1998; Yamaguchi et al. 1999; Renner et al. 2001; Cheng & Price 2007), which are inhibitory to elongation beyond the point of pausing. Transcription factors and features of the promoter influence recruitment, with nucleosomes also contributing to the process (for instance histone H2AZ reduces pausing relative to canonical histone H2A) (Weber et al. 2014; Jonkers & Lis 2015). This is often the rate-limiting step for transcription and it has been proposed that it acts as a quality checkpoint ensuring proper 5' capping of pre-mRNA and modifications of RNAP II itself before further transcription occurs (Adelman & Lis 2012). It may also help to maintain genes in an active state even when not being transcribed at that precise time by denying nucleosome occupancy of the region (Gilchrist et al. 2010) and, counterintuitively, pausing can be necessary for optimal transcription - for instance mutations in pausing regions of the heat shock protein 70 gene can actually reduce the heat shock response by rendering upstream promoter elements inaccessible to heat shock factor, which is the specific transcription factor associated with the process (Lee et al. 1992; Shopland et al. 1995).

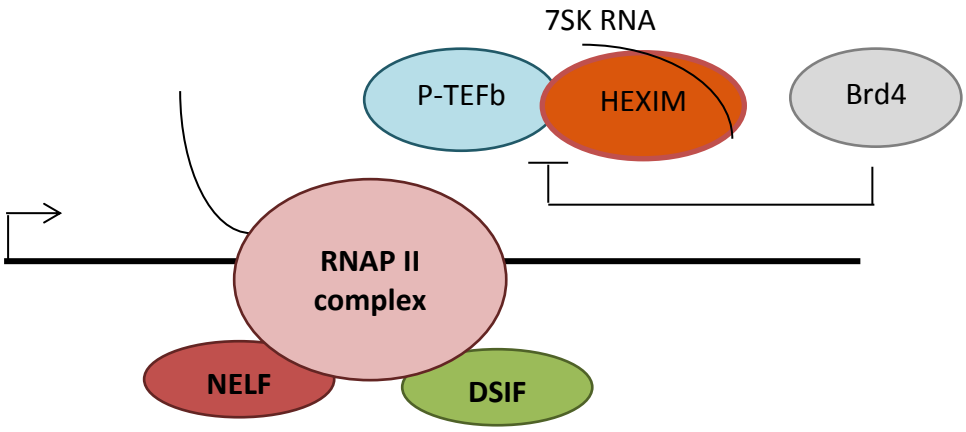
RNAP II is released by the action of the positive transcription elongation factor b (PTEF-b) complex, comprising cyclin dependent kinase 9 and one of three cyclins: T1, T2 or K

(typically one of the first two) (Peng et al. 1998; Fu et al. 1999; Chang & Li 2008; Yu et al. 2010). It phosphorylates NELF, removing it from the RNAP II complex (Fujinaga et al. 2004), and a subunit of DSIF - this process is required for productive elongation with DSIF remaining associated with RNAP II as it progresses (Yamada et al. 2006; Chen et al. 2009). RNAP II YSPTSPS repeats are also phosphorylated by P-TEFb at serine 2 of this sequence (Ramanathan et al. 2001). This mark accumulates during elongation and peaks downstream of polyadenylation sites, and other transcription associated cyclin dependent kinases (12 and 13) can also phosphorylate this residue (Bartkowiak et al. 2010; Bartkowiak & Greenleaf 2011; Zhou et al. 2012). Total absence of the mark is associated with inactive or paused polymerases (Muse et al. 2007). The phosphorylation at serine 5 can also be produced by cyclin dependent kinase 9 (Allen & Taatjes 2015) or cyclin dependent kinase 8 in the Mediator complex (Eick & Geyer 2013).

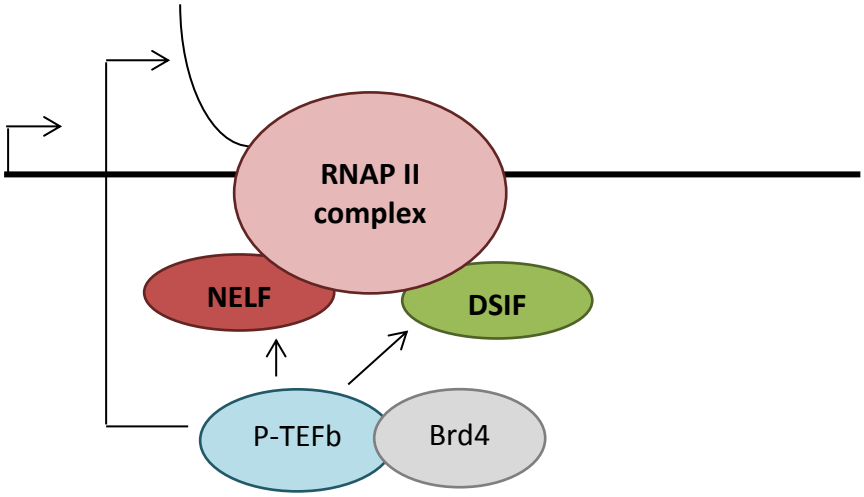
P-TEFb is highly regulated (Zhou et al. 2012) with the major mechanism involving being held in an inactive state by a complex of a protein called HEXIM and the RNAP III product 7SK RNA (Li et al. 2005; Barboric et al. 2005). Mechanisms exist to selectively release P-TEFb from this complex with the BRD4 pathway being an example often found in active chromatin (Yang et al. 2005; Wu & Chiang 2007; Hargreaves et al. 2009; Krueger et al. 2010). It is likely that HEXIM-sequestration is used to prevent P-TEFb aberrantly activating other paused RNAP II complexes, as the activity of the P-TEFb complex is not required to maintain high elongation speeds after this point (Zhou et al. 2012). Other steps of regulation at this initial pausing have been proposed - for instance some enhancers are also known to produce short RNAs that promote transcriptional activation and a proposed mechanism for their action is that they act as a decoy for NELF (Schaukowitch et al. 2014).

DRB has been known for a long time to inhibit RNAP II during elongation (Chodosh et al. 1989; Wada et al. 1998). It exerts its inhibitory activity via P-TEFb signalling - as it inhibits its cyclin dependent kinase 9 thereby preventing elongation. It also inhibits a variety of other kinases (Bensaude 2011). α-amanitin is another RNAP II inhibitor (Lindell et al. 1970) and operates throughout elongation as its mode of action is to bind between the two largest subunits of RNAP II, Rpb1 and Rpb2, and to trap the complex in a fixed conformation. This prevents incorporation of ribonucleotides into the elongating RNA strand (Bushnell et al. 2002; Brueckner & Cramer 2008).

a)



b)



c)

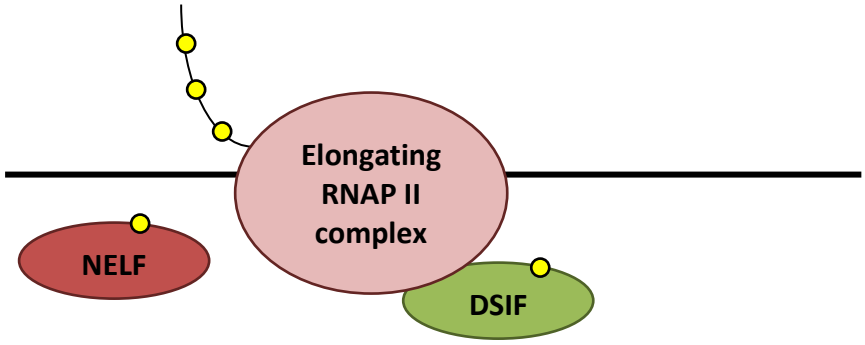


Figure 1.9 | **Schematic of RNA polymerase II being released from initial pausing.** P-TEFb consists of cyclin dependent kinase 9 and one of the cyclins H1, H2 or K. DSIF and NELF are also complexes. a) Brd4 binds P-TEFb, displacing the complex of HEXIM and 7SK RNA. b) P-TEFb phosphorylates RNAP II on Ser2 of the YSPTSPS repeats of its C-terminal tail, as well as NELF and DSIF. The transcriptional inhiibitional activity of DRB is due to its inhibition of P-TEFb. NELF dissociates after phosphorylation. c) The elongating RNAP II complex remains in association with DSIF and will accumulate phosphorylation marks on Ser5 on the YSPTSPS repeats as it elongates.

Pausing and arrest can also occur later on in the process (Zhou et al. 2012), if this pause is maintained (for instance by a lesion on the DNA) the polymerase complex may enter an arrest state and backtrack so that the position of the polymerase on the DNA is not aligned with the 3' end of the RNA it is transcribing (Gu & Reines 1995). TFIIS can rescue RNAP II by stimulating its endonuclease activity, realigning the relative positions of the DNA and transcribed RNA (Fish & Kane 2002). If the polymerase is not rescued then it may be marked for degradation (Sigurdsson et al. 2010). Other factors also promote RNAP II elongation via : TFIIF is responsible for converting paused RNAP II complexes back into active conformations (Price et al. 1989; Flores et al. 1989; Zhou et al. 2012), the proteins ELL1-3 (Zhou et al. 2012) suppress the pausing of RNAP II and the well known signalling molecule Myc mediates pause release (Rahl et al. 2010).

It is worth noting that given the diversity of RNAP II transcription there are many other methods by which elongation can be regulated. An example of this are super elongation complexes used for induction of rapidly induced genes. The contents of these complexes are diverse (Lin et al. 2010; Zhou et al. 2012; Whyte et al. 2013) but they are thought to be recruited to RNAP II by the RNAP II complex itself, Mediator and the Integrator complex (He et al. 2011; Takahashi et al. 2011; Gardini et al. 2014); and to function by regulating looping of DNA to allow enhancer sequences to promote transcription (Whyte et al. 2013; Hnisz et al. 2013) and by promoting P-TEFb activity (Jonkers & Lis 2015). Processes such as splicing and capping are also co-ordinated with elongation (Zhou et al. 2012).

The mechanism of termination for most mRNAs is dependent on polyadenylation signals, occuring 18-30 nucleotides downstream of it (Porrua & Libri 2015). The elongation complex remains associated with the RNA up to this point (Osheim et al. 1999; Osheim et al. 2002), necessitating cleavage of the 3' end of the nascent RNA. There are also less conserved downstream elements up to 30 nucleotides downstream of the cleavage site (MacDonald et al. 1994; Xiang et al. 2014) and some genes have further sequences from 40 to 100 nucleotides downstream (Hu et al. 2005). Cleavage and polyadenylation specificity factor (CPSF) recognises the polyadenylation site, couples polyadenylation to transcription (it accompanies elongating RNAP II) and cleaves the RNA at the appropriate site (Gilmartin & Nevins 1989; Takagaki et al. 1989; Bienroth et al. 1991; Dantonel et al. 1997). A variety of other proteins are associated with CPSF and with each other and encourage this process

(Takagaki et al. 1989; Gilmartin & Nevins 1991; Rüegsegger et al. 1996; Takagaki & Manley 1997; Sullivan et al. 2009; Ruepp et al. 2011). Poly-A polymerase then polyadenylates the RNA, with CPSF and nuclear poly(A)-binding protein 1 providing processivity (Bienroth et al. 1993; Wahle 1995).

Termination itself may be induced by the cleavages needed for by degradation of the downstream RNA by XRN2 (eventually "chasing down" RNAP II and inducing termination - the torpedo model) or by allosteric changes in the elongation complex induced by cleavage factor II, one of the proteins accompanying CPSF, decreasing processivity (West et al. 2004; Zhang & Gilmour 2006; Porrua & Libri 2015). Polymerase pausing after the polyadenylation signal may allow termination by either of these mechanisms (Porrua & Libri 2015). Regardless of whether the torpedo mechanism or the conformational change model is correct it has been proposed that at termination sites DNA:RNA hybrids (R-loops) form, resulting in the formation of repressive histone marks by the RNA interference pathway, polymerase stalling by a heterochromatin protein 1γ associated mechanism and a requirement for the R-loop unwinding protein senataxin for polymerase release (Skourti-Stathaki et al. 2011; Skourti-Stathaki et al. 2014).

Finally there are two known alternative RNAP II termination mechanisms (Porrua & Libri 2015). snRNA are terminated by being processed at a motif called the 3' box by members of the Integrator complex (Baillat et al. 2005; Ezzeddine et al. 2011; Porrua & Libri 2015); while some histone mRNAs are terminated by a poorly characterised mechanism at a stem loop structure which is polyadenylation independent, though it shares some machinery with the canonical mRNA termination pathway (Dominski et al. 2005).

1.5.3 RNA polymerase III (RNAP III)

RNAP III transcribes an eclectic variety of RNA species, generally of up to 400 bp in length (Schramm & Hernandez 2002). RNAP III can be specifically inhibited by the bacterial toxin tagetitoxin (Steinberg et al. 1990) and although other RNAP III inhibitors exist their selectiveness against other polymerases is not documented in the literature and cannot be assumed (Wu et al. 2003).

There are three distinct varieties of RNAP III promoter in metazoans: the type 1 promoter which is associated with the 5S gene (Bogenhagen et al. 1980a; Bogenhagen et al. 1980b);

the type 2 promoter which is associated with, amongst other things, adenovirus promoters (Fowlkes & Shenk 1980) and promoters for tRNA genes (Galli et al. 1981; Hofstetter et al. 1981; Sharp et al. 1981); and the type 3 promoter which is associated with a diverse range of genes including those encoding the small nuclear RNA (snRNA) component of the U6 spliceosome component (Krol et al. 1987; Kunkel & Pederson 1988; Das et al. 1988) and the RNA component of RNase P (Baer et al. 1989). There has also been a report of RNAP III successfully initiating transcription from RNAP II promoters (Duttke 2014), so there may be a degree of overlap in the function of the polymerases.

The type 1 promoters have an internal promoter, the internal control region, containing motifs known as the A box, the intermediate element and the C box (Bogenhagen 1985; Pieler, Appel, et al. 1985; Pieler, Oei, et al. 1985; Pieler et al. 1987). Type 2 promoters are also internal and most consist of an A and B box, with variable spacing, these components also encode structural features required for tRNA function (Allison et al. 1983; Schramm & Hernandez 2002). Type 3 promoters are external and found 5' of the coding sequence. They contain a distal sequence element (where transcription is activated), a proximal sequence element and a TATA box, which confers RNAP III specificity as mutation of it results in RNAP II driven transcription from the U6 snRNA locus (Mattaj et al. 1988; Hernandez & Lucito 1988; Kunkel & Pederson 1989; Lobo & Hernandez 1989).

Initiation of RNAP III transcription requires TFIIIB, containing TBP, BDP1 and either BRF1 for internal promoters or BRF2 for external promoters (Schramm et al. 2000; Oler et al. 2010; Moqtaderi et al. 2010; Barski et al. 2010; White 2011). Recruitment of TFIIIB is sufficient for RNAP III complex recruitment (Kassavetis et al. 1990; Marsolier et al. 1994; Chong et al. 2001) but the TFIIIC complex is required to recruit it to genes with internal promoters (Bieker et al. 1985; Setzer & Brown 1985; Schramm & Hernandez 2002). Type 1 promoters require an additional factor still as TFIIIA binds the internal control region and allows recruitment of TFIIIC (Engelke et al. 1980; Sakonju et al. 1981; Schramm & Hernandez 2002).

As RNAP III genes are short little control of the elongation step has been identified and it has a simple termination sequence, a chain of as few as four T residues (Bogenhagen & Brown 1981; Cozzarelli et al. 1983; Watson et al. 1984) in vertebrates. As some genes, such as that for lysine tRNA, contain this sequence the context around this tract affects termination -

flanking with AA usually results in RNAP III reading through the sequence but flanking it with GC results in efficient termination (Bogenhagen & Brown 1981; Cozzarelli et al. 1983; Goodier & Maraia 1998; Gunnery et al. 1999). Interrupted tracts of T have also been observed to act as terminators and unrelated non-canonical termination sequences have also been reported (Hess et al. 1985; Vnencak-Jones et al. 1985; Orioli et al. 2011).

The mechanism of eukaryotic RNAP III termination remains unclear - the involvement of specific termination proteins have been proposed, particularly the La protein, but this idea remains controversial (Arimbasseri et al. 2013).

1.6 The nucleolus

1.6.1 Structure, function and disease of the nucleolus

A central function of the nucleolus is ribosome biogenesis, the transcription and processing of pre-rRNA. The organisation of the nucleolus reflects this function, with a tripartite division into the fibrillar centres (FCs) surrounded by the dense fibrillar component (DFC), all of which are contained within a granular component (GC) marking the edge of the nucleolus (Junéra et al. 1995; Hernandez-Verdun 2006). It is thought that the FCs number varies with cell cycle phase (with more in G2 than G1) and that transcription begins at the junction between the FCs and DFCs (Hozák et al. 1994; Cmarko et al. 2000; Hernandez-Verdun 2006). Broadly speaking early processing of rRNA occurs in the DFCs and later processing occurs in the GCs (Biggiogera et al. 1989; Ginisty et al. 1998; Hernandez-Verdun 2006) - though as will be explained in chapter 1.6.2 it is thought that some rRNA maturation steps do occur in the nucleoplasm and even the cytoplasm.

The nucleolus is a highly dynamic structure (Raška et al. 2006), with its structure closely associated with its functions. In keeping with this it is actively disassembled during mitosis and reassembled in the subsequent G1-phase. During prophase of mitosis cyclin B1-cyclin dependent kinase 1 is stabilised and phosphorylates components of the nucleolar transcription machinery (Heix et al. 1998; Leung et al. 2004). After this the nucleolus is disassembled with portions of the RNAP I complex, such as UBF1, remaining associated with NORs (Roussel et al. 1996; Leung et al. 2004; Dundr et al. 2000) - regions on acrocentric chromosomes (chromosomes with the centromere near the chromosome end) where approximately 400 copies of the ribosomal DNA (rDNA) are located. Other components of

the transcription complex do not remain associated with these regions (Leung et al. 2004; Sirri et al. 2002). There are five acrocentric chromosomes in humans and studies have shown that there are five NORs (Savino et al. 2001).

In contrast, processing components and partially processed pre-rRNA do not retain any association with this region - with early rRNA processing proteins such as fibrillarin (found in the FC and DFC) and later acting ones such as B23 (found in the GC) dissociating at the same time (Leung et al. 2004). Nucleolar proteins that do not maintain NOR association are either released into the cytoplasm or associated with the perichromosomal region, surrounding non-centromeric regions of the chromosomes at this period of the cell cycle (Gautier et al. 1992; Boisvert et al. 2007).

When anaphase arrives cytoplasmic rRNA-processing proteins are packaged into hundreds of nucleolar-derived foci (Dundr et al. 1996; Dundr et al. 1997; Dundr & Olson 1998). Towards the end of the phase rRNA transcription is reactivated and both these foci and the perichromosomal region dissociate, with most their contents forming pre-nucleolar bodies on the surface of chromosomes (Dundr et al. 2000; Boisvert et al. 2007). Nucleolar proteins concentrate in these regions but nucleoli do not form from the pre-nucleolar bodies, rather proteins are released in set order - with early processing components such as fibrillarin being released earlier than proteins like B23 (Angelier et al. 2005). The DFC is therefore formed before the GC. NORs subsequently migrate together, resulting in fusion of these new nucleoli, at which point the functional nucleolus is assembled (Boisvert et al. 2007; Savino et al. 2001). It therefore follows that the number of NORs does not equal the number of nucleoli.

Despite the close relationship between ribosomal maturation and the structure of the nucleolus it is also involved in other cellular processes. For instance it is connected to cell cycle regulation as telomerase is bound to nucleolin and sequestered in the nucleolus until late S-phase in non-immortalised cells (Wong et al. 2002; Boisvert et al. 2007); and a pool of protein phosphatase 1γ is accumulated in nucleoli in interphase and released upon mitosis (Boisvert et al. 2007). Nucleoli have been proposed to be involved in processing RNAs other than pre-rRNA - with small nucleolar ribonucleoproteins (snRNPs) covalently modifying RNAs such as snRNA or the RNA component of RNase P and the nucleolus possibly acting as

a site of protein assembly with these RNAs (Boisvert et al. 2007). The mRNA editing enzyme ADAR2 is also known to operate within the nucleolus (Desterro et al. 2003; Vitali et al. 2005). A final important role for the nucleolus is that it has a key role as a stress-sensor and p53 activator, this is discussed further in chapter 1.6.3.

Unsurprisingly mutation in nucleolar or ribosomal-associated genes can cause disease - Roberts syndrome (a developmental disease) is associated with nucleolar fragmentation and is thought to be mediated via defects in rRNA production (Xu et al. 2013). Nucleolar fragmentation is the formation of multiple misshapen or "fragmented" nucleoli-like structures - possibly derived from enlarged, stressed nucleoli (Lewinska et al. 2014), initially described in yeast as a consequence of overexpression of NOP2 (de Beus et al. 1994) - this paper also demonstrated that fragmented nucleoli are more detached from the nuclear envelope than unfragmented nucleoli. Fragmentation has also been observed in cells with other defects (such as those lacking DNA methyltransferase 1 (Espada et al. 2007)), is a known sign of ageing in yeast (Sinclair et al. 1997) and has also been found to be associated with oxidative stress in yeast (Lewinska et al. 2014). The Espada study also demonstrated a disorganisation of rDNA within the nucleus in cells demonstrating fragmentation. In a study by Choesmel et al there appeared to be nucleolar fragmentation in cells treated with small interfering RNA (siRNA) against RPS19 (Choesmel et al. 2007), although this was not explicitly referred to in the text. These cells also demonstrated a defect in rRNA maturation.

Other nucleolar-associated genetic diseases include dyskeratosis congenita (mutation in dyskerin) and Diamond-Blackfan anaemia (mutation in the ribosomal protein RPS19) (Heiss et al. 1998; Draptchinskaia et al. 1999; Choesmel et al. 2007), mutations in ubiquitously expressed proteins with the phenotype expressed only in specific cell types.

Furthermore there are links between nucleolar dysfunction and neurodegenerative disease. For instance Alzheimer's is associated with increasing methylation of rDNA promoters (Pietrzak et al. 2011) and enlargement of the nucleoli has been observed in autopsies from asymptomatic Alzheimer's disease patients (Iacono et al. 2008). A notable result is that impairment of the essential RNAP I transcription factor TIF-1A in post-mitotic neurons in a mouse model resulted in mitochondrial dysfunction and oxidative damage associated with neurodegenerative diseases, such as ALS. The mice also demonstrated neurodegeneration,

although this was found in the hippocampus rather than the motor neurons (Yuan et al. 2005; Parlato et al. 2008).

Another possible link between the nucleolus and ALS is via the single most common cause of familial ALS - hexanucleotide expansion (GGGGCC) in the first intron of the c9orf72 gene (DeJesus-Hernandez et al. 2011; Renton et al. 2011). Some observations suggest the possibility of nucleolar involvement in c9orf72-mediated neurodegeneration. For instance dipeptides encoded by c9orf72 repeats have been found to bind nucleoli, to disrupt ribosomal biosynthesis and to result in cell death (Kwon et al. 2014). Meanwhile c9orf72 RNA has also been shown to induce nucleolar stress via formation of secondary structures such as DNA-RNA hybrids (R-loops) and G quadruplex DNA leading to sequestration of RNA-binding proteins, abortive transcription, impaired rRNA maturation and ultimately sensitisation of cells to stressors (Haeusler et al. 2014).

1.6.2 Maturation of ribosomal RNA (rRNA)

Mature cytoplasmic human ribosomes consist of the large 60S subunit and the small 40S subunit - each with a core of catalytic rRNA and associated ribosomal proteins. The 60S subunit contains the 28S, 5.8S and 5S rRNA species (as well as 46 ribosomal proteins) and the 40S subunit contains only the 18S rRNA species (and 33 ribosomal proteins). The process of ribosomal assembly is complex and occurs within the nucleolus, nucleoplasm and cytoplasm (Thomson et al. 2013). It is generally thought that processing begins in the nucleolus, progresses during movement through the nucleoplasm and that some RNA species complete processing in the cytoplasm (Tschochner & Hurt 2003) - however the majority of the process does occur within the nucleolus (Preti et al. 2013; Henras et al. 2015).

Transcription of the 47S rRNA subunit occurs as previously described. The resultant polycistronic transcript contains a long 5' external transcribed spacer (ETS1), followed by the sequence encoding 18S rRNA, two internal transcribed spacers (ITS1 and ITS2) - separated by the 5.8S rRNA sequence, the 28S rRNA sequence and finally a short 3' ETS (ETS2). As full length transcript can be isolated it is thought that maturation (at least in relation to cleavages) is post-transcriptional. Association with ribosomal proteins begins concurrently with this process and the particle assembling around the 47S ribosome as it is processed is

referred to as the 90S (Carron et al. 2011). This association is initially very dynamic, but components of the particles become more stable during ribosome maturation (Henras et al. 2015; Ferreira-Cerca et al. 2007). Defects in this process have been observed in c9orf72-linked ALS patients (Haeusler et al. 2014).

In the first step of processing exonucleases act on sites 01/A' (Miller & Sollner-Webb 1981; Craig et al. 1987) and 02 (Gurney 1985), removing the ETS2 sequence and slightly truncating the ETS1 sequence. The former process involves many snoRNPs, including the U3 snoRNP (Enright et al. 1996). It has been observed that disruption of site 01/A' cleavage does not hinder further rRNA maturation (Henras et al. 2015).

This generates the 45S pre-rRNA which can then be processed near simultaneously either by exonucleases at sites A0 and 1, generating the 41S pre-rRNA (which contains the coding sequences, ITS1 and ITS2) (Hannon et al. 1989; Rouquette et al. 2005; Mullineux & Lafontaine 2012) or can be processed by an endonuclease at site 2, generating the 30S pre-rRNA (which contains the 18S coding sequence, ETS1 and a portion of ITS1) and the 32.5S pre-rRNA (containing the remainder of ITS1, 5.8S, ITS2 and 28S) (Idol et al. 2007; Mullineux & Lafontaine 2012). After this the 41S species is processed at site 2 or the 30S species is processed at sites A0 and 1, resulting in production of the 21S species (containing 18S and a portion of ITS1) and the 32.5S species (Henras et al. 2015). At this point the 90s pre-ribosomal particle is considered split into pre-40S (18S) and pre-60S (28S, 5.8S and later 5S) particles (Carron et al. 2011). The A0 and 1 sites are proximal (thought to form a stem structure (Renalier et al. 1989)) and it is thought that the fibrillarin-containing U3 snoRNA (Lapeyre et al. 1990; Kass et al. 1990) plays a key part in chaperoning of the RNA folding to allow this proximity - though many other factors are implicated in this step of processing (Enright et al. 1996).

21S is processed by 3' exonucleolytic trimming into the 21S-C species (Idol et al. 2007) and then by cleavage by an endonuclease at site E to generate 18S-E (Rouquette et al. 2005) - the proteins bystin/ENP1, RPS19 and the exosomal RRP6 are presumably involved in this process as knockdown of any of these results in 21S-C accumulation (Idol et al. 2007; Carron et al. 2011; Preti et al. 2013). 18S-E is then digested by a 3' exonuclease up to residue G5551 (this intermediate is called 18S-E/24) and action of the endonuclease hNOB1 at site 3 is

responsible for the final maturation step of 18S rRNA, converting 18S-E into the mature 18S species. Polyuridation has been observed at the 18S-E/24 intermediate and it is possible that this represents either an alternative pathway for 18S maturation or marking of RNA for degradation (Preti et al. 2013). During these stages of the process the pre-rRNA species are transported from the nucleus, with conversion of 18S-E to 18S rRNA occurring in the cytoplasm (Rouquette et al. 2005; Preti et al. 2013). It is thought that the exportin Crm1 is involved in nuclear export of pre-ribosomes (Gleizes et al. 2001; Thomas & Kutay 2003), though it was also shown to have a function upstream of nuclear export in ribosome maturation (Rouquette et al. 2005) - adding some ambiguity to its role.

The 32.5S species generated from the 41S or 45S species is acted on by XRN2 exonuclease on the 5' end, generating the 32S species (Henras et al. 2015). The 5.8S rRNA exists in short and long forms in eukaryotes, details of how this is regulated remains unclear in mammalian cells though XRN2 is required (Preti et al. 2013) and it has been observed that depletion of the protein BOP1 in mouse cells eliminates production of the short form 5.8S (Sloan et al. 2013). In *S. cerevisiae* this process is coupled with maturation of the 3' end of the 28S sequence (Kufel et al. 1999), there is some evidence of this being the case in higher eukaryotes also (Peculis & Steitz 1993).

After this an endonuclease acts at site 4, generating the 12S (containing 5.8S and a portion of ITS2) and 28.5S (containing 28S and part of ITS2) (Farrar et al. 2008). The processing of 28.5S is simple, with action of XRN2 on the 5' of the 28.5S being the final step of 28S maturation (Wang & Pestov 2011).

In contrast processing of 12S to 5.8S is a multi-step process involving multiple exonucleases. 12S is processed to 7S, requiring the exosome (Preti et al. 2013; Tafforeau et al. 2013; Schilders et al. 2007) and the nucleolar exonuclease ISG20-L2 (Couté et al. 2008). Processing of 7S also requires the exosome and involves at least two steps, as depletion of the exosomal protein RRP6 results in accumulation of an intermediate called 5.8S+40 (Tafforeau et al. 2013). A final intermediate consisting of 5.8S with a few extra nucleotides (sometimes dubbed 6S as in Fig 1.1) is processed by the 3' exonuclease ERI1 as demonstrated in mice (Ansel et al. 2008) and is widely conserved amongst eukaryotes (Gabel & Ruvkun 2008). This final step has been shown to be cytoplasmic in yeast (Thomson & Tollervey 2010) and

evidenced to be so in *X. laevis* (Trotta et al. 2003), though it has not been verified in mammalian cells.

These represent the major pathways of rRNA species maturation in humans though other pathways do exist, such as cleavage of the 45S species at site E to generate the 36S species (a substrate for XRN2 exonuclease). This intermediate is quite abundant in mouse cells (Wang & Pestov 2011) but is very minor in humans (Preti et al. 2013).

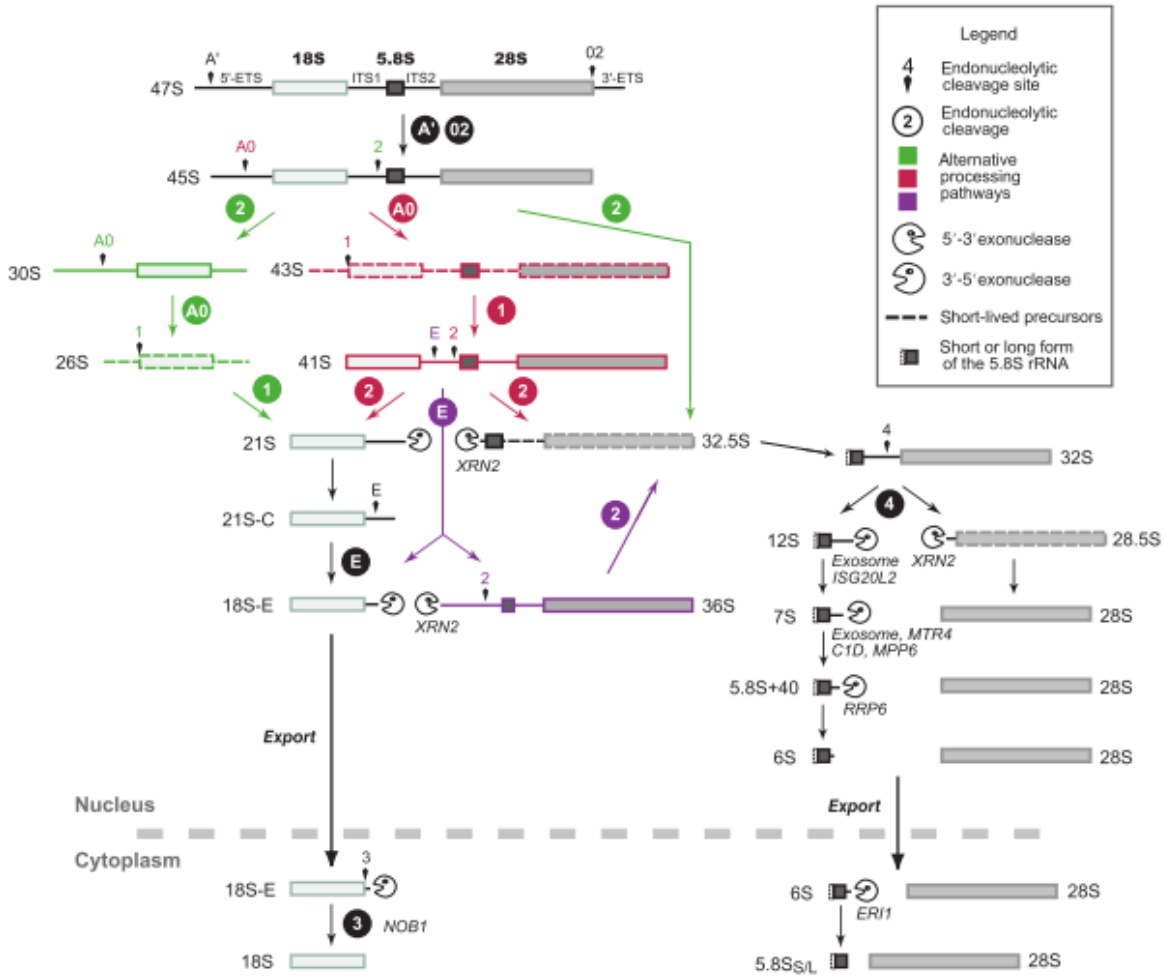


Figure 1.10 | **Diagram demonstrating the cleavages required to generate mature rRNA in Homo sapiens.** rRNA maturation includes a complex, branching sequence of cleavages and modifications. Reused under the Creative Commons Attribution Non-Commercial License (Henras et al. 2015).

Further modifications in structure and protein association occur after cytoplasmic export (Thomson et al. 2013) and almost all ribosomal proteins are essential for the rRNA processing previously described (Robledo et al. 2008), though their actual function largely remains unclear. A variety of other proteins are also involved in the process including ATPases, GTPases, RNA helicases, kinases (Kressler et al. 2010) and a variety of proteins of unknown function containing RNA-binding domains, such as the RRM domain, or protein-interaction domains (Henras et al. 2015). Which proteins are involved and what role they play is more well characterised in yeast but the vast majority of nucleolar yeast proteins have been shown to be present in human cells (Andersen et al. 2005; Hinsby et al. 2006) and many mammalian proteins of these kinds have been found involved in rRNA maturation

(Schäfer et al. 2006; Zemp et al. 2009; Zemp et al. 2014), including some that perform functions that are not utilised in lower eukaryotes (Srivastava et al. 2010).

snoRNPs are intimately involved in this maturation process and perform two major functions: RNA chaperoning (Hughes 1996; Sharma & Tollervey 1999; Henras et al. 2015) to allow the cleavages previously described and RNA modification on specific residues (using base pairing to target the modification). The latter function is more common and can be subdivided further into two activities: ribose methylation by box C/D RNPs (catalysed by fibrillarin (Toiiervey et al. 1993; Galardi et al. 2002)) and formation of pseudouridine (ψ) bases through uridine isomerisation by box H/ACA RNPs (catalysed by dyskerin (Ni et al. 1997; Ganot et al. 1997; Zebarjadian et al. 1999)). Both classes of snoRNPs are named after motifs in their snoRNA. Base methylation has also been observed in yeast (Henras et al. 2015).

A quality control pathway known as the nucleolar surveillance pathway is also known to exist in yeast which marks pre-rRNA for degradation by way of polyadenylation (LaCava et al. 2005; Houseley & Tollervey 2006; Dez et al. 2006) - how these pre-rRNAs are chosen remains obscure. Although most research into this pathway has been performed in yeast, it is thought to exist in higher eukaryotes also. In mouse cells where RNAP I is inhibited at the elongation stage by actinomycin D (ActD) aberrant pre-rRNA transcripts have been observed to be polyadenylated by PAPD5 (an orthologue of a yeast nucleolar surveillance pathway protein) and degraded by the exosome (Shcherbik et al. 2010). XRN2 is also thought to be involved (Wang & Pestov 2011). It has also been proposed that a translation-like cycle is involved in preventing immature ribosomes from initiating translation (Strunk et al. 2012).

In humans 5S rRNA is already mature immediately after transcription (Lodish et al. 2000).

1.6.3 Nucleolar disruption

The nucleolus is observed to undergo morphological changes in response to stress. Changes in morphology have been observed to occur as a result of events such as DSBs or hypoxia, but these changes are typically mediated via transcriptional inhibition - with ATM and the von Hippel-Lindau protein acting as intermediaries in the cases of DSBs and hypoxia (Kruhlak et al. 2007; Boulon et al. 2010).

Inhibition of RNAP II with DRB has been shown to induce a reversible morphological change (Scheer & Benavente 1990; Le Panse et al. 1999) where rRNA transcription sites are extruded into the nucleoplasm to form what are dubbed nucleolar necklaces. The terms nucleolar unravelling, nucleolar disruption and nucleolar fragmentation (distinct from the phenomenon discussed previously) have been used to describe this process. The term "nucleolar disruption" will be used throughout this document. Nucleolar necklaces contain "beads" which represent sites of transcription (Granick 1975b; Scheer et al. 1984; Le Panse et al. 1999) and contain components of transcription and early processing, such as the rDNA itself, rRNA, the RNAP I complex (including UBF1) and fibrillarin (Louvet et al. 2005). It has been shown that in this process DRB does not substantially decrease RNAP I transcription but does impair rRNA processing (Granick 1975a). It was also observed that later processing machinery of the GC, such as the RNA chaperone B23 (Lindström 2011), form shrunken masses that are proximal to and surrounded by the nucleolar necklace. The granular masses were observed to not contain any RNA with a 28S sequence, and normal nucleolar structure (and function) was restored between 30 and 60 minutes after removal of DRB (Louvet et al. 2005). It is likely that nucleolar disruption is due to the action of DRB (indirectly) on RNAP II transcription rather than as a direct consequence of its CK2 inhibition as mutating away the CK2 phosphorylation site on B23 does result in formation of B23 shrunken masses but does not result in formation of the nucleolar necklace (Louvet et al. 2006). In addition α-amanitin, an inhibitor of RNAP II that works via a different mechanism to DRB, has been observed to produce nucleolar necklaces, while RNAP I inhibition with low dose ActD does not (Haaf & Ward 1996).

There are, however, subtle differences in the morphological changes in the nucleus between different RNAP II inhibitors: For example DRB is observed to affect the nucleolus but not the coiled bodies (CBs), compartments containing large numbers of snRNPs (Carmo-Fonseca et al. 1992), whereas α-amanitin is observed to induce a large increase in the number and intensity of CBs and to result in their localisation to the nucleolar necklace, though not to the fibrillarin-containing beads (Haaf & Ward 1996), patterns consistent with this have been observed with high dose ActD (inhibiting RNAP II) also (Carmo-Fonseca et al. 1992).

It has been proposed that p53 induction can occur via inhibition of RNAP II and subsequent nucleolar disruption. The proposal that RNAP II inhibition was involved emerged from

observations that cells deficient in removing UV photolesions from transcribed genes induce p53 activity at a lower UV dose. It was later proposed that nucleolar disruption rather than RNAP II inhibition itself was in experiments showing that microirradiation of individual nucleoli (i.e. nuclear regions lacking RNAP II-associated genes) did not induce stabilisation of p53 but that microinjection of antibodies against UBF1, disrupting nucleoli without observable damage, did (Rubbi & Milner 2003). A variety of disparate pathways are thought to converge on nucleolar disruption (explaining how diverse stresses result in similar p53 responses), but nucleolar disruption is not thought to be a completely universal mediator of p53 activation - as some stresses such as bleomycin treatment can activate p53 without affecting the nucleolus' morphology (Boulon et al. 2010).

Subsequent studies have illustrated multiple mechanisms by which the nucleolus can induce p53 activation. A key short-term response involves B23, which has been observed to stabilise p14ARF by binding it, promoting its nucleolar localisation and preventing its association with nucleoplasmic ubiquitin ligases (Chen et al. 2010). p14ARF can then bind and inhibit the activity of human double minute 2 (Hdm2), a major negative regulator of p53, and therefore stabilise p53. B23 is also upregulated in response to oncogenic stress (Boulon et al. 2010).

The ribosomal protein RPL11 is also known to signal p53 in response to defects in rRNA processing. The protein is typically NEDDylated (a ubiquitin-like modification) but in response to stress it is de-NEDDylated and accumulates in the nucleoplasm, allowing interaction with Hdm2 - resulting in the stabilisation of p53 and its own degradation (Hölzel et al. 2010; Sundqvist et al. 2009). There are therefore plausible mechanisms by which transcriptional stress can lead to nucleolar disruption and from there to cell death.

1.7 The phosphatidylinositol 3-kinase-related kinase (PIKK) family

The phosphatidylinositol 3-kinase related kinase (PIKK) family is an atypical family of kinases, with a degree of sequence similarity to the phosphatidylinositol 3-kinase (PI3K) family of lipid kinases (Lempiäinen & Halazonetis 2009). The PIKK family contains six proteins of varying function: ataxia-telangiectasia mutated (ATM), ataxia and Rad3-related (ATR), DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), mammalian target of rapamycin (mTOR), suppressor of morphogeneis in genitalia 1 (SMG1) and

transformation/transcription domain-associated protein (TRRAP). All of these proteins are kinases (Lovejoy & Cortez 2009) except for TRRAP which contains a kinase domain but did not retain its kinase activity through evolution (Vassilev et al. 1998; McMahon et al. 1998), and kinase activity of the family can be inhibited by caffeine (Blasina et al. 1999; Sarkaria et al. 1999; Block et al. 2004; Reinke et al. 2006). All PIKKs are large and contain a FRAP, ATM, TRRAP (FAT) domain consisting of repeats of the huntingtin, elongation factor 3, A subunit of protein phosphatase 2A and TOR1 (HEAT) motif (Lovejoy & Cortez 2009). HEAT repeats are of key importance to protein-protein interactions - for instance ATM and DNA-PKcs bind and have their kinase activity stimulated by Nbs1 (You et al. 2005) and the Ku70/Ku80 heterodimer (Spagnolo et al. 2006), respectively, at these repeats. PIKKs also contain a small FAT, C terminus (FATC) domain at the C terminus, and as the name of the family would imply, a kinase-like domain between the FAT and FATC domains (Lovejoy & Cortez 2009) as well as a PIKK-regulatory domain (PRD) between the kinase and FATC domains (Mordes et al. 2008). The remainder of the proteins sequence of PIKKs consist primarily of more HEAT repeats but are otherwise divergent (Perry & Kleckner 2003).

The FRAP, ATM and TRRAP (FAT) domain is implicated in modulation of kinase activity, for example a complex of FK506-binding protein (FKBP12) and rapamycin bind to mTOR in this region - potently inhibiting it (Stan et al. 1994; Chen et al. 1995; Lempiäinen & Halazonetis 2009). Similarly inactive ATM is found in multimers where the FAT and kinase domains interact - autophosphorylation induces dissociation of multimers and activation of ATM by disrupting this interaction (Bakkenist & Kastan 2003).

One role of the FATC domain is to mediate association of the Tip60 complex (which contains TRRAP) to ATM - the complex acetylates and activates ATM after DNA damage. Deletion of ATM's FATC domain led to abolition of this activity (Sun et al. 2005; Jiang et al. 2006). Small changes in the FATC domain can also drastically affect kinase activity - a single residue deletion from the domain In mTOR abolishes kinase activity (Lempiäinen & Halazonetis 2009) and point mutation of a key leucine residue in SMG1 can reduce kinase activity by over 90%, though other mutations produced less dramatic results (Morita et al. 2007).

The PRD domain is essential for kinase function but is less sensitive to small changes relative to the FATC domain - some small deletions have no effect or even enhance kinase activity

(Sekulić et al. 2000; Mordes et al. 2008), with the conserved C-terminal half of the domain being a site of protein-protein interaction and post-translational modification. The PRD domain of ATM is the region acetylated by the FATC-interacting Tip60 complex (Sun et al. 2005; Jiang et al. 2006; Sun et al. 2007), in ATR kinase activity is enhanced by interaction with topoisomerase II-binding protein 1 at this domain (Kumagai et al. 2006; Mordes et al. 2008) and in mTOR a downstream target phosphorylates two sites in the PRD, though the effects on activity of these modifications remain unclear (Chiang & Abraham 2005; Holz & Blenis 2005).

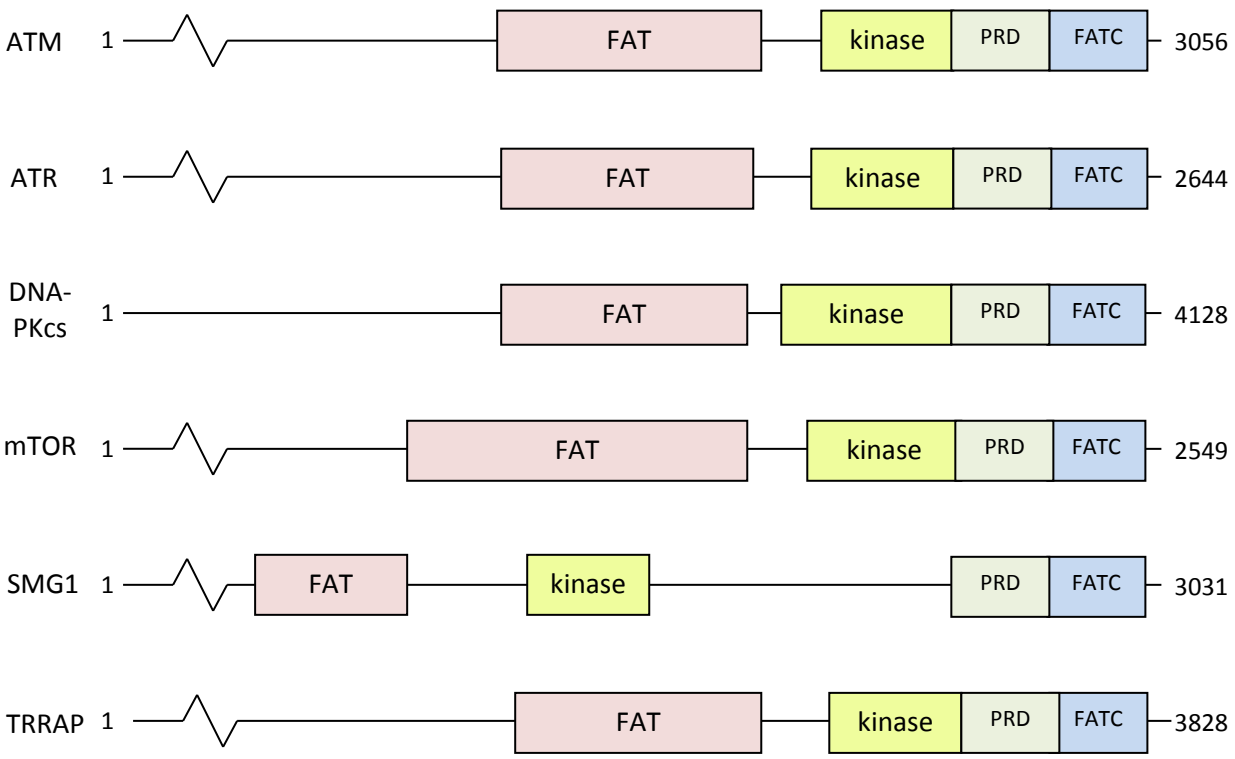


Figure 1.11 | **Comparison of primary structures of the PIKK family.** All members of the family have similar domains, though TRRAP's kinase domain does not retain kinase activity.

1.7.1 Ataxia-telangiectasia mutated (ATM)

The canonical method of ATM activation via Nbs1 of the MRN complex is outlined in chapter 1.4.2. There are, however, downstream effects of ATM activation not discussed in that section - for instance cells from patients lacking ATM demonstrate radiosensitivity, as would be expected, but also have defects in with the multiple cell cycle checkpoints (Maréchal & Zou 2013). This indicates the role of ATM in formation of cell cycle checkpoints. Indeed a proteomic screen of potential ATM targets (and ATR targets, as the targets of these kinases overlap substantially) demonstrated many targets related to the cell cycle and DNA replication as well as to DNA damage repair (Matsuoka et al. 2007).

Chk2 is phosphorylated by ATM on threonine 68 (and other sites) and forms a dimer (Lee & Chung 2001; Schwarz et al. 2003; Wu & Chen 2003), which then triggers additional conformational changes into active monomers and removal of the initial ATM phosphorylations (Ahn et al. 2002). Many of its verified substrates are involved in one of four processes - DNA damage repair itself, cell cycle regulation, apoptosis or p53 signalling (including p53 itself), and many of these substrates are also direct ATM targets. Other functions such as repression of RNAP I transcription after damage (Kruhlak et al. 2007) have

been described and the protein also has DNA damage response-independent functions that will not be summarised here such as a role in stability of telomeres (Zannini et al. 2014).

One mechanism of action of Chk2 is to phosphorylate cdc25A (a phosphatase) and thereby mark it for degradation. This prevents dephosphorylation of cyclin dependent kinase 2 and provides a block for the G1/S checkpoint (Falck et al. 2001). At the G2/M checkpoint Chk2 phosphorylates cdc25C, allowing it to be bound and transported out of the cytoplasm via interaction with 14-3-3 proteins (Dalal et al. 1999). This prevents cdc25 from activating the cyclin B1/cyclin dependent kinase 1 complex needed for the checkpoint (Takizawa & Morgan 2000). Five other pathways by which Chk2 mediates cell cycle checkpoints have been described but it is unknown whether these are DNA damage response associated or not (Zannini et al. 2014).

p53 is another major target of ATM, along with Hdm2 (Maya et al. 2001) and Chk2, both of which interact with it. It has been reported that the Chk2 interaction phosphorylates serine 20, possibly encouraging p53 dissociation from Hdm2 and therefore its stability (Chehab et al. 2000; Shieh et al. 2000; Hirao et al. 2000) though these findings have since been disputed (Zannini et al. 2014). In contrast the direct action of ATM on p53 (on serine 15) stimulates its transactivation activity (Dumaz & Meek 1999), some of the cell cycle control functions of ATM derive from this - for instance expression of the cyclin dependent kinase inhibitor p21 (inhibiting the G1/S transition) is promoted by ATM promoted p53-mediated transcription (Derheimer & Kastan 2010).

Another downsteam target of ATM is structural maintenance of chromosomes 1 (SMC1) SMC1 forms part of the cohesin complex, binds BRCA1 (and is therefore thought may have a role in DNA damage repair) and forms part an ATM-dependent Chk2-independent branch of the intra-S phase checkpoint (Yazdi et al. 2002). This pathway Is dependent on prior ATM activity on both Rad50 and Nbs1 of the MRN complex (Buscemi et al. 2001; Yazdi et al. 2002; Gatei et al. 2011). BRCA1 itself is also targeted by ATM, with differential phosphorylation resulting in arrest of different cell cycle checkpoints - serine 1387 modification is required for intra-S arrest (Xu et al. 2002) whereas serine 1423 is associated with G2/M arrest (Xu et al. 2001). BRCA1 is also a target of Chk2 at a different residue (Lee et al. 2000).

ATM can indirectly promote apoptosis by stabilising p53 both directly and via HDMX. ATM (directly and via Chk2) phosphorylate HDMX, a protein that marks p53 for ubiquitination and degradation by the proteasome (Zannini et al. 2014) and this causes HDMX to be retained in the nucleus by 14-3-3 proteins - these proteins and Chk2 stimulate ubiqutination and degradation of HDMX and therefore, indirectly, stabilisation of p53 (L. Chen et al. 2005; Pereg et al. 2006; LeBron et al. 2006). Chk2 also promotes transcription of pro-apoptotic genes by effects on p53 and E2F-1 (Stevens et al. 2003; Powers et al. 2004).

ATM is known to impact on SSB repair as well - it stabilises TDP1, promoting SSB repair by promoting its interaction with XRCC1 and DNA ligase IIIα (Das et al. 2009; Pommier et al. 2014). Other targets of ATM that are related to SSB repair or base excision repair include DNA ligase 3α (Dong & Tomkinson 2006) and APLF (Iles et al. 2007) and ATM can affect DNA damage repair by affecting chromatin dynamics, for example via phosphorylation of KRAB-associated protein 1 - relaxing chromatin and allowing access of DNA damage repair enzymes (Ziv et al. 2006; Goodarzi et al. 2008),

There is at least one MRN-independent ATM activation method. ATM can be oxidised into an active disulphide-linked dimer that does not target H2AX (Guo et al. 2010), ATM has since been linked to an antioxidant response mediated by the pentose phosphate metabolic pathway (Cosentino et al. 2011). Other stimuli that are known to stimulate ATM independently of MRN are hypoxia (Bencokova et al. 2009) and hyperthermia (Hunt et al. 2007), and it has been proposed that SSBs can also stimulate ATM (Khoronenkova & Dianov 2015). Collapsed replication forks, a trigger of ATR activity, will also trigger ATM if ATR signalling is compromised (Chanoux et al. 2009).

There is in fact significant overlap in targets and crosstalk between ATM and ATR. In *Xenopus* ATM can also phosphorylate DNA topoisomerase II binding protein 1 (TopBP1), a component of ATR signalling, on serine 1131. The importance of this modification will be discussed in chapter 1.7.2. (Hashimoto et al. 2006; Yoo et al. 2007). ATM may be involved in further signalling events regulating ATR signalling (Shin et al. 2012). The crosstalk is so substantial that ATM has even been known to phosphorylate checkpoint kinase 1 (Chk1), often thought of as a specific target of ATR, under certain conditions (Gatei et al. 2003; Helt et al. 2005).

1.7.2 Ataxia- and Rad3-related (ATR)

It is thought that the molecular structure that results in ATR activation is a junction between single stranded and double stranded DNA (MacDougall et al. 2007). These junctions can be generated at DNA replication forks if DNA polymerases and helicases become uncoordinated from each other, such as if the polymerases are inhibited (Walter & Newport 2000; Byun et al. 2005), resected ends at DSBs (Lee et al. 1998) and as intermediates in other varieties of DNA repair such as nucleotide excision repair, mismatch repair and the long-patch pathway used in base excision repair and single strand break repair (Shiotani & L Zou 2009). The amount of stimuli that can trigger ATR activation is therefore much wider than for ATM.

As previously mentioned in the context of HR, RPA binds single stranded DNA. ATR interacting protein (ATRIP) can in turn interact with RPA at multiple sites (Zou & Elledge 2003; Ball & Cortez 2005; Myers & Cortez 2006; Namiki & Zou 2006; Ball et al. 2007) and with ATR via its N-terminal HEAT repeats (Ball & Cortez 2005). At DSBs ATR signalling is stimulated by ATM and MRN complex activity (Jazayeri et al. 2006; Myers & Cortez 2006), with long stretches of single stranded DNA encouraging (via ATM and nuclease activity) a switch from ATM to ATR signalling (Shiotani & Lee Zou 2009) though it is still possible to activate ATR at resected DSBs in the absence of ATM (Tomimatsu et al. 2009). This explains the observation that ATM is quickly activated at DSBs but that ATR activation is much slower and much more prominent in the S and G2 phases (when HR would be operating) (Jazayeri et al. 2006).

ATR signalling requires the loading of two protein complexes onto DNA, these require RPA but do not require the ATR-ATRIP complex to be loaded (Kondo et al. 2001; Melo et al. 2001; Zou et al. 2002; Zou & Elledge 2003; Ellison & Stillman 2003; Majka et al. 2006). One is a ring shaped complex resembling PCNA (Shiotani & L Zou 2009) known as the 9-1-1 complex for its subunits RAD9, RAD1 and HUS1. The other complex, the RAD17 complex, loads the 9-1-1 complex onto DNA (Ellison & Stillman 2003; Shiotani & L Zou 2009) - it is analogous in function to the RFC complex which loads PCNA onto DNA and contains some of the same subunits. However the RFC complex loads PCNA onto 3' double-stranded/single-stranded DNA junctions whereas the RAD17 complex loads 9-1-1 onto the 5' equivalent

which is associated with stressed replication forks and DNA resection at DSBs (Ellison & Stillman 2003; Zou & Elledge 2003; Majka et al. 2006).

Localisation at single stranded DNA stimulates phosphorylation of ATR at threonine 1989, in the FAT domain, and promotes ATR stimulation by TopBP1 (Liu et al. 2011). This protein can also be recruited by RAD9 of the 9-1-1 complex in a phosphorylation dependent manner (Lee et al. 2007; Delacroix et al. 2007). It was previously mentioned that in *Xenopus* ATM can phosphorylate TopBP1 on serine 1131. ATR can also do this, and the effect of TopBP1 phosphorylation at this site is that TopBP1's association with ATR/ATRIP is enhanced. This implies a positive feedback loop, amplifying ATR signalling (Hashimoto et al. 2006; Yoo et al. 2007). TopBP1 activation of ATR in practice is strongly correlated with DNA damage, and it interacts with the 9-1-1 complex in both phosphorylation dependent and independent modes (Lee & Dunphy 2010).

A key effector kinase, phosphorylated by ATR and phosphorylating many downstream targets, is Chk1. Its phosphorylation occurs in a multi-step processes, with ATR and another kinase phosphorylating claspin (Kumagai & Dunphy 2003; Hae et al. 2006), a protein that stabilises, and is stabilised by, Chk1 (Kumagai & Dunphy 2000; Chini et al. 2006; Yang et al. 2008). This inhibits degradation of claspin in G2, when it would normally be degraded (Mailand et al. 2006; Peschiaroli et al. 2006; Bassermann et al. 2008), and thereby allowing Chk1 to be stabilised by claspin in both S and G2 phases. This in turn allows ATR to modify Chk1 itself and stimulates its activity (Liu et al. 2000; Kumagai et al. 2004; Mamely et al. 2006).

ATR signalling (including via Chk1) is associated with processes such as regulation of DNA repair, cell-cycle arrest, senescence and apoptosis - it is thought that there is significant redundancy between these its signalling and that of ATM (Maréchal & Zou 2013) and many targets, such as p53, are the same (Tibbetts et al. 1999; Weber & Ryan 2015). ATR is also required for cell survival and regulates both the intra-S-phase checkpoint and the G2/M checkpoint to promote genome stability. ATM also contributes to these processes (Brown & Baltimore 2003; Weber & Ryan 2015). ATR signalling is also well known for leading to stabilisation and restart of stalled replication forks (Weber & Ryan 2015).

Another interesting target of ATR is H2AX, generating the γH2AX modification and potentially recruiting ATM, demonstrating further cross talk between the pathways (Ward & Chen 2001). ATR has also been reported to phosphorylate DNA-PKcs and ATM (Yajima et al. 2006; Stiff et al. 2006).

1.7.3 DNA-dependent protein kinase, catalytic subunit (DNA-PKcs)

As with ATM, DNA-PKcs has been shown to stabilise TDP1 and encourage SSB repair by phosphorylation at serine 81 (Das et al. 2009; Pommier et al. 2014) and it has also been proposed that it may be able to activate Chk2 (Li & Stern 2005; Shang et al. 2010). Other functions shown *in vivo* are covered within chapter 1.4.2, as it is mostly involved with NHEJ.

1.7.4 Mammalian target of rapamycin (mTOR)

Mammalian target of rapamycin (mTOR) is a PIKK that can form into two distinct complexes mTOR complex 1 (mTORC1) or mTORC2, each containing several subunits of which about half are present in both complexes (Laplante & Sabatini 2012). As the name of the protein implies rapamycin is a potent inhibitor of mTOR - however this inhibition is indirect and conditional. Rapamycin itself binds the FK506-binding protein (FKBP12) (Koltin et al. 1991) and a complex of rapamycin with FKBP12 directly inhibits mTOR in the mTORC1 complex only - the mTORC2 complex is insensitive to acute rapamycin treatment (Laplante & Sabatini 2012). This could explain why rapamycin fails to inhibit some activities of the protein, such as autophosphorylation at serine 2481 (Peterson et al. 2000). Many analogues of rapamycin with differing properties are commercially available, referred to as rapalogues, and some of these such as Ku0063794 inhibit both mTORC1 and mTORC2 (García-Martínez et al. 2009).

mTORC1 activation is largely mediated through the GTPase-activating protein for Rheb, the tuberous sclerosis 1/2 (TSC1/2) heterodimer. Rheb binds to guanosine triphosphate (GTP) directly and potently stimulates mTORC1 activity, while TSC1/2 converts Rheb into an inactive, GDP bound state (Inoki et al. 2003; Tee et al. 2003). Akt signalling and hypoxia can also activate or inhibit mTORC1 directly (respectively) and signalling from amino acids is a pre-requisite for activation of mTORC1 (Wang et al. 2007; Vander Haar et al. 2007; Thedieck et al. 2007; Sancak et al. 2007; Gwinn et al. 2008; Laplante & Sabatini 2012).

mTORC1 is known to be activated by a wide range of different signalling pathways such as insulin signalling or Wnt (Inoki et al. 2002; Potter et al. 2002; Inoki et al. 2006) and can be

deactivated by heat stress or hypoxia (Takahara & Maeda 2012). In the longer term mTORC1 signalling can also be decreased via p53 transcriptional control of signalling proteins (Stambolic et al. 2001; Feng et al. 2005; Budanov & Karin 2008). mTORC2 activation is much more poorly characterised - it is known that unlike mTORC1 it is insensitive to nutrient status but like mTORC1 it is sensitive to insulin signalling (Laplante & Sabatini 2012). It is also known to require PI3K signalling and have some dependency on ribosomes (Zinzalla et al. 2011).

mTORC1 has a key role in metabolic control, upregulating glycolysis and lipogenesis via the transcription factors sterol regulatory binding element protein and hypoxia inducible factor 1α (Düvel et al. 2010; Garelick & Kennedy 2011). It is also implicated in regulation of autophagy (Martina et al. 2012) and osmotic stress responses (Ortells et al. 2012) through transcriptional regulation; and has a role in RNA splicing (Han et al. 2010; Goh et al. 2010; Hsu et al. 2011) and localisation (Dai et al. 2011).

The complex also has a key role in regualating metabolism across the body. For instance it is involved in determination of satiety as it is activated in the hypothalamus in response to leptin (Cota et al. 2006) and has been reported to inhibit expression of appetite inducing signalling molecules such as neuropeptide Y and agouti-related peptide (Blouet et al. 2008; Cota et al. 2008). mTORC1 activation is also associated with an increase in the size and number of pancreatic β cells and an increase in insulin release (Pende et al. 2000; Shigeyama et al. 2008; Rachdi et al. 2008) but excessively high flux through mTORC1 is also associated with in promoting insulin resistance in various tissues (Um et al. 2004; Khamzina et al. 2005; Tremblay et al. 2007).

mTOR in mTORC2 has a far less well characterised role but is thought to regulate the cytoskeleton (Sarbassov et al. 2004; Jacinto et al. 2006), metabolism, cell cycle arrest and apoptosis (Laplante & Sabatini 2012).

The mechanisms by which mTORC1 regulates these pathways are either translational or transcriptional. There are three ways in which it exerts translational control: signalling through regulation of transcriptional initiation via phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and via the ribosomal S6 kinase 1 (S6K1) (which also affects transcription), and promotion of translation of mRNA containing the

terminal oligopyrimidine track in the 5' untranslated region (TOP) motif. These will be described in order except for regulation of TOP containing mRNAs where the mechanisms remain obscure - with the previous hypothesis, that it was regulated by S6K1 signalling, being disproven (Tang et al. 2001).

Hypophosphorylated 4E-BP1 binds strongly to eukaryotic initiation factor 4E (eIF4E) and prevents its interaction with eIF4G (Marcotrigiano et al. 1999). Hyperphosphorylated 4E-BP1 loses this interaction, and allows interaction between eIF4E and eIF4G. This allows formation of the eIF4F complex and recognition of mRNA caps, leading to translational initiation (A.C. Gingras et al. 2001). 4E-BP1 release is regulated by hierarchical series of phosphorylation events at set residues - it is unclear how many of these sites mTOR may phosphorylate but rapamycin has been shown to strongly affect phosphorylation status at, amongst other residues, serine 65 and threonine 70 (Mothe-Satney et al. 2000; A.C. Gingras et al. 2001), which are important sites for eIF4E release (Karim et al. 2001). Translational regulation by this method favours translation of mRNAs with structured 5' regions though specific motifs (such as TOP, mentioned previously) may also involve other layers of regulation (A.C. Gingras et al. 2001).

S6K1 is activated by mTORC1 - full activation of the enzyme requires phosphorylation at multiple sites with mTORC1 phosphorylating at threonine 389 and (indirectly) at threonine 229. Both of these sites are critical but not sufficient for S6K1 function (Martin & Blenis 2002). S6K1 then affects translation through multiple pathways, for instance it is one of multiple kinases (Holz et al. 2005; Shahbazian et al. 2006) that can phosphorylate eIF4B and encourage it to bind eIF4A, increasing its affinity for ATP (Rogers et al. 2002) and resulting in formation of the eIF4F complex (Lachance et al. 2002).

The kinase also regulates ribosome biogenesis - S6K1 is proposed to promote RNAP I transcription by (indirectly) upregulating UBF expression, in conjunction with more short term upregulation of the process by an unknown kinase (Hannan et al. 2003; Nader et al. 2005). As its name implies S6K1 phosphorylates ribosomal protein S6 - though the significance of this action is unclear (Iadevaia et al. 2014).

Finally mTORC1 can exert transcriptional control independently of S6K1 by phosphorylating TIF-1A (promoting rRNA synthesis) and therefore providing a crucial link between nutrient

status and ribosome biogenesis (Mayer et al. 2004). RNAP III activity is also promoted by mTORC1 signalling via phosphorylation of the transcriptional inhibitor Maf1 (releasing RNAP III) (Shor et al. 2010). mTORC1 may also be implicated in pre-rRNA processing as rapamycin treatment both impairs synthesis of and induces decay of mature rRNA, though it does not affect nucleolar morphology (Iadevaia et al. 2012).

1.7.5 Suppressor of morphogenesis in genitalia 1 (SMG1)

The canonical role of suppressor of morphogenesis in genitalia 1 (SMG1) is in the nonsense-mediated decay pathway, otherwise known as the mRNA surveillance or turnover pathways (Nicholson et al. 2010). This pathway mediates decay of mRNAs containing premature termination codons and exists in most eukaryotes (Culbertson & Leeds 2003) but is also implicated in degrading some mRNAs lacking these (Lewis et al. 2003; Stalder & Mühlemann 2008) - implying a role in both mRNA quality control but also in regulation of gene expression.

The core protein complex consists of up frameshift proteins (UPF) 1-3. UPF1 is an RNA helicase (Czaplinski et al. 1995; Bhattacharya et al. 2000), UPF3 binds the exon-junction complex during splicing and UPF2 binds the complex together (Lykke-Andersen et al. 2000). SMG1 is also found at the exon-junction complex (Kashima et al. 2006). Activity of the core complex requires rounds of phosphorylation and dephosphorylation (Ohnishi et al. 2003); the former activity requires SMG1 (the kinase itself (Yamashita et al. 2001)), UPF2 and UPF3 (Nicholson et al. 2010), whereas the latter activity is dependent on SMG5, SMG6 and SMG7 - with protein phosphatase 2A performing the enzymatic activity (Ohnishi et al. 2003; Chiu et al. 2003; Nicholson et al. 2010). Other proteins with unclear function exist, such as SMG8 (Nicholson et al. 2010).

SMG1 is also implicated in the DNA damage response, being responsive to ultraviolet (UV) radiation, ionising radiation (IR) (Brumbaugh et al. 2004) and oxidative stress. In the latter case it is thought to phosphorylate p53 prior to ATM (Gehen et al. 2008) - SMG1 phosphorylates p53 at serine 15 in cells depleted of UPF1 or UPF2, indicating that its role in the DNA damage response is not dependent on other components of the nonsense mediated decay pathway (Gewandter et al. 2011). Depletion of SMG1 also increases the

rate of apoptotic cell death mediated by tumour necrosis factor-α implicating SMG1 in cytokine signalling (Oliveira et al. 2008).

In the telomeres UPF1, SMG1 and SMG6 are enriched and depletion of the proteins resulted in telomere damage and SMG1 was also shown to be negatively associated with production of telomeric repeat-containing RNA (Azzalin & Lingner 2006; Azzalin et al. 2007; Nicholson et al. 2010). Although the role of this RNA species is unclear it is thought that it is displaced from binding the chromosome ends (Cusanelli & Chartrand 2015) rather than being causative for telomere loss.

1.7.6 Transformation/transcription domain-associated protein (TRRAP)

TRRAP is a kinase-dead PIKK (Vassilev et al. 1998; McMahon et al. 1998) notable for being a common component of histone acetyltransferase complexes (Murr et al. 2007), which acetylate histone tails and allow access to the DNA for a variety of cellular processes such as transcription (Brown et al. 2000; Carrozza et al. 2003). Although this is its most well known role it has also been located to the p400 chromatin remodelling complex (Fuchs et al. 2001) and the MRN complex (Robert et al. 2006), neither of which have histone acetyltransferase activity, and has been shown to associate with other molecules such as c-myc (McMahon et al. 1998), β-catenin (Sierra et al. 2006) and p53 (Ard et al. 2002).

It is also possible that TRRAP, in complex with Tip60, can activate ATM via its FATC domain (Sun et al. 2005) but studies have determined that it is not necessary for ATM activation as TRRAP-depleted cells can still activate ATM (Murr et al. 2006).

1.8 Phosphodiesterases (PDEs)

The cyclic nucleotide phosphodiesterases (PDEs) hydrolyse the secondary messenger molecules cyclic 3',5'-adenosine monophosphate (cAMP) and cyclic 3',5'-guanosine monophosphate (cGMP), antagonistic to the activities of adenylyl and guanylyl cyclases, and act as homodimers. There are eleven families of PDEs (PDEs 1-11), some of which contain multiple proteins, and some proteins of which contain multiple splice variants that may differ in tissue specificity or subcellular localisation. Of the PDE families PDEs 4, 7 and 8 are cAMP specific; PDEs 5, 6 and 9 are cGMP specific and the remainder are dual function, though not necessarily equally competent at cAMP and cGMP hydrolysis. PDE10 enzymes have a preference for cAMP for instance (Keravis & Lugnier 2012). The nomenclature of

PDEs are such that the family is assigned an Arabic numeral, the member within a family a letter and different splice variants or the proteins arising from them with an additional numeral. PDE expression and activity are regulated by a variety of processes with no clear patterns emerging among the superfamily as a whole (Omori & Kotera 2007). This is perhaps to be expected as the N-termini of the different PDE families vary substantially, even though the catalytic domain is largely conserved. It is notable, however, that cGMP can act as an inhibitor of PDE3, and that many of the families have phosphorylation sites for cAMP or cGMP dependent protein kinase (PKA/PKG), downstream of adenylyl and guanylyl cyclases, suggesting a degree of autoregulation of cAMP/cGMP pathways (Keravis & Lugnier 2012).

These features can be illustrated using the PDE8 family as an example. There are two members of the family, each with five splice variants (Wang, Wu, Egan, et al. 2001; Gamanuma et al. 2003) - PDE8A and PDE8B. Two splice variants of PDE8B are PDE8B1 and PDE8B3 and these differ in tissue specificity - PDE8B1 predominates over PDE8B3 in the thyroid gland but the converse is true in the brain (Hayashi et al. 2002). PDE8A and PDE8B also differ in tissue distribution. PDE8B is most highly expressed in the thyroid with far weaker expression in other tissues whereas PDE8A is robustly expressed in a range of tissues (Fisher et al. 1998; Hayashi et al. 1998; Keravis & Lugnier 2012). The N-terminal regions of PDE8 proteins also contain two domains the other PDE families lack, the REC and PAS domains. The role of these are unclear and some splice variants lack one or both domains but this demonstrates the heterogeneity of regulatory elements in PDEs (Wang, Wu, Egan, et al. 2001; Hayashi et al. 2002). There are also putative phosphorylation sites for PKA and PKG on PDE8 proteins (Keravis & Lugnier 2012).

Caffeine has long been known to act as a broad acting PDE inhibitor (Sutherland & Rall 1958; Essayan 2001) and this activity is also present in many other chemically similar methylxanthines such as theophylline and theobromine (Butcher & Sutherland 1962). Caffeine has varied activities beyond just being a PDE inhibitor (Daly 2000) and so other broad acting PDE inhibitors tend to be used. One such inhibitor is 3-isobutyl-1-methylxanthine (IBMX), another methylxanthine which inhibits all families of PDEs except for the PDE8 and PDE9 families (Daly 2000; Omori & Kotera 2007). These two families have very high IC_{50} values for IBMX (Huai et al. 2004; H. Wang et al. 2008) likely due to a point

mutation from phenylalanine to tyrosine present in both families. When this mutation was reversed in PDE8A the enzyme became an order of magnitude more IBMX sensitive (H. Wang et al. 2008). There are also a variety of inhibitors available to act on specific families of PDEs (Boswell-Smith et al. 2006), though it is common for them to inhibit other PDE families at higher concentrations. For instance dipyridamole has an IC_{50} of around 1-2 μM for PDE5 (depending on isoform) (Wang, Wu, Myers, et al. 2001) but also inhibits many other PDE families (Jackson et al. 2007).

Naturally the activities of PDEs inhibit the cAMP/cGMP signalling pathways, in some cases they can effectively be the main regulator of this process, for instance in cardiac myocytes where cyclases are constitutively active regulation of cyclic nucleotide levels relies primarily on PDE activity (Mongillo et al. 2004). Events downstream of cAMP or cGMP generation are largely mediated via PKA or PKG; though both molecules also directly interact with some cation channels (Fimia & Sassone-Corsi 2001; Feil & Kemp-Harper 2006; Zaccolo & Movsesian 2007) and a major component of cAMP signalling is via the Epac proteins, cAMP activated guanine nucleotide exchange factors that act on Ras-related GTPases (Grandoch et al. 2010). The cyclic nucleotides can also directly affect PDE activity and allow crosstalk between the cAMP and cGMP pathways as cGMP is stimulatory for PDE2 and inhibitory for PDE3 (Keravis & Lugnier 2012).

PKA exists in multiple isoforms, all found in an inactive tetrameric state where two regulatory subunits inhibit two catalytic subunits, and cAMP induces a conformational change in the regulatory subunit that encourages dissociation of the tetramer (Taylor et al. 1990). PKA may also require phosphorylation by the constitutively active 3-phosphoinositide-dependent kinase 1 (Cheng et al. 1998; Casamayor et al. 1999; Nirula et al. 2006). A key function of PKA are activation of the specific transcription factors CREB and NFκB (Shaywitz & Greenberg 1999; Fimia & Sassone-Corsi 2001). PKG is found as a homodimer in its inactive state with cGMP binding inducing a conformational change and its activity is associated with calcium homeostasis and modulation of calcium sensitivity of proteins (Francis et al. 2010) though it is also thought to have many roles that overlap with PKA (Pearce et al. 2010). There are also multiple PKG genes and alternative splicing variants (Lucas et al. 2000). The activities of PKA and PKG on PDEs can vary depending on the target. For instance PKA phosphorylation of the calmodulin and calcium dependent PDE1 reduces

its affinity for calcium and so inhibits it but PKA activity stimulates PDE3 and PDE4 (Oki et al. 2000; Zaccolo & Movsesian 2007).

The variety of activities of the directly cAMP-activated Epac proteins in different tissue types is vast and activation has been known to signal down through several distinct pathways. The two Epac proteins are also differentially localised in the cell - with Epac1 broadly being cytoplasmic or located to internal membranes and Epac2 usually found in regions of the plasma membrane (Grandoch et al. 2010). Their immediate functions are to act as guanine exchange factors for the Ras superfamily members Rap1, Rap2 (de Rooij et al. 1998; Kawasaki et al. 1998), R-Ras (López De Jesús et al. 2006) and Ras itself (Métrich et al. 2008).

Localisation of components of the cyclic nucleotide signalling pathways can be vital and can allow maintenance of different pools of cAMP and cGMP in different regions of the cell. In the aforementioned cardiac myocytes PDE4 and PDE2 proteins were found to regulate responses to an external stimulus while PDE3 proteins were found to regulate a distinct pool of cAMP (Mongillo et al. 2004; Mongillo et al. 2006; Rochais et al. 2006). Scaffolding proteins can have a key role in this - for example A-kinase anchoring proteins, which are responsible for PKA localisation (Wong & Scott 2004), can form cyclic nucleotide signalling complexes localised to a particular subcellular location. Two examples include a complex of mAKAP, PKA, PDE4D3, ERK3 and Epac1 found at the nuclear envelope of rat cardiomyocytes (Dodge-Kafka et al. 2005) and a complex of AKAP79/150, PKA, Akt and Epac2A to the plasma membrane in mouse primary cortical neurons (Nijholt et al. 2008).

2. Materials and Methods

2.1 Reagents and solutions

2.1.1 Solutions

1x diethylpyrocarbonate (DEPC)-treated water – DEPC (Sigma-Aldrich) added to 0.1% (v/v) to distilled water, mixed and left at room temperature for 24 hours, then autoclaved. Stored at room temperature.

1x phosphate buffered saline (PBS) - made centrally for the centre by support staff. 137mM sodium chloride, 0.3mM potassium chloride, 10mM disodium phosphate, 1.7mM monopotassium phosphate made up in one litre of distilled water and adjusted to pH 7.4. Autoclaved and stored at room temperature.

10% (w/v) SDS in water - made centrally for the centre by support staff.

10x TBS - communal lab solution. 10mM Tris base, 1.4M sodium chloride made up in five litres of distilled water. Stored at room temperature.

EDTA (pH 7.0) - Ethylenediaminetetraacetic acid (EDTA) (Thermo-Fisher Scientific) was made up to concentration in DEPC-treated water, then pH adjusted to 7.0. Stored at room temperature.

EDTA (pH 8.0) - as above, but pH adjusted to 8.0.

5x Laemmli buffer (LB) – communal lab solution. A solution of 250mM Tris HCl (adjusted to pH 8), 10% SDS, 0.5% bromophenol blue (Thermo-Fisher Scientific), 500mM dithiothreitol and 50% glycerol made up in distilled water.

2% (w/v) methylene blue in 70% ethanol - the desired mass of methylene blue (Thermo-Fisher Scientific) was added to the desired volume of ethanol (Thermo-Fisher Scientific), pre-diluted to 70% (v/v) in distilled water. Stored at room temperature.

Methylene blue solution for northern blots - 0.02% methylene blue was dissolved in a 0.3M aqueous solution of sodium acetate (in DEPC-treated water) and pH adjusted to 5.5.

10x 3-(N-morpholino)propanesulfonic acid (MOPS) electrophoresis buffer - 0.2M MOPS (Sigma-Aldrich), 20mM sodium acetate (BDH chemicals), 10mM EDTA (pH 8.0). Not autoclaved before use. Stored at room temperature, protected from light.

1x northern blot prehybridisation solution - 0.5M sodium phosphate (pH 7.2), 1mM EDTA (pH 7.0), 7% (w/v) SDS. The solution was autoclaved and 10g/l bovine serum albumin (BSA) (Thermo-Fisher Scientific) added after it had cooled. Stored at room temperature and heated to 42°C before use.

1x northern blot soaking solution - 0.05M sodium hydroxide (Thermo-Fisher Scientific) made up in DEPC-treated water.

1x PBSTS - TWEEN-20 (Sigma-Aldrich) added to 0.1% (v/v) to 1x PBS, sodium dodecyl sulphate (SDS) (Sigma-Aldrich) added to 0.02% (w/v), mixed and stored at room temperature.

2x RNA Gel Loading Dye - purchased from Thermo-Fisher Scientific.

0.5M sodium phosphate (pH 7.2) - 1M solutions of monosodium phosphate (NaH_2PO_4 - Sigma-Aldrich) and disodium phosphate (Na_2HPO_4 - Sigma-Aldrich) were prepared and stored at room temperature. 280ml of NaH_2PO_4 was mixed with 720ml of Na_2HPO_4 to produce a litre of pH 7.2 sodium phosphate and this was diluted to concentration with distilled water.

20x SSC solution - 3M sodium chloride (Thermo-Fisher Scientific), 0.3M sodium citrate (Thermo-Fisher Scientific), made up to final volume with distilled water. pH adjusted to 7.0 and solution autoclaved. Stored at room temperature, and appropriate dilutions made from 20x stock.

50x TAE - communal lab solution. 2M Tris base, 50mM EDTA (pH 8) and 1.75% glacial acetic acid (v/v) made up in one litre of distilled water. Diluted to 1x in distilled water and stored at room temperature.

1x TBST - 10x TBS diluted to 10% (v/v) in distilled water, TWEEN added to 0.1% (v/v), mixed and stored at room temperature.

10x TOWBIN - communal lab solution. 25mM Tris base 192mM glycine made up in five litres of distilled water. Stored at room temperature.

0.2% (v/v) Triton X-100 - Triton X-100 (Sigma-Aldrich) added to 0.2% (v/v) to 1x PBS, mixed, heated to 65°C and then stored at room temperature.

10x western blot running buffer - communal lab solution. 250mM Tris base (Thermo-Fisher Scientific), 2.5M glycine (Thermo-Fisher Scientific), 35mM SDS made up in five litres of distilled water. Stored at room temperature and diluted to 1x in distilled water before use.

1x western blot transfer buffer – 10x TOWBIN diluted to 10% (v/v) and methanol (Thermo-Fisher Scientific) added to 10% (v/v) in distilled water. Used at room temperature immediately.

1x western blot stripping solution - 62.5mM Tris base (pH 6.7) in 20ml 10% SDS.

2.1.2 Tissue culture materials

10% foetal calf serum (FcS) DMEM - Dulbecco's Modified Eagle Medium (Gibco - ThermoFisher Scientific - 21969-035) supplemented with 10% (v/v) pre-filtered foetal calf serum (PAN-Biotech), 1% L-glutamine (Corning) and 1% penicillin-streptomycin (Corning). L-glutamine and penicillin-streptomycin are filtered through a gamma sterilised 0.22µm Millex-GP syringe filter (Merck-Millipore - SLGP033RS). Stored at 4°C.

15% FcS DMEM - Dulbecco's Modified Eagle Medium (Gibco - ThermoFisher Scientific - 21969-035) supplemented with 15% (v/v) pre-filtered foetal calf serum (PAN-Biotech), 1% L-glutamine (Corning) and 1% penicillin-streptomycin (Corning). L-glutamine and penicillin-streptomycin are filtered through a gamma sterilised 0.22µm Millex-GP syringe filter (Merck-Millipore - SLGP033RS). Stored at 4°C.

0.1% FcS DMEM - Dulbecco's Modified Eagle Medium (Gibco - ThermoFisher Scientific - 21969-035) supplemented with 0.1% (v/v) pre-filtered foetal calf serum (PAN-Biotech), 1% L-glutamine (Corning) and 1% penicillin-streptomycin (Corning). L-glutamine and penicillin-streptomycin are filtered through a gamma sterilised 0.22µm Millex-GP syringe filter (Merck-Millipore - SLGP033RS). Stored at 4°C.

10% FcS MEM - Minimum Essential Medium (Gibco - ThermoFisher Scientific – 21096-022) supplemented with 10% (v/v) pre-filtered foetal calf serum (PAN-Biotech), 1% L-glutamine (Corning) and 1% penicillin-streptomycin (Corning). L-glutamine and penicillin-streptomycin are filtered through a gamma sterilised 0.22µm Millex-GP syringe filter (Merck-Millipore - SLGP033RS). Stored at 4°C.

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OPTI-MEM - purchased in solution from Gibco/Thermo-Fisher Scientific (31985-070).

1% (v/v) PFA in 1xPBS - 16% (v/v) Paraformaldehyde (formaldehyde) aqueous solution (Electron Microscopy Sciences) serially diluted to 4%, and then to 1% in 1xPBS. Stored at -20°C, used at room temperature.

4% (v/v) PFA in 1xPBS - 16% (v/v) Paraformaldehyde (formaldehyde) aqueous solution (Electron Microscopy Sciences) diluted to 4% in 1xPBS. Stored at -20°C, used at room temperature.

0.25% (w/v) trypsin in 1xPBS - the desired mass of trypsin (PAN Biotech - P10-025100P) solubilised in a small volume of 1x PBS, then filtered through a gamma sterilised 0.22µm Millex-GP syringe filter (Merck-Millipore - SLGP033RS) into a larger volume of 1x PBS to make a final concentration of 0.25% (w/v).

2.1.3 Reagents

2.1.3.1 Vehicles

Dimethyl sulphoxide (DMSO) - purchased from Sigma-Aldrich.

2.1.3.2 Dyes and labels

4',6-diamidino-2-phenylindole (DAPI) - desiccated solid (Thermo-Fisher Scientific) made up to 10mg/ml in distilled water. Stored at -20°C. Diluted to 0.05µg/ml in 1x PBS before use.

Ethynyl uridine (EU) - desiccated solid from Click-iT® RNA Alexa Fluor® 488 Imaging Kit (Invitrogen – ThermoFisher Scientific - C10329) made up to concentration with distilled water. Stored at -20°C.

Hoechst 33258 - desiccated solid (Sigma-Aldrich) made up to concentration with distilled water. Stored at -20°C.

2.1.3.3 Inhibitors

3-isobutyl-1-methylxanthine (IBMX) - desiccated solid (Sigma-Aldrich) made up to concentration with DMSO. Stored at -20°C.

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) - routinely kept as a communal stock (Sigma-Aldrich) solution in the lab, solvent is DMSO. Stored at -20°C.

α-amanitin - routinely kept as a communal stock solution (Sigma-Aldrich) in the lab, solvent is water. Stored at -20°C.

Actinomycin D (ActD) - desiccated solid (Sigma-Aldrich) made up to concentration with DMSO. Stored at -20°C.

ATR Kinase Inhibitor II - made up from solid (Calbiochem) in DMSO. Stored at -20°C.

Caffeine - solid (Sigma-Aldrich) made up to concentration in cell culture medium. Used on the same day as preparation.

Camptothecin (CPT) - a communal stock solution in the lab (Sigma-Aldrich), solvent is DMSO. Serially diluted 1mM stock (in DMSO) used for most experiments. Stored at -20°C.

CX5461 - desiccated solid (Selleck Chemicals) made up to concentration with DMSO. Stored at -20°C.

Dipyrimadole - desiccated solid (Sigma-Aldrich) made up to concentration with DMSO. Stored at -20°C.

Etoposide - routinely kept as a communal stock solution (Sigma-Aldrich) in the lab, solvent is DMSO. Stored at -20°C.

Hydrogen peroxide - purchased in solution from Thermo-Fisher Scientific.

Hydroxyurea (HU) - desiccated solid (Sigma-Aldrich) made up to concentration with water. Stored at -20°C.

Ku0063794 (mTOR inhibitor) – made up from solid (Tocris) in DMSO. Stored at -20°C.

Ku55933 (ATM inhibitor) - a communal stock solution in the lab, made up from solid (Tocris) in DMSO. Stored at -20°C.

Ku58948 (PARP inhibitor) - a communal stock solution in the lab, made up from solid (AstraZeneca) in DMSO. Stored at -20°C.

NU7441/Ku57788 (DNA-PKcs inhibitor) - made up from solid (Stratech) in DMSO. Stored at -20°C.

PI-103 - made up from solid (Tocris) in DMSO. Stored at -20°C.

Theophylline - solid (Sigma-Aldrich) made up to concentration in cell culture medium. Used on the same day as preparation.

Wortmannin - made up from solid (Sigma-Aldrich) in DMSO. Stored at -20°C.

2.1.3.4 Other reagents

Doxycycline - desiccated solid doxycycline hyclate (Sigma-Aldrich) made up to concentration with distilled water. Stored at -20°C.

Insulin - made up from solid (Sigma-Aldrich) in distilled water containing 1% glacial acetic acid (Thermo-Fisher Scientific). Stored at -20°C.

Lipofectamine RNAiMax transfection reagent - purchased from Invitrogen (Thermo-Fisher Scientific - 13378-150).

Metafectane Pro - purchased from Biontex (T040).

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Rabbit polyclonal anti-Phospho-4E-BP1 (Thr37/46) - used at a 1:1000 dilution for western blots - primary incubation overnight for western blots. Catalogue number #9459 from Cell Signalling Technology. Stored at -20°C.

Mouse monoclonal anti-actin (Sigma) - used at a 1:1000 dilution for western blots - primary incubation for one hour for western blots. Catalogue number A4700 from Sigma-Aldrich. Stored at -20°C. Used for all actin blots except those noted below.

Mouse monoclonal anti-β actin (ProteinTech) - used at 0.426µg/ml for western blots - primary incubation for one hour for western blots. Catalogue number 66009-1-Ig from ProteinTech. Stored at -20°C. Used in Figs 6.6c.

Rabbit polyclonal anti-CENPF - used at 2.5-5µg/ml (concentration of antibody varies by batch) for immunofluorescence. Catalogue number ab5 from Abcam. Stored at 4°C.

Rabbit polyclonal anti-Phospho-Chk1 (Ser345) - used at a 1:500 dilution for western blots - primary incubation overnight for western blots. Catalogue number 2344 from Cell Signalling Technology. Stored at -20°C.

Rabbit polyclonal anti-Phospho-Chk2 (Thr68) - used at a 1:500 dilution for western blots - primary incubation overnight for western blots. Catalogue number 2661 from Cell Signalling Technology. Stored at -20°C.

Mouse monoclonal anti-fibrillarin - used at a 1:250 dilution for immunofluorescence. Catalogue number ab4566 from Abcam. Stored at 4°C.

Rabbit polyclonal anti-fibrillarin - used at 3.6-4 µg/ml (concentration of antibody varies by batch) for immunofluorescence. Catalogue number ab5821 from Abcam. Stored at 4°C.

Rabbit polyclonal anti-fused-in-sarcoma (FUS) (Novus) - used at 2.5µg/ml for both immunofluorescence and western blot - primary incubation overnight for western blots. Catalogue number NB100-565 from Novus, part of Bio-Techne. Stored at 4°C.

Rabbit polyclonal anti-FUS (ProteinTech) - used at 1.23µg/ml for immunofluorescence. Catalogue number 11570-1-AP from ProteinTech. Stored at 4°C.

Mouse monoclonal anti-FUS (Santa Cruz) - used at 0.5µg/ml for both immunofluorescence and western blotting - primary incubation overnight for western blots. Catalogue number sc-47711 from Santa Cruz Biotechnology. Stored at 4°C.

Rabbit polyclonal anti-FUS (Sigma) - used at a 1:400 dilution for immunofluorescence. Catalogue number HPA008784 from Sigma-Aldrich. Stored at 4°C.

Mouse monoclonal anti-Phospho-γH2AX (Ser139) - used at a 1:1000 dilution for immunofluorescence. Catalogue number 05-636 from Merck-Millipore. Stored at 4°C.

Mouse monoclonal anti-nucleophosmin/anti-B23 - used at 0.5µg/ml for immunofluorescence. Catalogue number FC-61991 from Invitrogen, ThermoFisher Scientific. Stored at 4°C.

Rabbit polyclonal anti-Phospho-p53 (Ser15) - used at a 1:5000 dilution for western blots - primary incubation overnight for western blots. Catalogue number #9284 from Cell Signalling Technologies. Stored at -20°C.

Rabbit polyclonal anti-phosphodiesterase 8A (PDE8A) - used at 0.1µg/ml for western blots - primary incubation overnight. Catalogue number ab109597 from Abcam. Stored at -20°C.

Mouse monoclonal anti-poly ADP ribose (10-H) - used at 1:200 for western blots - primary incubation overnight. Catalogue number ab14459 from Abcam. Stored at 4°C.

Mouse monoclonal anti-PARP1 - used at 0.2µg/ml for western blots - primary incubation overnight. Catalogue number MCA1522G from AbD Serotec - Biorad. Stored at 4°C.

Rabbit polyclonal anti-Phospho-Rb (Ser807/811) - used at 1:1000 for western blots - primary incubation overnight for western blots. Catalogue number #9308 from Cell Signalling Technology. Stored at -20°C.

Rabbit polyclonal anti-Phospho-RPA32 (Ser4/Ser8) - used at 0.2µg/ml for western blots - primary incubation overnight for western blots. Catalogue number A300-245A from Bethyl Laboratories. Stored at -20°C.

Rabbit polyclonal anti-TDP43 (ProteinTech) - used at 0.613µg/ml for western blots - primary incubation overnight for western blots. Catalogue number 10782-2-AP from ProteinTech. Stored at 4°C.

2.1.4.2 Secondary antibodies

Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) - used at 2µg/ml as a secondary antibody in immunofluorescence. Catalogue number A-31628 from Invitrogen, Thermo-Fisher Scientific. Stored at 4°C.

Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) - used at 2µg/ml as a secondary antibody in immunofluorescence. Catalogue number A-11001 from Invitrogen, Thermo-Fisher Scientific. Stored at 4°C.

Alexa Fluor 555-conjugated goat anti-rabbit IgG (H+L) - used at 2µg/ml as a secondary antibody in immunofluorescence. Catalogue number A-21429 from Invitrogen, Thermo-Fisher Scientific. Stored at 4°C.

Alexa Fluor 555-conjugated goat anti-mouse IgG (H+L) - used at 2µg/ml as a secondary antibody in immunofluorescence. Catalogue number A-21422 from Invitrogen, Thermo-Fisher Scientific. Stored at 4°C.

Alexa Fluor 647-conjugated goat anti-rabbit IgG (H+L) - used at 2µg/ml as a secondary antibody in immunofluorescence. Catalogue number A-21244 from Invitrogen, Thermo-Fisher Scientific. Stored at 4°C.

HRP-conjugated goat anti-rabbit IgG - used at a 1:5000 dilution as a secondary antibody for western blots. Catalogue number P0448 from Dako.

HRP-conjugated rabbit anti-mouse IgG - used at 1:5000 dilution as a secondary antibody for western blots. Catalogue number P0260 from Dako.

2.1.5 siRNAs

Control siRNA - ON-TARGETplus Non-targeting Control Pool purchased from Dharmacon (GE Life Sciences - D-001810-10-05). Resuspended in RNAse-free distilled water.

Fused-in-sarcoma (FUS) siRNA - siGENOME SMARTpool purchased from Dharmacon (GE Life Sciences - M-009497-02). Resuspended in RNAse-free distilled water.

Phosphodiesterase 8A (PDE8A) siRNA - siGENOME SMARTpool purchased from Dharmacon (GE Life Sciences – M-003824-04-0005). Resuspended in RNAse-free distilled water.

2.1.6 Enzymes

RNA Qualified (RQ1) Dnase - purchased from Promega (M610A) along with 10x buffer for the enzyme (M198A). Stored at -20°C.

T4 Polynucleotide kinase - purchased from New England Biolabs (M0201) along with 10x buffer for the enzyme (B0201). Stored at -20°C.

2.1.7 Northern blot probes

All sourced from Eurogentec.

5'-ETS-1b DNA oligonucleotide - AGACGAGAACGCCTGACACGCACGGCAC

18S DNA oligonucleotide -TTTATTCCTCTAGATAGTCAAGTTCGACC

28S DNA oligonucleotide - CCCGTTCCCTTGGCTGTGGTTTCGCTAGATA

2.2 Tissue culture

2.2.1 Cell lines and media

Cell line	Media
1BR (hTERT)	15% FcS MEM
A549	15% FcS DMEM
FUS patient (R521H heterozygous) fibroblasts (hTERT)	10% FcS MEM
FUS patient sibling (WT) fibroblasts (hTERT)	10% FcS MEM
Hela LAP-FUS (WT/R521G mutant)[1]	10% FcS DMEM
Hela LAP-TDP43 (WT)[2]	10% FcS DMEM
NM720 (DNA-PKcs patient fibroblasts) (hTERT)[3]	15% FcS MEM

[1] Details of cell line the same as the Hela LAP-TDP43 cells, with expression of LAP-tagged fused-in-sarcoma (FUS) rather than TDP43. Courtesy of Don Cleveland.

[2] Hela cells containing tetracycline-inducible trans-activating response region DNA-binding protein with a molecular mass of 43kDa (TDP43) with an N-terminal localisation-affinity-purification (LAP) tag, an N-terminal myc tag and a C-terminal HA tag; at the Flp Recognition Target site. The LAP tag consists of green fluorescent protein (GFP), a 6xhistidine tag and a PreScission protease cleavage sequence (Ling et al. 2010). Courtesy of Don Cleveland.

[3] Patient exhibited multiple heterozygous mutations in the PRKDC gene (encoding DNA-dependent protein kinase, catalytic subunit (DNA-PKcs)) with low DNA-PKcs expression and minimal DNA-PKcs activity (Woodbine et al. 2013).

TDP1±/± mouse embryonic fibroblasts	10% FcS DMEM
U2OS	10% FcS MEM
XRCC1±/± mouse embryonic fibroblasts	15% FcS DMEM

Table 2.1 | **Media used for each mammalian cell line.** Notes for individual cell lines in footnotes.

2.2.2 Cell line maintainance

Cells were grown in the media previously indicated in Corning tissue culture flasks with vented caps, at T75 size or T175 size depending on the number of cells required at any given time. These flasks were stored in NuAire DH Autoflow incubators at 37°C and 5% CO_2.

To prevent overconfluence the cells were periodically split. This entailed a wash in 1x PBS at room temperature and then treated with 0.25% trypsin at 37°C until they had detached from the matrix of the flask. At this point the trypsinisation reaction was quenched with addition of FcS-containing media at an equal volume to that of the trypsin solution, and the solution was moved to 30ml Greiner Bio-One containers and centrifuged at 440 x g for five minutes at room temperature.

The resultant pellet was then resuspended in the appropriate media for the cell line and a fraction of the cells transferred to a tissue culture flask containing either 10ml (T75) or 25ml (T175) of media.

2.2.3 Seeding cells for experiments

When cells were required for an experiment, the cells were split as previously described and the cells counted using a Marienfield haemocytometer after resuspension of the cell pellet. If microscopy of the cells was required then autoclaved coverslips (unless otherwise stated 16mm diameter, 0.13mm thickness - Thermo-Fisher Scientific) were placed in the appropriate wells of the plate before seeding.

The desired amount of cells were then transferred into an appropriate cell culture dish and allowed to settle for approximately 48 hours before being used in the experiment. The dishes used were Nuncleon four well dishes (1.9cm^2/well - Thermo-Fisher Scientific), Costar six well plates (9.5cm^2/well - Corning), Costar 24-well plates (1.9cm^2/well - Corning), 100mm TC-treated culture dishes (55cm^2/well - Corning), Nunc 140mm vented tissue tissue culture dishes (145cm^2/well - Thermo-Fisher Scientific) or 35mm glass bottom dishes (no. 2, uncoated, γ irradiated) (MatTek). Microscopy experiments were performed in four, six or 24

well plates; transfections in six well plates; experiments for western or northern blots in six well or 140mm plates; UV microirradiation in glass bottom dishes; and clonogenic survival assays in 100mm dishes.

In experiments involving cells containing tetracycline-inducible transgenes, cells were seeded as described but had their media replaced with media containing 2µg/ml doxycycline the next day. They were then allowed to settle for either 24 or 48 hours, to allow time for transgene expression.

In experiments involving cell-cycle arrest, cells were seeded as described but had their media replaced with 0.1% FcS media (appropriate for their cell line) and were left in the incubator for four days before any experiments with the cells proceeded.

2.2.4 Fixation protocol

Cells were fixed by application of 1% PFA for five minutes (ten minutes for experiments started after 19/10/15) where immunofluorescence of endogenous FUS was to be performed later. If this was not the case they were fixed using 4% PFA for fifteen minutes. In either case they were then washed three times in 1x PBS and stored in their dishes at 4°C until required.

2.3 Chemical treatments of live mammalian cells

Any chemical treatment on cells was performed with the equivalent volume of solvent on control cells, and cells were washed in 1x PBS three times before reintroduction of media in recovery experiments. In experiments involving multiple chemical treatments the first treatment was applied for the time indicated in table 2.2 and then remained in the media during the second chemical treatment, unless otherwise stated.

The exception to this is when doxycycline was used to induce transgene expression in Hela cell lines for microscopy, where doxycycline was included in the media during all experiments.

Unless otherwise stated the chemical treatments used were as follows:

Chemical	Concentration	Treatment time
α-amanitin	2µg/ml	16 hours
Actinomycin D	5nM or 4µM	3 hours
ATR Kinase Inhibitor II	10µM	1 hour
Caffeine	20mM	1 hour
CPT	4µM	45 minutes
CX5461	10µM	3 hours
Dipyridamole	10µM-300µM	1 hour
Doxycycline	2µg/ml	24 hours
DRB	266µM	30 minutes
Etoposide	20µM	30 minutes
EU	1mM	30 minutes
Hoechst 33258	10µg/ml	30 minutes
Hydrogen peroxide	10mM	10 minutes
Hydroxyurea	2mM	16 hours
IBMX	1mM-5mM	1 hour
Insulin	400nM	15 minutes
Ku0063794 (mTOR inhibitor)	1µM	1 hour
Ku55933 (ATM inhibitor)	10µM	1 hour
Ku58948 (PARP inhibitor)	1µM	1 hour
NU7441 (DNA-PKcs inhibitor)	10µM	1 hour
PI103	10µM	1 hour
Theophylline	20mM	1 hour
Wortmannin	200nM	1 hour

Table 2.2 | **Conditions and concentrations used for chemical treatments of mammalian cell lines.** Cells were incubated under the conditions described in chapter 2.2.2 during chemical treatment.

2.4 Clonogenic survival assays

2.4.1 Protocol

Cells were seeded in 10cm wells, in duplicate for each dose of treatment and cell density except for untreated or mock treated controls - which were seeded in triplicate. The cells were allowed to settle for twenty four hours before being treated and allowed to grow for

twelve days (patient fibroblasts) or nineteen days (control fibroblasts) in the incubator. Cells treated with camptothecin (CPT) had the media containing the chemical kept on them until fixation.

Cells were then briefly washed in distilled water before being stained for one hour in a solution of 2% methylene blue in 70% ethanol. The methylene blue solution was removed and the plates were thoroughly washed out with water before being left to dry overnight.

The number of colonies was then counted manually by looking at the colonies under a bright light and marking the underside of the plate to locate each colony. These marks were then counted using a Bartholomew Scientific Ltd. Stuart Colony Counter SC6 and the raw data plotted in Microsoft Excel. Where cells were plated at multiple cell densities per dose, the result from the higher cell density was used.

2.4.2 Cell numbers seeded

Cells intended for use in clonogenics that were to be irradiated were seeded at the numbers shown in Table 2.2 and those intended for CPT treatment were seeded at the numbers shown in Table 2.3.

Dose of ionising radiation	Cells seeded per plate
0 Gray	1000
1 Gray	1000
2 Gray	1000 and 2000
4 Gray	2000 and 4000
6 Gray	4000 and 8000

Table 2.3 | **Cells seeded and doses used in clonogenic survival assays (ionising radiation).** Amount of cells seeded per plate for each dose of ionising radiation used.

Dose of CPT	Cells seeded per plate
0nM	1000
2nM	1000
4nM	1000
6nM	1000 and 4000
8nM	4000
10nM	4000 and 8000

Table 2.4 | **Cells seeded and doses used in clonogenic survival assays (camptothecin).** Amount of cells seeded per plate for each dose of camptothecin used.

2.5 Immunofluorescence

2.5.1 Permeabilisation

Fixed cells were permeabilised by application of 0.2% Triton X-100 for two minutes prior to immunolabelling.

2.5.2 Immunolabelling

Permeabilised cells were washed once in 1x PBS and then blocked by application of 5% BSA (in PBS) to the cells for one hour at room temperature, on a rocker. Primary antibodies diluted to the appropriate concentration in 1% BSA (in PBS) were then applied and the cells left on a rocker at 4°C overnight. Cells were then washed three times in 1x PBSTS for five minutes per wash. Secondary antibodies were applied at the appropriate concentration (again diluted in 1% BSA in PBS) for one hour at room temperature before cells were washed another three times in 1x PBSTS.

All stages from the application of secondary antibodies onwards were conducted protected from light as much as possible. When GFP-expressing cells were immunolabelled the entire

process was performed protected from light. Diluted primary antibodies were preserved by adding 10% (w/v) thiomersal (Thermo-Fisher Scientific) to 1 part in 500 to the antibody containing 1% BSA solution, followed by storage at 4°C and reuse - up to twice.

2.5.3 DAPI staining and mounting

Immunolabelled cells were treated with 0.05µg/ml DAPI for five minutes, then washed three times in 1x PBS for five minutes per wash before being mounted.

VectaShield (Vector Labs - H-1000) mounting media was applied to the slide and the coverslip applied cell-side down into the VectaShield before being secured in place by application of nail varnish around the edge of the coverslip. Once the varnish had dried the slides were stored protected from light at 4°C until required.

2.6 RNA imaging

2.6.1 EU labelling

EU labelling was performed by application of EU to $4x10^4$ cells at a final concentration of 1mM. Cells incubated in this solution for 45 minutes at 37°C and were then fixed in 4% PFA for 15 minutes.

2.6.2 Click Chemistry

Click Chemistry was performed following the protocol of the Click-iT® RNA Alexa Fluor® 488 Imaging Kit with minor modifications. These were: using my own fixation protocol, using 0.2% Triton X-100 rather than 0.5% Triton X-100 for permeabilisation, reducing the volume of reaction cocktail to be used per coverslip by half, and staining DNA with DAPI rather than Hoechst 33342.

2.7 Irradiation

γ irradiation was introduced using a Gamma Cell 1000 (Atomic Energy of Canada Ltd.), which contains a Cs^{137} source. X-ray irradiation was performed using an AGO HS MP1 X-ray machine.

2.8 Microscopy

2.8.1 Image acquisition

Images in experiments not involving UVA tracking were acquired using a Zeiss Axioplan 2 equipped with Plan-NEOFLUAR 10x/0.30, Plan-NEOFLUAR 40x/0.75 and Plan-APOCHROMAT 100x/1.4 Oil DIC lenses, except in experiments where Z-stack functions were required in

which case images were acquired on an Olympus IX73 equipped with an MPlanApo N 100x/1.40 Oil lens. All images, unless otherwise stated, were taken at 100x magnification.

The Zeiss Axioplan 2 initially ran SimplePCI as its image acquisition software, and images acquired prior to September 2014 were taken using this software and saved in 24-bit JPEG format.

All later images were taken using MicroManager and saved in the OME-TIFF format.

2.8.2 Image processing for presentation

Unless otherwise stated composite TIFF images for presentation were processed using a macro running in ImageJ 1.47t. This macro sets the mapping from pixel values to display values in a batch of images to be the same, allowing direct comparison of signal strength between these images, before converting the composite image to the RGB colour format. The batches of images to be processed were grouped by cell line and by experiment.

TIFF images not processed in this manner were processed with a macro that set the contrast per colour channel per image to its maximal value, save for Z-stacked images where this was done manually. It is noted where this method of presentation was used.

2.8.3 Quantifying fluorescence

Nuclear fluorescence was quantified using SimplePCI, with the fluorescence of a minimum of 35 nuclei measured per data point. Regions of the image corresponding to nuclei had their fluorescence in the green channel measured, with a corresponding number of non-nuclear regions of the image being used to measure (and subtract) background fluorescence.

The regions corresponding to nuclei were selected by thresholding in the blue (DAPI) channel followed by a thresholding by size to exclude small regions of blue signal not corresponding to nuclei. If necessary manual selection was then used to exclude nuclei that were not fully within the image, include nuclei that the algorithm had missed, or separate pairs of nuclei the algorithm had misidentified as one single region of fluorescence.

2.8.4 Foci counting

Foci were manually counted through the eyepiece of the Zeiss Axioplan 2. The foci from 25 cells were counted per data point in experiments quantifying γH2AX foci. In all other experiments the foci from 50 cells per data point were counted.

2.8.5 3D projection

3D projections were made using inbuilt functions of ImageJ, using the brightest value projection method and without deconvolution. Images were manually reset to maximum contrast per image prior to projection.

2.9 UVA laser tracking

2.9.1 Seeding and pre-sensitisation

$2x10^5$ cells per dish were seeded onto glass-bottom dishes (MatTek) and allowed to settle for 24 hours. Cells were pre-sensitised to UV laser tracking by addition of 10µg/ml Hoechst 33258 diluted in the appropriate medium for the cell line, 30 minutes prior to laser tracking.

2.9.2 Laser tracking at fixed timepoints

Individual cells were irradiated with a UVA laser (351nm, 0.44 J/m^2) focused through a 40x/1.2-W objective using a Zeiss Axiovert equipped with LSM 520 Meta and Carl Zeiss AIM LSM software. The laser tracks were introduced over an area of approximately 12 µm x 0.1 µm at 30 second intervals for 5 minutes, after which the cells were fixed in 4% PFA for 15 minutes. Immunofluorescence was then performed using polyclonal FUS antibodies from ProteinTech and the laser tracked cells visualised using the Zeiss Axioplan 2.

2.9.3 Laser tracking in real time

Individual GFP-positive cells were irradiated using the parameters previously stated, and pictures taken on irradiation and every 15 seconds thereafter. Fluorescence quantification for these experiments were performed within the Carl Zeiss AIM LSM software but compiled and analysed in Microsoft Excel.

2.10 Western blots

2.10.1 Preparation

Cells were harvested for western blots by either trypsinising or scraping. After this step the cells were counted, centrifuged at 440 x g and resuspended in 1ml 1x PBS. The cells were then centrifuged at 44000 x g, the PBS removed and the pellet resuspended in fresh PBS.

The cells were centrifuged at 44000 x g a final time, the PBS removed and the pellet flash frozen in liquid nitrogen. Pellets were stored at -80°C. Cell pellets for RNA extraction were also prepared in this way, though omitting counting the cells, as RNA quantity could be more directly and accurately assayed at a later stage.

Some experiments were performed in suspension - when this was the case the cells were counted prior to the experiment and there was no trypsinisation or scraping step.

Some experiments also had cells harvested by direct lysis in 1x LB after a single 1x PBS wash.

2.10.2 SDS-PAGE

Protein samples were resolved by SDS-PAGE. In order to do this cells were run dissolved in 1x LB at 120V on a 10% polyacrylamide 0.1% SDS gel with a 5% polyacrylamide 0.1% SDS stacking gel, in a tank filled with 1x running buffer. Precision Plus Protein™ Dual Color Standards (Bio-Rad) were also run to determine protein sizes.

2.10.3 Transfer

Wet electrotransfer was used to immobilise proteins onto a 0.45µm nitrocellulose membrane (Thermo-Fisher Scientific). Gels and nitrocellulose were enclosed in a transfer cassette, loaded into a tank containing 1x transfer buffer and proteins electrophoretically transferred at 10V over 999 minutes. An ice pack and a magnetic bead were used to keep the buffer cool and well circulated within the tank.

2.10.4 Ponceau staining and probing

Nitrocellulose membranes were stained using Ponceau Red (Sigma-Aldrich) for two minutes and then washed three times in distilled water to assess relative protein loading levels. Samples were re-run using adjusted volumes if samples were noticeably misloaded by visual inspection.

If the protein loading was satisfactory then the membranes were cut (if required) and were then washed in 1x TBST until no Ponceau Red staining remained visible. The membranes were then blocked for an hour at room temperature using either 5% milk powder (Co-operative) or 5% BSA in TBST - typically the latter was used if phospho-antibodies were to be used in later steps of the protocol.

Primary antibodies were diluted in the blocking solution and applied to the membrane either overnight at 4°C, or for an hour at room temperature - depending on the antibody. The primary antibodies, with the exception of anti-actin antibodies, were preserved using thiomersal and kept at 4°C for later reuse. The membranes were then washed in TBST for fifteen minutes, ten minutes and then twice for two minutes. Secondary antibodies, again diluted in blocking solution, were then applied for one hour at room temperature and then removed before development.

2.10.5 Development

Blots were developed using Amersham ECL Western Blotting Reagent (GE Life Sciences), using either Amersham Hyperfilm ECL (GE Life Sciences) for weaker chemiluminescent signals or Carestream MXBE film (Carestream) for stronger signals, to reduce saturation. Some membranes were reprobed for different proteins after development, if the goat anti-rabbit secondary antibody was used then the membrane was either left in blocking solution at 4°C overnight before application of a new primary (mouse) antibody. If the rabbit anti-mouse secondary antibody had been used then the membrane was stripped before reprobing.

2.10.6 Stripping

Membranes were incubated with stripping solution for 15 minutes at room temperature and then washed with TBST three times before being reblocked and reprobed.

2.11 siRNA transfection

All experiments with siRNA transfections included an additional transfection with control siRNA.

2.11.1 Double transfection protocol

Double transfections were performed on A549 cells, per transfection 10µl 20µM smartPOOL siRNA was gently mixed into 200µl OPTI-MEM and 12µl metafectane pro mixed into a separate 200µl of OPTI-MEM - both were incubated at room temperature for five minutes.

The two mixtures were then combined and incubated together for half an hour at room temperature. $1x10^5$ A549 cells were plated per well in a six well plate and the transfection mixture introduced immediately afterward to make a total volume of 2ml per well.

After 24 hours incubation at 37°C the media was removed from the cells, the cells were washed and the media replaced. A new transfection was then performed on the same cells and the cells were used in experiments after a further 48 hours at 37°C.

2.11.2 Reverse transfection protocol

Reverse transfections were performed on U2OS cells, per transfection 50µl 1µM smartPOOL siRNA was gently mixed into 75µl OPTI-MEM and 2.7µl RNAiMax mixed into a separate 125µl of OPTI-MEM - both were incubated at room temperature for five minutes.

An autoclaved coverslip (22x22mm, 0.13mm thickness - Marienfield) was placed into wells of a six well plate where cells intended for microscopy were to be seeded. The two mixtures were then combined and incubated together within the wells of a six well plate for twenty minutes at room temperature. $2x10^5$ U2OS cells were plated per well directly on top of the mixture, the total volume per well made up to 2ml with complete media and the mixture well mixed. The cells were used for experiments after 48 hours incubation at 37°C.

2.12 RNA extraction

RNA extraction from flash frozen cell pellets was performed using the Qiagen RNeasy Mini Kit (Qiagen – 74104) using spin columns as described in the protocol provided, with an additional 15 minute RQ1 DNAse treatment in 1x DNase buffer between the first and second wash with buffer RPE and the addition of an additional buffer RPE wash after this.

After extraction small quantities of RNA were analysed using a NanoDrop ND-1000 Spectrophotometer and run at 80V on a 0.7% agarose (Thermo-Fisher Scientific) gel in 1xTAE, using freshly-cleaned equipment reserved for RNA use to check for degradation.

2.13 Northern blots

2.13.1 Running RNA samples

RNA was run on a 1% agarose-formaldehyde gel, this was cast in two steps. Per 100ml of gel to be cast, 1g of agarose was dissolved in 72ml distilled water by heating. This solution was then allowed to cool to 55°C before addition of 10ml 10x MOPS electrophoresis buffer and 18ml formaldehyde (Sigma-Aldrich) before being poured for casting.

Equivalent amounts (by weight) of RNA were mixed in a 1:1 ratio with 2x RNA loading buffer, heated to 70°C and briefly spun on a centrifuge. During this process the gel was

placed in a tank of 1x MOPS electrophoresis buffer and run at 5Vcm^{-1} for 5 minutes prior to the RNA being loaded. The gel was then run overnight at the same voltage, using a Pharmacia Biotech pump to circulate the buffer.

2.13.2 Transfer to membrane

After being run the gel was soaked in 65°C DEPC-treated water three times for five minutes in order to remove the formaldehyde, before being soaked for twenty minutes in 1x northern blot soaking solution and for forty minutes in 20x SSC. The RNA was transferred to a membrane of Amersham Hybond XL (GE Healthcare) overnight using capillary action, 20x SSC was used as the transfer buffer.

At this point the RNA was cross-linked to the membrane by exposure to 120 mJ/cm^2 UV radiation in a UVP CL-1000 UV crosslinker and was then gently shaken in 6x SSC for five minutes before being dried. The membrane was stained by a solution of methylene blue to visualise the RNA before being destained in a 0.2x SSC, 0.1% SDS solution for fifteen minutes at room temperature and then dried prior to blotting.

2.13.3 Preparation of labelling probe

To label each probe for northern blotting an enzymatic reaction was prepared containing 1µl of 10µM oligonucleotide probe, 5µl 10x T4 polynucleotide kinase buffer, 2µl T4 polynucleotide kinase, 2µl ^{32}P ATP (Perkin Elmer, 10mCurie/ml) and 40µl nuclease-free water. This was incubated behind a plexiglass shield for one hour at 37°C before being run down a G-25 Illustra Micro Spin (GE Healthcare - 27-5325-01) column at 855 x g for 3 minutes. The filtered oligonucleotide was then added to 10ml pre-heated pre-hybridisation solution.

2.13.4 Northern blot

The membrane was incubated for an hour at 42°C (18S, 28S probes) or 40°C (5'-ETS-1 probe) in 10ml pre-hybridisation solution in a hybridisation oven. At this point the solution was replaced with the 10ml of pre-hybridisation solution containing the oligonucleotide probe and incubation continued overnight. After incubation the membrane was washed once in a 1x SSC, 1% SDS solution for ten minutes at hybridisation temperature, followed by three washes in a 0.5x SSC, 0.1% SDS solution also at hybridisation temperature for ten minutes each. The membrane was then dried, placed on flat saran wrap and left to expose a Fuji phosphor screen. The time of exposure varied depending on the probe - for the strong 28S

and 18S probes one hour was sufficient whereas the 5'-ETS-1b probe required 72 hours. The phosphor screen was then scanned using a Fujifilm FCA-5100 phosphor imager. The membrane was reprobed, or disposed of.

2.13.5 Intensity quantification

Regions of signal had their intensity analysed from within ImageJ, along with the intensity of a region of no signal in the same lane. The background intensity was then subtracted from that of the signal in Microsoft Excel before being used in any calculations.

2.14 Statistics

Statistical analysis was performed using Microsoft Excel and consisted of paired, two-tailed Student's t-tests for pairs of data; or two-way analysis of variance (ANOVA) when comparing series of data. One-way ANOVA was also used to compare data within a series. Error bars on graphs represent the standard error of the mean of the data.

3. Characterisation of a FUS-ALS patient cell line and investigation into roles of FUS

FUS is a multi-functional protein and is thought to be involved in many key cellular processes such as double strand break repair (DSBR) (Baechtold et al. 1999), transport of mRNAs between the nucleus and cytoplasm (Zinszner, Sok, et al. 1997), transcription (Bertolotti et al. 1996) and splicing (Calvio et al. 1995). A disruption of any one of these processes could potentially be pathogenic to cells and possibly have some relevance in the process of ALS pathogenesis.

Therefore characterisation of an immortalised fibroblast line derived from a FUS-ALS (fused-in-sarcoma amyotrophic lateral sclerosis) patient, harbouring a heterozygous R521H point mutation in FUS, began. The intention of this was to determine if any cellular processes were defective within this cell line as any defective processes found in patient cells could be implicated in FUS-ALS pathogenicity. A fibroblast line derived from an unaffected sibling and immortalised at the same time by the same method (via human telomerase reverse transcriptase - hTERT) was used as a control for these experiments. Both cell lines were a kind gift from the lab of Majid Hafezparast.

Models of FUS pathology have been generated in mice and one such model appears to successfully recapitulate features of FUS-ALS (Mitchell et al. 2013). However there are also mouse models which have exhibited radiosensitivity (Kuroda et al. 2000) or chromosomal instability (Hicks et al. 2000) instead and the defects described in the Kuroda and Hicks mice are suggestive of a deficiency in DNA damage repair. Given these data it was decided that the initial focus for characterising our FUS-ALS patient cells would be to assess any sensitivity they may have had to DNA damage.

3.1 Clonogenic survival assays on FUS-ALS patient cells in response to camptothecin (CPT) and (IR)

A common technique to investigate survival defects of cell lines is the clonogenic survival assay, by which cells are seeded in small numbers on plates and allowed to grow to form colonies. During this period cells may be subjected to chemical or radiological treatment and the sensitivity of different cell lines to a given treatment may be assessed by comparing their capacities to form colonies afterwards (Puck & Marcus 1956).

This assay was performed on the R521H FUS patient fibroblasts, with fibroblasts from an unaffected sibling which had been immortalised in the same fashion (via human telomerase reverse transcriptase - hTERT) used as a control.

Two DNA damaging agents were utilised. The first was camptothecin (CPT), which inhibits topoisomerase I (TOP1) by locking the enzyme into an intermediate state where it is covalently linked to nicked DNA - a TOP1 cleavage complex (TOP1cc). CPT-induced TOP1ccs are reversible but can be converted into irreversible strand breaks in response to collisions with the replication machinery (Furuta et al. 2003; Hisang et al. 1989), or the transcription machinery (Wu & Liu 1997). The second damaging agent used was ionising radiation (IR) which induces a mixture of single and double-stranded breaks (Bradley & Kohn 1979).

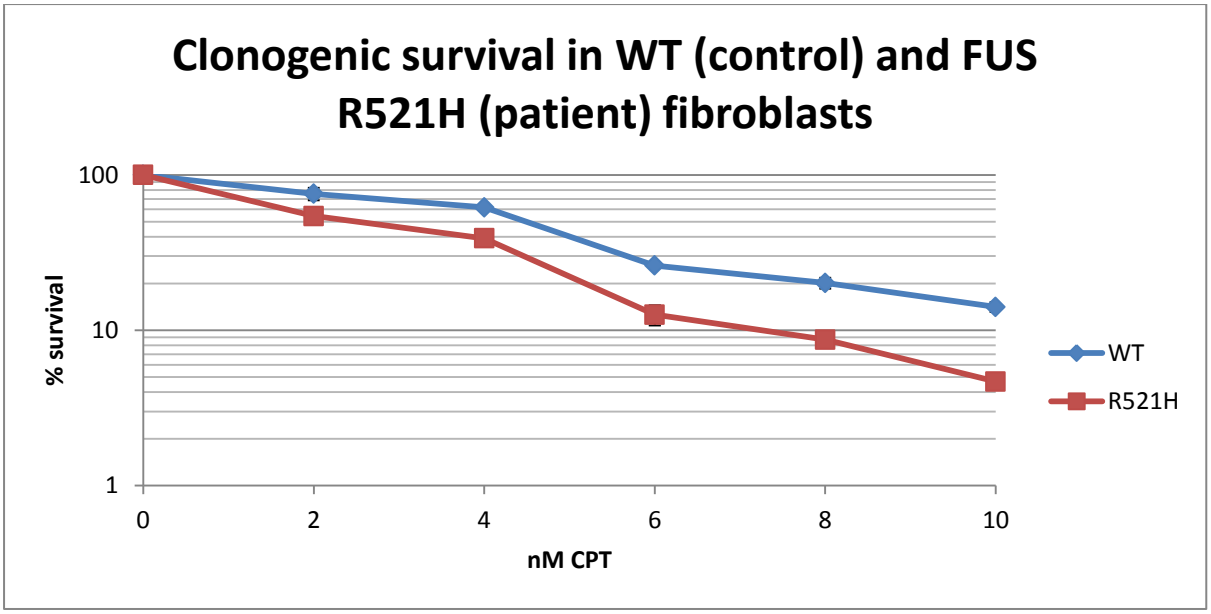


Figure 3.1 | **Clonogenic survival assays of patient and sibling control fibroblasts in response to CPT.** In response to chronic CPT treatment patient (R521H) cells showed a statistically significant (by two-tailed ANOVA at 95% confidence intervals) defect in colony formation relative to control (WT) cells. n=3.

Cells were seeded onto plates in duplicate (triplicate for untreated or mock-treated control cells) in the numbers shown in tables 3.1 and 3.2. After 24 hours the cells had CPT added to their media and were grown for twelve (patient fibroblasts) or nineteen (control fibroblasts) days. Colonies were counted after fixation and staining with methylene blue.

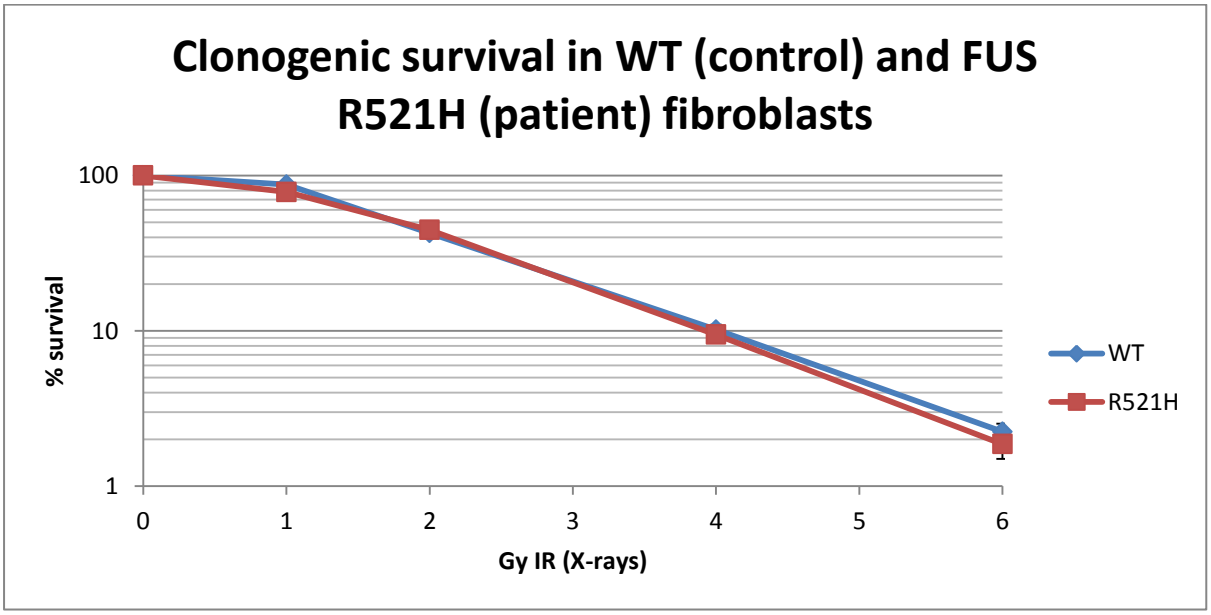


Figure 3.2 | **Clonogenic survival assays of patient and sibling control fibroblasts in response to IR.** In response to acute IR treatment patient (R521H) cells showed no statistically significant defect in colony formation relative to control (WT) cells. n=3.

Cells were seeded onto plates in duplicate (triplicate for untreated or mock-treated control cells) in the numbers shown in tables 3.1 and 3.2. After 24 hours the cells were irradiated and grown for twelve (patient fibroblasts) or nineteen (control fibroblasts) days. Colonies were counted after fixation and staining with methylene blue.

Dose of ionising radiation	Cells seeded per plate
0 Gray	1000
1 Gray	1000
2 Gray	1000 and 2000
4 Gray	2000 and 4000
6 Gray	4000 and 8000

Table 3.1 | **Cells seeded and doses used in clonogenic survival assays (ionising radiation).** Amount of cells seeded per plate for each dose of ionising radiation used.

Dose of CPT	Cells seeded per plate
0nM	1000
2nM	1000
4nM	1000
6nM	1000 and 4000
8nM	4000
10nM	4000 and 8000

Table 3.2 | **Cells seeded and doses used in clonogenic survival assays (camptothecin).** Amount of cells seeded per plate for each dose of camptothecin used.

Treatment with CPT resulted in a lower percentage of patient fibroblasts forming colonies relative to the sibling control (Fig 3.1), indicating a sensitivity of the patient line to the chemical. However no such difference was observed in response to IR (Fig 3.2).

3.2 Recruitment of FUS to damage sites by UVA laser microirradiation

As FUS was considered a candidate for involvement in the DNA damage response, its recruitment to sites of damage was investigated by way of UV microirradiation. Microirradiation is a well-established technique used to examine proteins involved in DNA damage (Walter et al. 2003) where a small area of a cell is irradiated and the relocation of proteins to the site can be examined. If the protein being investigated is tagged (for instance with green fluorescent protein (GFP)) then kinetic information about its recruitment to sites of microirradiation could be gathered in real time.

As such a series of transgenic Hela cell lines containing FUS fused to an N-terminal localisation-affinity-purification (LAP) tag, containing GFP, were utilised for this experiment.

The LAP-tag also contains histidine and myc tags, though these were not used for any experiments. These cell lines are hereafter referred to as LAP-FUS Helas.

LAP-FUS expression in these cells was controlled via the tetracycline inducible system which works by including a gene of interest (in this case LAP-tagged FUS) downstream of a promoter (in this case the cytomegalovirus promoter) and a tetracycline operator sequence. Normally the tetracycline operator sequence is bound by the tetracycline repressor protein, preventing transcription of the transgene downstream, but in the prescence of tetracycline (or doxycycline) a conformational change occurs in the tetracycline repressor protein preventing its binding the operator sequence and therefore allowing transcription to proceed. Otherwise identical cells containing trans-activating response region DNA-binding protein with a molecular mass of 43kDa (TDP43) fused to the GFP-containing tag instead of FUS are documented in the literature (Ling et al. 2010) and were used in later experiments.

A range of different LAP-FUS Hela cell lines harbouring different FUS-ALS causative mutations were available with the cell lines carrying wild-type (WT) and R521G mutant FUS being used for this experiment. The R521G mutant was chosen as there was no R521H mutant available in these cell lines. Therefore a cell line with a mutation on the same residue as in the patient fibroblasts was favoured. Western blots of the cell lines chosen were taken to ensure that the tetracycline-inducible expression of FUS could be attained in both lines.

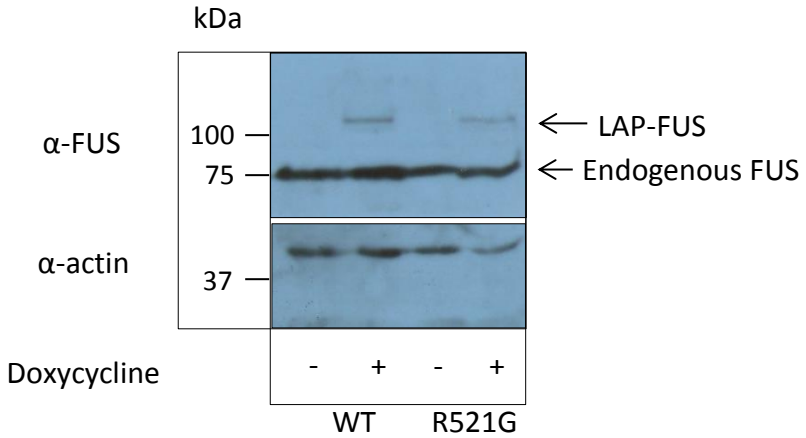


Figure 3.3 | **Expression of LAP-FUS.** Doxycycline induction of LAP-FUS transcription produces modest expression in both WT and mutant cells. Approximately $5x10^5$ cells per lane.

LAP-FUS Helas were seeded, allowed to settle and induced with doxycycline before being harvested for western blot analysis two days later.

Western blot analysis of the LAP-FUS Hela lines demonstrated that expression of tagged exogenous FUS was modest (Fig 3.3). With exogenous FUS expression verified the real-time laser tracking experiment was set up.

The conditions used were UVA irradiation at 351nm, introducing 0.44 J/m^2 of energy into cells pre-sensitised to damage with the intercalating agent Hoechst 33258. These conditions would be expected to yield primarily SSBs (Ferrando-May et al. 2013), DSBs (Walter et al. 2003) and oxidative damage (Kielbassa et al. 1997); though cyclobutane pyrimidine dimers (CPDs) (Dinant et al. 2007) have also been detected under similar conditions.

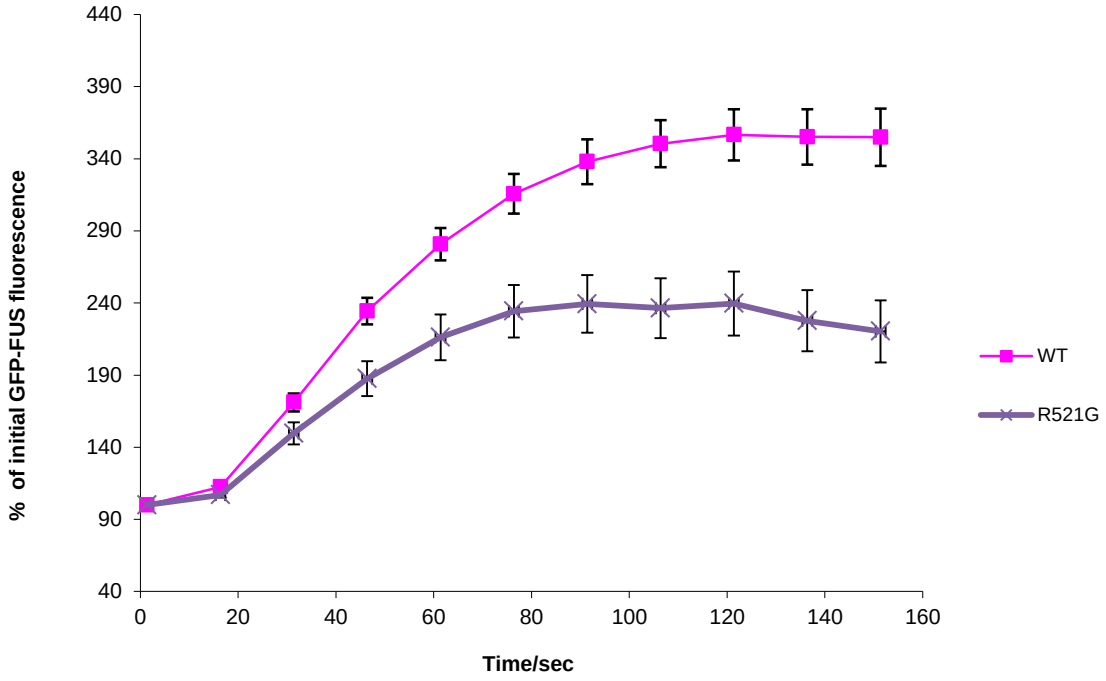


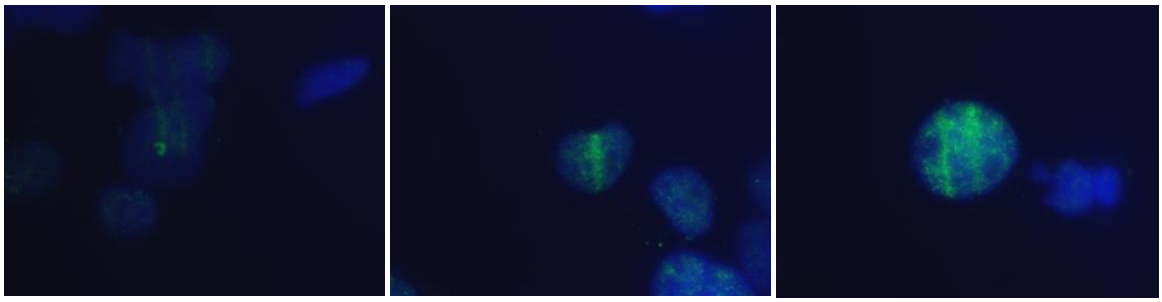
Figure 3.4 | **Recovery of fluorescence after laser microirradiation in cells expressing GFP-FUS.** In cells harbouring either wild type or mutant (R521G) LAP-tagged FUS. R521G cells show a statistically significant impairment in recruitment to laser tracks (by two-tailed ANOVA at 95% confidence intervals). n=4.

LAP-FUS Helas were seeded onto glass bottomed dishes and allowed to settle before addition of doxycycline. After another twenty four hours Helas were pre-sensitised to damage with Hoechst 33258 half an hour prior to UVA irradiation at 351nm - introducing 0.44 J/m^2 of energy. Only GFP-expressing LAP-FUS Helas were irradiated and fluorescence at the site of damage was measured in real time.

The data showed that GFP-FUS was recruited to sites of microirradiation and moreover cells harbouring the R521G FUS mutation demonstrated a marked defect (Fig 3.4) in recruitment to the sites of damage potentially indicating a role for FUS in DNA damage repair and of the

R521 residue for its function in the process. Recruitment of GFP-FUS was rapid and maximal recruitment occurred within two minutes, consistent with the kinetics of poly ADP ribose polymerase (PARP) activity. Similar experiments were repeated by others in the lab, with or without PARP inhibitor and demonstrated a dependence of this recruitment on the PARP1 protein (Rulten et al. 2014).

In order to verify that this recruitment was not an artefact of FUS overexpression microirradiation experiments were performed, using the same laser and pre-sensitisation conditions, in a cell line lacking exogenous FUS (U2OS). As there would therefore be no GFP-tagged FUS these experiments required processing by immunofluorescence (IF) and could not yield real time kinetic data.



Immediately post-irradiation | 2.5 minutes post-irradiation | 5 minutes post-irradiation

Figure 3.5 | **laser microirradiation recruits FUS in U2OS cells.** Endogenous FUS tracking to sites of UVA laser damage, detected with IF using antibodies from ProteinTech (green channel). The blue channel is DAPI (a nuclear stain).

U2OS cells were seeded and allowed to settle before irradiation under the same conditions as laid out in figure 3.4. Three individual cells were irradiated two and a half minutes apart before a 15 minute fixation in 4% paraformaldehyde (PFA), generating timepoints for 0, 2.5 and 5 minutes post-irradiation. Cells were processed by IF and 4',6-diamidino-2-phenylindole (DAPI) stained prior to microscopy.

The cells showed a clear recruitment of endogenous FUS to sites of laser microirradiation (Fig 3.5) - demonstrating that the recruitment of the exogenous GFP-tagged protein was not an artefact of FUS overexpression. Similar data replicating these microirradiation results, and demonstrating a PARP1 dependence of FUS recruitment to sites of microirradiation, are published in the paper by Rulten et al. 2014 on which I am a co-author.

3.3 Measuring DNA damage repair capacity in FUS-ALS patient by the γH2AX assay

Microirradiation studies can demonstrate recruitment of proteins to sites of damage, but this does not necessarily mean that there is any physiologically relevant activity of a recruited protein at these sites. In addition to this estimations of the amount of radiation damage that UVA microirradiation (with pre-sensitisation) can introduce in a localised area varies wildly, from only a few Gy (Bekker-Jensen et al. 2006) to several tens or even hundreds of Gy (Paull et al. 2000; Splinter et al. 2010), largely depending on the method of estimation used. At the higher estimations there would be a serious risk of generating artefactual data - particularly with proteins such as FUS that are known to bind nucleic acids (Crozat et al. 1993). This is because high local doses of damage would be expected to result in activation of PARP enzymes and production of substantial amounts of PAR - a signalling molecule which is structurally similar to nucleic acids (Gibson & Kraus 2012). As mentioned previously FUS recruitment to sites of microirradiation is PARP1 dependent (Rulten et al. 2014) and therefore it can be plausibly hypothesised that FUS may be engaged in a non-physiological recruitment to sites of damage due to the high concentrations of the nucleic acid-like molecule PAR at the microirradiation site.

For these two reasons it is necessary to compliment the microirradiation data with data from another DNA damage repair assay such as the γH2AX foci counting assay - a simple quantitative measurement of DNA damage from DSBs (Rogakou et al. 1998; Löbrich et al. 2010) whereby cells are treated with a DNA damaging agent, fixed at set timepoints after damage and counted after IF for phosphorylated histone H2AX. This assay was performed using patient fibroblasts and with damage introduced by CPT and by IR as with the clonogenic survival assays. Etoposide (VP16) was also included as FUS has recently been proposed to be a key component in the early stages of DNA damage repair - a paper demonstrated decreased γH2AX response to etoposide in cells depleted of FUS by knockdown with small hairpin RNA (Wang et al. 2013).

A key difference between these fibroblasts and motor neurons which die in ALS are that the fibroblasts replicate but in a developed brain the motor neurons do not. Although adult neurogenesis occurs in humans it is restricted to specific regions of the hippocampus and lateral ventricles under normal conditions (Ming & Song 2011) and is not thought to occur in the motor neurons that degrade in ALS. Furthermore ALS is thought to be a degenerative

rather than developmental disease with sporadic cases typically presenting over the age of 40 and with even the most aggressive familial forms of the disease, such as that arising from the FUS P525L mutation, typically presenting in the late teens and early 20s (Shang & Huang 2016). As such replication-associated DNA damage in neural precursor cells is unlikely to be of serious relevance to ALS pathology.

Therefore for this assay the patient cells (and the WT controls) were arrested in the G1 phase of the cell cycle by serum starvation before DNA damage was applied. This is to minimise the effects of replication-associated DNA damage repair on the data and for the same reason cells were costained both for γH2AX and the cell cycle marker centromere protein F (CENPF) during IF. CENPF is present at low levels in cells in the G1 or G0 phases but accumulates during S phase before reaching a maximum at G2/M (Landberg et al. 1996) - it is therefore often used as a simple marker of cell proliferation and cells expressing high levels of CENPF were excluded from foci counts in the following data.

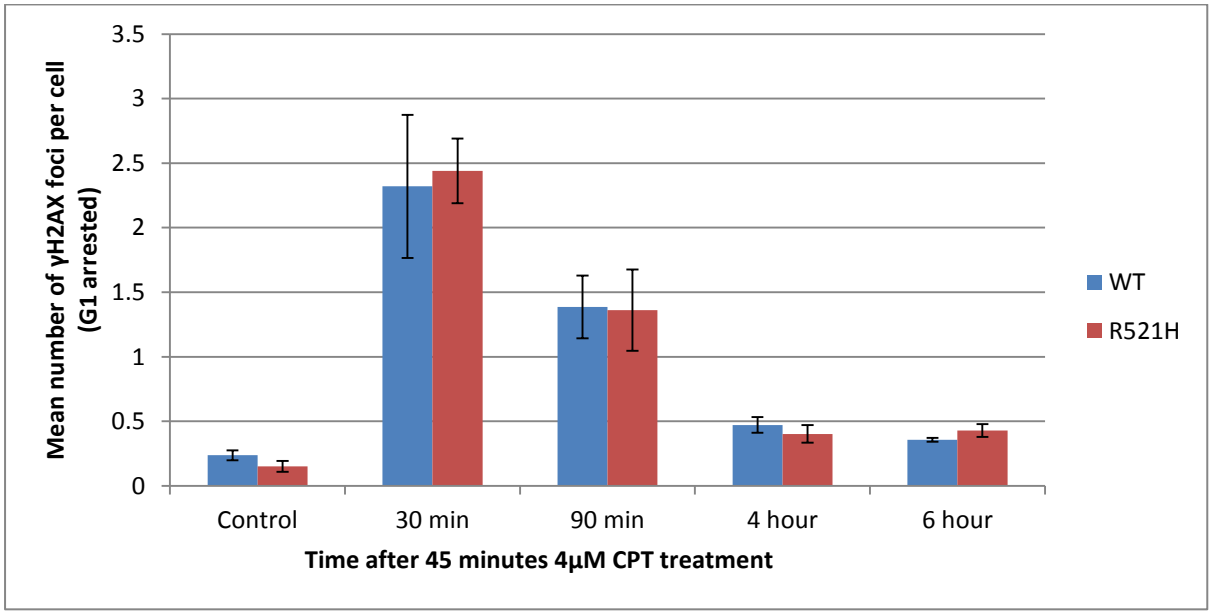


Figure 3.6 | **γH2AX assays of patient and sibling control fibroblasts treated with CPT.** Patient cells demonstrate no statistically significant defect in DNA damage repair in response to CPT by this assay. n=4.

Cells were seeded and allowed to settle before being thoroughly washed and serum starved. Four days later the cells were damaged with 4μM CPT for 45 minutes, washed three times in PBS and allowed to recover for set timepoints before being fixed. Cells were then subjected to IF for phosphorylated H2AX and for CENPF, and foci counting was performed, excluding cells with high CENPF expression.

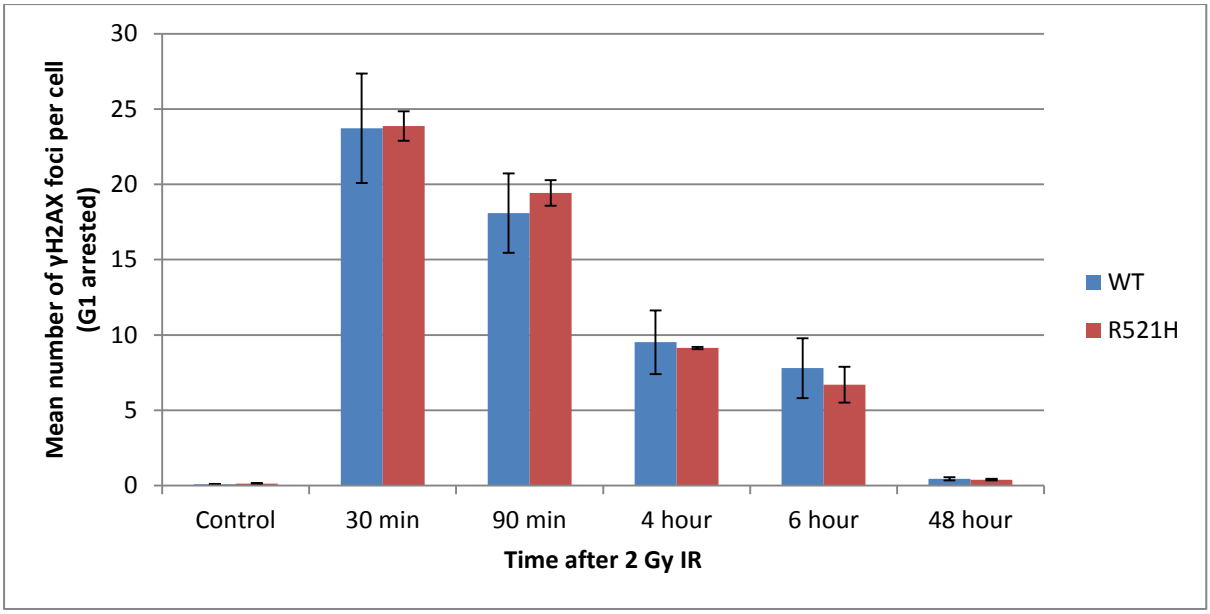


Figure 3.7 | **γH2AX assays of patient and sibling control fibroblasts treated with IR.** Patient cells demonstrate no statistically significant defect in DNA damage repair in response to IR by this assay. n=3.

Cells were treated as in Figure 3.6, with 2 Gy IR treatment instead of CPT treatment.

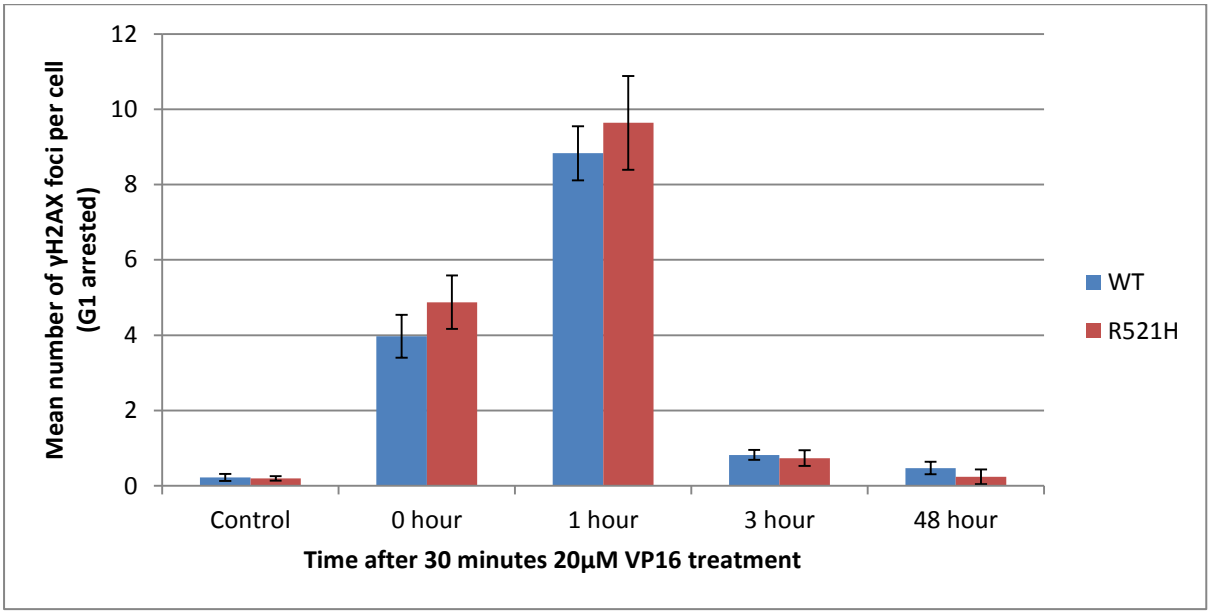


Figure 3.8 | **γH2AX assays of patient and sibling control fibroblasts treated with etoposide/VP16.** Patient cells demonstrate no statistically significant defect in DNA damage repair in response to VP16 by this assay. n=3.

Cells were treated as in Figure 3.6, with 20μM etoposide treatment for 30 minutes instead of CPT treatment.

Surprisingly, given previous data, the cells demonstrated no statistically significant defect in recovery from DNA damage in response to CPT (Fig 3.6), IR (Fig 3.7) or etoposide (Fig 3.8). Therefore there is no effect detectable by this assay of the R521H mutation on DNA damage repair.

3.4 Examining nucleolar fragmentation in FUS-ALS patient cells by immunofluorescence

The data from the γH2AX assays contradicted the data from the laser microirradiation studies. This suggested that a defect in DNA damage repair was unlikely to account for the survival defect noted in the clonogenic survival assays - although a defect in the process could not be definitively be excluded it would have been expected that a defect in DNA damage repair severe enough to result in statistically significant cell death would be detectable on a γH2AX assay.

Therefore other processes in which FUS is implicated were considered to account for the increased cell death in the FUS R521H fibroblast line. Transcription was considered a likely candidate as CPT is also a transcriptional inhibitor as well as a DNA damaging agent (Bendixen et al. 1990). Furthermore if FUS were involved in restarting transcription after transcriptional inhibition or stress then a defect in this process due to a FUS mutation could be a plausible reason for the FUS R521H fibroblasts being sensitive to CPT but insensitive to IR. Moreover neurons are known to be very transcriptionally active (Flangas & Bowman 1970; Sarkander & Uthoff 1976) so a defect in restarting transcription after stress could differentially affect neurons and therefore be more likely to result in cell death in these cells *in vivo*.

An estimated 60% (Warner 1999) of transcription is performed by RNA polymerase I (RNAP I) - the polymerase responsible for transcription of ribosomal RNA (rRNA) (Scheer & Rose 1984; Scheer et al. 1984), save the 5S rRNA transcript (Parker & Roeder 1977), and the polymerase which operates in the nucleolus. The nucleolus is also a key mediator in cellular stress responses and undergoes morphological changes in response to some varieties of stress, including transcriptional stress (Boulon et al. 2010). For this reason nucleoli of the patient cells were subjected to transcriptional inhibition and examined for morphological changes during recovery.

The nucleolus consists of three main compartments: the fibrillar centres (FCs), which are surrounded by the dense fibrillar component (DFC), which is in turn embedded into the granular component (GC). Primary rRNA transcripts are located at the junction between the FCs and the DFC, and migrate out to the GC while being processed in the DFC and GC (Louvet et al. 2005).

B23, also known as nucleophosmin or NPM1, is a component of the GC and is thought involved in many cellular processes (Lindström 2011). As the other components of the nucleolus are embedded in the GC, B23 is used as a marker for the nucleolus as a whole. Fibrillarin has multiple roles, one of which is operating in the U3 snoRNP complex which is involved in the earliest steps of mammalian rRNA maturation (Lapeyre et al. 1990; Kass et al. 1990). It is therefore is used as a marker of the FCs and DFC, of which it is a component (Rodriguez-Corona et al. 2015), and often also as a marker for transcriptional activity in the nucleolus as pre-rRNA processing is co-transcriptional (Preti et al. 2013). B23 and fibrillarin were used as nucleolar markers in the IF for the following experiments for the GC and the FCs/DFCs respectively.

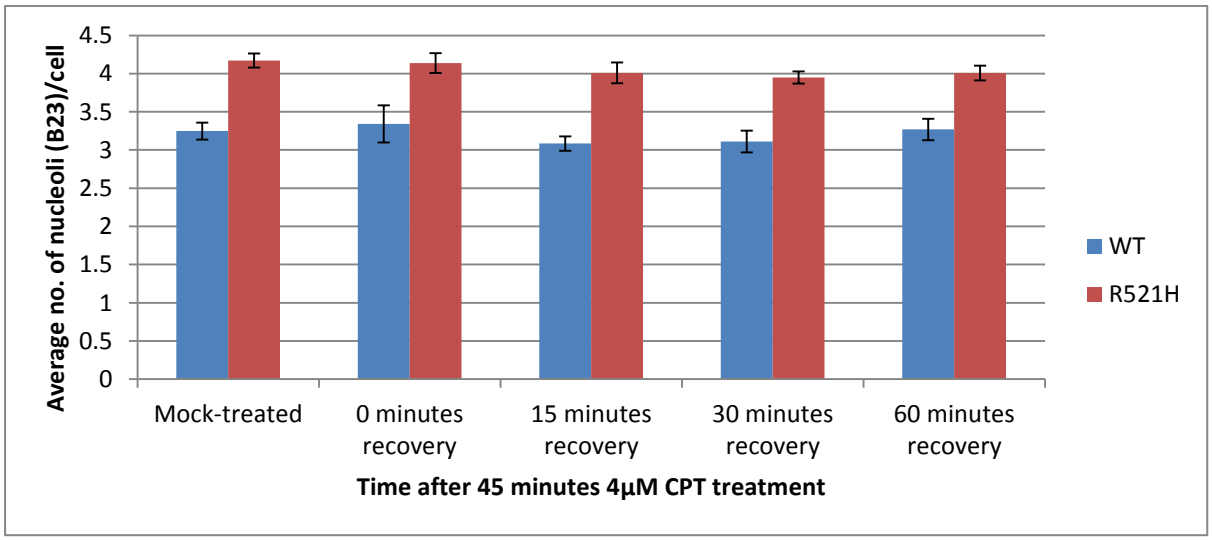
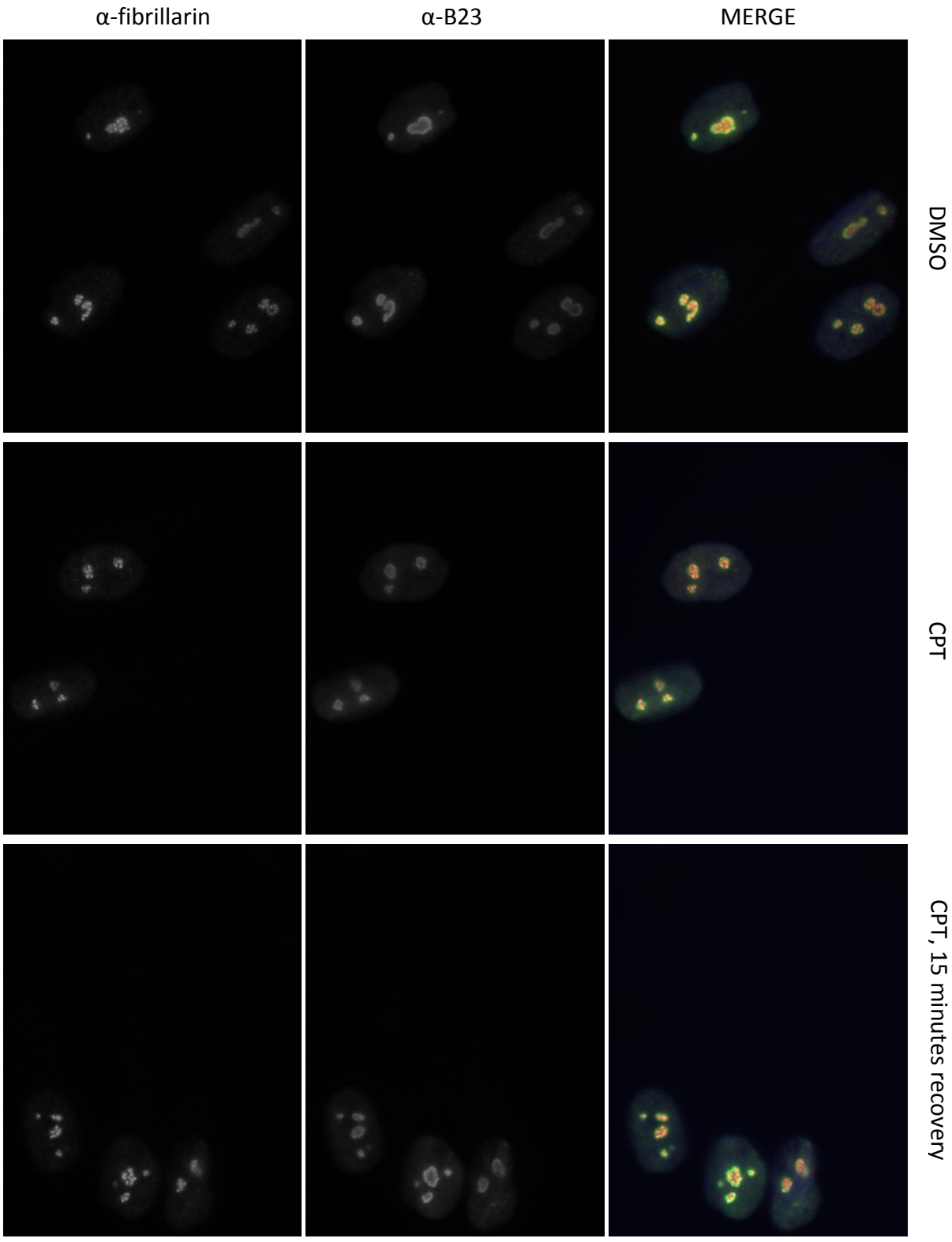


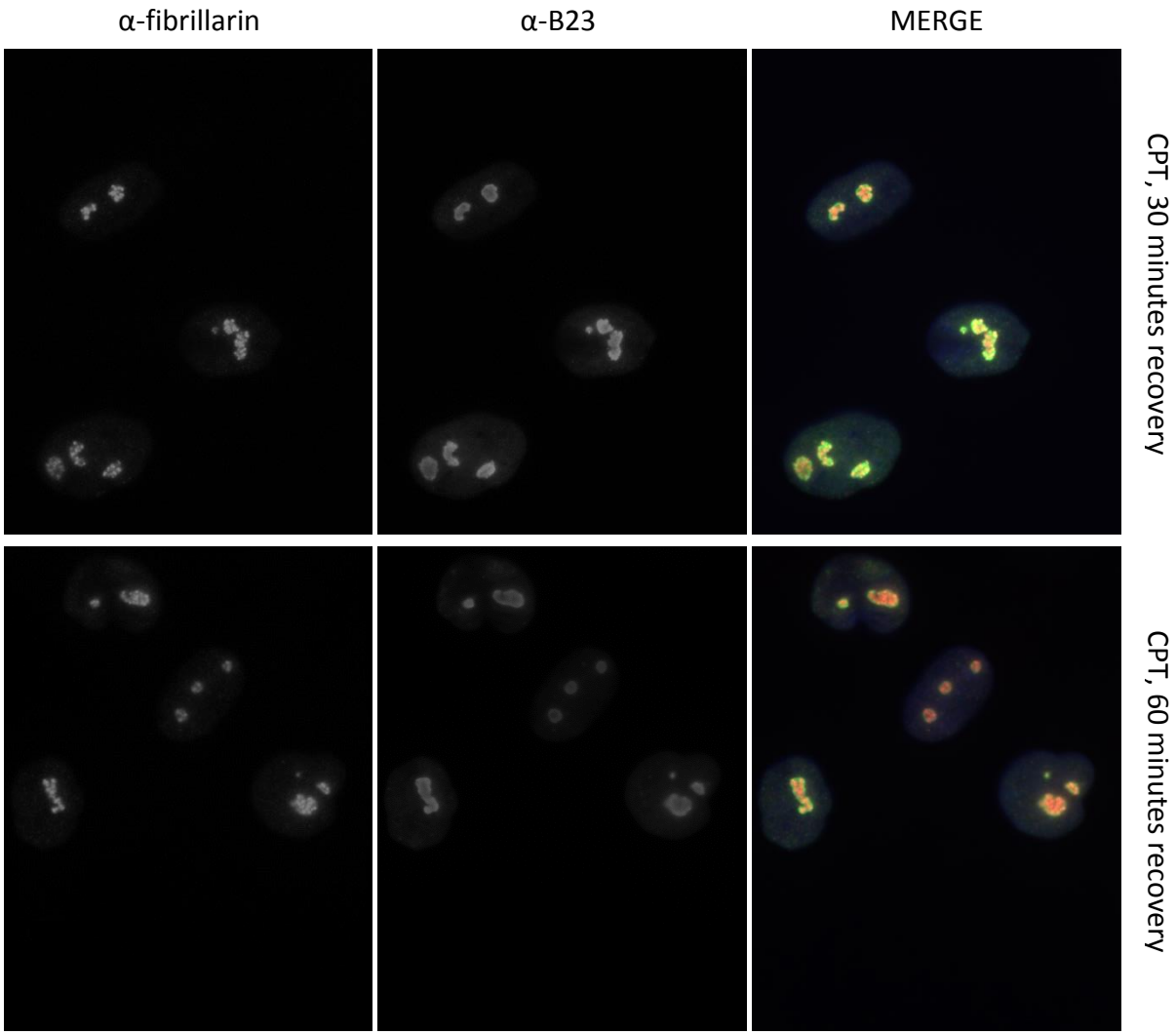
Figure 3.9a | **Comparison of nucleolar fragmentation between patient and control fibroblasts.** The number of nucleoli, as measured by B23 were counted per cell after 4µM CPT treatment and set recovery times. Patient cells demonstrated a statistically significant (by two-tailed ANOVA at 95% confidence intervals) increase in the number of nucleoli counted, suggesting nucleolar fragmentation. This difference was constitutive and not affected by CPT treatment.

Cells were seeded and allowed to settle before treatment with 4µM CPT for 45 minutes. They were then washed three times in PBS and allowed to recover for set timepoints before being fixed and processed by IF.

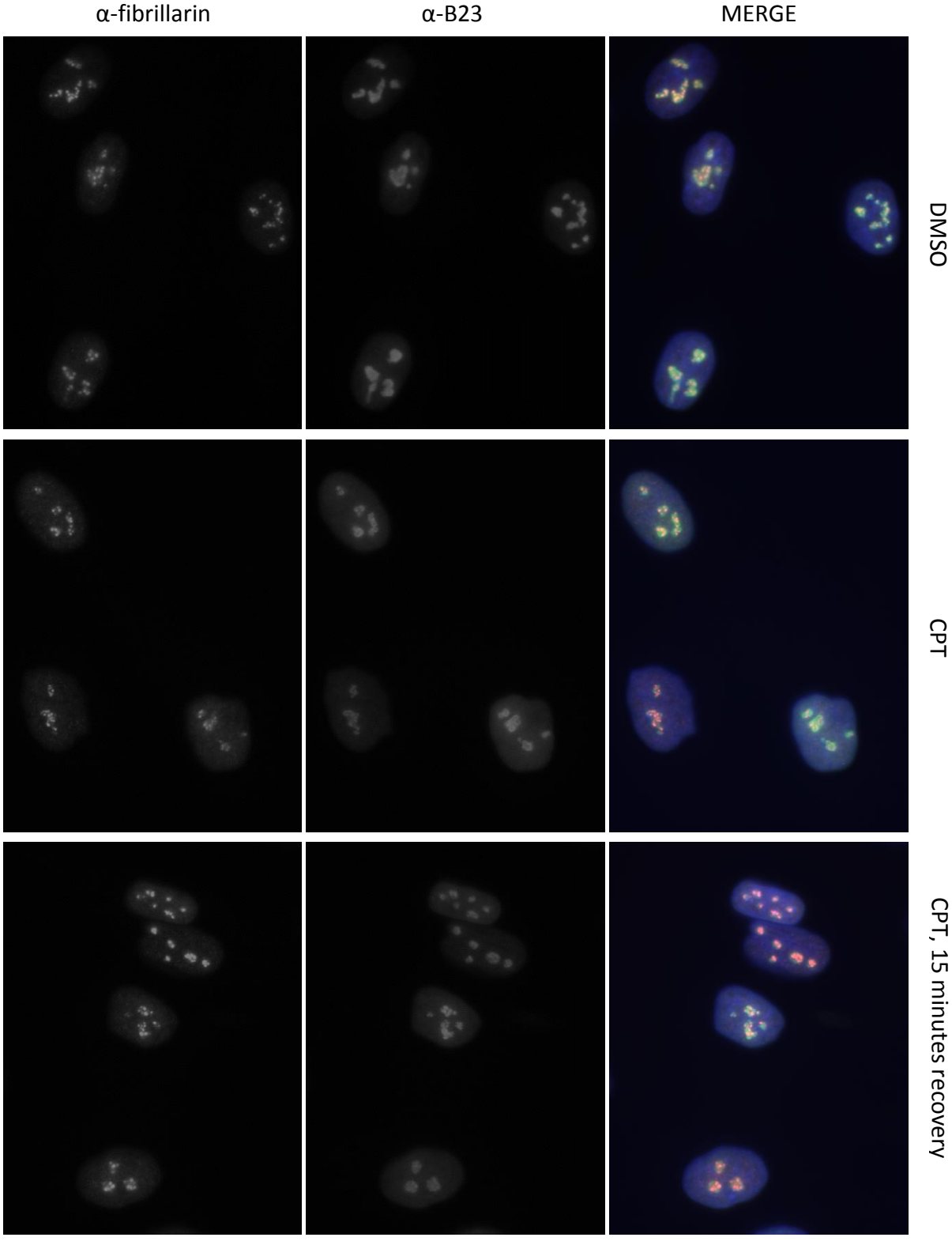
WT fibroblasts



WT fibroblasts



Patient fibroblasts



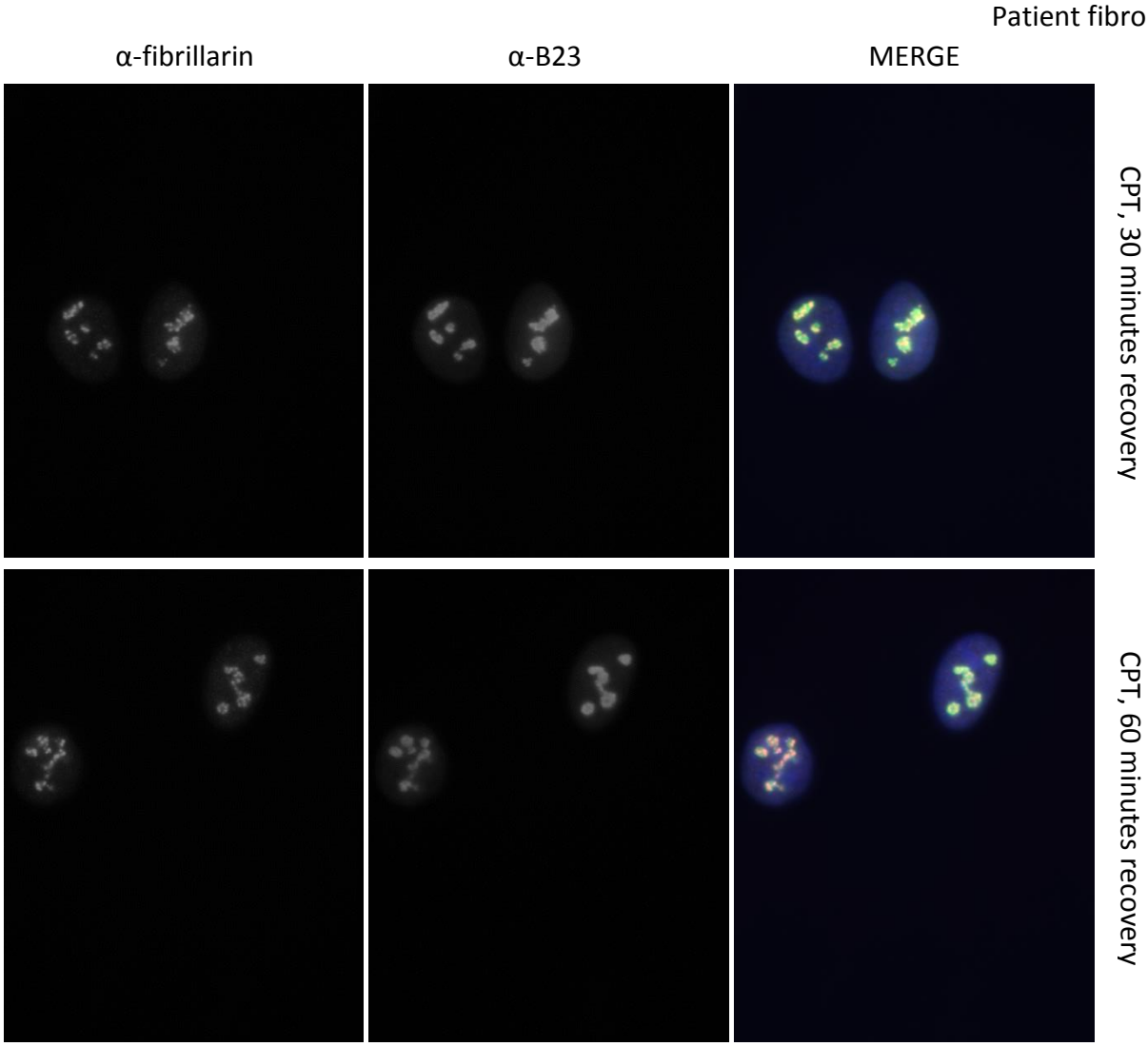


Figure 3.9b | **Comparison of nucleolar fragmentation between patient and control fibroblasts.** Representative images of cells counted in figure 3.9a.

It was observed that patient line appeared to constitutively have more nucleoli per cell as marked by B23 relative to control cells, indicating nucleolar fragmentation, but that CPT treatment did not seem to affect fragmentation (Fig 3.9a). Nucleolar fragmentation involves formation of multiple misshapen nucleolar-like structures, possibly from enlarged and stressed nucleoli (Lewinska et al. 2014), and has previously been linked to accelerated ageing in yeast (Sinclair et al. 1997) and Roberts syndrome in humans (Xu et al. 2013). In contrast there was a modest decrease in the number of foci of fibrillarin per cell upon CPT treatment (recovering within an hour after CPT withdrawal) - indicating a decrease of transcriptional activity in the nucleolus but there was no statistically significant difference in this response between the patient line and the control (Fig 3.10a).

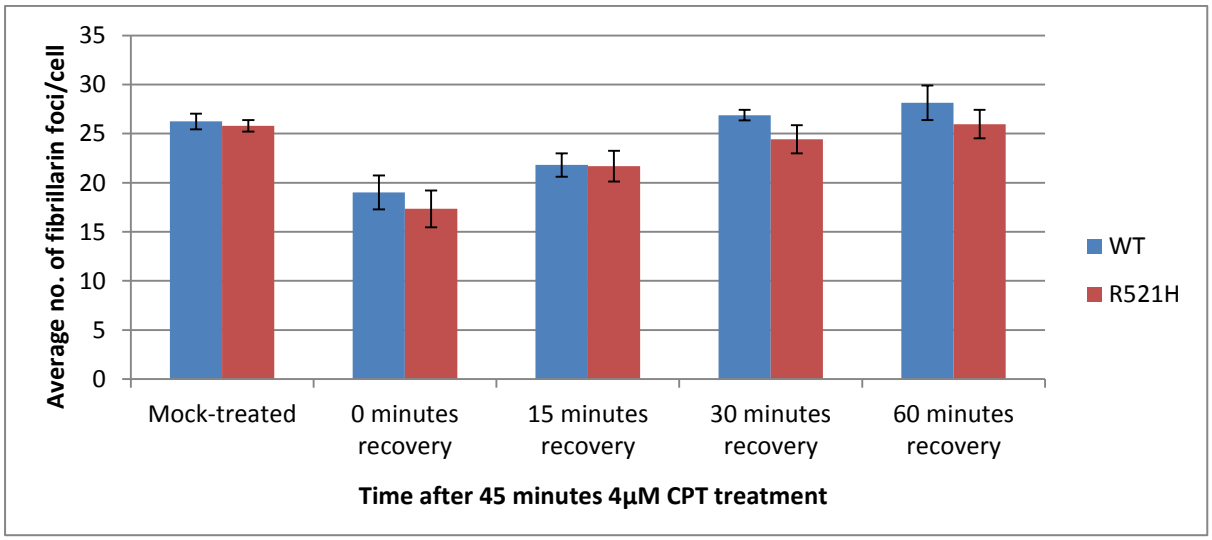
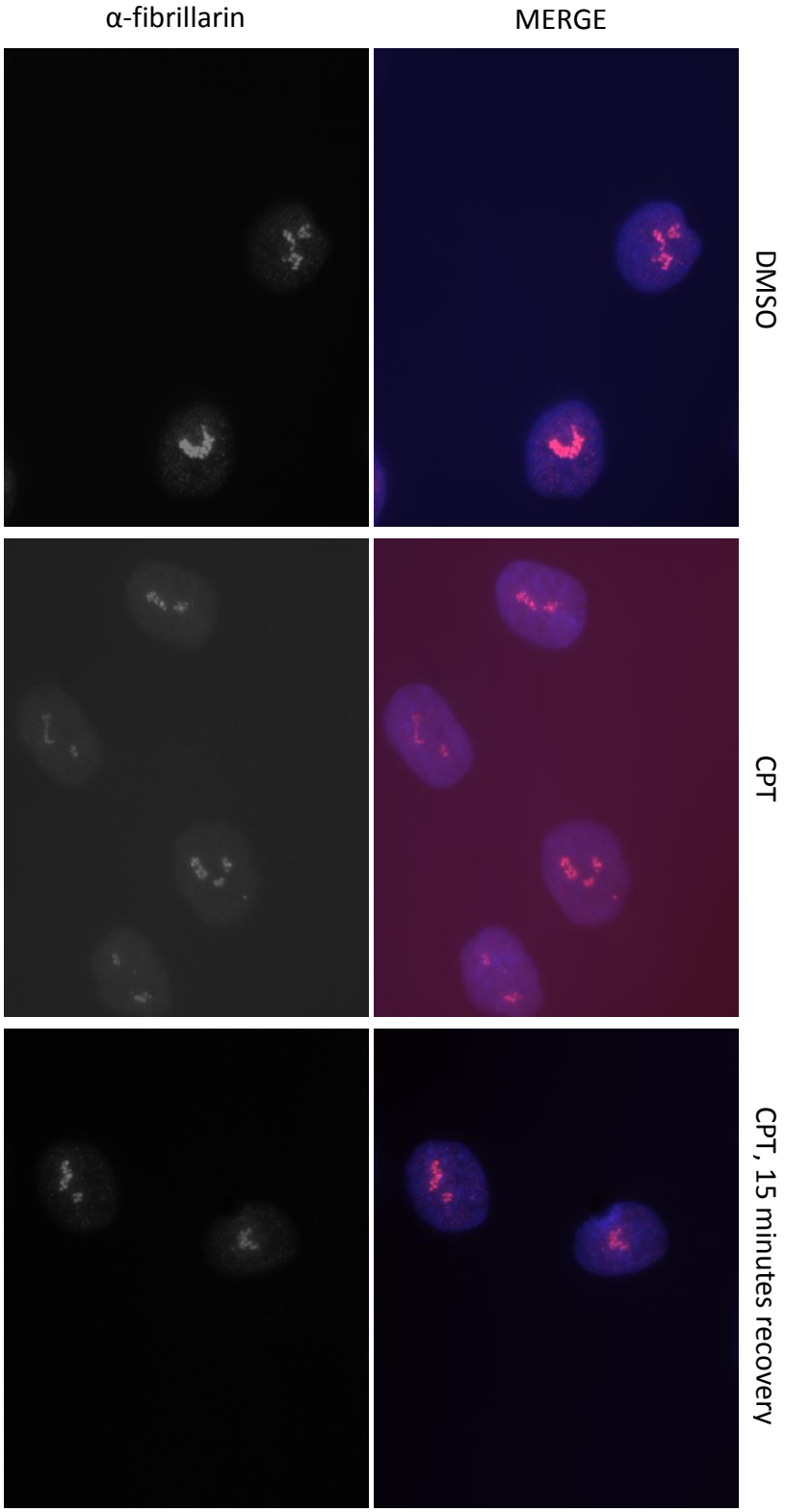


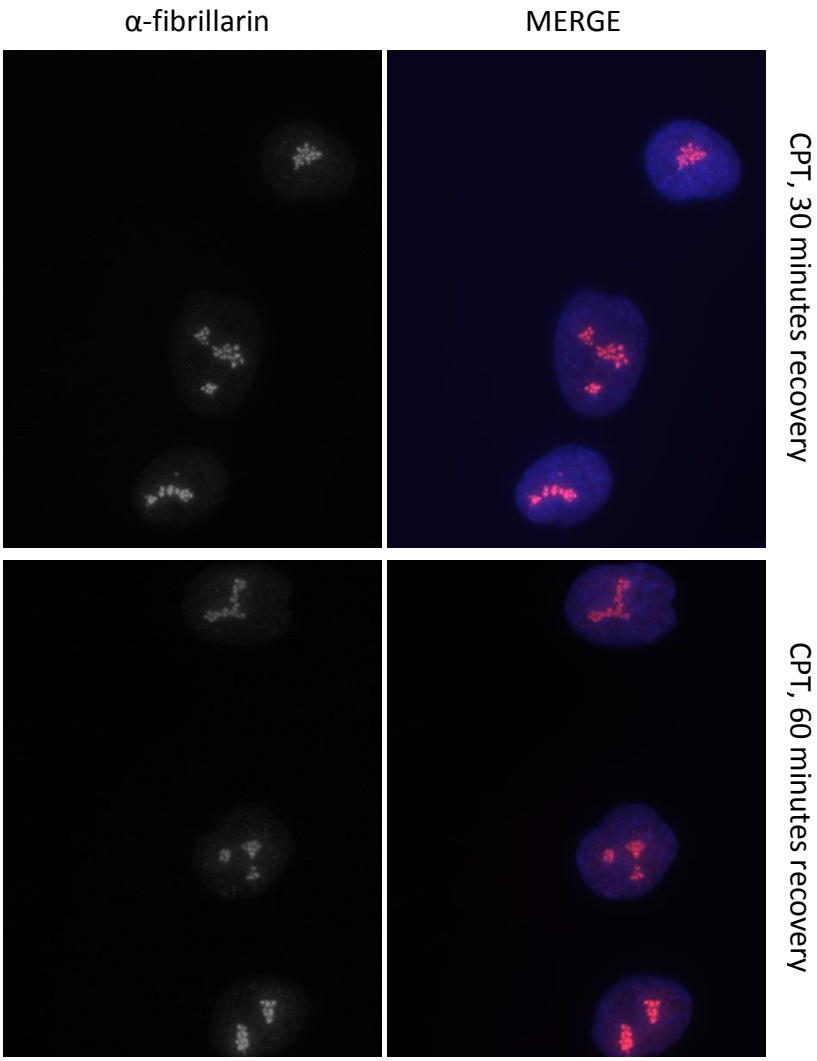
Figure 3.10a | **Comparison of nucleolar transcription sites between patient and control fibroblasts.** The number of fibrillarin foci were counted per cell after 4µM CPT treatment and set recovery times. CPT treatment induced a modest decrease in FCs/DFCs in both patient and control fibroblasts.

Cells were seeded and allowed to settle before treatment with 4µM CPT for 45 minutes. They were then washed three times in PBS and allowed to recover for set timepoints before being fixed and processed by IF.

WT fibroblasts



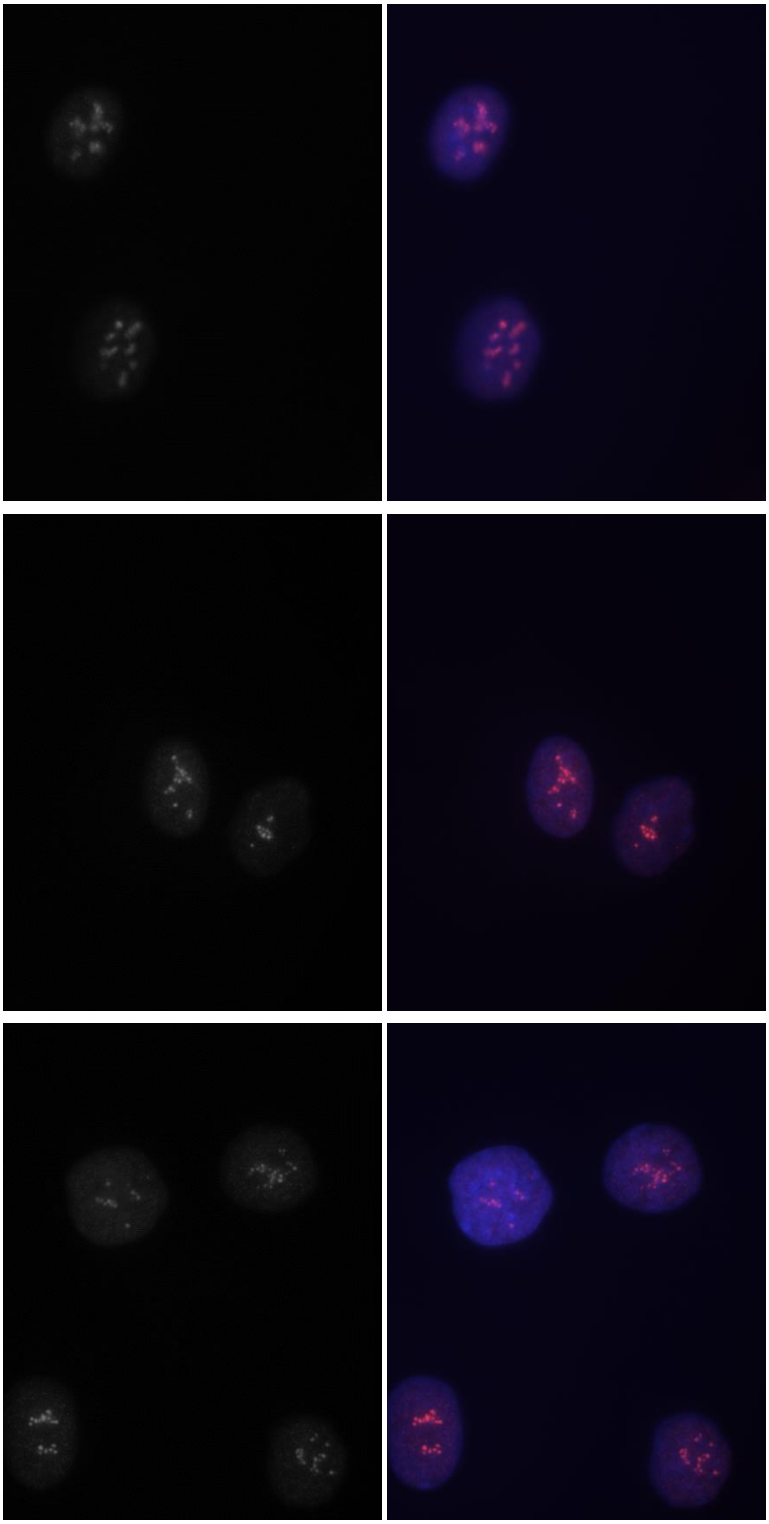
WT fibroblasts



Patient fibroblasts

α-fibrillarin

MERGE



DMSO

CPT

CPT, 15 minutes recovery

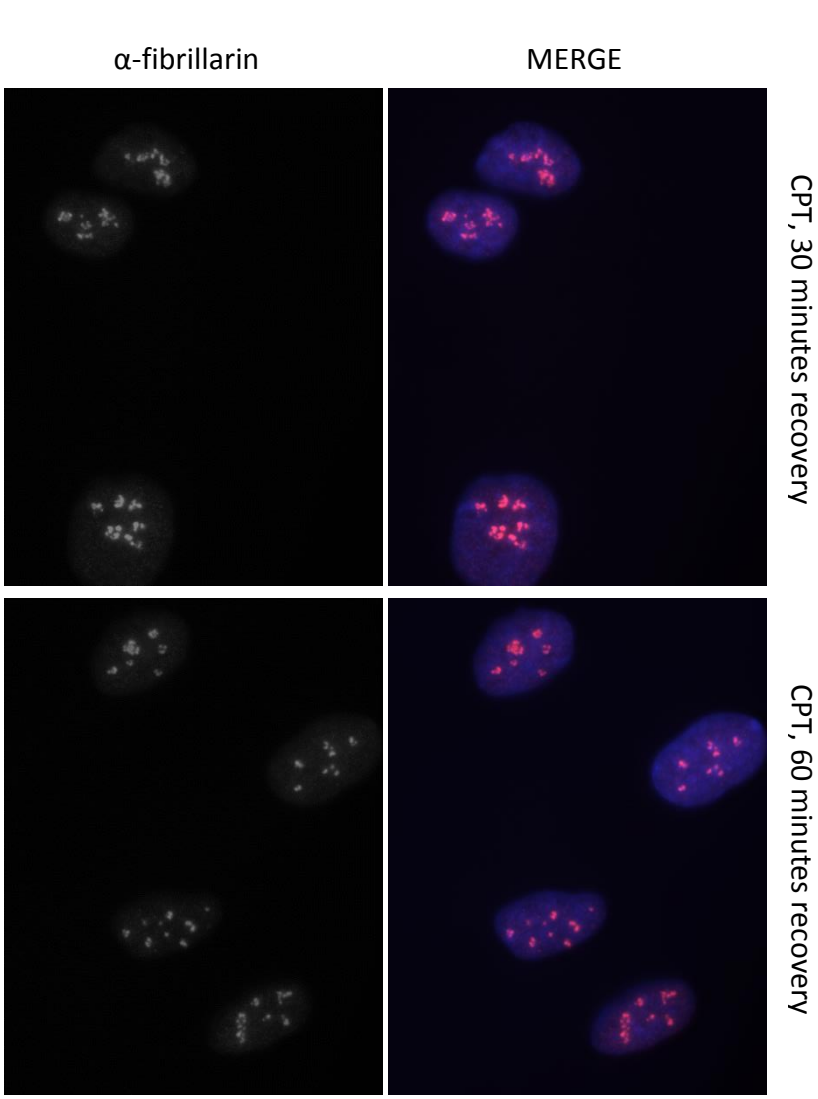


Figure 3.10b | **Comparison of nucleolar transcription sites between patient and control fibroblasts.** Representative images of cells counted in figure 3.10a.

This experiment was modified and repeated in the wild-type and R521G LAP-FUS lines. As the amount of fibrillarin foci did not seem affected by the R521H mutation and nucleolar fragmentation did not seem affected by CPT treatment the new experiment was simplified - the nucleoli were counted using only B23 IF (counting in doxycycline-induced cells expressing GFP-tagged FUS) and were counted without any CPT treatment.

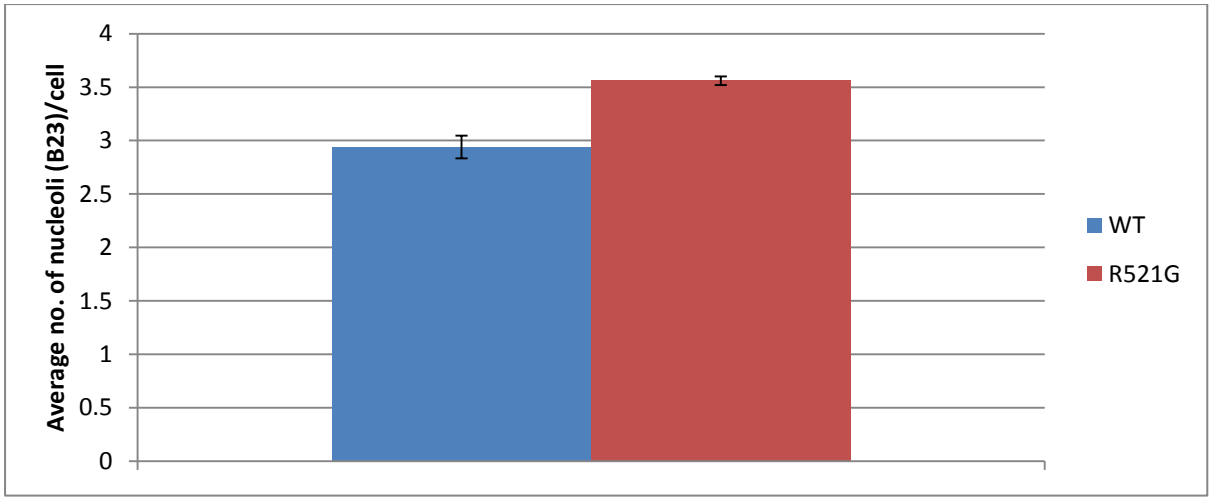


Figure 3.11a | **Comparison of nucleolar fragmentation between WT and R521G LAP-FUS Helas.** The number of nucleoli per cell, as measured by B23, were counted. Mutant cells demonstrated a statistically significant (by Student's t-test - p value = 0.03014439) increase in the number of nucleoli counted.

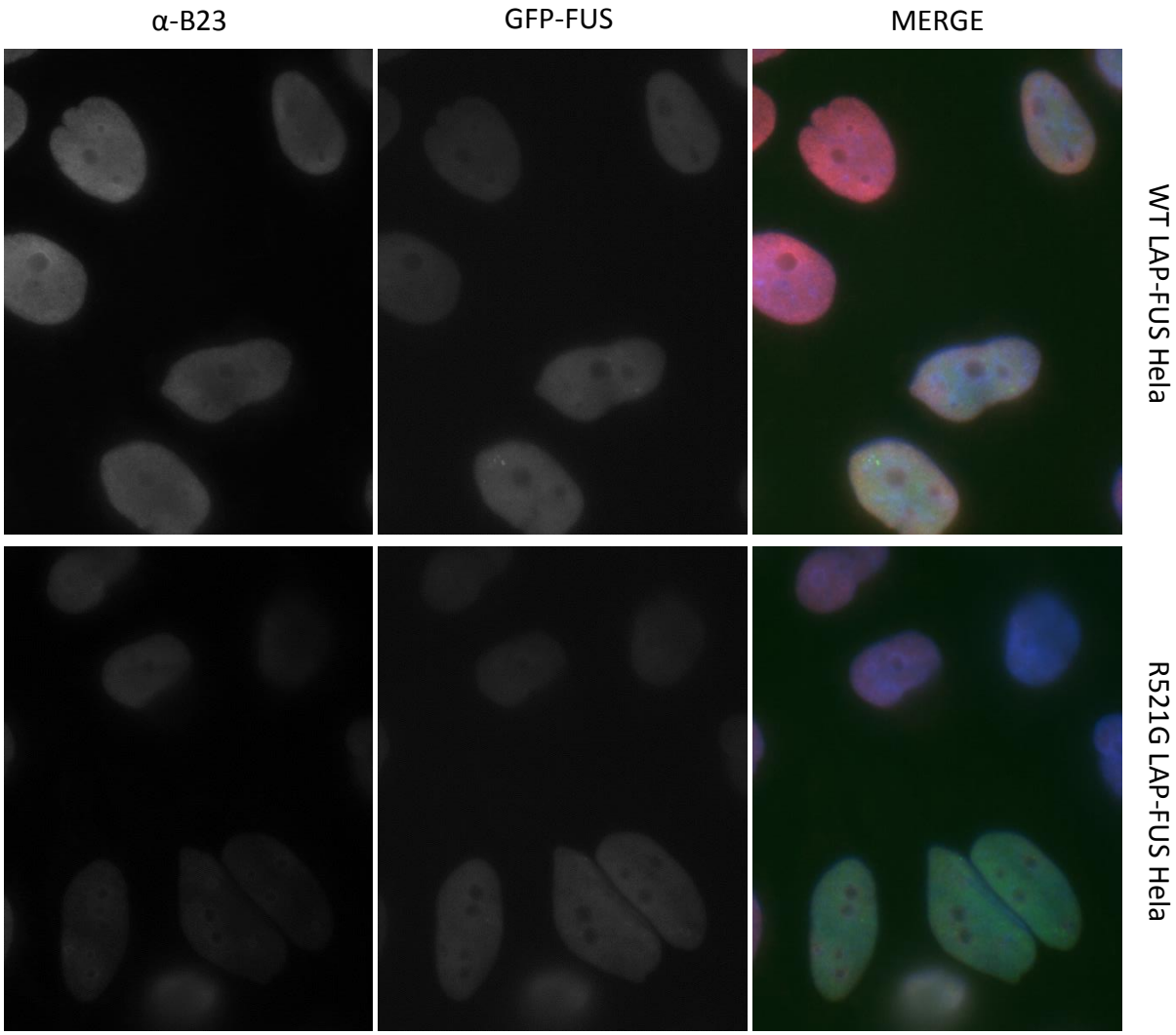


Figure 3.11b | **Comparison of nucleolar fragmentation between WT and R521G LAP-FUS Helas.** Representative images of cells counted in figure 3.11a.

The data showed increased nucleolar fragmentation in the R521G mutant, though to a lesser extent than in the fibroblasts (Fig 3.11a), indicating that more than one FUS-ALS mutation can cause this phenotype. Genetic dosage may play a role in this discrepancy as the patient fibroblasts have one wild type FUS allele to one mutant allele, while the R521G Hela has two wild type alleles to one mutant allele in cells expressing exogenous FUS.

Curiously there was also substantial nucleoplasmic expression of B23 in both the LAP-FUS Hela lines. There is normally a fraction of nucleoplasmic B23 but this fraction is small and the protein is primarily nucleolar (Lindström 2011) and such high nucleoplasmic expression is unusual. This aberrant expression of B23 was consistently observed in these cell lines in subsequent experiments. It has been observed that B23 translocates to the nucleoplasm under stress conditions in order to activate p53 (Boulon et al. 2010) so excessive nucleoplasmic B23 may simply be a feature of these Helas. This staining pattern still allowed easy identification of the nucleolus as B23 was excluded from the FCs and DFC - therefore use of the B23 antibody to identify nucleoli continued despite its excessive nucleoplasmic expression in the LAP-FUS Helas.

It was evident that FUS mutation could cause nucleolar fragmentation, but it was unclear if differing levels of FUS proteins could do so. Therefore a small interfering RNA (siRNA) knockdown of FUS was performed against a common cell line (A549 - derived from a lung carcinoma (Giard et al. 1973)) and the nucleoli of cells transfected with FUS siRNA compared to those transfected with control siRNA.

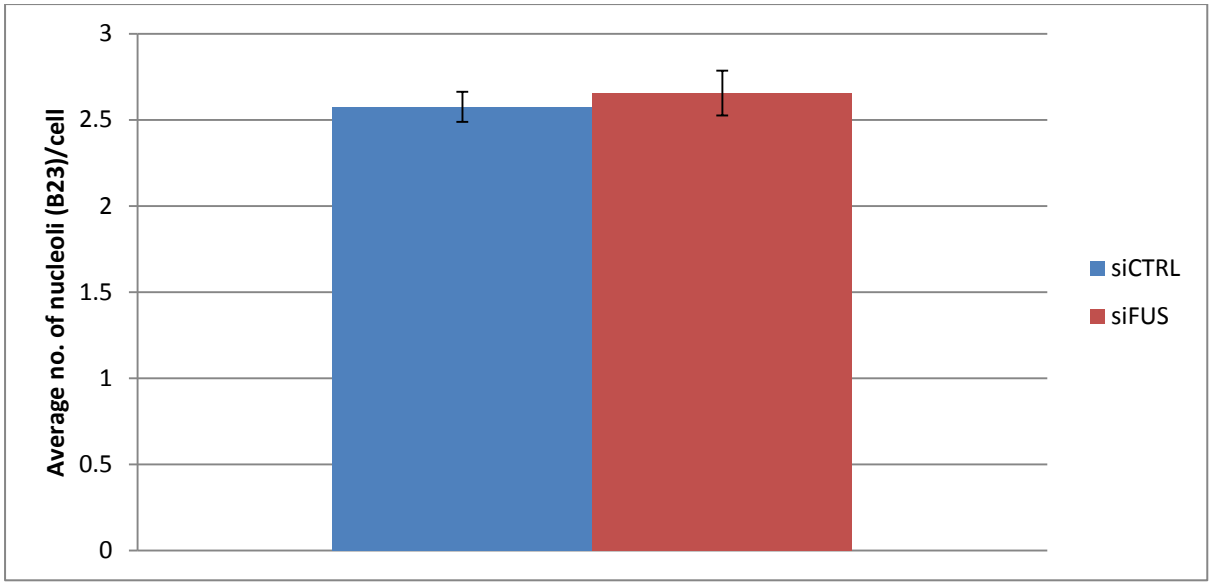


Figure 3.12a | **Comparison of nucleolar fragmentation between A549 cells treated with control siRNA or FUS siRNA.** The number of nucleoli per cell, as measured by B23, were counted. There was no statistically significant diference in nucleoli counts between cells treated with control siRNA or siRNA against FUS. n=3.

A549 cells were subjected to double siRNA transfection with control siRNA or siRNA directed against FUS before being probed for B23 and fibrillarin by IF, or collected for a western blot.

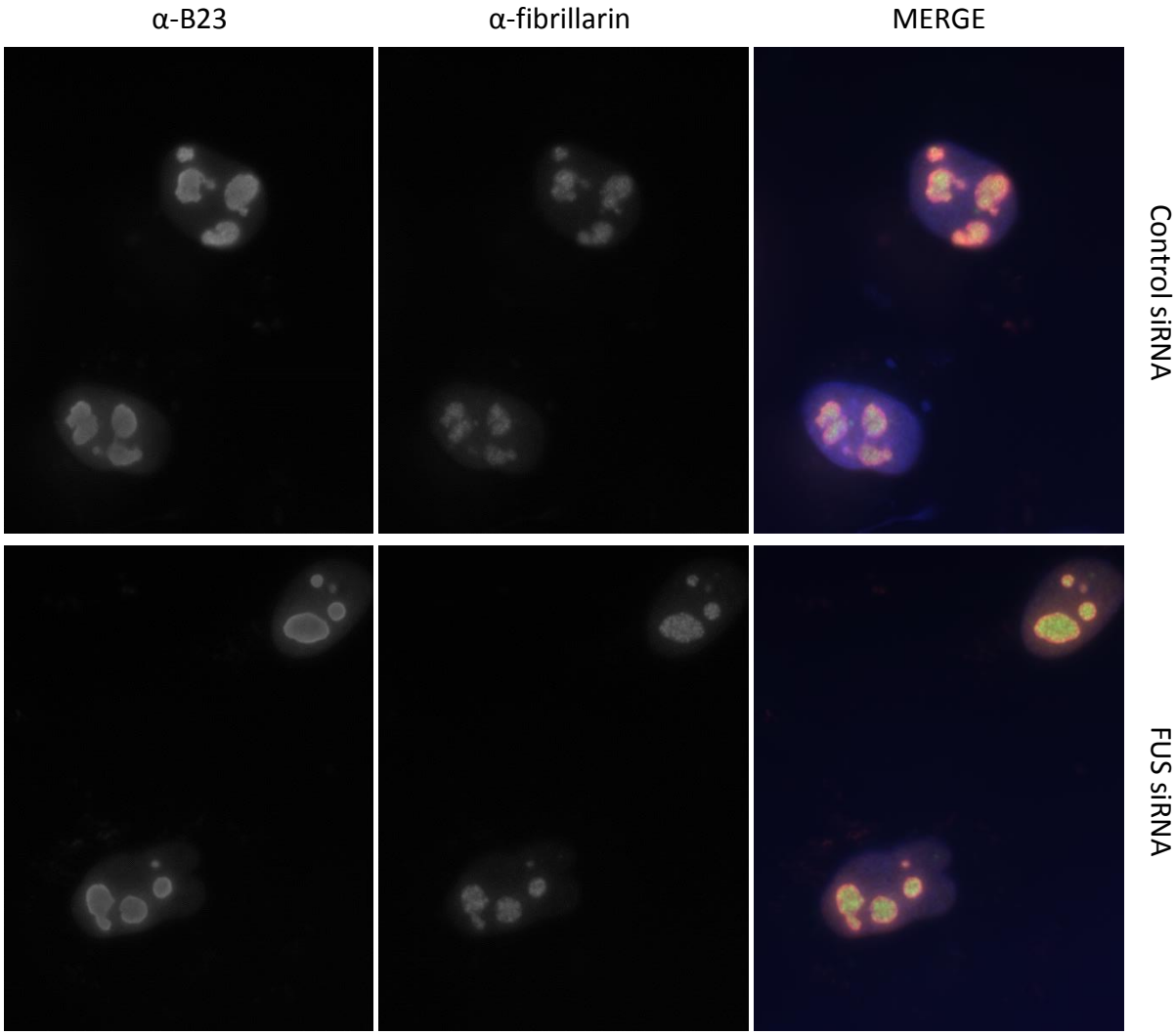


Figure 3.12b | **Comparison of nucleolar fragmentation between A549 cells treated with control siRNA or FUS siRNA.** Representative images of cells counted in figure 3.12a.

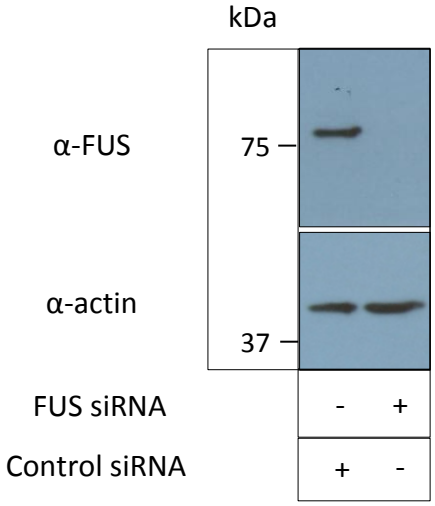


Figure 3.12c | **Comparison of nucleolar fragmentation between A549 cells treated with control siRNA or FUS siRNA.** Western blot demonstrating successful knockdown of FUS. Approximately $5x10^5$ cells per lane.

A549s transfected with siRNA against FUS demonstrated near-identical B23 staining relative to cells transfected with control siRNA (Fig 3.12a) indicating that nucleolar fragmentation is related to the presence of a FUS mutation rather than to a deficit of functional FUS protein. Validation of the siRNA knockdown is shown in Fig 3.12c.

3.5 Measurement of transcriptional recovery after DNA damage using ethnyl uridine (EU) RNA labelling

With a nucleolar defect observed in patient cells, it was decided to assay transcription directly using ethnyl uridine (EU) labelling - in this technique cells are introduced to EU, an analogue of RNA containing an exposed alkyne group which is readily integrated into RNA as it is transcribed. After fixation a copper-catalysed click chemistry reaction is used to label the EU, and so the RNA, with a fluorophore (Jao & Salic 2008). The intensity of fluorescence can then be used to measure global transcription in labelled cells.

It was expected that cells that have a defect in various cellular processes may take longer, or be unable to, restart transcription after exposure to transcriptional stress, such as inhibition. For instance cells with a mutation that affected the speed or efficiency of initiating elongation may demonstrate this defect. As such a control experiment was performed using mouse embryonic fibroblasts (MEFs) defective in tyrosyl-DNA phosphodiesterase 1 (TDP1) or X-ray repair cross-complementing protein 1 (XRCC1). TDP1 is the enzyme responsible for removal of stalled TOP1 from TOP1ccs (Yang et al. 1996) and XRCC1 has been shown to recruit TDP1 (amongst other proteins) to SSBs (Plo et al. 2003). Both of these cell lines would be expected to be defective at restarting transcription after CPT treatment, as they would take longer to repair DNA damage prior to restarting of transcription or even be unable to do so altogether. Therefore the transcriptional capacity of these cells was examined by EU labelling at set timepoints after CPT treatment.

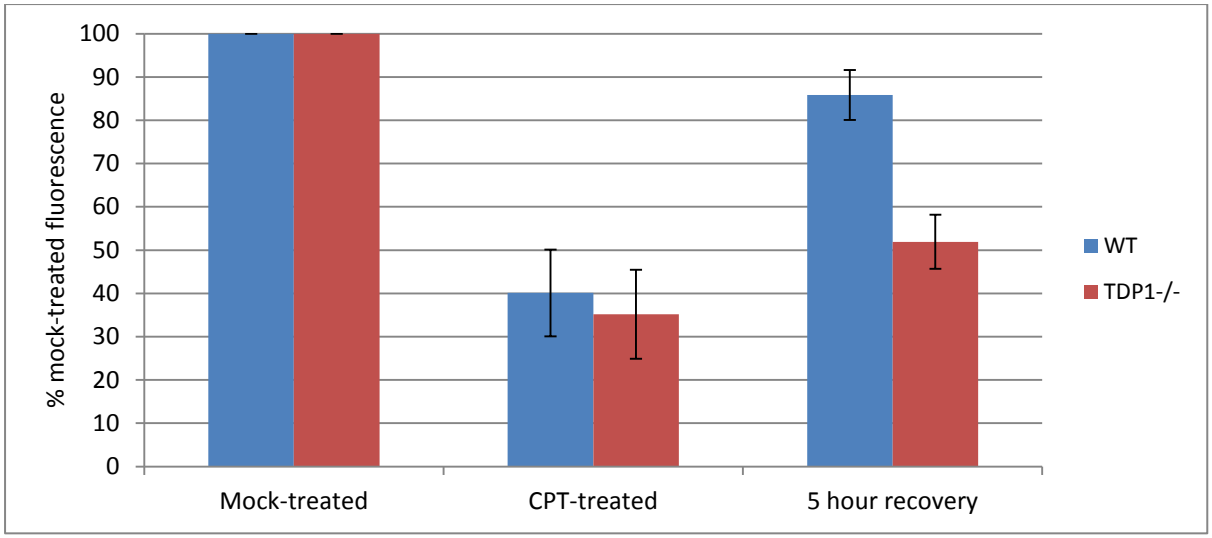
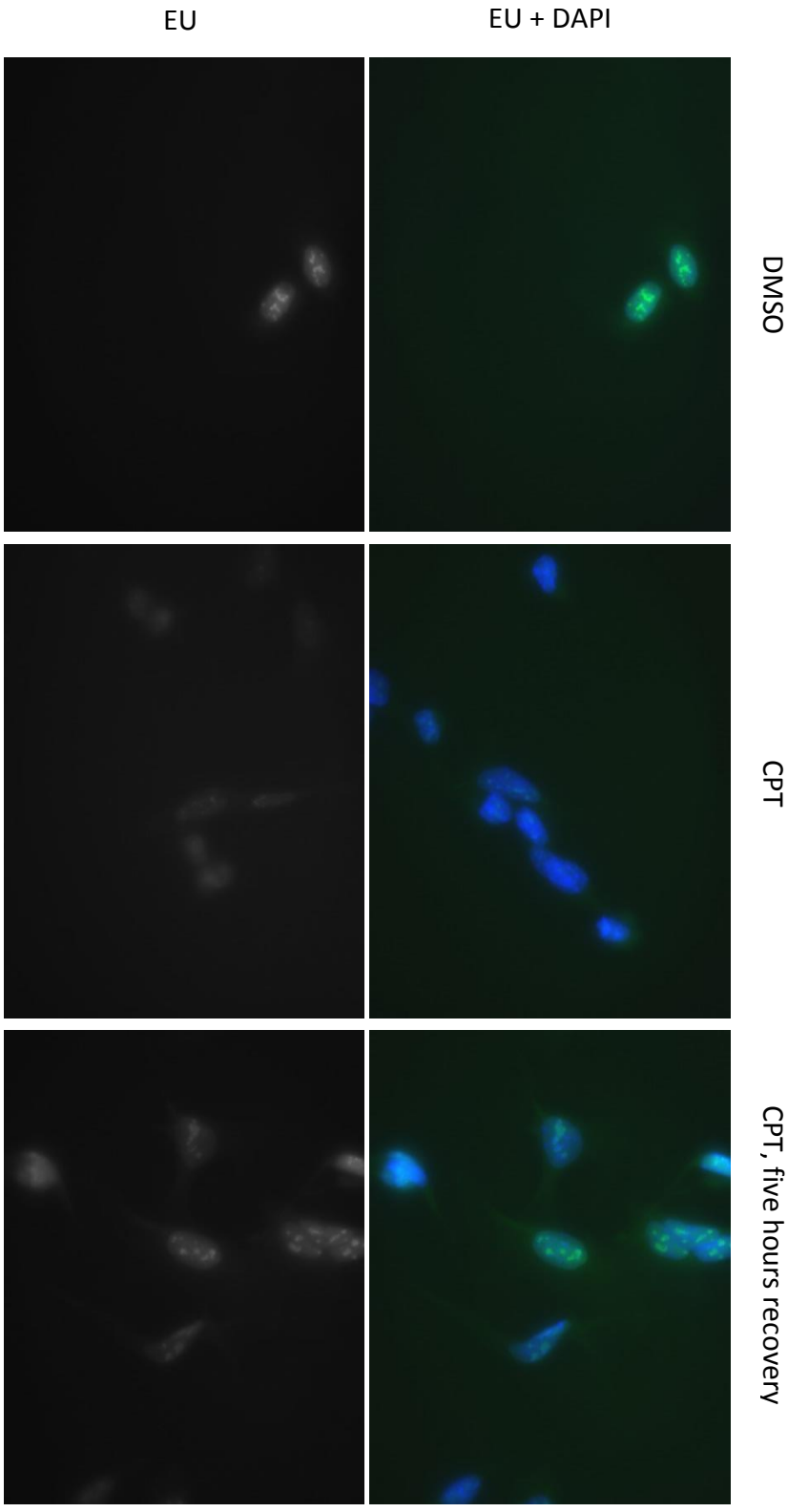


Figure 3.13a | **Measuring recovery of global transcription after CPT treatment in TDP1 null MEFs.** Mutant cells demonstrated a statistically significant (by Student's t-test - p value = 0.035238) decrease in global transcription after treatment with CPT and five hours recovery. n=3.

Cells were treated with 4µM CPT for 45 minutes total. The cells not allowed recovery time were co-incubated with EU for the final 30 minutes and the cells allowed recovery time were incubated with EU for 30 minutes five hours after removal of the CPT. All cells were then fluorescently labelled using the Click-iT® RNA Alexa Fluor® 488 Imaging Kit before microscopy. A negative control of TDP MEFs unlabelled with EU but processed using the Imaging Kit was also generated (Fig 3.13c).

WT MEFs



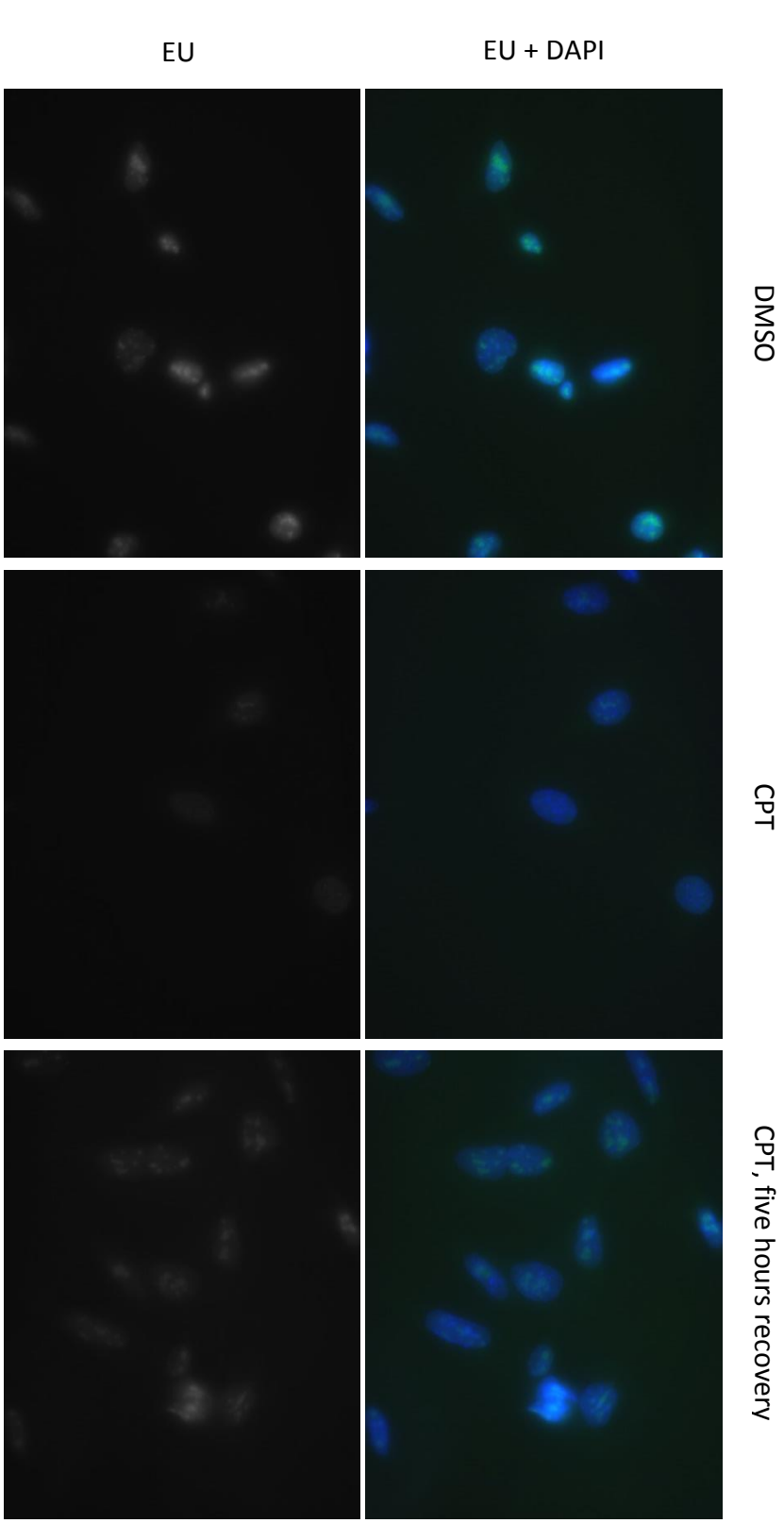


Figure 3.13b | **Measuring recovery of global transcription after CPT treatment in TDP1 null MEFs.** Representative images of cells used for figure 3.13a (taken at 40x magnification).

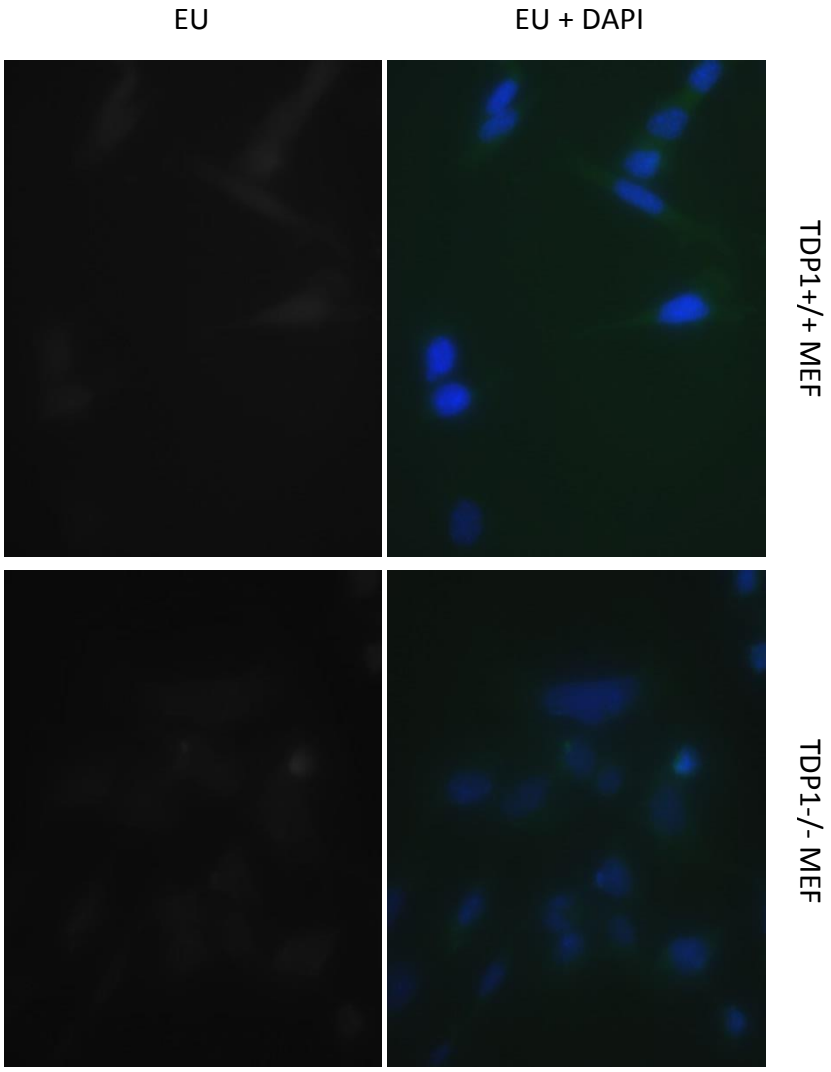


Figure 3.13c | **Measuring recovery of global transcription after CPT treatment in TDP1 null MEFs - non-EU labelled control.** Non-EU labelled negative control to demonstrate requirement for EU in RNA imaging (taken at 40x magnification).

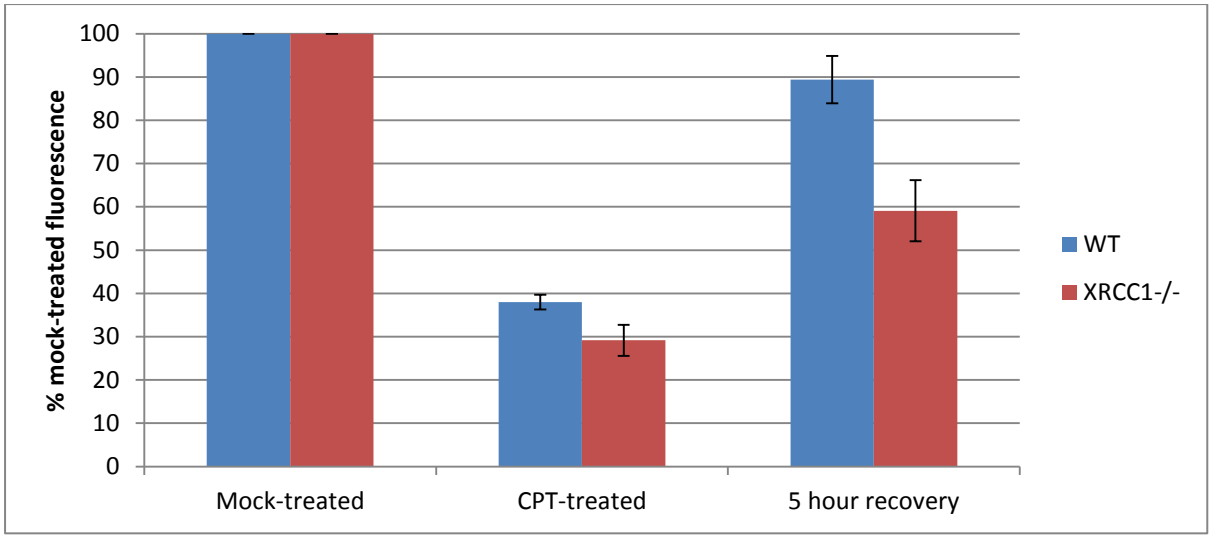
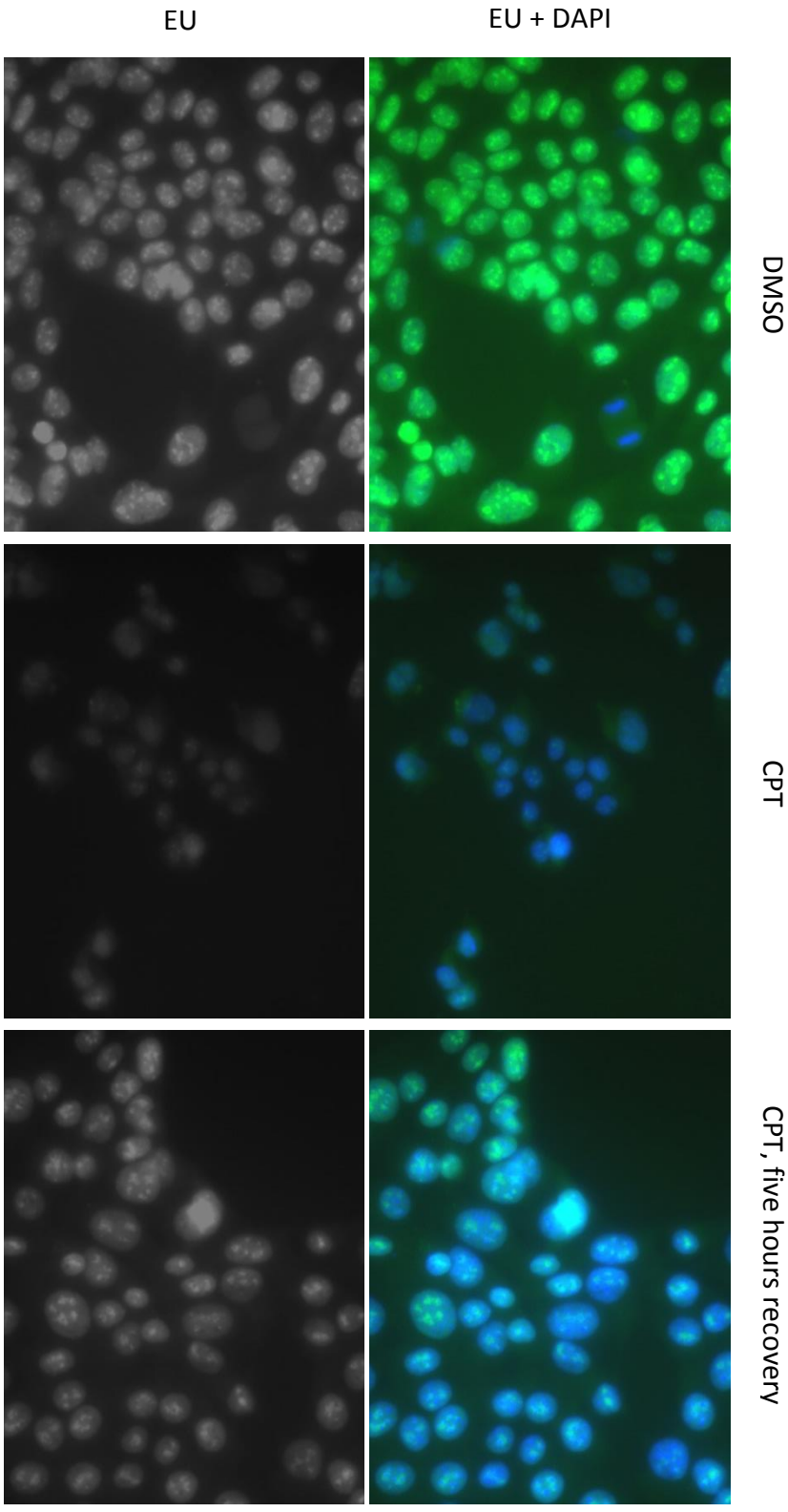


Figure 3.14a | **Measuring recovery of global transcription after CPT treatment in XRCC1 null MEFs.** Mutant cells demonstrated a statistically significant (by Student's t-test - p value = 0.034536) decrease in global transcription after treatment with CPT and five hours recovery. n=5.

Cells were treated with 4µM CPT for 45 minutes total. The cells not allowed recovery time were co-incubated with EU for the final 30 minutes and the cells allowed recovery time were incubated with EU for 30 minutes five hours after removal of the CPT. All cells were then fluorescently labelled using the Click-iT® RNA Alexa Fluor® 488 Imaging Kit before microscopy.

WT MEFs



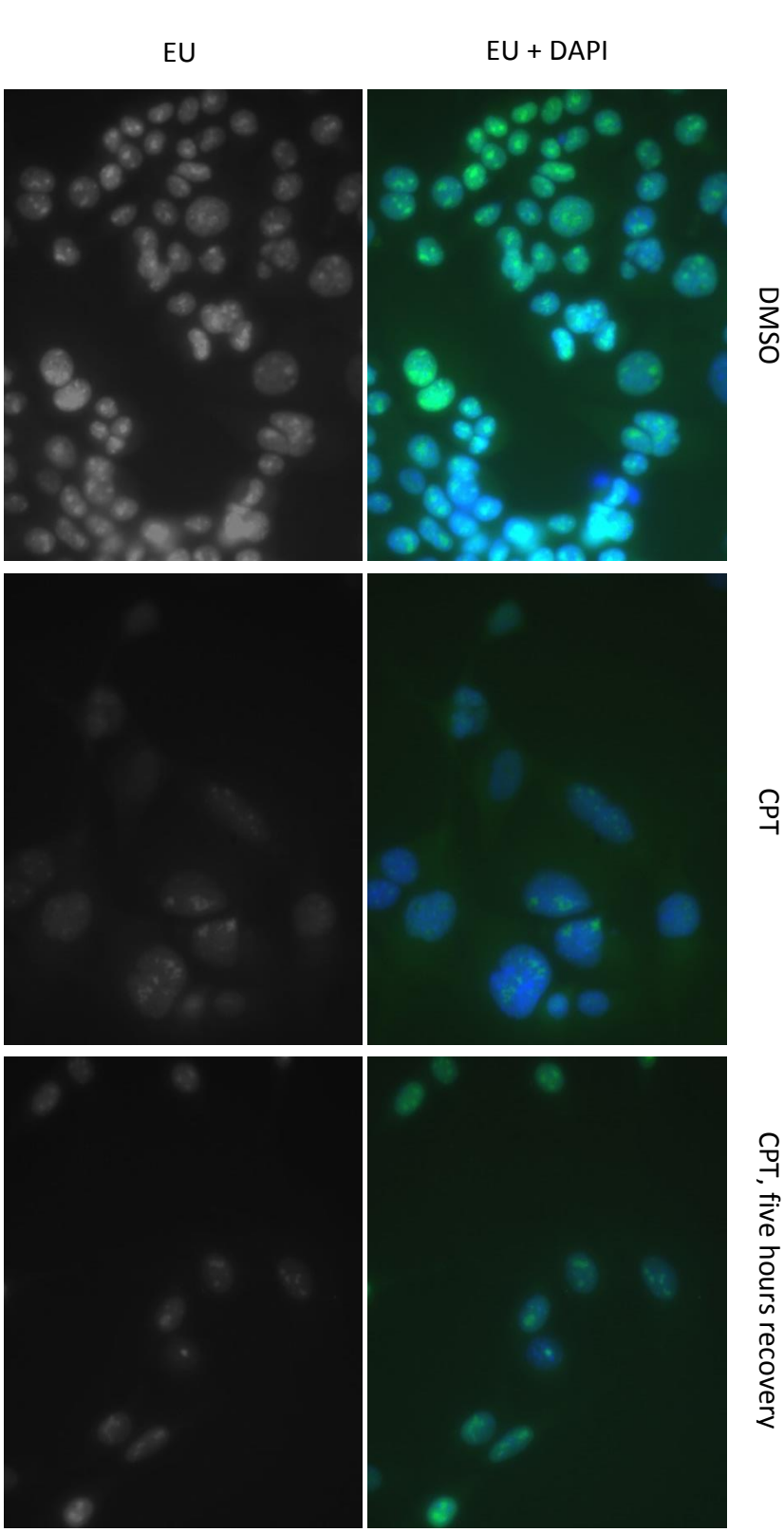


Figure 3.14b | **Measuring recovery of global transcription after CPT treatment in XRCC1 null MEFs.** Representative images of cells used for figure 3.14a (taken at 40x magnification).

In both sets of mutant MEFs global transcription was greatly and significantly reduced after recovery relative to their wild-type littermate controls - acting as a positive control (Figs 3.13a, 3.14a). With the positive control performed the transcriptional recovery assay was repeated using patient fibroblasts and their wild-type sibling controls.

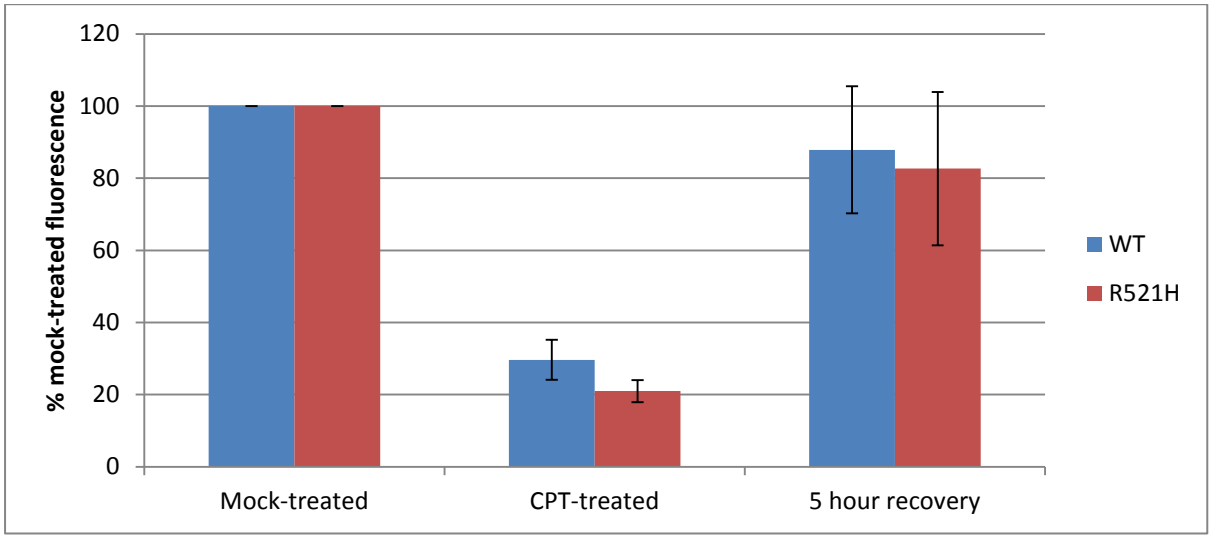
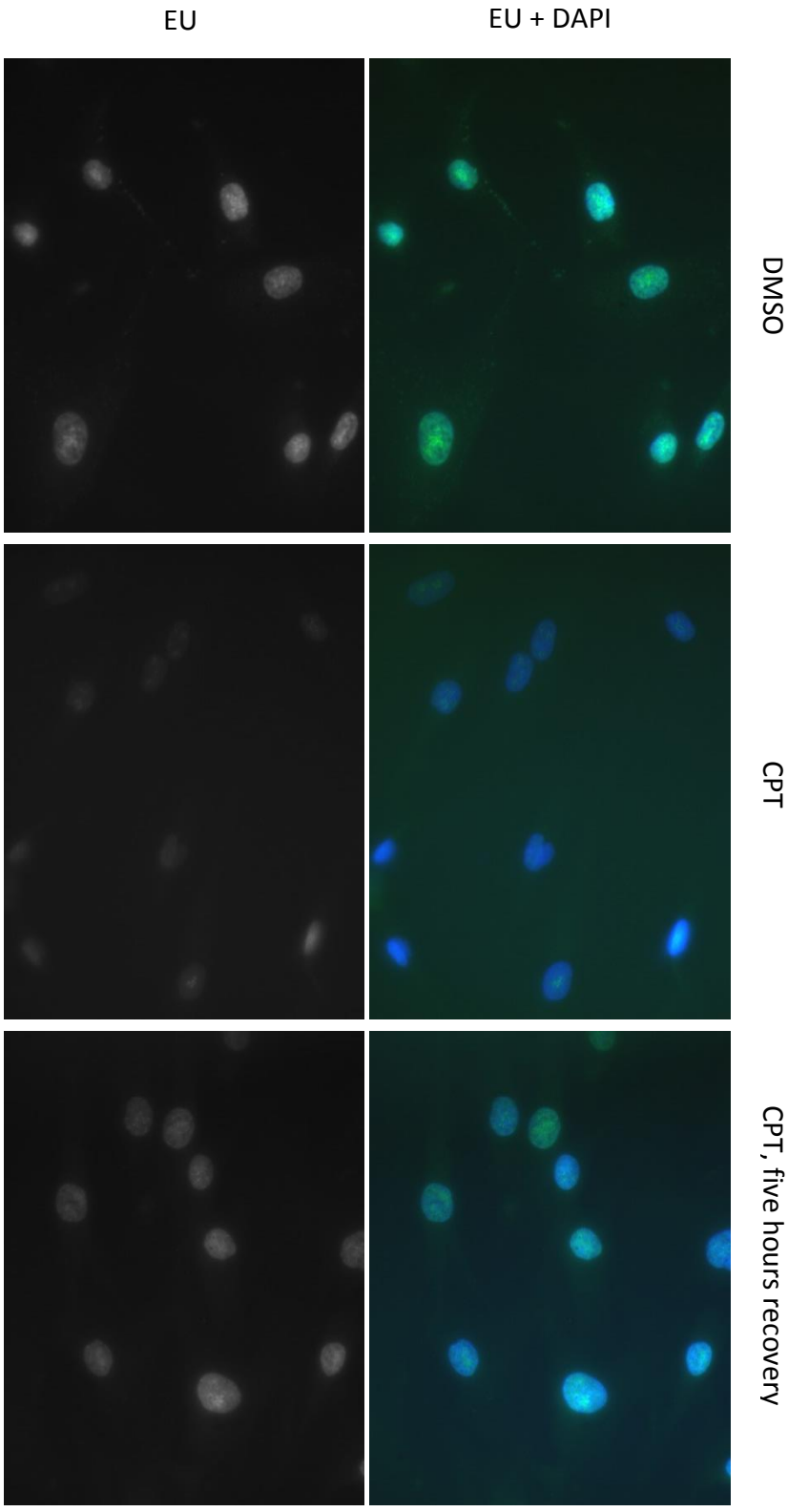


Figure 3.15a | **Measuring recovery of global transcription after CPT treatment in FUS patient fibroblasts and controls.** No statistically significant defect in recovery of global transcription was observed.

Cells treated as in figure 3.14a.

WT fibroblasts



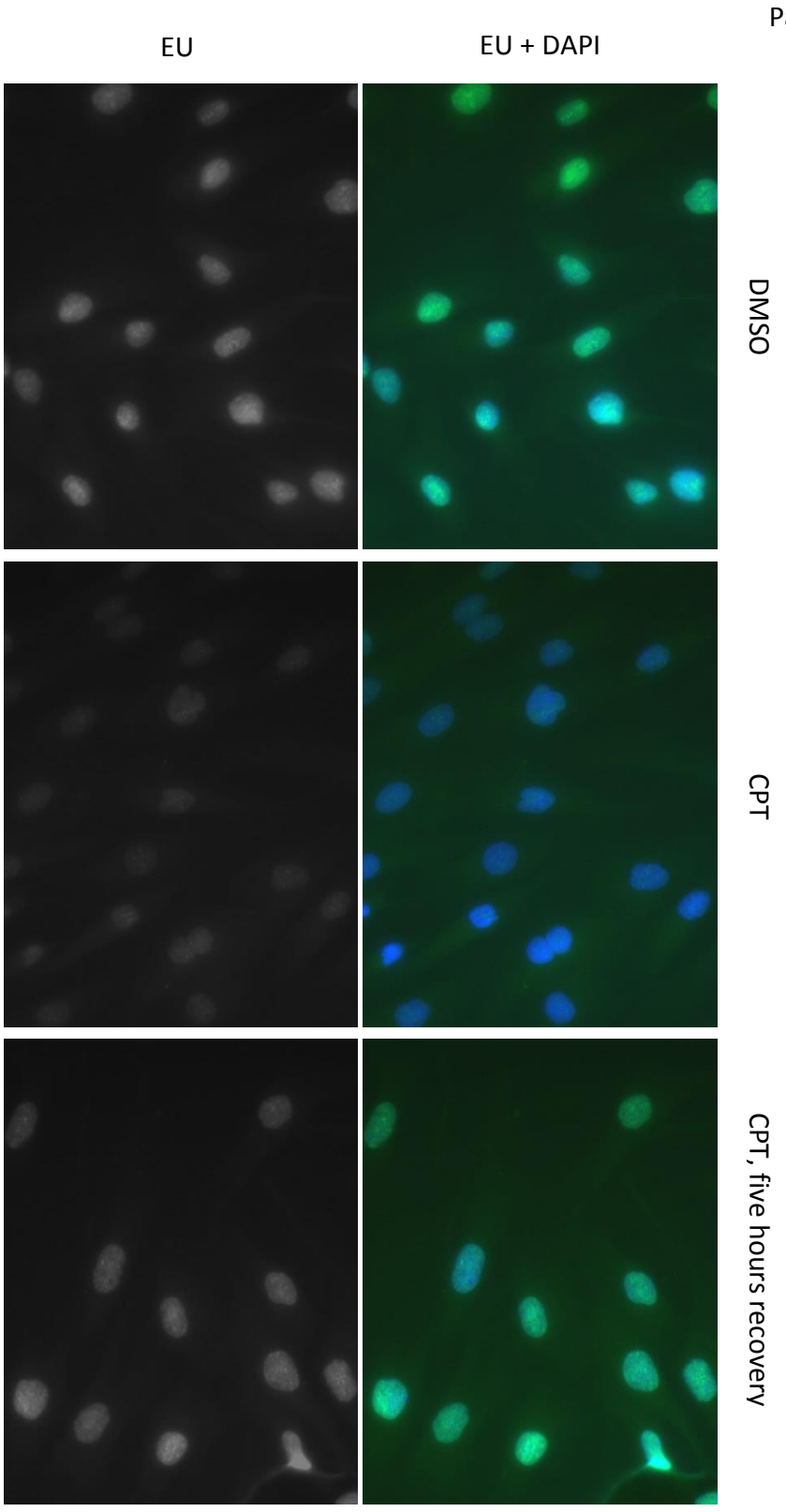


Figure 3.15b | **Measuring recovery of global transcription after CPT treatment in FUS patient fibroblasts and controls.** Representative images of cells used for figure 3.15a (40x magnification).

Unlike the MEFs the fibroblasts demonstrated no detectable defect in recovery from transcriptional stress (Fig 3.15a). It was proposed that there may have been a more subtle, transient defect and so the experiment was repeated with additional intermediate timepoints.

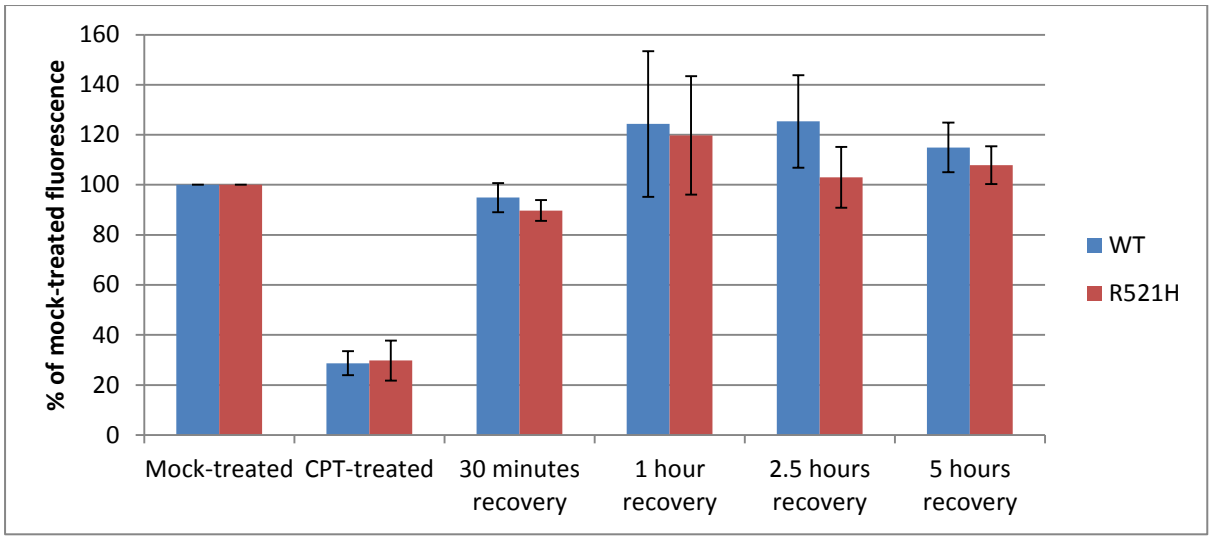
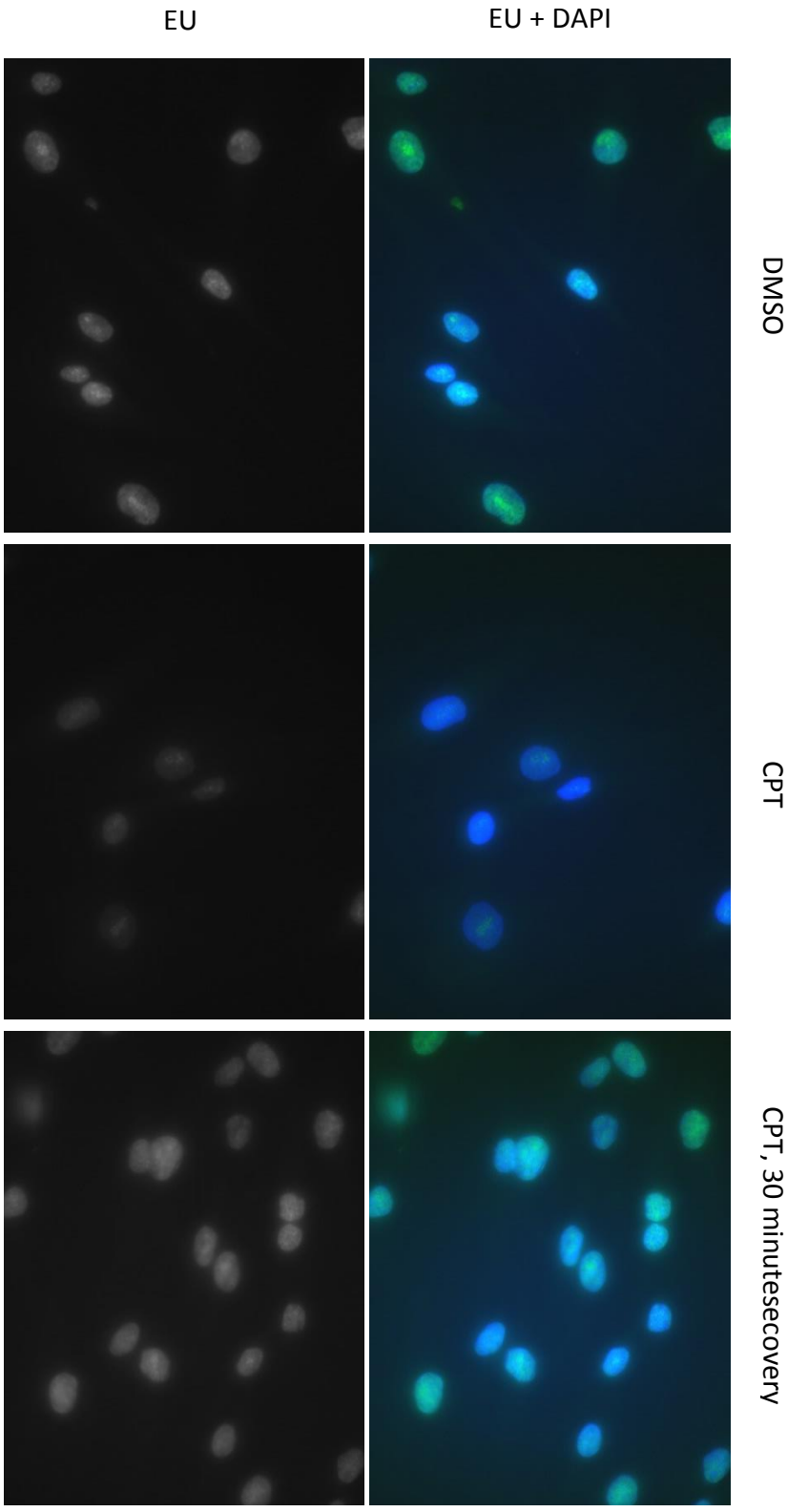


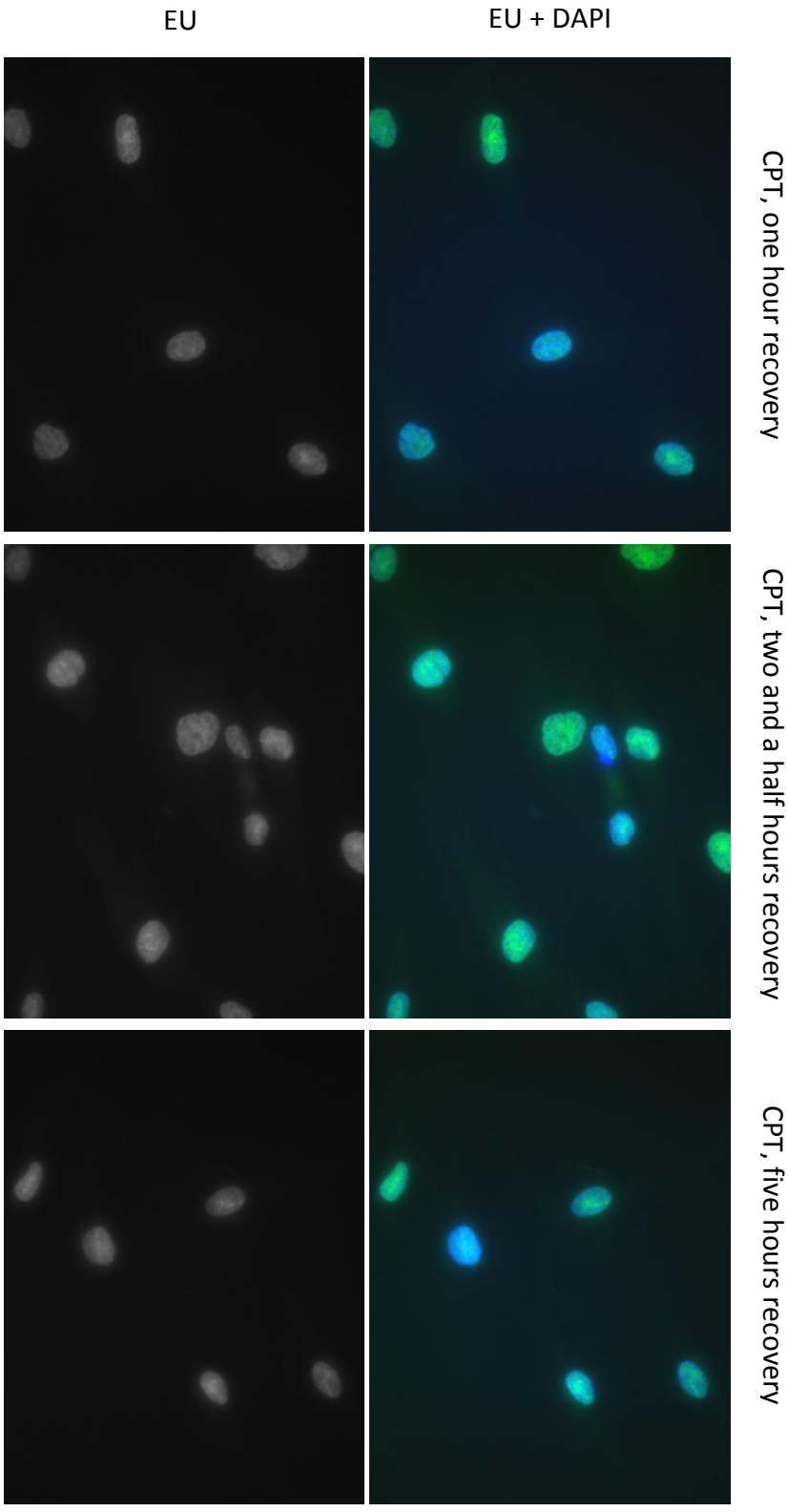
Figure 3.15c | **Measuring recovery of global transcription after CPT treatment in FUS patient fibroblasts and controls.** With intermediate recovery timepoints – still no statistically significant defect in recovery of global transcription was observed. n=3.

Cells were treated as in figure 3.14a, with additional recovery timepoints.

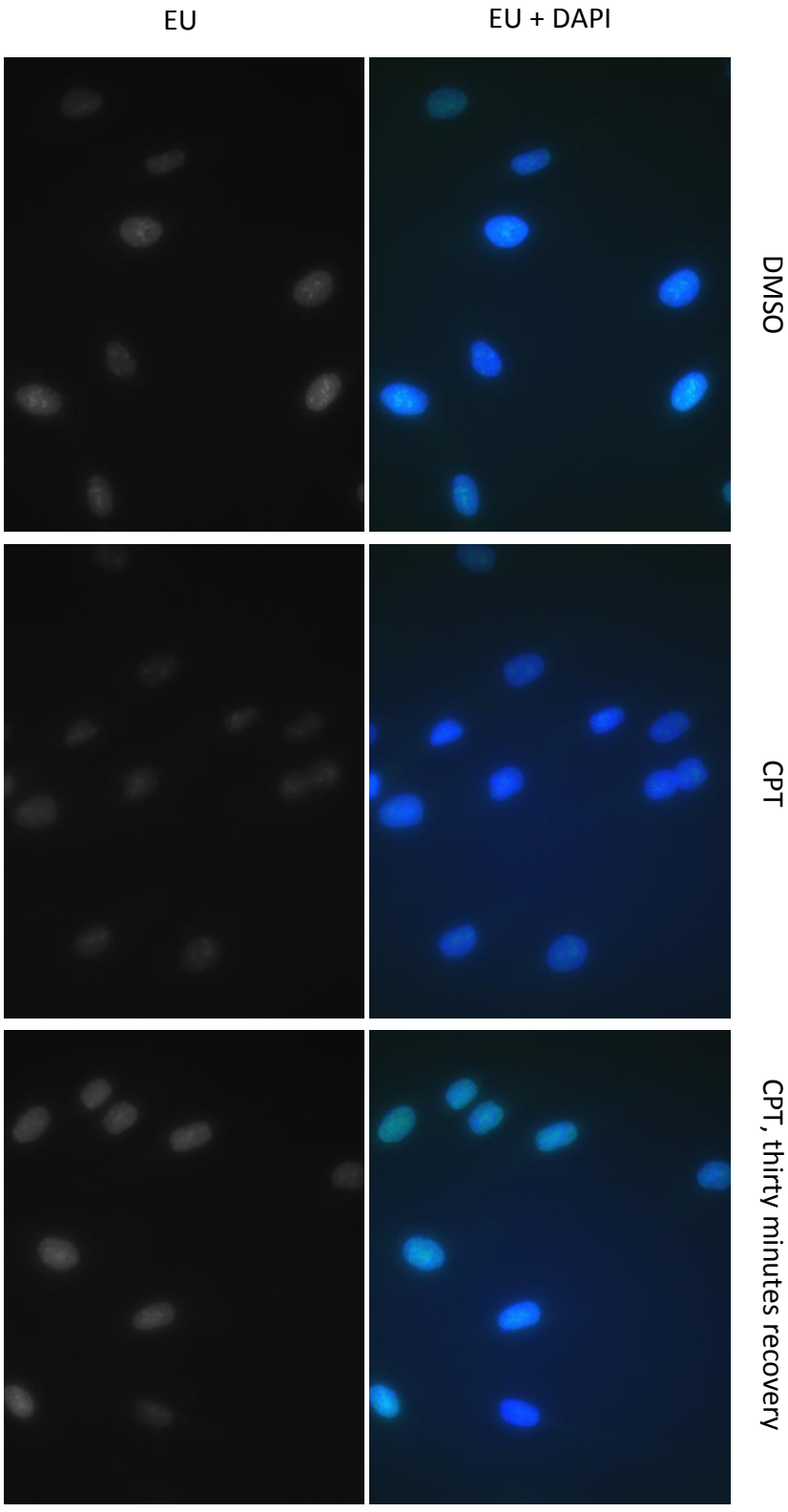
WT fibroblasts



WT fibroblasts



Patient fibroblasts



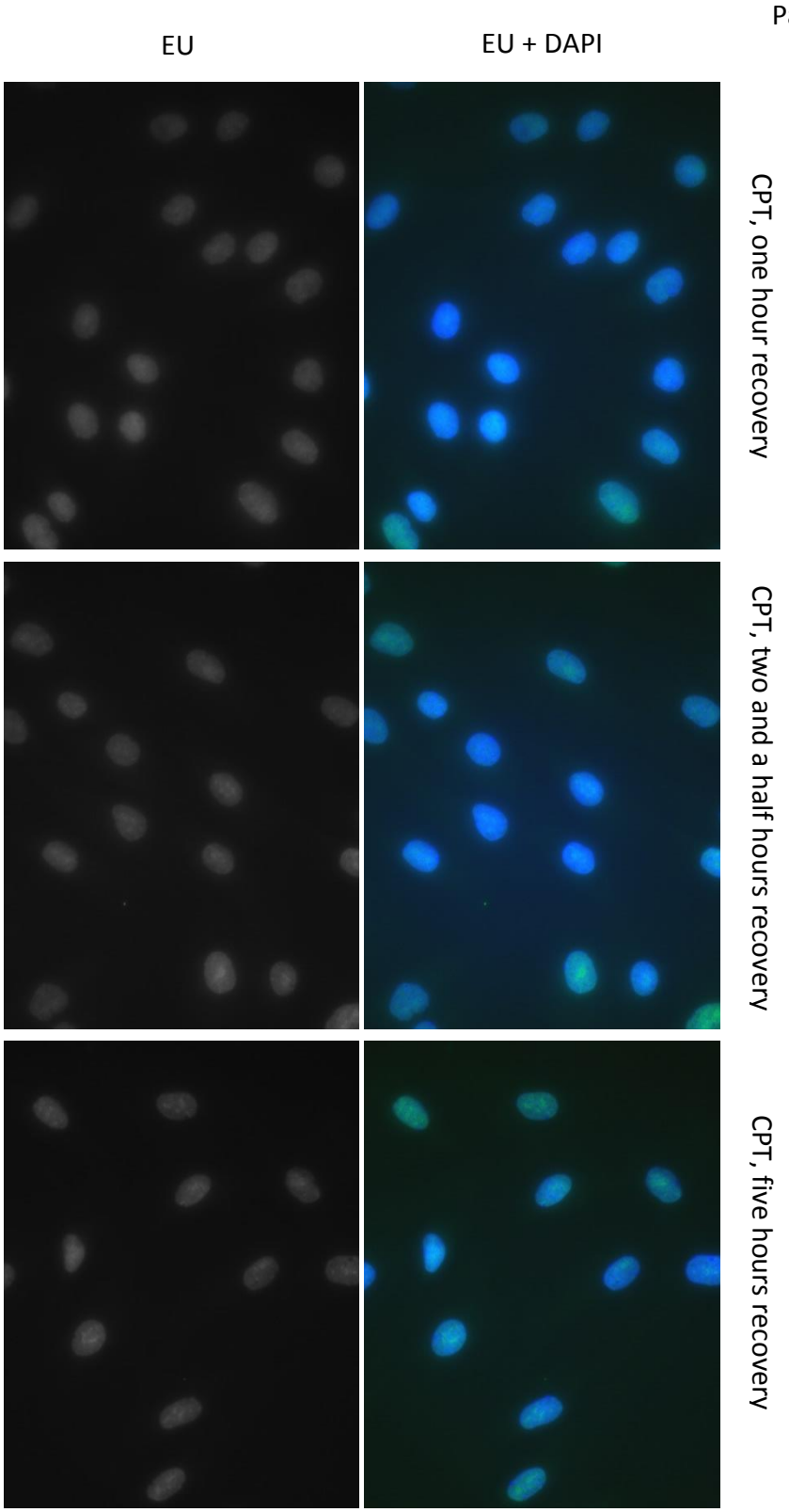


Figure 3.15d | **Measuring recovery of global transcription after CPT treatment in FUS patient fibroblasts and controls.** Representative images of cells used for figure 3.15c (40x magnification).

No transient defect in restarting of transcription could be detected in the patient fibroblasts (Fig 3.15c) indicating that the fibroblasts had no defect in this process that could be observed with this technique.

3.6 rRNA maturation in FUS-ALS patient cells

As no defect in transcription could be found another nucleolar function was examined. There are many cellular processes in which the nucleolus is involved, such as stress signalling (Rubbi & Milner 2003), but as FUS is a nucleic acid binding protein a nucleolar process directly involving nucleic acids was focused on next - rRNA maturation. Furthermore a defect in this process has been previously observed in ALS patient cells albeit in cells harbouring c9orf72 hexanucleotide expansions rather than mutations in FUS (Haeusler et al. 2014).

Ribosomes in humans contain four rRNA species - the 18S, 28S, 5.8S and 5S transcripts. Of these all but the 5S transcript are encoded in tandem repeats around which nucleoli form, are transcribed by RNA polymerase I (RNAP I) and are initially transcribed on the 47S pre-rRNA transcript. The 47S transcript undergoes a series of complex, branching processing steps in order to produce and export the 18S, 28S and 5.8S rRNAs. Three probes were used in this experiment - the 18S and 28S probes recognise sequences within the mature rRNAs, which are also present in the 47S transcript; whereas the 5'-ETS-1b (external transcribed spacer) probe recognises a sequence that is removed after cleavage of the 01/A' site - typically the first step in rRNA maturation (Preti et al. 2013; Carron et al. 2011). Measurement of the ratio of signal intensity from 5'-ETS-1b versus the 18S and/or 28S ribosomes therefore provides a relatively simple measure of a cell line's capacity for ribosomal maturation - with a higher ratio implying a defect in maturation. This can be analysed by northern blotting.

Patient and control fibroblasts were harvested for RNA extraction using the Qiagen RNeasy Mini Kit was used for RNA extraction. The quality of this RNA was assessed on a NanoDrop machine and by visual inspection after being run on an agarose gel (Fig 3.16).

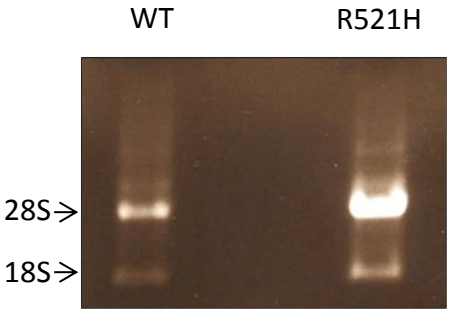


Figure 3.16 | **Quality control of RNA extracted from patient and control fibroblasts.** RNA from patient fibroblasts (right) and control fibroblasts (left). Although the total amount of RNA recovered varied substantially between the lines both showed an intensity of the 28S (upper) band of approximately twice that of the 18S (lower) band. Both RNA species also demonstrated an A260/A280 ratio (absorbance at 260 nm over absorbance at 280) of 2.08 - very close to the theoretical ratio of pure RNA at 2.10.

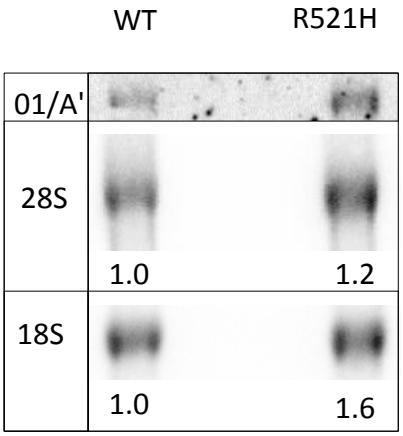


Figure 3.17 | **Northern blots measuring 5'ETS1-b (01/A'), 18s and 28S RNA species in patient and control fibroblasts.** The numbers underneath the bands depict the ratio of 5'-ETS1-b signal (adjacent to the 01/A' site) relative to the RNA species shown in the band, normalised to WT. Patient fibroblasts demonstrated a modest increase in 5'-ETS1-b signal relative to 28S or 18S signal, suggesting a small defect in rRNA maturation.

RNA was run on a formaldehyde gel (the same amount of RNA from each cell line), as described in materials and methods, and the gel transferred to an Amersham Hybond XL membrane by capillary action and crosslinked to it by UV irradiation. Membranes were incubated with oligonucleotide probes overnight and a phosphor screen used to visualise the radioactive signals - with an hour's exposure for the 28S and 18S probes and exposure for seventy two hours with the 5'-ETS-1b probe prior to scanning. The same membranes were reprobed with multiple oligonucleotides, with the 5'-ETS-1b probe exposed first, followed by the 28S probe and then the 18S probe. The scanned images then had the intensity of their bands measured in ImageJ and ratios of intensity were calculated in Microsoft Excel.

The data in Fig 3.17 demonstrates a modestly increased ratio of 5'-ETS-1b to 28S and to 18S in patient fibroblast lines relative to sibling lines. This is consistent with patient cells having a mild inherent defect in rRNA maturation. However due to the difference being relatively

slight (with the ratio being only 1.2-1.6x larger in the patient cells than in the wild type) the experiment should be repeated in future to ensure statistical significance and verify this result.

3.7 Conclusion

On the basis of these data it can be observed that patient fibroblasts clearly demonstrate two phenotypes: sensitivity to CPT treatment and constitutively increased fragmentation of the nucleoli - suggestive of a defect in one or more nucleolar processes. Recovery of transcription (the bulk of which is nucleolar) after DNA damage appeared unimpaired in patient cells relative to wild type controls but a possible defect in rRNA maturation was observed - although further studies may be required to verify this finding.

The combination of fragmented nucleoli and a defect in rRNA maturation has been previously observed in Hela cells treated with siRNA against the ribosomal protein RPS19 (Choesmel et al. 2007) so it is a possibility that these two phenotypes are linked. However neither a clear explanation for the CPT sensitivity of the cells nor a clear link between the nucleolar defects and ALS pathogenesis can be established from this data alone.

The involvement of DNA damage repair processes was initially investigated but the data does not suggest any clear defect in these processes in these FUS-ALS patient cells. Although Hela cells expressing a similar FUS mutant to that in the patient cells demonstrated defective recruitment of FUS to sites of laser damage there were no defects in DNA damage repair found in the patient cells using the γH2AX assay and clonogenic survival assays demonstrated a survival defect in response to CPT but not to IR. This suggests that it is not DNA damage as such that confers CPT sensitivity to the patient cells but another feature of the chemical - perhaps its activity as a transcriptional inhibitor.

Together these data show clear nucleolar defects in these FUS-ALS patient cells but do not demonstrate any clear evidence of a defect in DNA damage repair in the cells.

4. Relocalisation of FUS in response to transcriptional stress

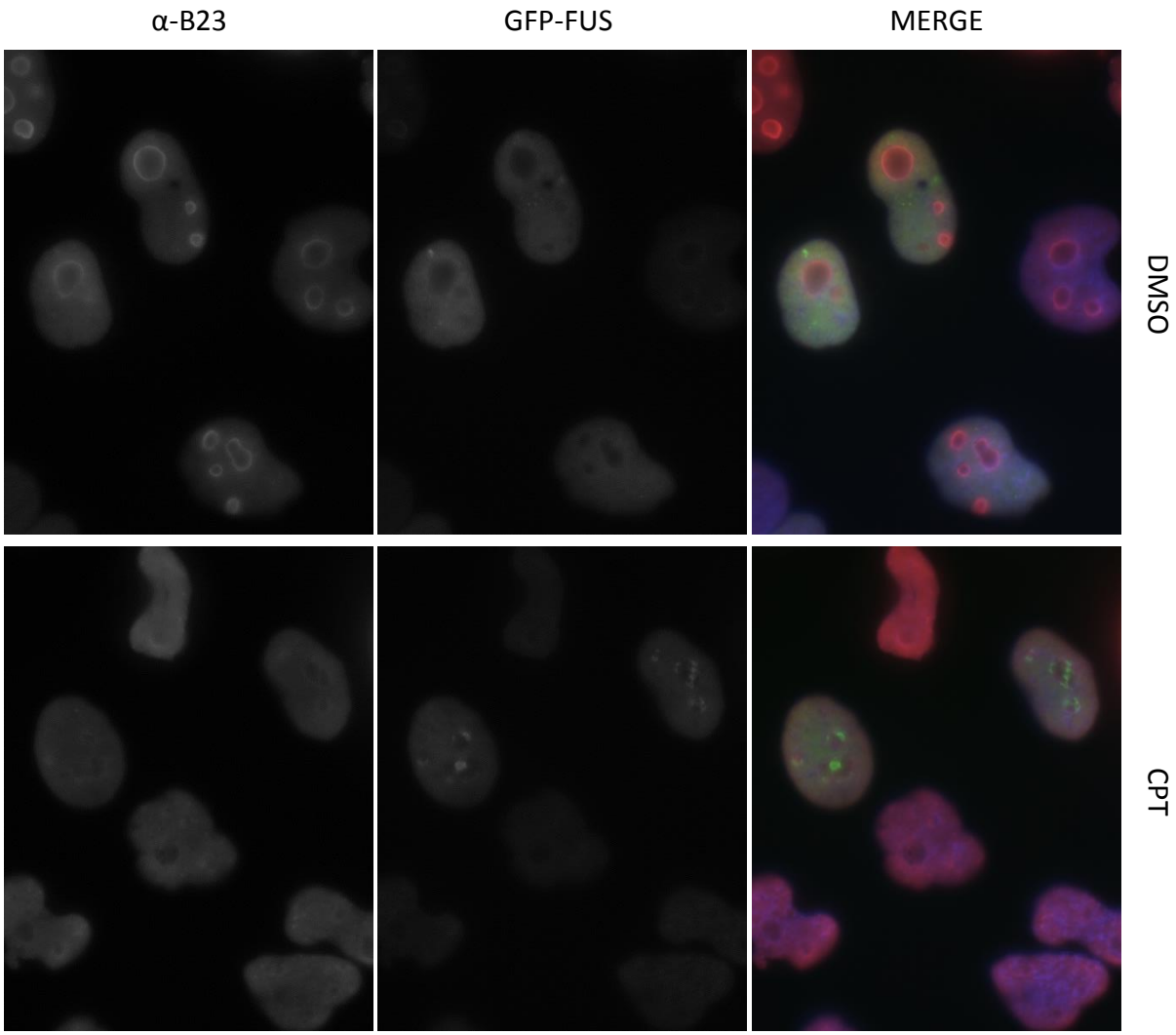
FUS had been seen to localise to sites of laser microirradiation (Figs 3.4 and 3.5), suggesting a recruitment to sites of DNA damage. This data in itself does not necessarily demonstrate a physiological relevance for FUS moving to these sites however particularly as, as previously discussed, laser microirradiation is also estimated by some to produce extremely high levels of localised DNA damage which may result in artefactual data.

Moreover there was no detectable defect in DNA damage repair in FUS-ALS patient cells by the γH2AX assay (Figs 3.6-3.9) and this casts doubt on the idea of FUS having physiological relevance in the process of DNA damage repair. As some proteins involved in DNA damage repair have been observed to form foci at sites of DNA damage in response to IR or chemical treatment, including XRCC1 (El-Khamisy et al. 2003) and the Ku70/80 complex (Britton et al. 2013), subsequent experiments were designed to see if FUS could also form foci.

4.1 FUS relocalisation in response to CPT in an overexpression system

As an initial experiment into possible FUS relocalisation in response to chemically-induced DNA damage doxycycline-induced LAP-FUS Helas were CPT treated and subjected to B23 IF.

WT LAP-FUS Helas



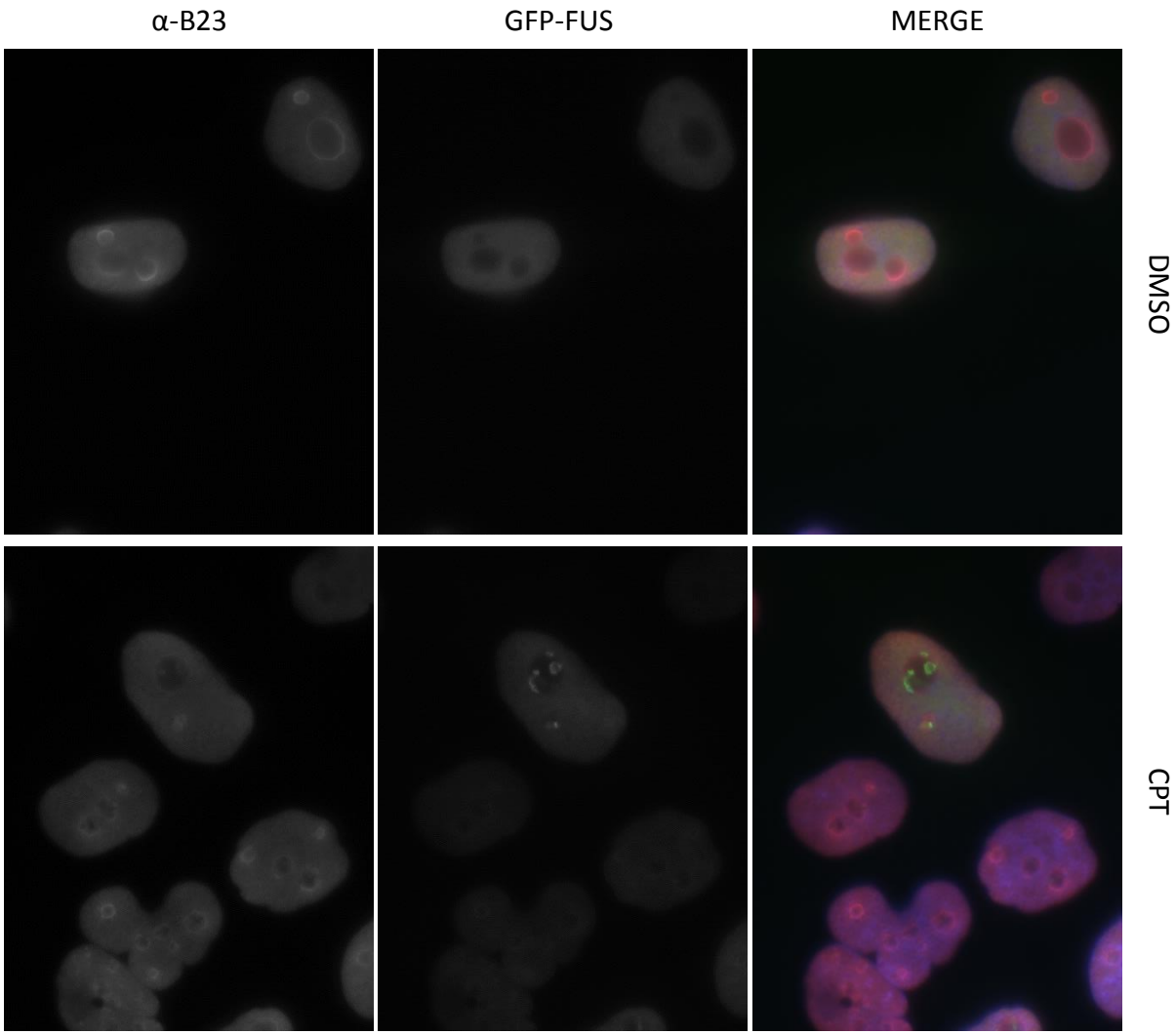


Figure 4.1a | **FUS focus formation in response to CPT in Hela LAP-FUS cells.** Clear nucleolar foci of FUS within B23 signal are observed after CPT treatment.

Upon CPT treatment a clear relocalisation of FUS was observed (Fig 4.1a) - hereafter referred to as foci. Instead of the pan-nuclear foci observed with XRCC1 or Ku80 it appears as though FUS is recruited to the nucleolus (as marked by B23). This is suggestive of FUS being recruited not to sites of DNA damage but to the nucleolus for an unknown purpose. There is one possible previous report of FUS foci in the literature (Zinszner, Immanuel, et al. 1997) in which nucleolar aggregates of FUS were observed after chemical treatment and detergent extraction, with the nucleolar localisation of the protein being dependent on the aggregation-prone N terminus of FUS. However the chemical treatment used induced inhibition of RNA polymerase II (RNAP II) rather than DNA damage. Although CPT was not utilised in this paper it is worth noting that CPT is both a DNA damaging agent and a

transcriptional inhibitor (Bendixen et al. 1990), so it is possible that the foci observed in Fig 4.1 are the same as described in the Zinszner paper.

The experiment pictured in Fig 4.1 was repeated, in the wild-type FUS Hela line only, with IF for fibrillarin performed instead of for B23. This was to provide additional verification that FUS was localising to the nucleolus due to the unusually high nucleoplasmic B23 signal consistently observed in these cell lines.

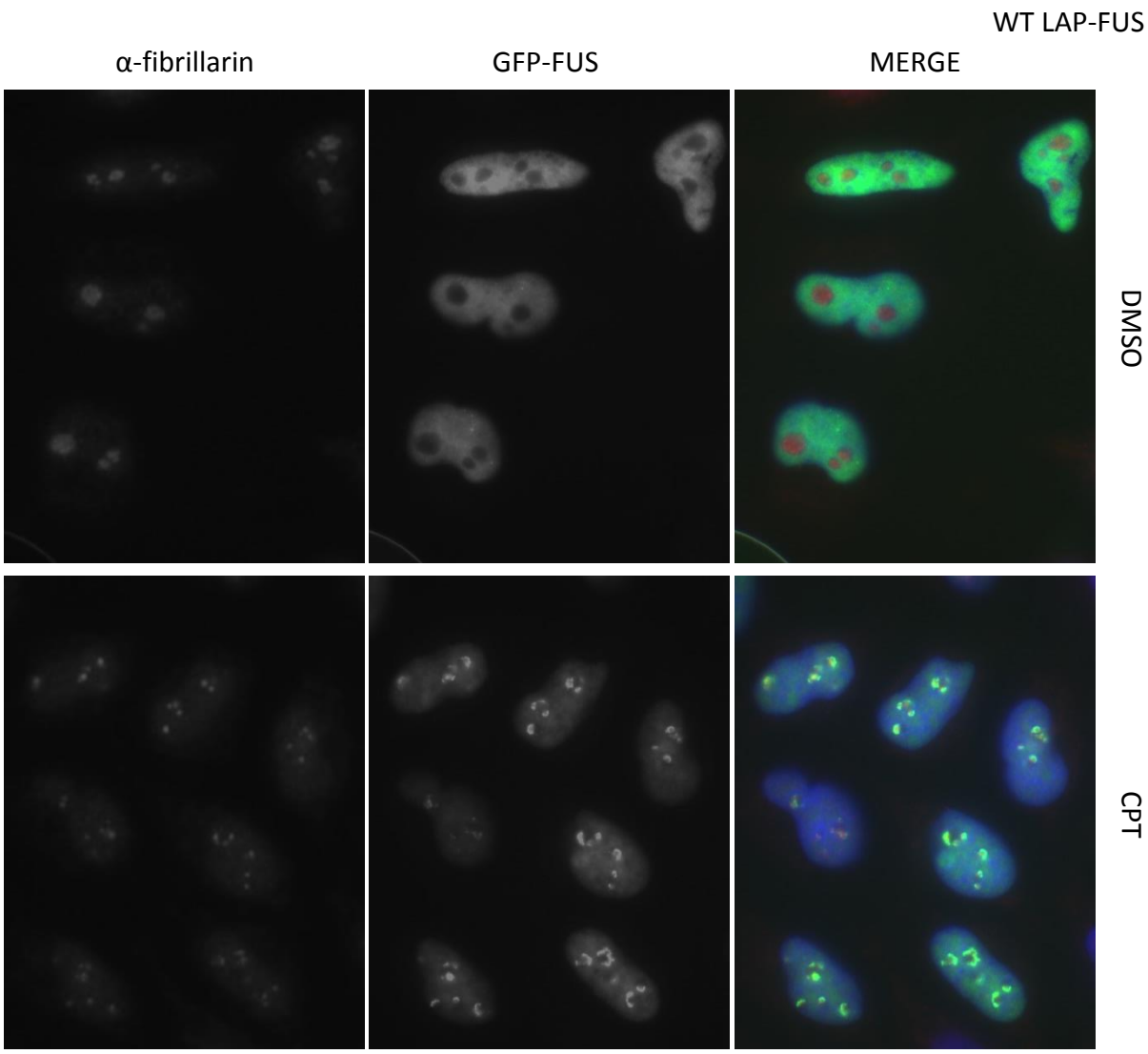
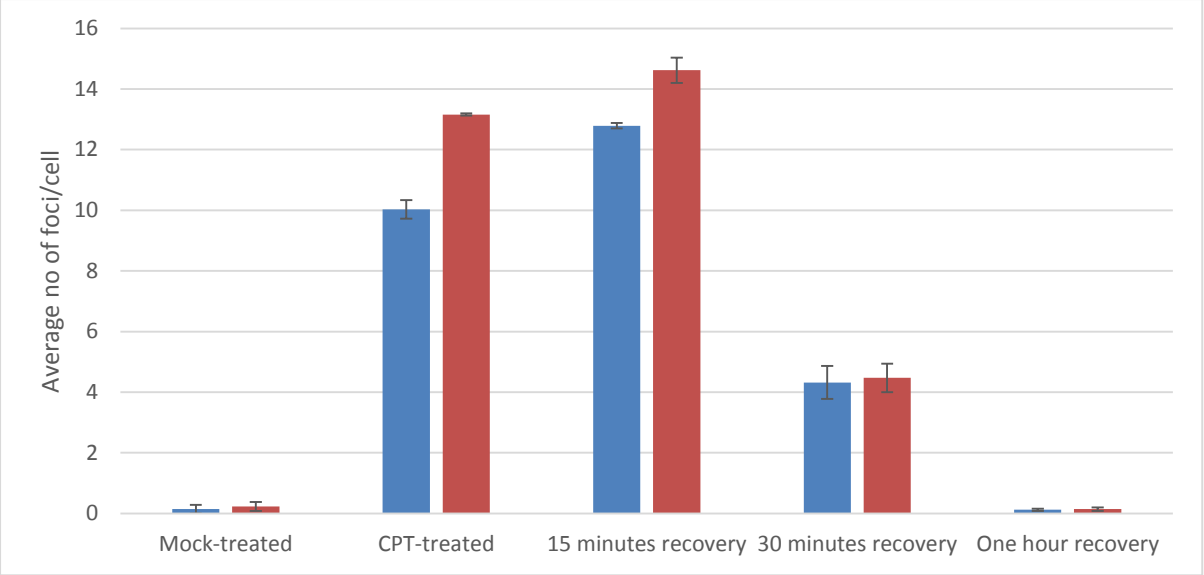


Figure 4.1b | **FUS focus formation in response to CPT in Hela LAP-FUS cells.** Clear nucleolar foci of FUS proximal to fibrillarin signal are observed after CPT treatment. Images taken using SimplePCI system.

This additional experiment (Fig 4.1b) also demonstrated FUS recruitment to the nucleolus although it was ambiguous as to whether the foci co-localised with or were proximal to fibrillarin. A recovery experiment was set up to see if these CPT-induced foci were reversible.



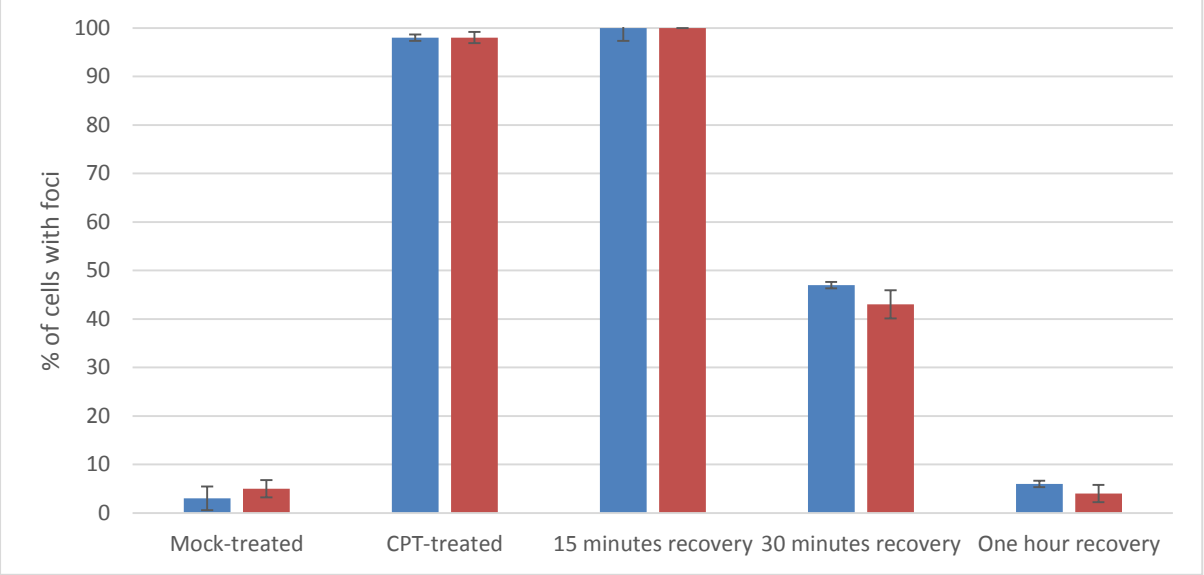
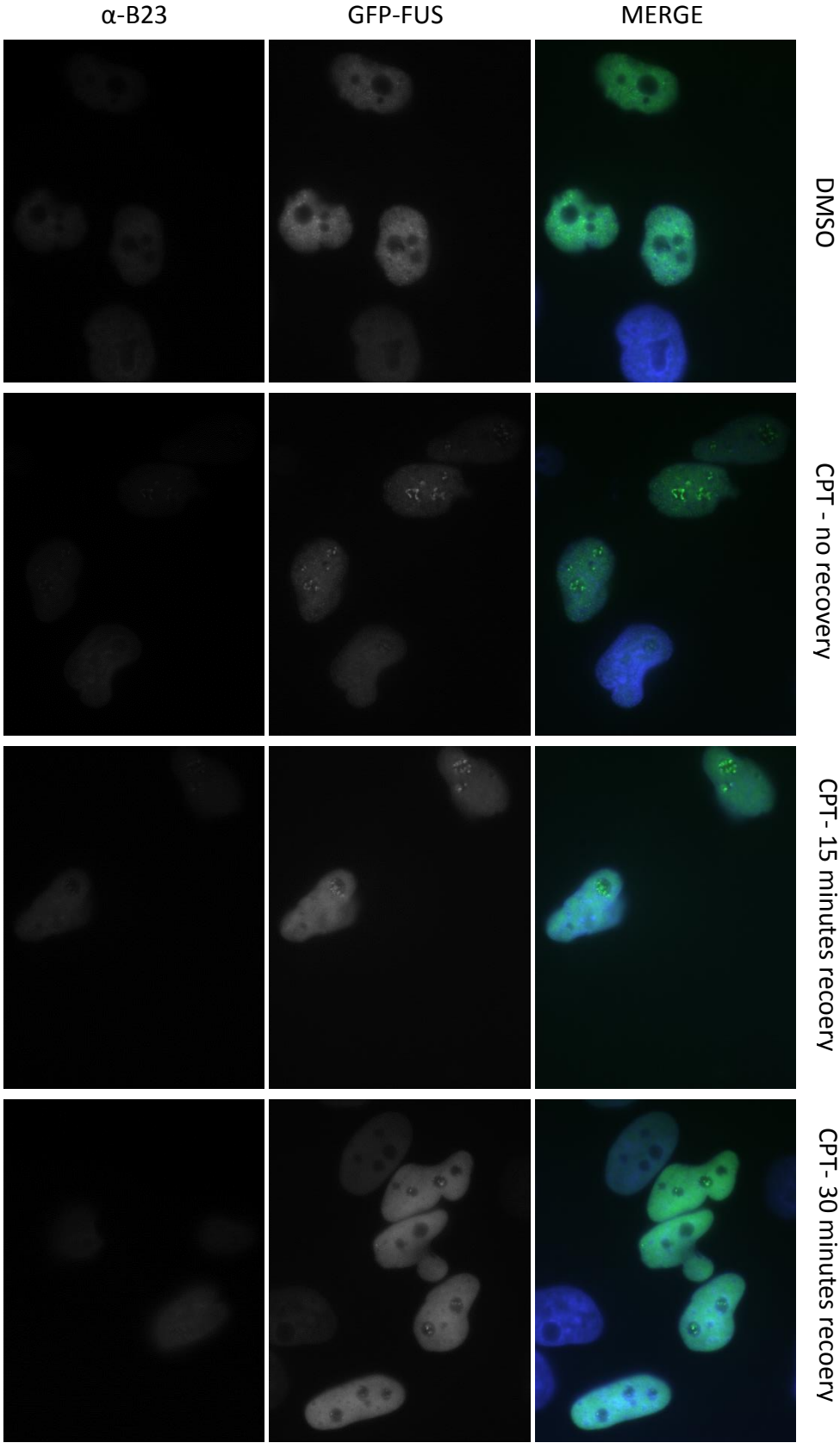
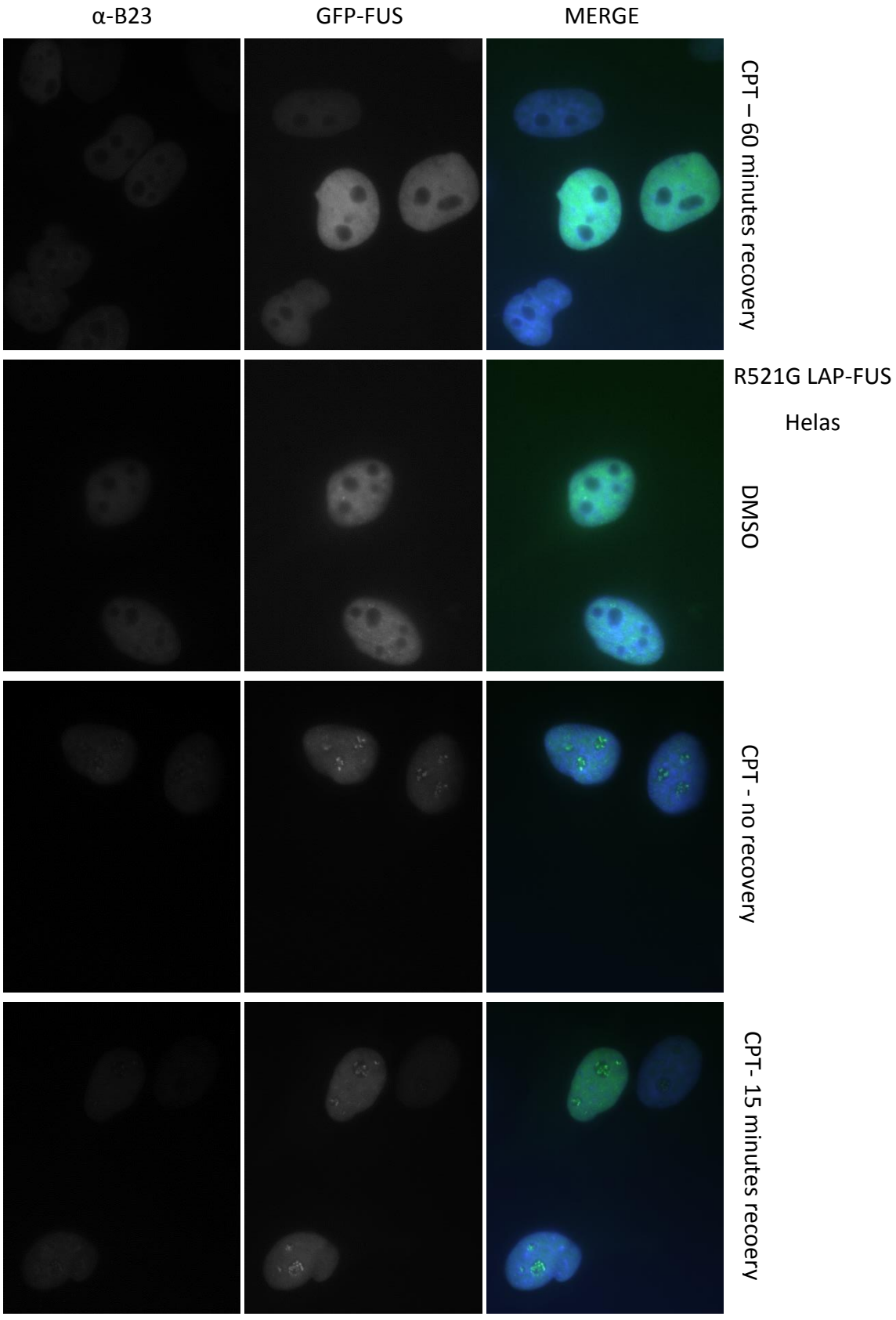


Figure 4.2a | **FUS foci counting in Hela LAP-FUS cells after varying recovery times.** Foci are formed after CPT treatment but are readily reversible and are resolved between half an hour and one hour after chemical treatment is withdrawn. Helas expressing the R521G mutant of FUS demonstrate more foci per cell than controls (by two-tailed ANOVA at 95% confidence intervals). The proportion of cells with foci does not vary significantly between these cell lines. n=3.

Cells were damaged with 4µM CPT for 45 minutes, washed three times in PBS and allowed to recover for set timepoints before being fixed and processed by IF.

WT LAP-FUS Helas




α-B23
GFP-FUS
MERGE
CPT – 60 minutes recovery
R521G LAP-FUS
Helas
DMSO
CPT - no recovery
CPT- 15 minutes recoery

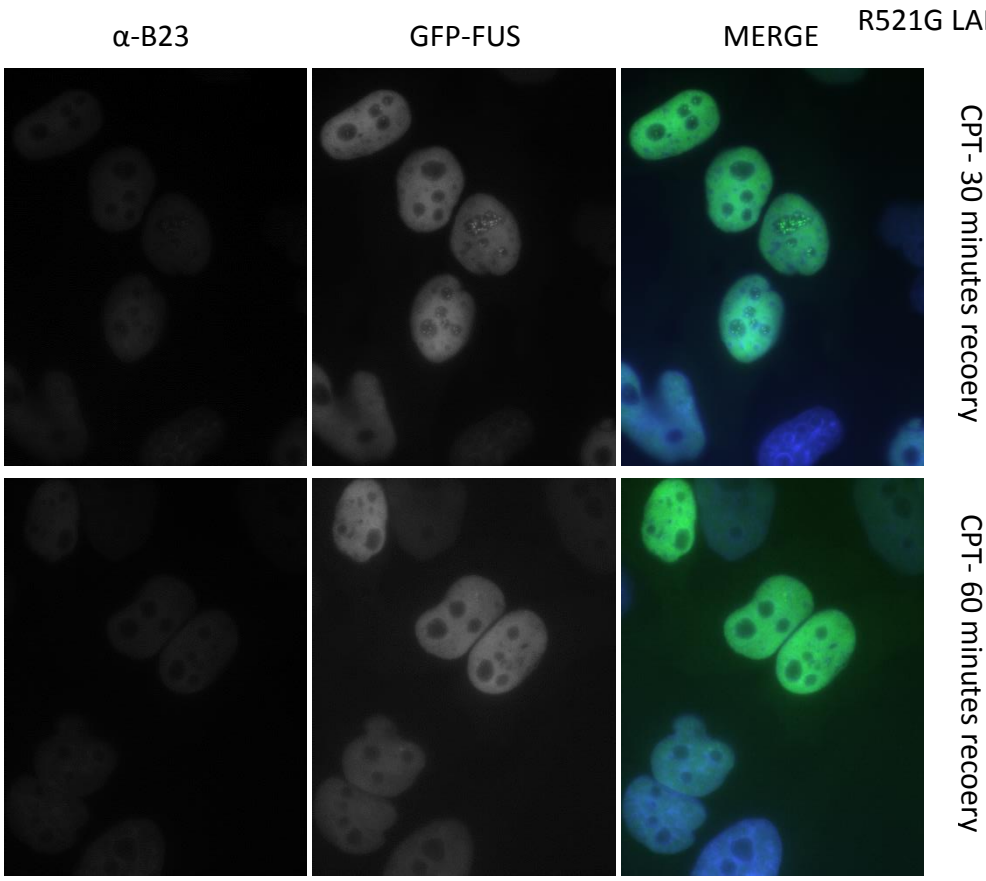


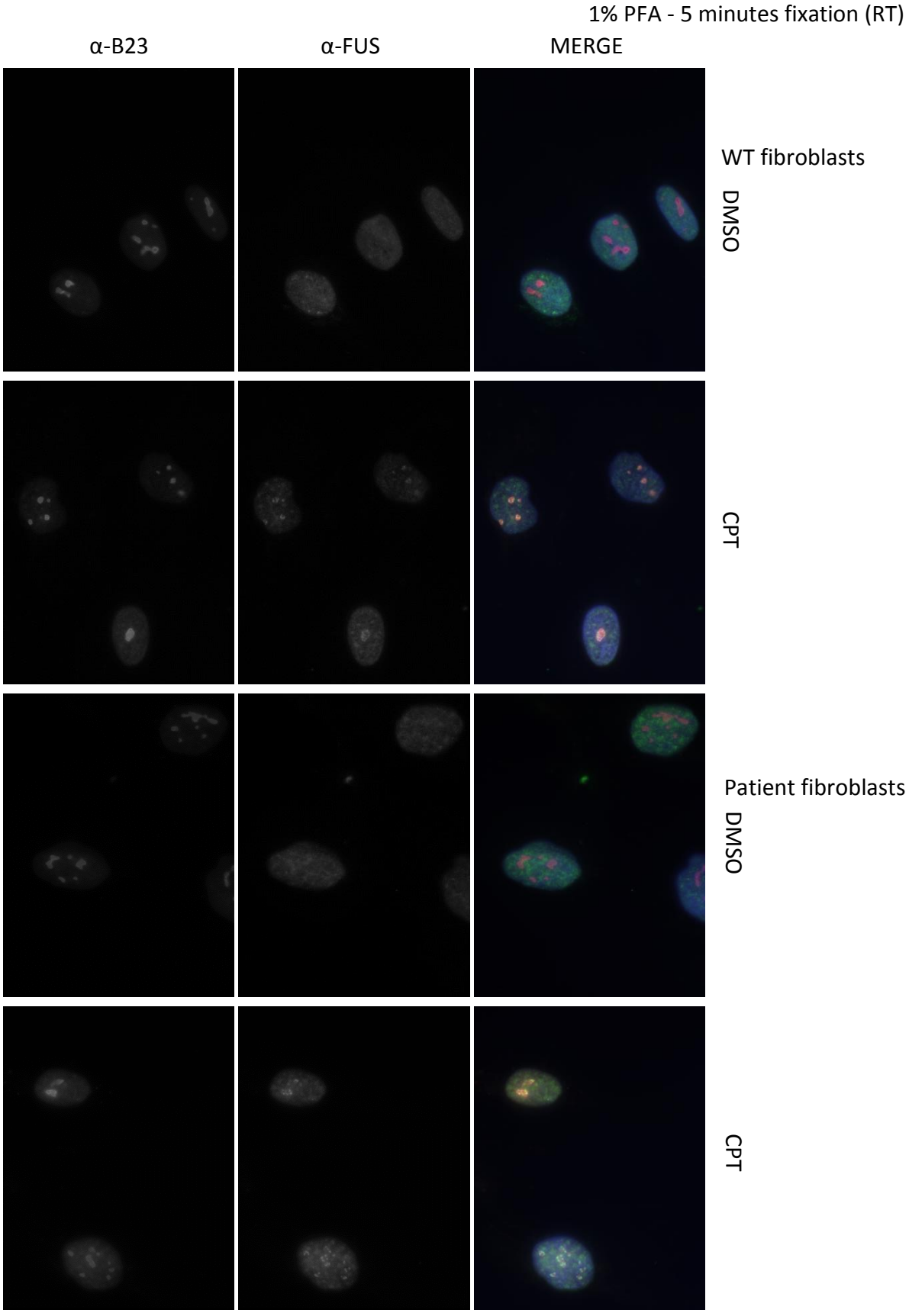
Figure 4.2b | **FUS foci counting in Hela LAP-FUS cells after varying recovery times.** Representative images of cells counted for figure 4.1a. Contrast set to maximum per image, rather than maximum per image set.

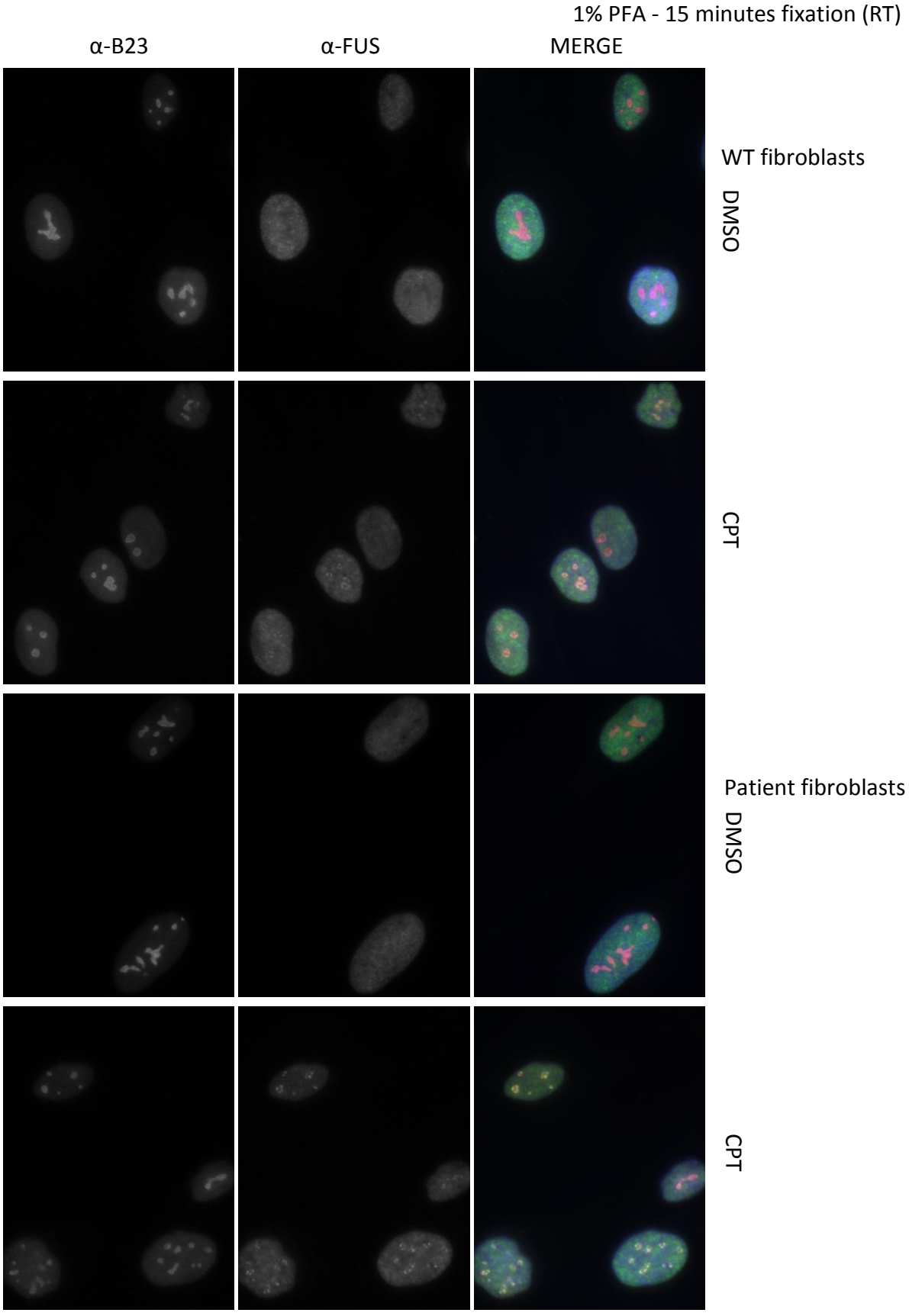
The key observations were that FUS foci are reversible on removal of CPT and that R521G mutant cells had on average significantly more foci per cell though the proportion of cells with foci did not differ from wild type (Fig 4.2). It is possible that the increased number of FUS foci is related to the increased nucleolar fragmentation (Fig 3.10) in these cells though the magnitude of the effect is greater.

4.2 Endogenous FUS relocalisation in response to CPT

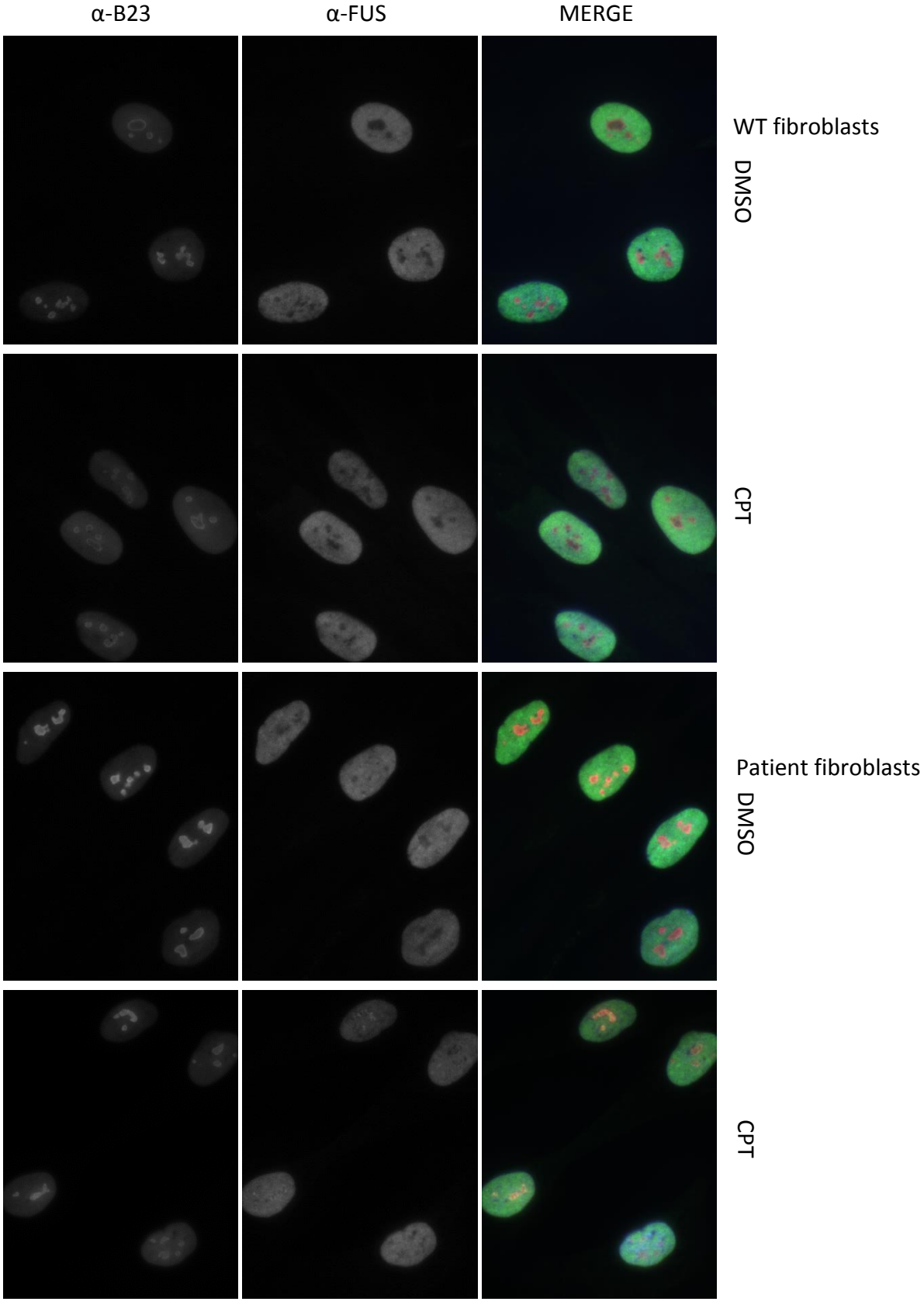
The FUS foci observed in Figs 4.1 and 4.2 were foci of GFP-FUS rather than endogenous FUS. This left open the possibility of the foci being an artefact of the GFP tag of FUS or of overexpression (although GFP-FUS expression was modest (Fig 3.3)). IF of CPT-treated cells initially did not show any foci of FUS (data not shown) however this does not conclusively indicate that endogenous FUS foci cannot form as foci of some proteins cannot be observed under standard fixation conditions - for instance Ku80 foci are only visible after detergent extraction (Britton et al. 2013).

As such protocols were adjusted to see if endogenous foci could be observed. The two variables that were adjusted were paraformaldehyde (PFA) concentration in the fixation process and fixation length - all previous fixations had been performed in 4% PFA for 15 minutes. The experiment designed was to treat patient and control fibroblasts with CPT but to vary the conditions of fixation between every combination of 1% or 4% PFA, and 5 or 15 minute fixation in order to attempt to visualise endogenous FUS foci using IF.


1% PFA - 5 minutes fixation (RT)
α-B23
α-FUS
MERGE
WT fibroblasts
DMSO
CPT
Patient fibroblasts
DMSO
CPT


1% PFA - 15 minutes fixation (RT)
α-B23
α-FUS
MERGE
WT fibroblasts
DMSO
CPT
Patient fibroblasts
DMSO
CPT

4% PFA - 5 minutes fixation (RT)



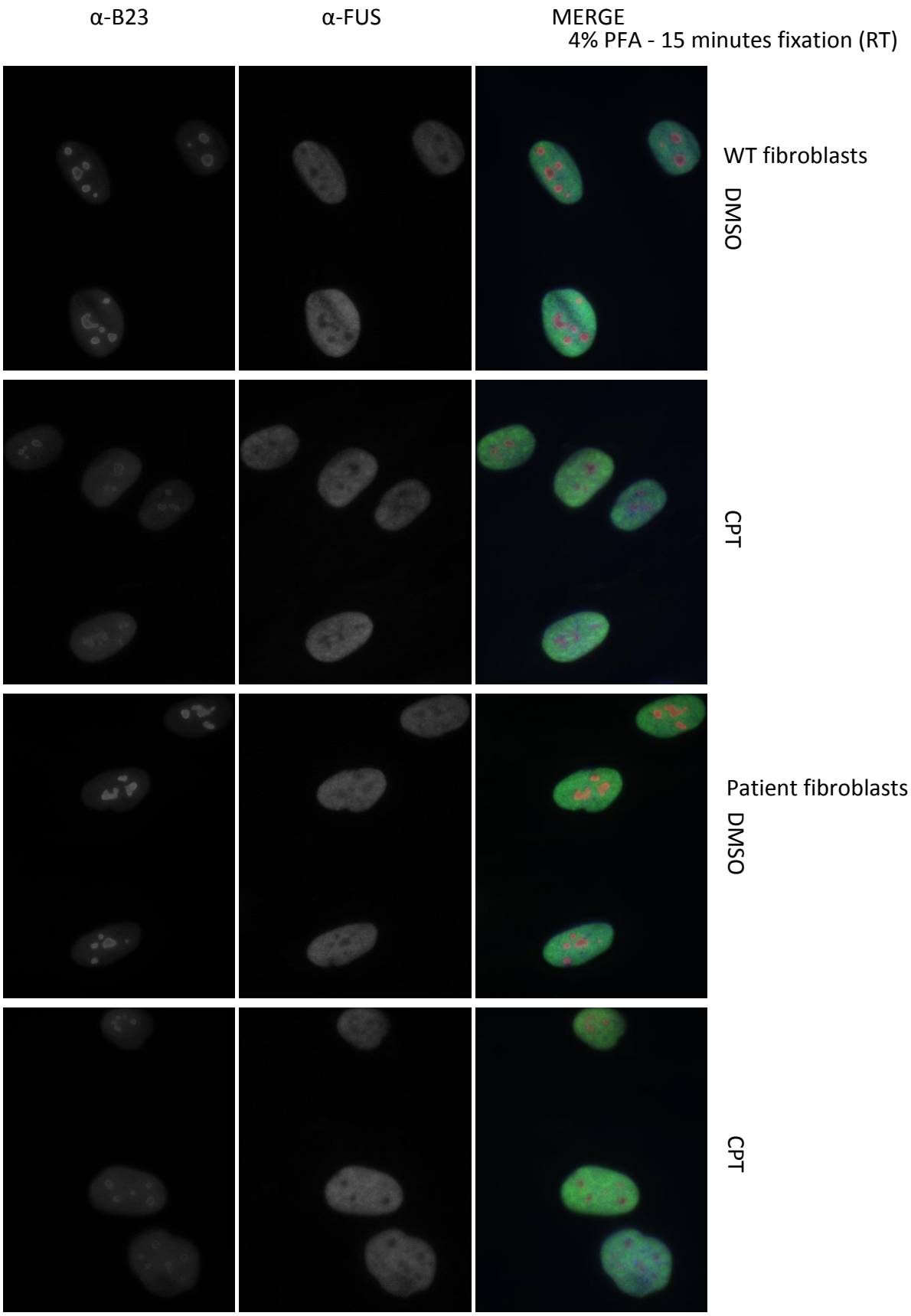


Figure 4.3 | **endogenous FUS foci in CPT-treated patient fibroblasts (R521H) or a sibling control fibroblast line (WT).** Endogenous foci are sensitive to fixation conditions and do not appear in most cells fixed in 4% PFA.

Adjusting these two variables did indeed allow visualisation of endogenous foci by IF (Fig 4.3), as the foci appeared to be sensitive to fixation conditions. No FUS foci were visible in the majority of CPT-treated cells treated with 4% PFA, though occasionally foci would be visible in individual cells after 5 minutes fixation. In contrast fixation with 1% PFA showed the vast majority of CPT-treated cells forming FUS foci, with fixation for 5 minutes being most reliable at showing FUS foci. Therefore this was adopted as the standard fixation protocol for experiments involving using IF to locate endogenous FUS foci (the antibody used, from Novus, was also used for all subsequent FUS IF experiments unless otherwise stated). 4% PFA fixation for 15 minutes was used for all other experiments. A downside of these conditions were that fibrillarin could not be visualised by IF using this milder fixation protocol (data not shown) and no conditions that allowed for visualisation of endogenous FUS foci and of fibrillarin were found.

Punctate patterns of FUS in the nucleoplasm could also be observed in some samples - these were present intermittently and their presence did not seem to be related to CPT treatment. Harsher fixation conditions appeared to slightly reduce this punctate background, so in later experiments fixation time was adjusted to ten minutes in 1% PFA.

As the Hela LAP-FUS R521G cell line had demonstrated increased foci per cell relative to wild types, it was of interest if the patient line demonstrated a similar effect relative to its control. It was also investigated if endogenous foci were reversible, as GFP-FUS foci were. Therefore foci of endogenous FUS were counted after CPT treatment and at set recovery timepoints, using the new fixation protocol.

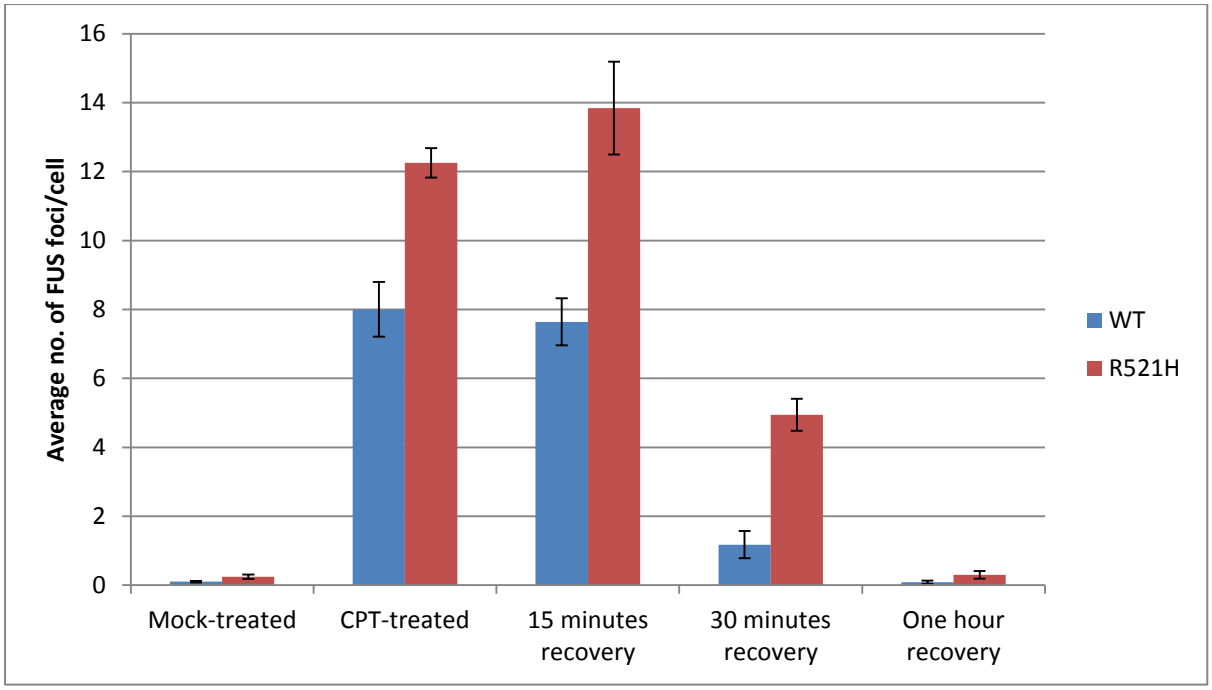


Figure 4.4 | **foci counting with recovery in patient and control fibroblasts.** Endogenous FUS foci are reversible and are resolved within an hour. Patient cells demonstrate significantly more foci per cell (by two-tailed ANOVA at 95% confidence intervals). n=3.

The results of this experiment showed that endogenous FUS focus formation was reversible and that patient fibroblasts produced more foci per cell than wild-type controls, with slower resolution of foci after removal of CPT (Fig 4.4). Mutant LAP-FUS Helas also produced more foci per cell, though to a lesser extent (Fig 4.2). If the increase in foci per cell was a downstream consequence of nucleolar fragmentation then this would make sense as the patient fibroblasts had more nucleolar fragmentation relative to its control than the R521G Hela had relative to the WT Hela.

This recovery experiment was later repeated, controlling for the cell cycle by also co-staining for CENP-F during IF. As described previously this protein is highly expressed in S phase and G2/M phase but not in G1 or G0.

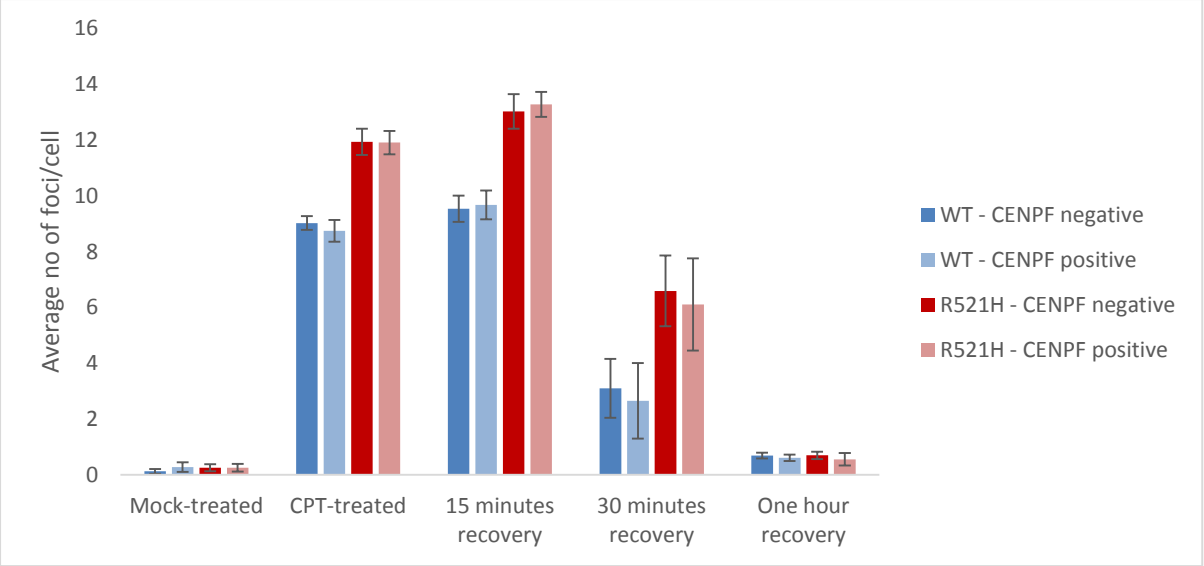
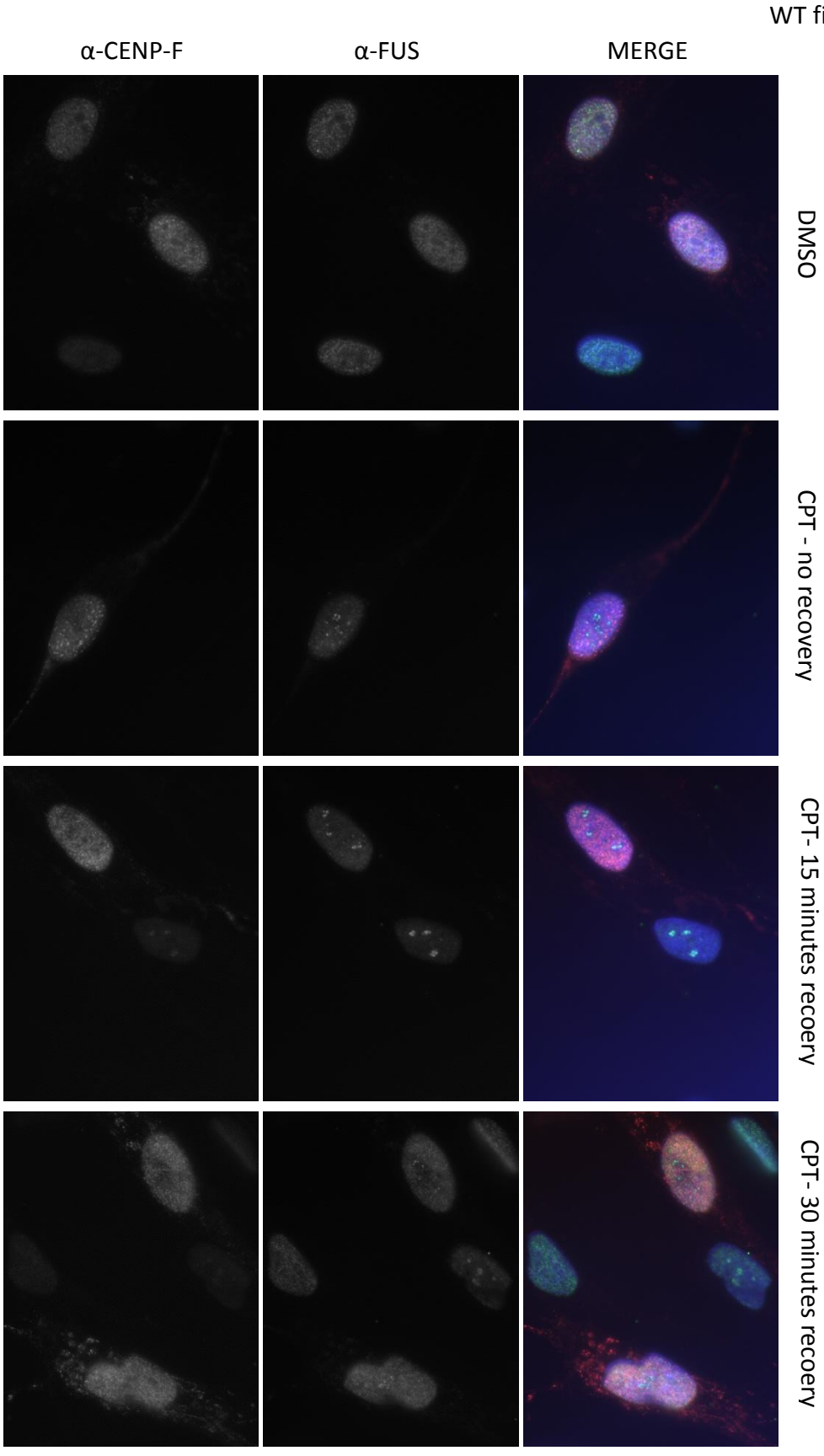
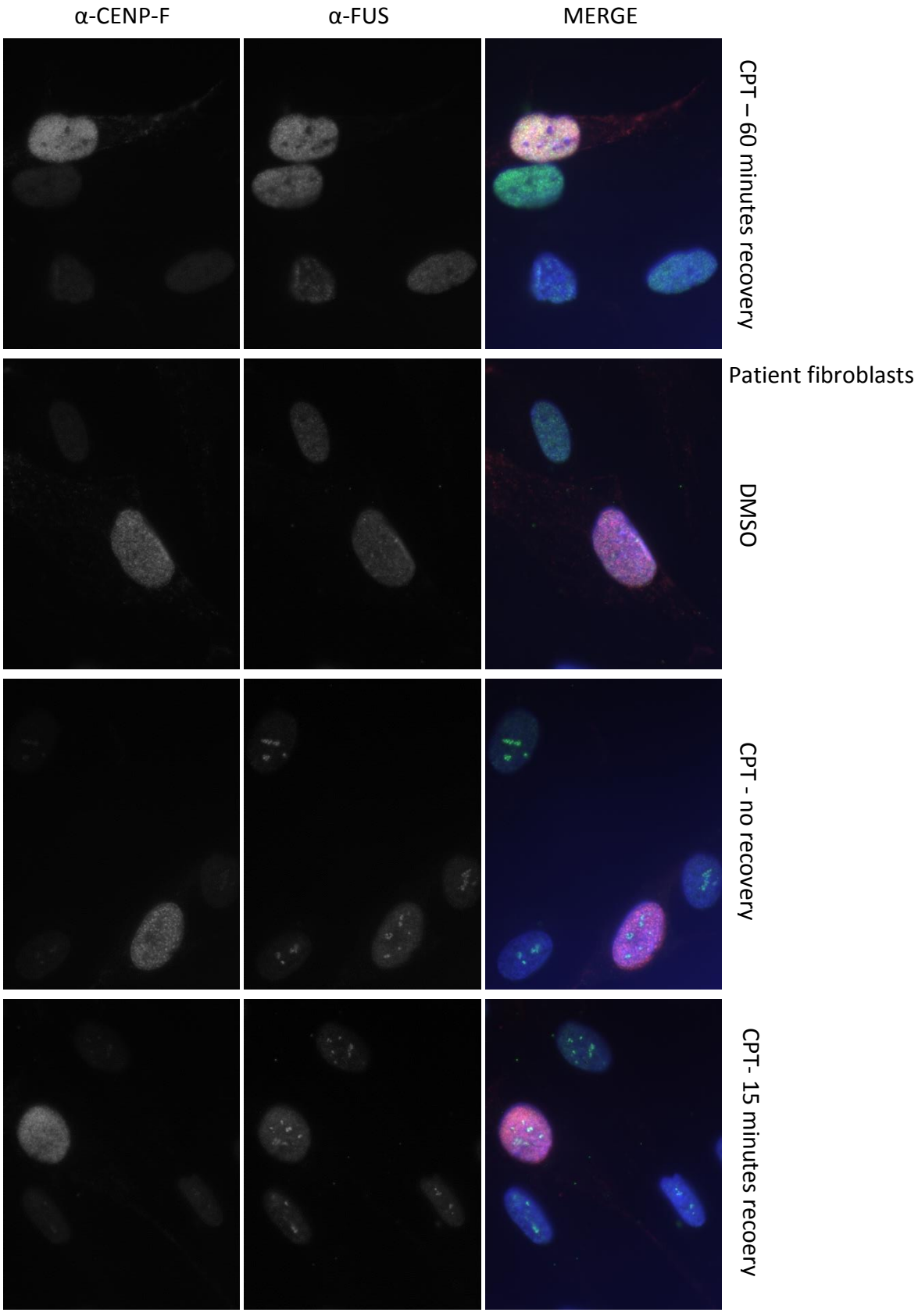


Figure 4.5a | **foci counting with recovery in patient and control fibroblasts.** Cells in earlier phases of the cell cycle show no significant difference in FUS foci formation relative to those in later stages of the cell cycle. n=3.


WT fibroblasts
α-CENP-F
α-FUS
MERGE
DMSO
CPT - no recovery
CPT- 15 minutes recoery
CPT- 30 minutes recoery


α-CENP-F
α-FUS
MERGE
CPT – 60 minutes recovery
Patient fibroblasts
DMSO
CPT - no recovery
CPT- 15 minutes recoery

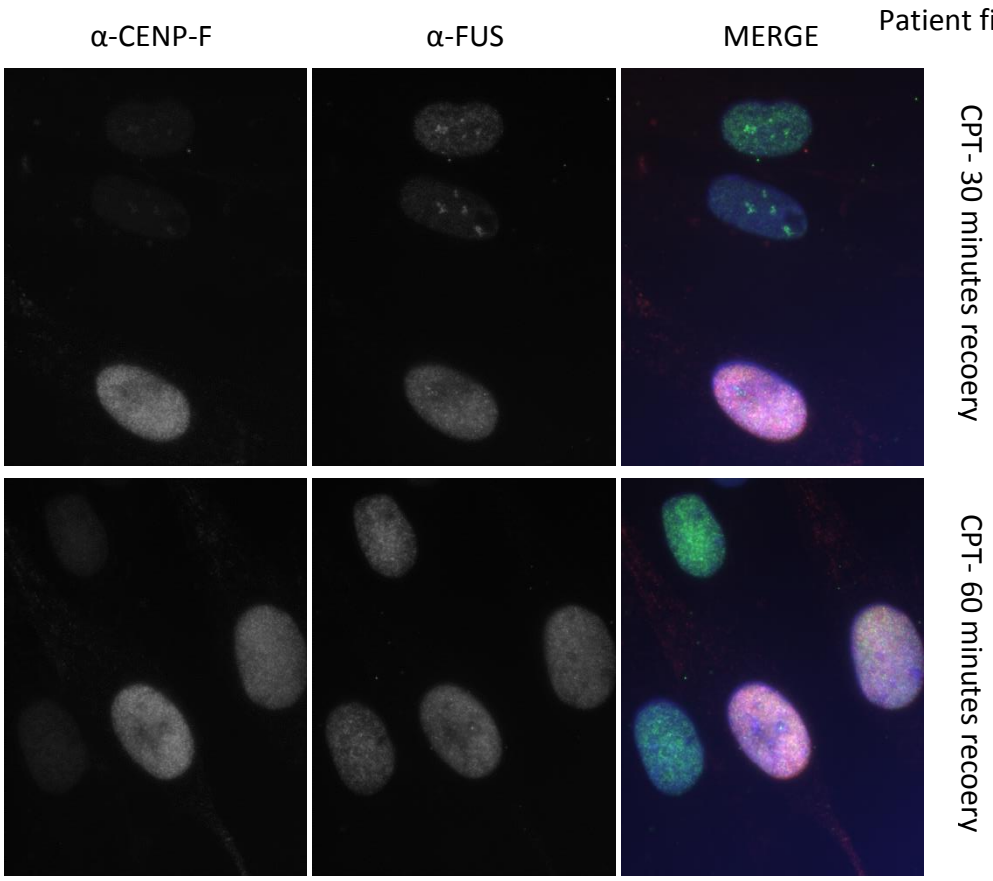


Figure 4.5b | **foci counting with recovery in patient and control fibroblasts.** Representative images of cells counted for figure 4.5a. Contrast set to maximum per image, rather than maximum per image set.

No difference was observed in FUS foci count between CENP-F positive and CENP-F negative cells (Fig 4.5). This data minimises any effect of the replication process on FUS foci count. This also implies that FUS foci do not represent sites of DNA damage. If this were the case then those cells which had undergone any replication during CPT incubation (a substantial proportion of CENP-F positive cells) would have been expected to have vastly more FUS foci than CENP-F negative cell as they would be subjected to both replication and transcription-associated CPT-induced DSBs. It follows that the role of CPT as a transcriptional inhibitor is likely to be more important for the relocalisation of FUS than its role as a DNA damaging agent.

These endogenous foci had only been observed in the fibroblast lines so in order to ensure that these were not just a feature of these fibroblasts other cell lines were treated with CPT, fixed and subjected to FUS IF.

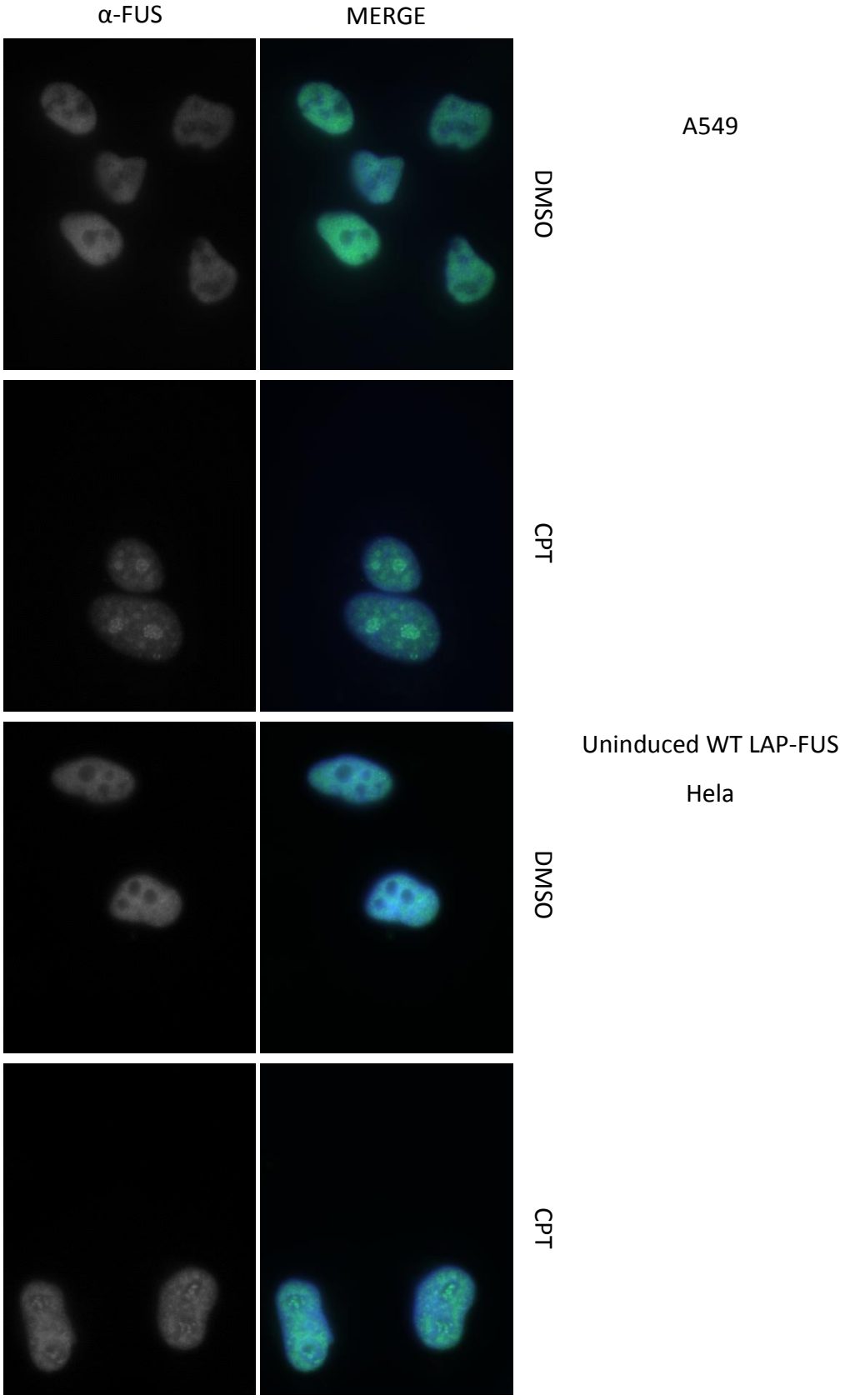


Figure 4.6 | **FUS foci in additional cell lines.** FUS foci are observed in A549 cells and in WT LAP-FUS Helas that have not been induced to express exogenous GFP-FUS.

A549 cells as well as LAP-FUS WT Hela cells (without doxycycline induction) produced FUS foci (Fig 4.4) and, crucially, foci were also observed in WT cortical neurons harvested from CD1 mice (Ryan Green, personal communication) - indicating they can form in neuronal tissue. Later experiments also confirmed that foci form in U2OS cells (Fig 6.4), 1BR cells and cells derived from a patient suffering DNA-PKcs deficiency (Fig 5.6f).

To investigate something of the nature of the FUS foci cells were treated with CPT and subjected to detergent pre-extraction. Detergent pre-extraction with Triton can be used to remove water-soluble protein from the nucleus of cells (Capco et al. 1982) and by implication proteins and structures that can be observed by immunofluorescence after a detergent pre-extraction are associated (directly or indirectly) with water-insoluble structures within the cells including chromatin (Fey et al. 1986) but also RNA species and the nuclear matrix (Carter et al. 1991).

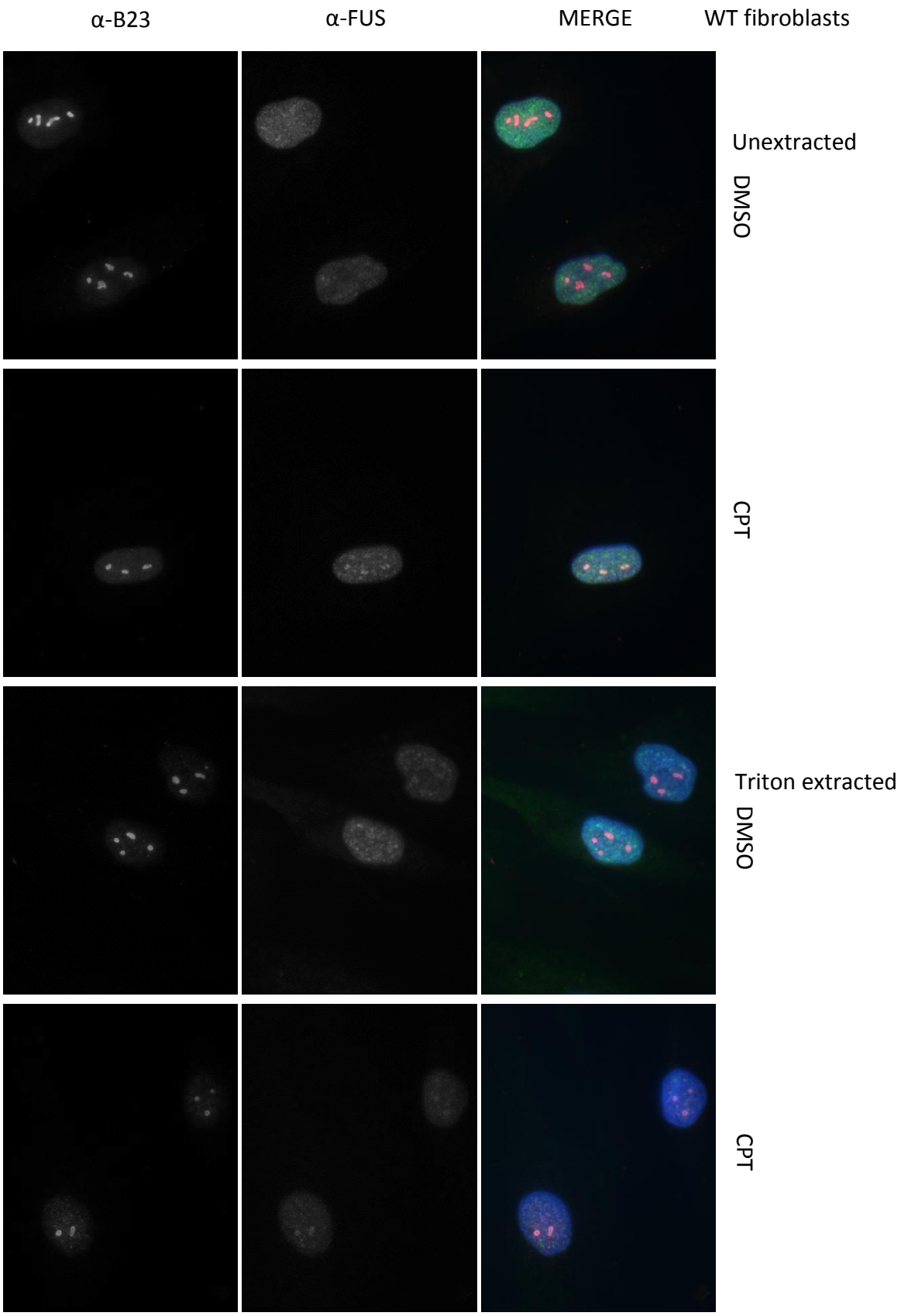


Figure 4.7 | **Triton extraction of FUS foci in patient fibroblasts or control cells.** Nucleolar FUS is resistant to Triton extraction, potentially indicating a degree of chromatin or RNA association. Images artificially brightened by 30%. Cells were CPT treated and then treated at room temperature with either PBS or 0.2% Triton X-100 prior to fixation and IF.

FUS foci appeared to be retained after Triton extraction indicating their association with these structures (Fig 4.5) and a possibility of their being associated with chromatin or with RNA species.

4.3 Relocalisation of FUS in response to inhibition of RNA Polymerase II (RNAP II)

With the data generated at this point not indicating any role of DNA damage in FUS focus formation it was hypothesised that CPT producting FUS foci was due to its activity as a transcriptional inhibitor. In order to test this fibroblasts were tested with other RNA polymerase inhibitors. Initially two inhibitors of RNAP II were utilised: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Sehgal et al. 1976) and α-amanitin (Lindell et al. 1970).

A control experiment was also set up to confirm DRB and α-amanitin's specificity as RNAP II inhibitors. RNAP II is nucleoplasmic and responsible for transcribing messenger RNA (mRNA) and most non-coding RNAs (ncRNA) (Viktorovskaya & Schneider 2015) therefore RNAP II inhibitors will reduce transcription primarily in the nucleoplasm rather than the nucleolus. This can be visualised with EU labelling followed by click chemistry as described in chapter 3.

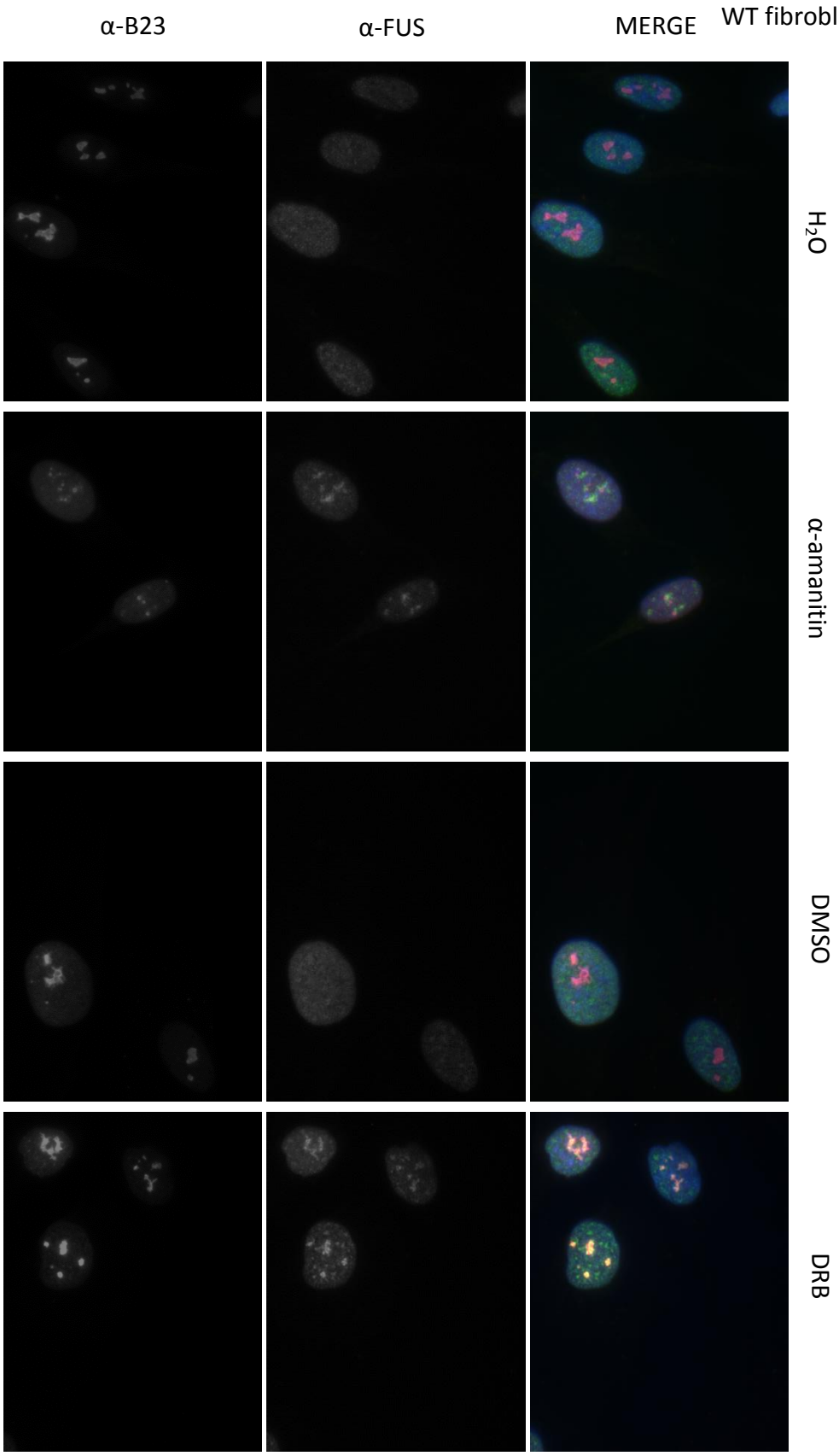


Figure 4.8a | **endogenous FUS foci formed in response to the RNAP II inhibitors DRB and α-amanitin.** Both RNAP II inhbitors resulted in formaton of FUS foci. The shrunken B23 masses proximal to FUS foci in α-amanitin treated cells are consistent with the disordering of nucleoli known to occur in response to RNAP II inhibition. DRB was applied at 266μM for 30 minutes. α-amanitin was applied at 2μg/ml for 16 hours.

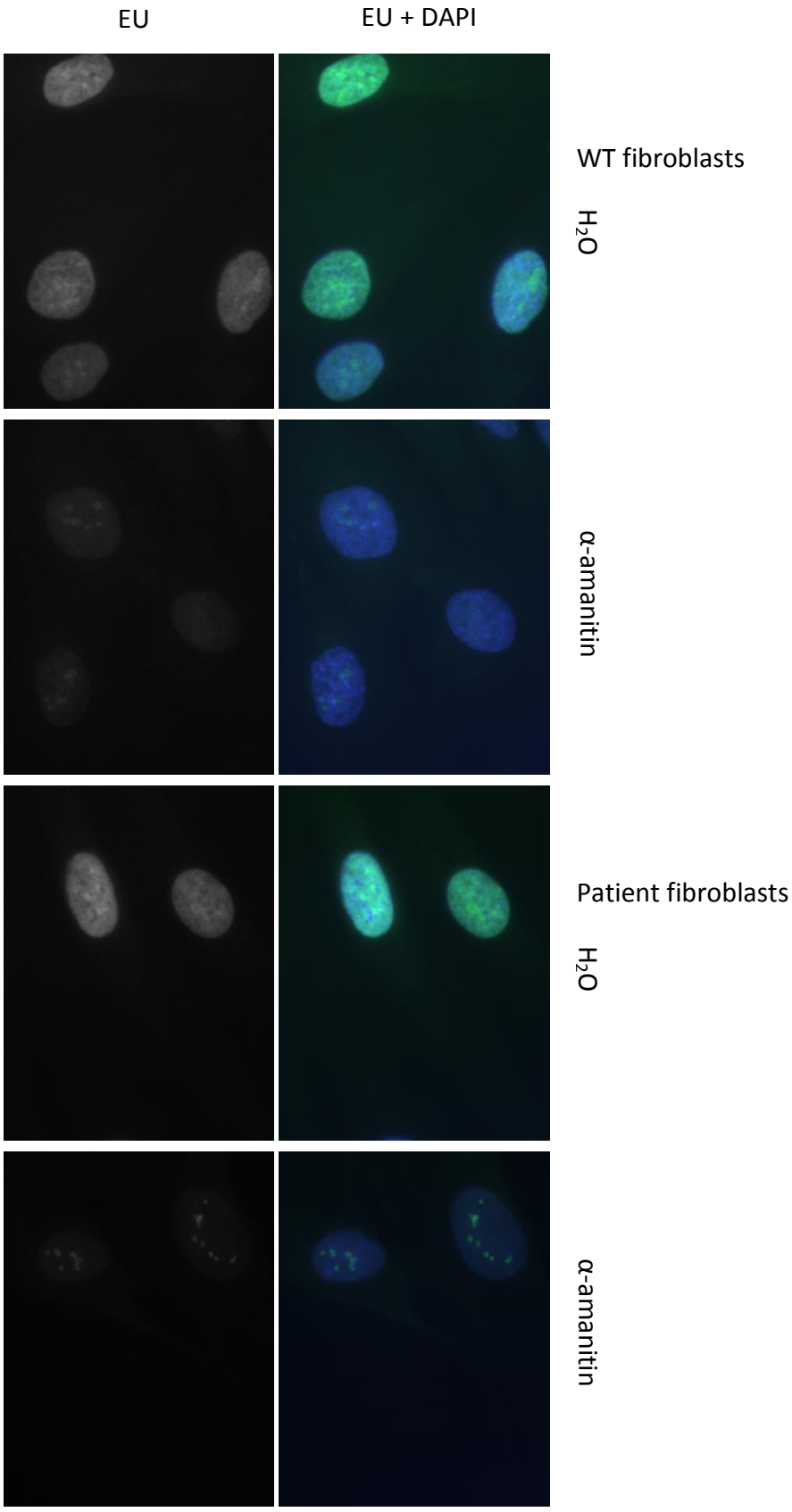


Figure 4.8b | **α-amanitin reduces nucleoplasmic transcription, as measured by EU labelling.** α-amanitin treated cells demonstrate a decrease in transcription primarily in the nucleoplasm.

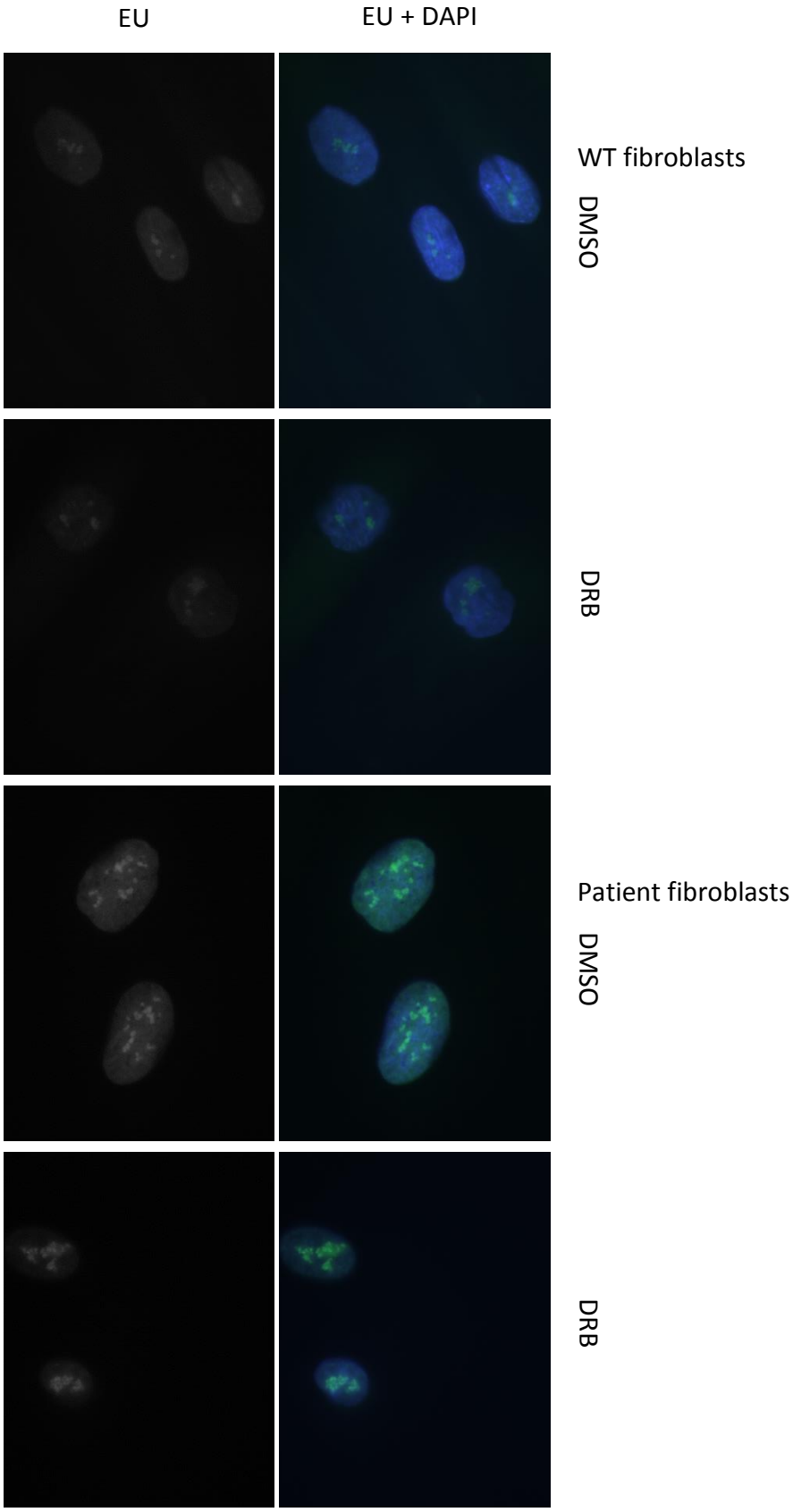


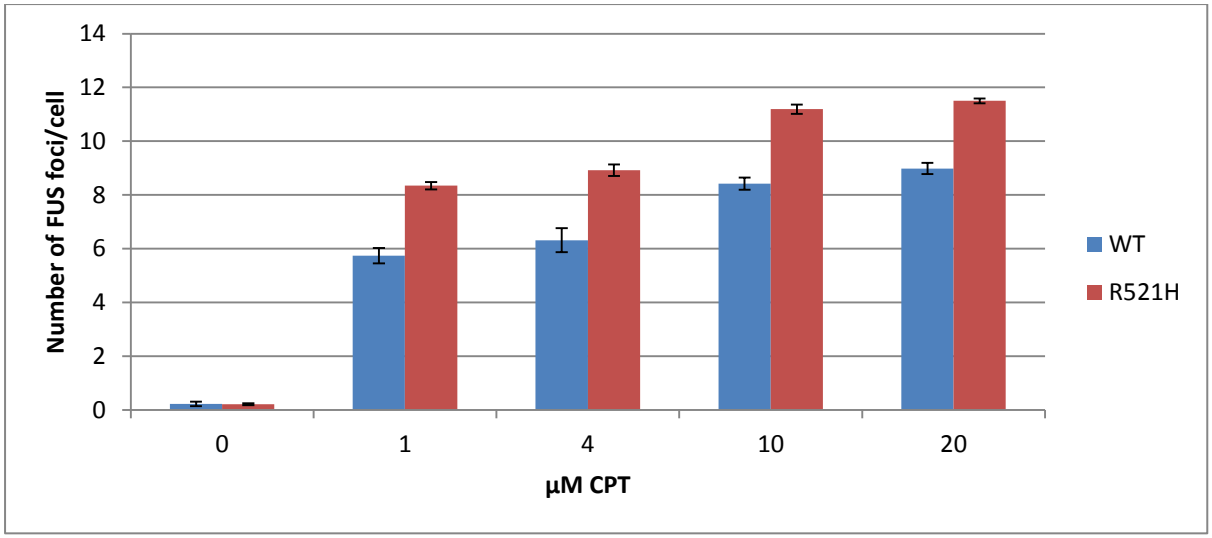
Figure 4.8c | **DRB reduces nucleoplasmic transcription, as measured by EU labelling.** DRB treated cells demonstrate a decrease in transcription primarily in the nucleoplasm rather than in the nucleolus.

Both inhibitors were confirmed to primarily inhibit nucleoplasmic transcription in Figs 4.8b and 4.8c. DRB also produced a relocalisation of FUS similar to CPT. However α-amanitin produced FUS foci that were proximal to, but not colocalising with, areas of B23 signal. These areas of B23 signal were also shrunken relative to water treated controls (Fig 4.8a). The difference in localisation of FUS in response to these two inhibitors was not unexpected. Transcriptional inhibition can cause disassembly of the nucleolus - resulting in formation of a shrunken mass of late rRNA processing proteins such as B23 (Louvet et al. 2005) with a proximal nucleolar necklace containing transcription sites (Granick 1975a; Granick 1975b; Scheer et al. 1984).

It would be expected that the α-amanitin treated cells would undergo this disassembly due to their long treatment time, even if DRB treated cells contained intact nucleoli after their relatively short treatment. It is therefore likely that the varying locations of FUS foci in DRB and α-amanitin treated cells represent the varying degrees of nucleolar disruption in the cells - with FUS recruited to transcription sites in the nucleolar necklace rather than to the B23-containing mass in the α-amanitin treated cells. This could indicate FUS associating with the transcription or early RNA processing machinery, rather than being associated with later processing.

DRB- and α-amanitin-induced foci have also been observed in CD1 neurons (Ryan Green, personal communication). These cells underwent DRB treatment at a higher dose and with a longer incubation period, 300μM DRB for the period of one hour and produced DRB-associated foci proximal to shrunken masses of B23 - consistent with nucleolar disruption.

Whether the extent of foci formation correlated with the extent of RNAP II inhibition was unclear so titrations of the three RNAP II inhibitors used were set up in patient and control fibroblasts and the foci in treated cells counted. The same control cells (treated with DMSO) were used for the CPT and DRB titrations as the two chemicals are both soluble in the same vehicle and have similar incubation times.



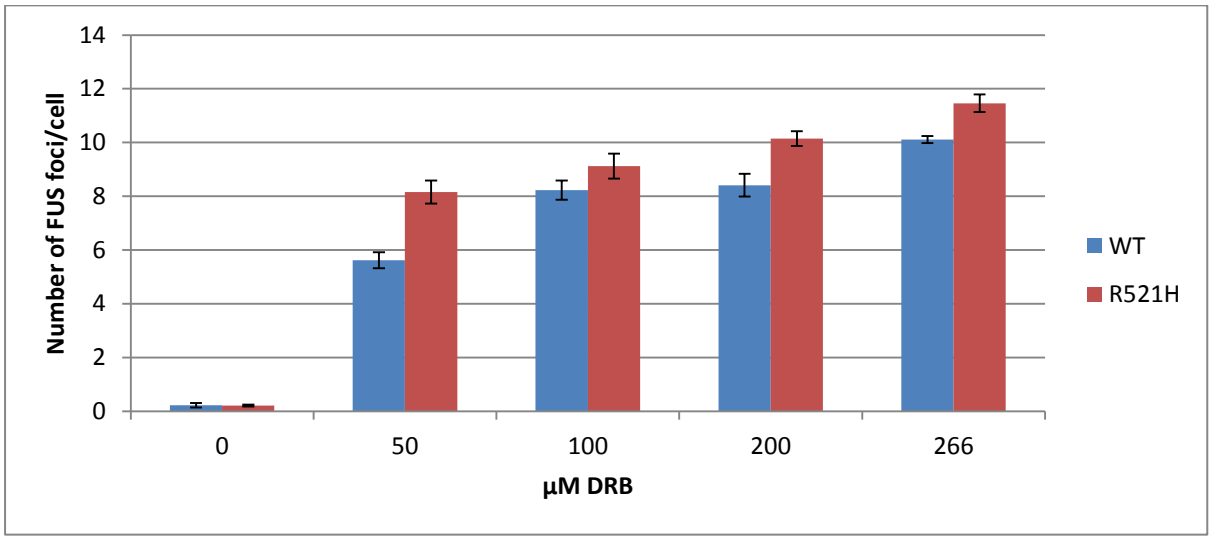


Figure 4.9 | **titration of CPT and DRB and their effect on FUS foci counts.** Increasing the concentration of either RNAP II inhibitor increases the number of FUS foci produced per cell. These data were statistically significant (by one-tailed ANOVA at 95% confidence intervals) for both WT and R521H fibroblasts and the R521H fibroblast line also produced significantly more (by two-tailed ANOVA at 95% confidence intervals) foci than the WT line. n=3.

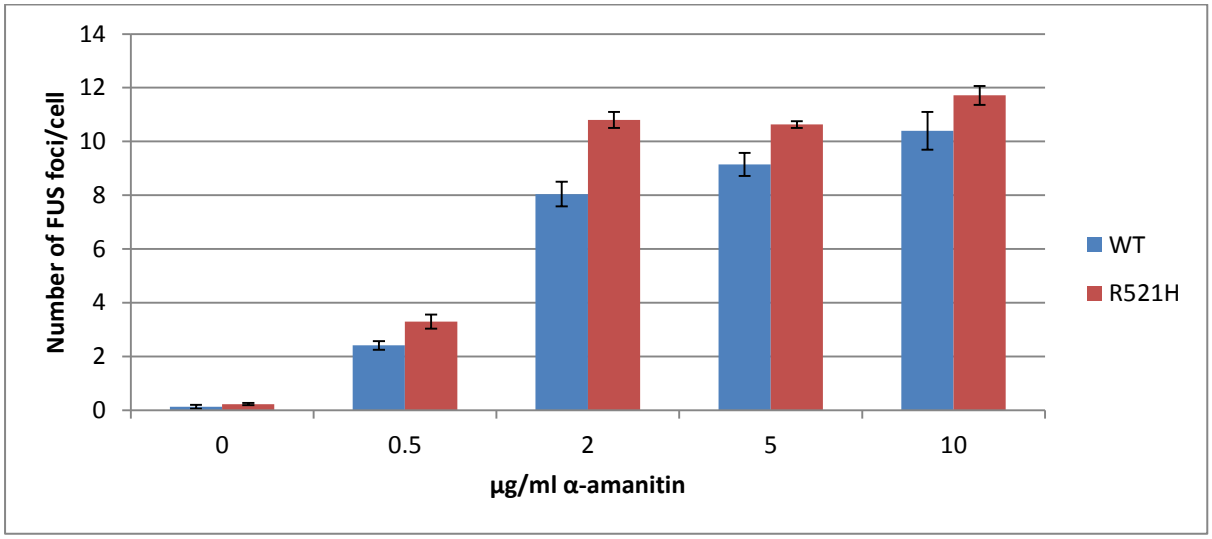


Figure 4.10 | **titration of α-amanitin and its effect on FUS foci counts.** Increasing α-amanitin concentrations increases the number of FUS foci produced per cell and the percentage of cells with foci. These data were statistically significant (by one-tailed ANOVA at 95% confidence intervals) for both WT and R521H fibroblasts and the R521H fibroblast line also produced significantly more (by two-tailed ANOVA at 95% confidence intervals) foci than the WT line. n=3.

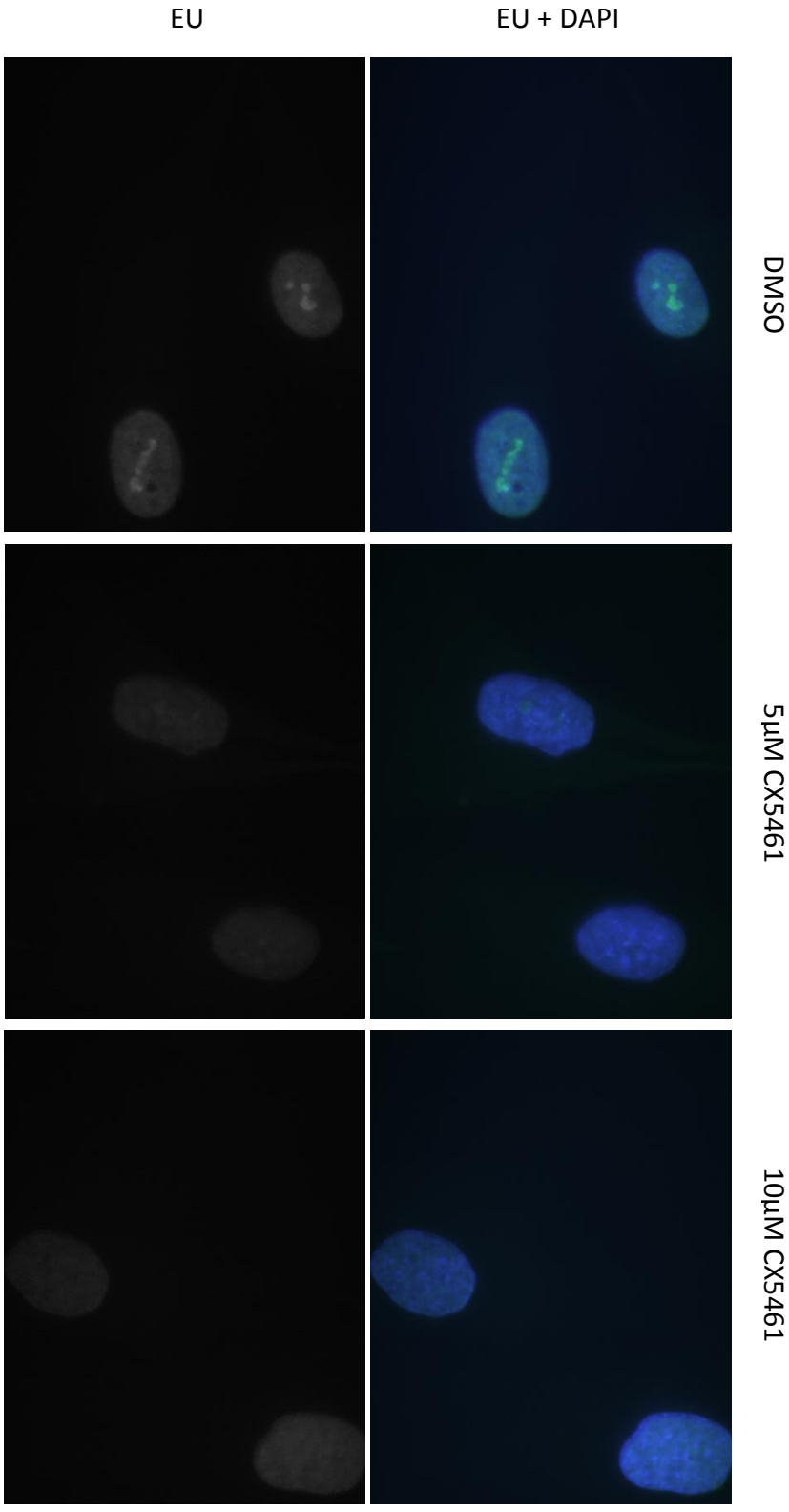
These titrations revealed that increased doses of any of these three RNAP II inhibitors resulted in an increase of focus formation per cell - further linking RNAP II inhibition with the process of FUS relocalisation (Fig 4.9 - CPT and DRB, Fig 4.10 - α-amanitin).

4.4 The impact of RNAP I inhibition on FUS relocalisation

The previous data strongly suggested that formation of FUS foci could be induced on RNAP II inhibition but did not indicate whether inhibition of other nuclear RNA polymerases could have the same effect. There are two other nuclear RNA polymerases in humans but of these the nucleoplasmic RNA polymerase III (RNAP III) could not be investigated independently using small molecule inhibitors as the only selective RNAP III inhibitor, tagetitoxin (Steinberg et al. 1990), was not commercially available at the time of the experiments. Another RNAP III inhibitor was commercially available but its selectivity for RNAP III over other RNAP enzymes could not be verified in the literature (Wu et al. 2003).

Therefore the next polymerase investigated was the nucleolar RNA polymerase I (RNAP I). The selective RNAP I inhibitor CX5461 (Haddach et al. 2012) was chosen for use in this study, and control experiments using EU labelling and click chemistry were used to verify its selectivity (Fig 4.11c).

WT fibroblasts



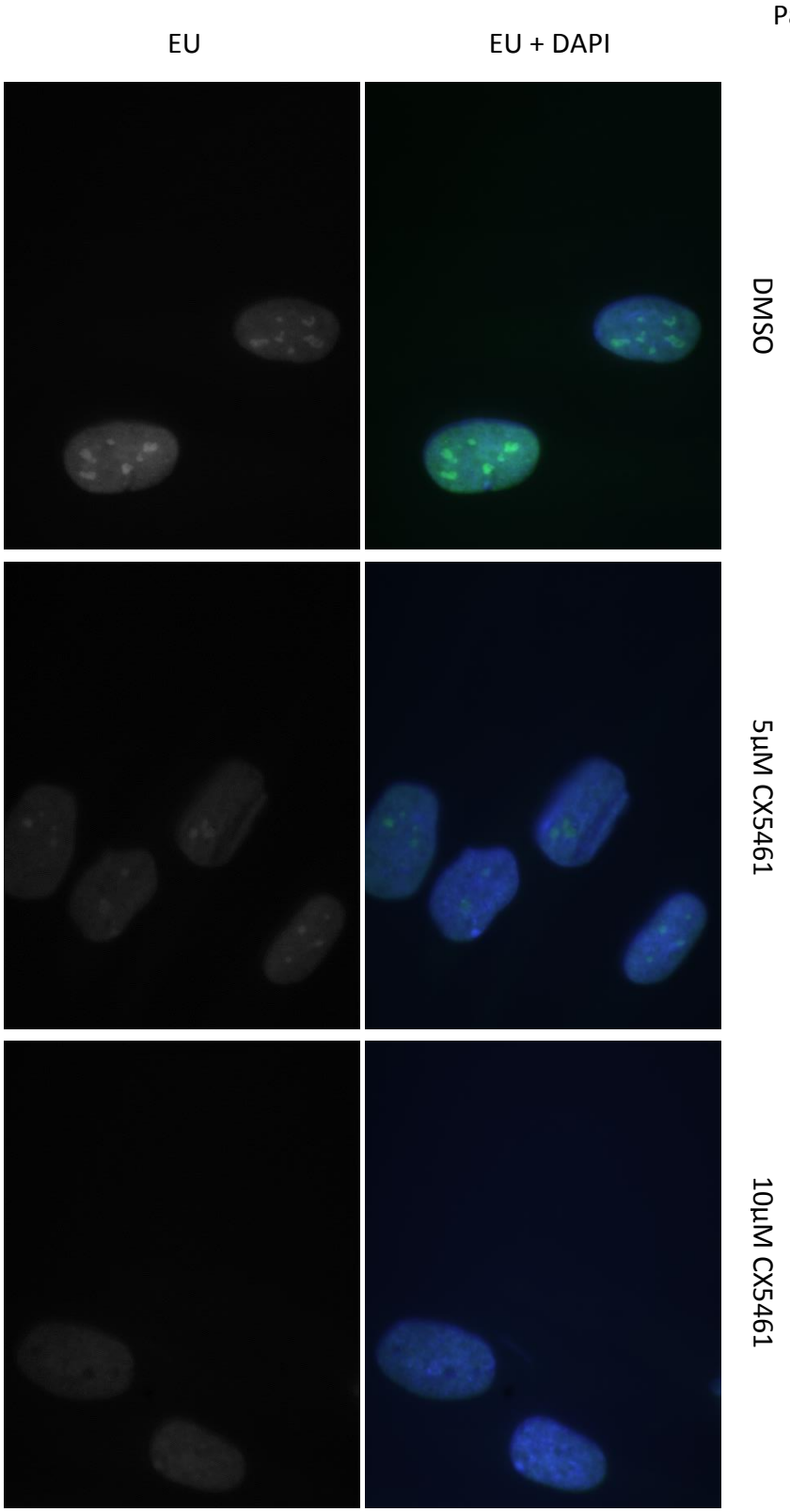


Figure 4.11a | **Controls to test the selectivity of CX5461, as measured by EU labelling.** CX5461 did not seem to have large effects on nucleoplasmic transcription but completely ablated nucleolar transcription at 10μM.

RNAP I is a nucleolar polymerase and nucleolar transcription was clearly ablated after three hours 10μM CX5461 treatment while nucleoplasmic transcription was largely unaffected (Fig 4.11a) - indicating that the inhibitor is RNAP I selective. Therefore this concentration of CX5461 was chosen for experiments to see if RNAP I inhibition could produce FUS foci. DRB-treated cells were also included as a positive control. Finally cells treated with CX5461 followed by DRB were included in order to see if pre-inhibition of RNAP I impacted on RNAP II-induced FUS foci formation.

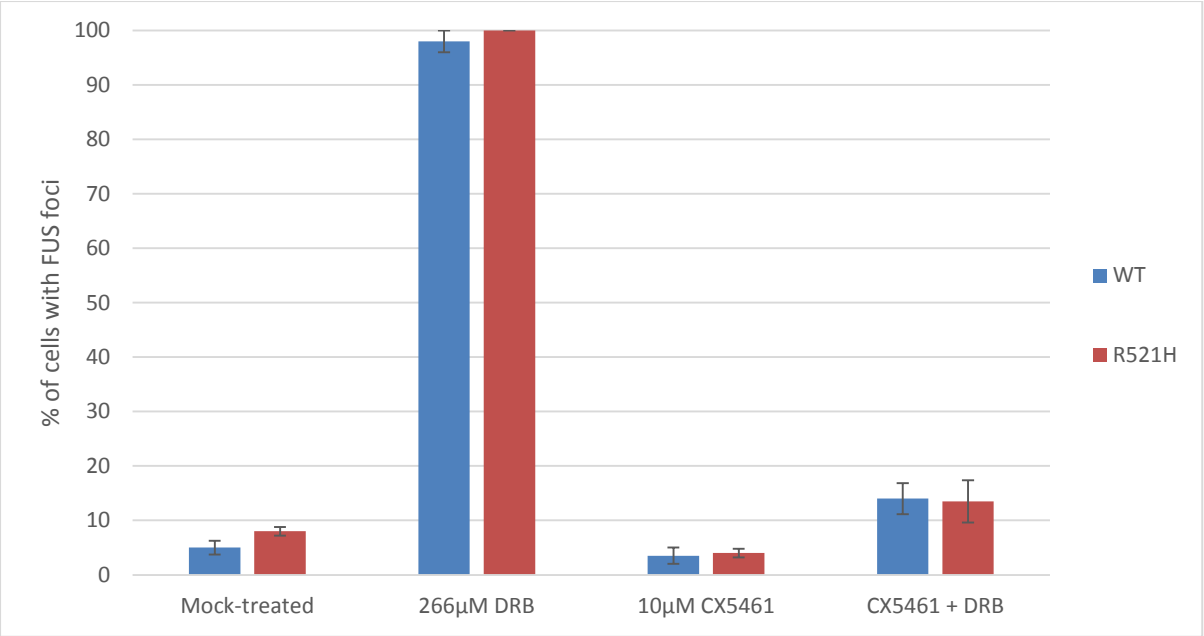
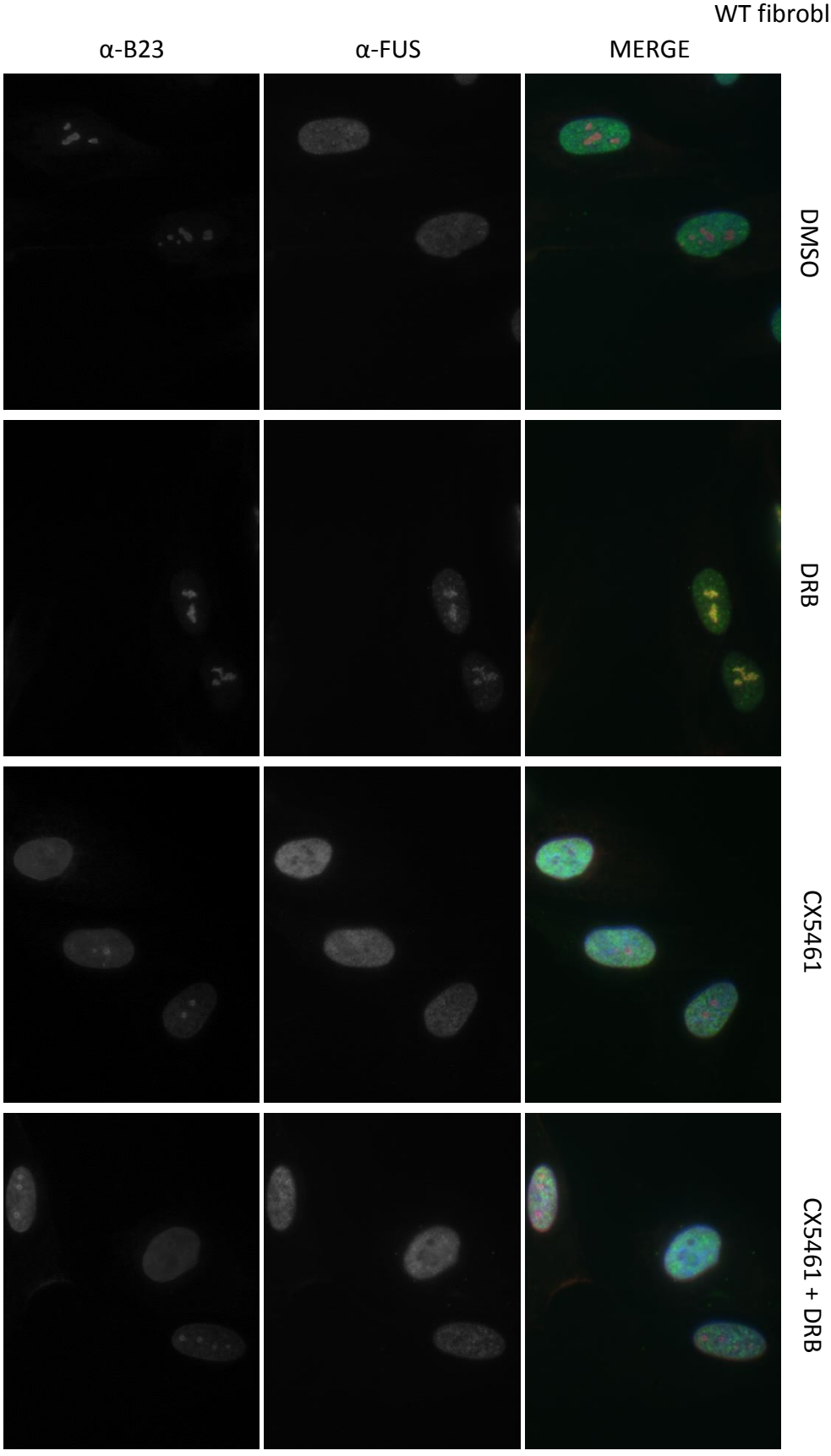


Figure 4.11b | **endogenous FUS foci do not form in response to transcriptional inhibition of RNA Polymerase I.** CX5461 treatment does not produce FUS foci, but does prevent their formation by subsequent DRB treatment. n=4.

Cells treated with both CX5461 and DRB were treated for 3 hours total with CX5461 (10μM), with DRB (266μM) added 2.5 hours into the CX5461 incubation.


WT fibroblasts
α-B23
α-FUS
MERGE
DMSO
DRB
CX5461
CX5461 + DRB

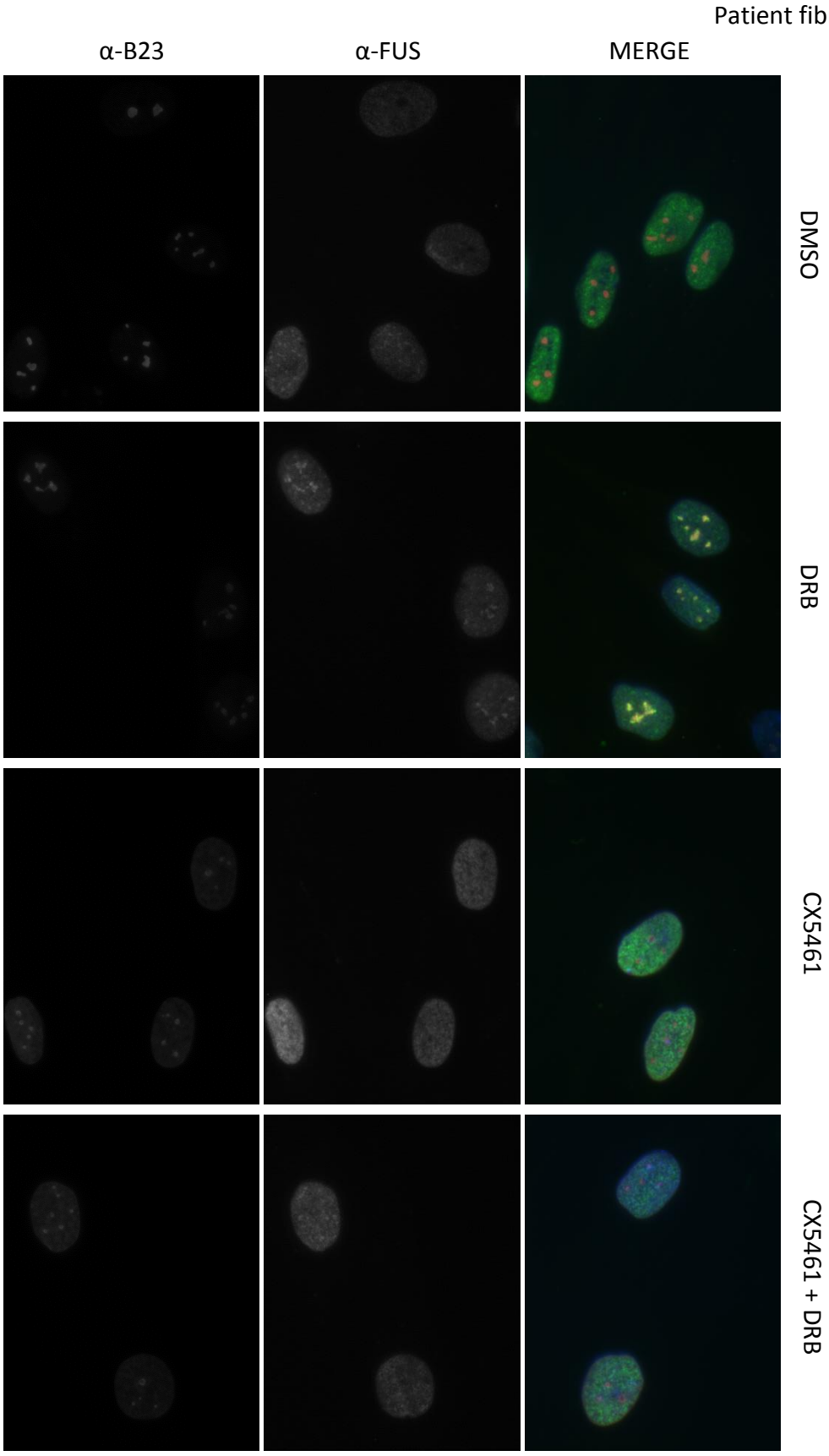


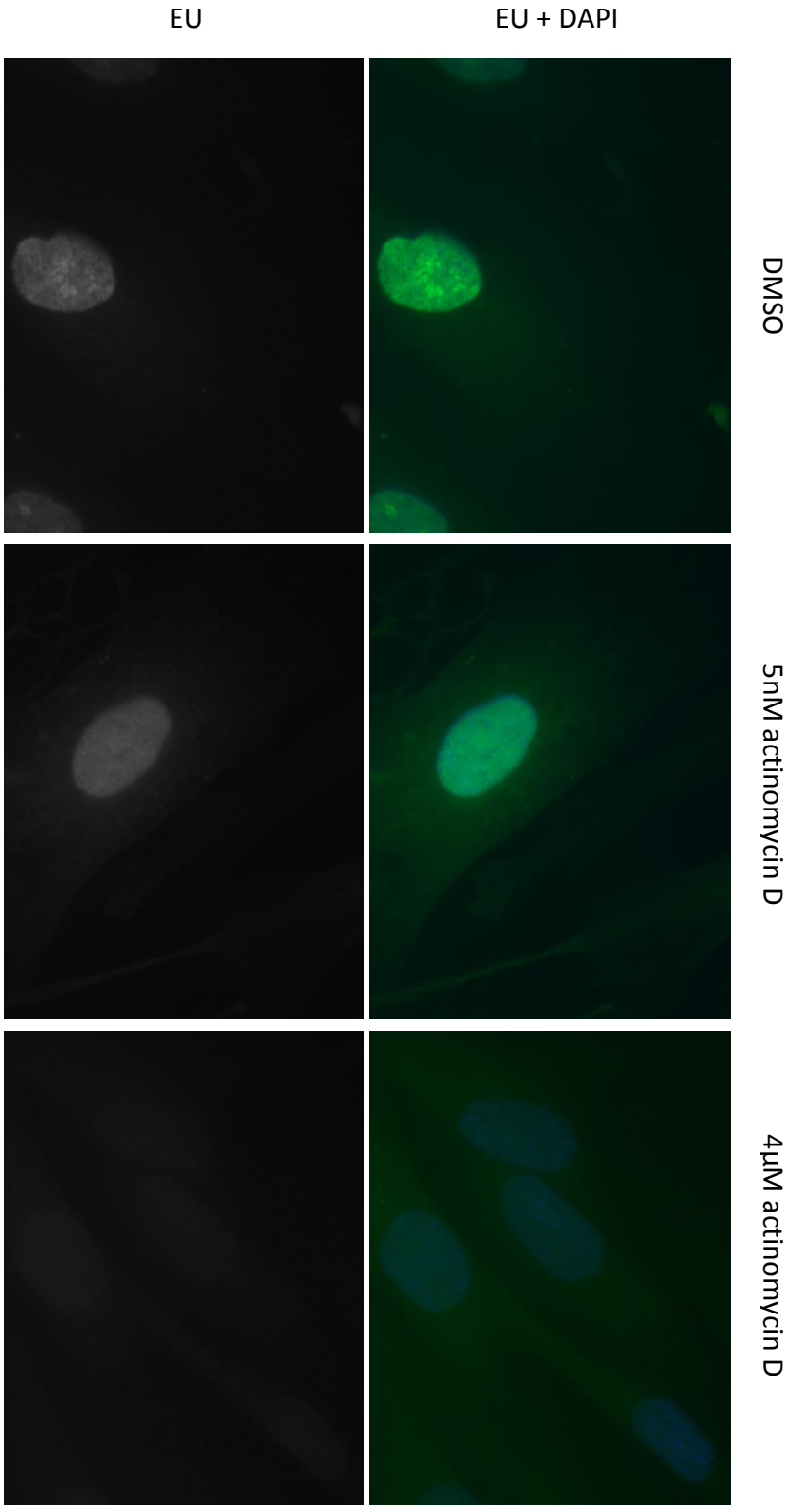
Figure 4.11c | **endogenous FUS foci do not form in response to transcriptional inhibition of RNA Polymerase I.** CX5461 treatment does not produce FUS foci, but does prevent their formation by subsequent DRB treatment. Representative images used for chart in figure 4.11b.

Cells treated with CX5461 did not produce FUS foci - indicating that the process of focus formation is not associated with RNAP I inhibition. CD1 neurons also did not produce foci in response to CX5461 (Ryan Green, personal communication). Interestingly DRB treatment produced foci, as expected, but pre-inhibition of RNAP I prior to DRB treatment appeared to prevent the vast majority of this foci formation (Fig 4.11).

A hypothesis regarding these data could be that FUS foci are associated with nucleolar pre-rRNA in the forming nucleolar necklace and that its relocalisation is contingent on (direct or indirect) binding to pre-rRNA. If this was the case then it would be expected that FUS could not relocalise after RNAP I pre-treatment as CX5461 treatment would deplete the cells of the pre-rRNAs to which FUS would localise.

To corroborate the roles of RNAP II and RNAP I in foci formation cells were also treated with differing concentrations of actinomycin D (ActD). This chemical inhibits both RNAP I and RNAP II, the former in the nM range of concentrations and the latter in the µM range (Jao & Salic 2008), an EU labelling experiment was set up to verify this.

WT fibroblasts



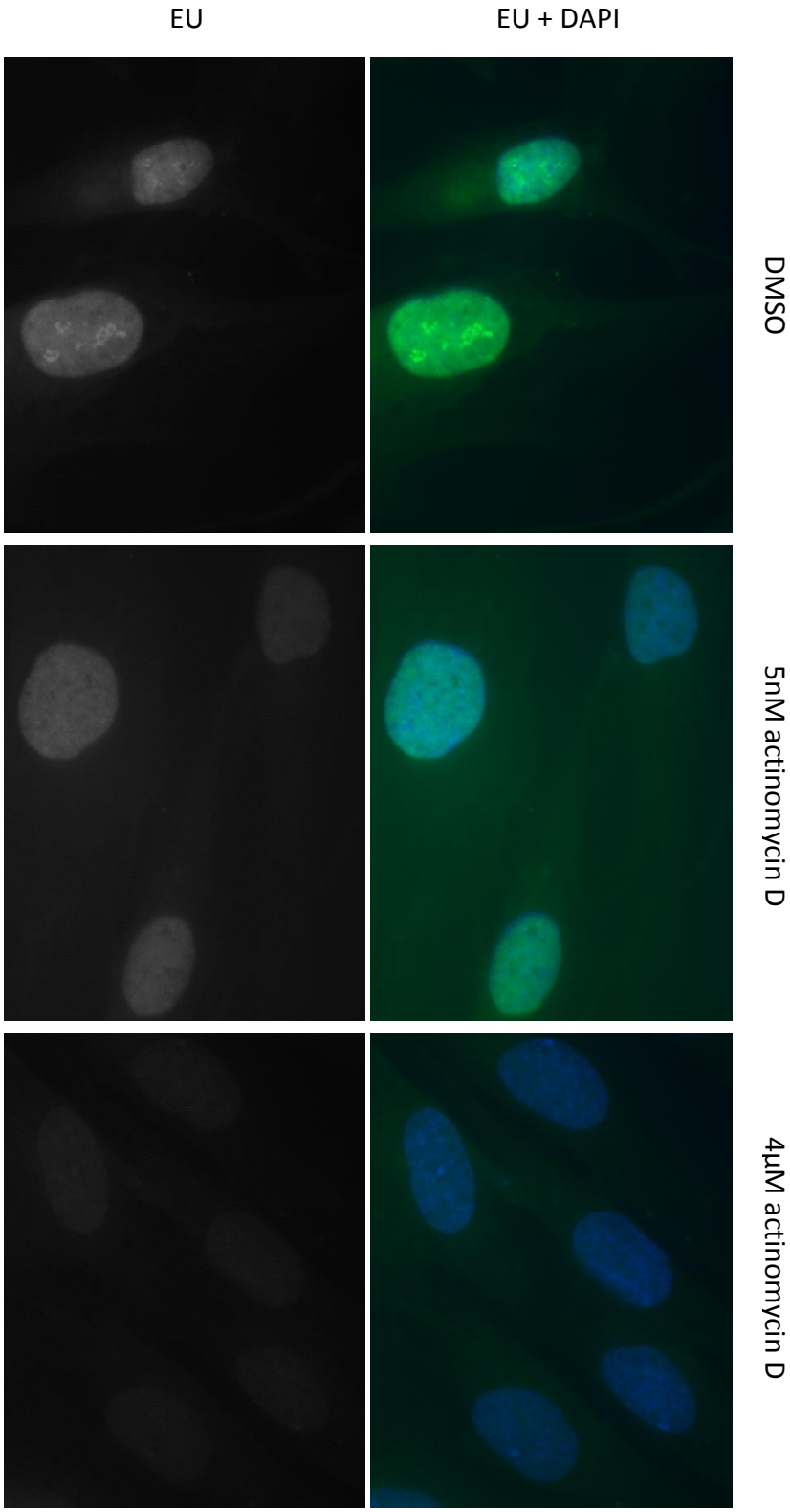
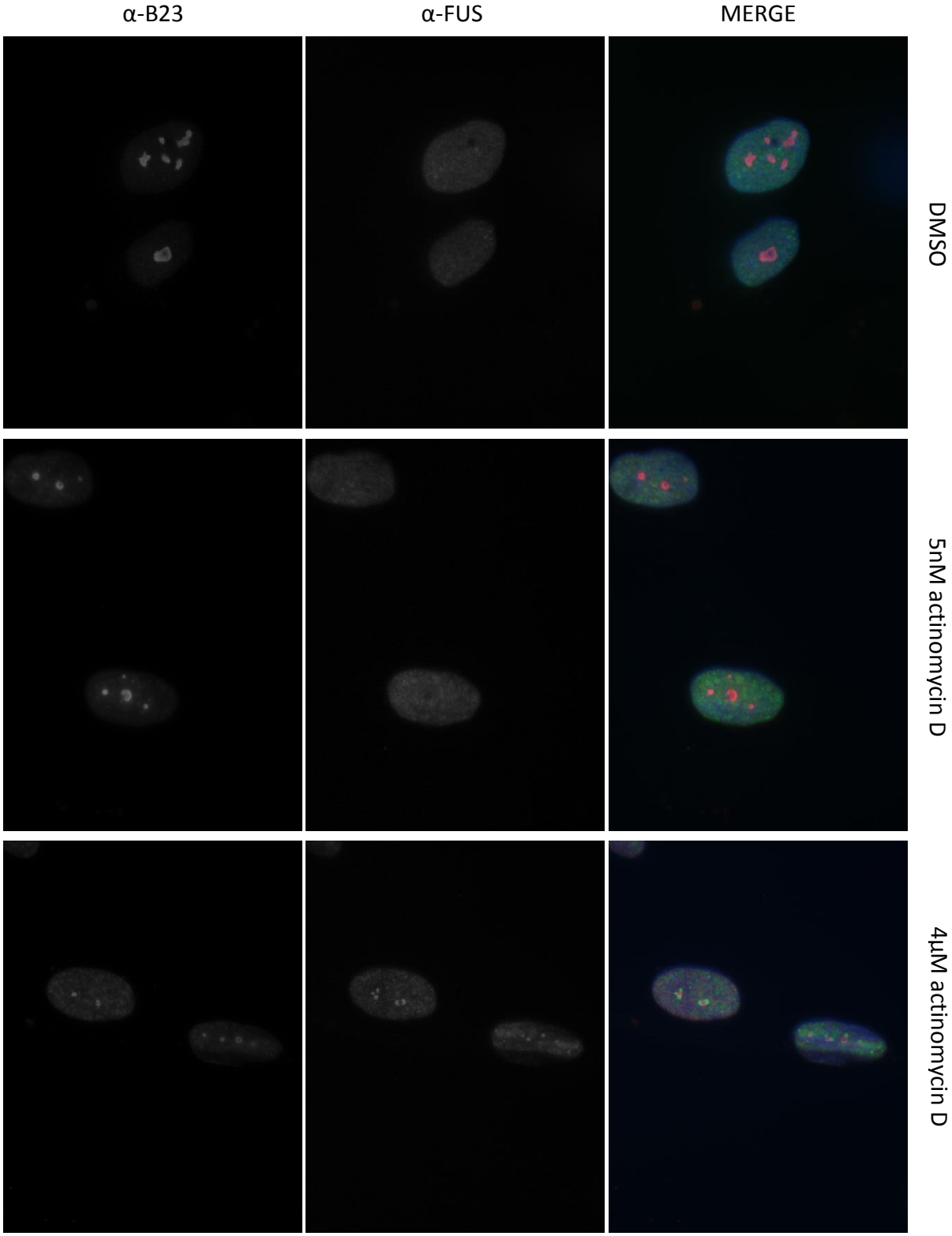


Figure 4.12a | **Actinomycin D reduces nucleolar transcription at low concentrations and all nuclear transcription at high concentrations, as measured by EU labelling.** 5nM actinomycin D clearly abolishes nucleolar transcription but has no effects on nucleoplasmic transcription. In contrast, 4µM actinmycin D abolishes both.

Cells were treated with actinomycin D at concentrations of 5nM (expected to inhibit RNAP I only) or 4µM (expected to also inhibit RNAP II) for three hours, then EU labelled and processed with click chemistry.

The specificity of actinomycin D for RNAP I at nanomolar concentrations was verified by this experiment, which also confirmed that the inhibitor works on both RNAP I and RNAP II at micromolar concentrations (Fig 4.12b). The length of ActD treatment was three hours. A foci counting experiment with ActD was set up using these conditions to investigate whether inhibiting both RNAP II and RNAP I at approximately the same time would result in FUS foci formation.

WT fibroblasts



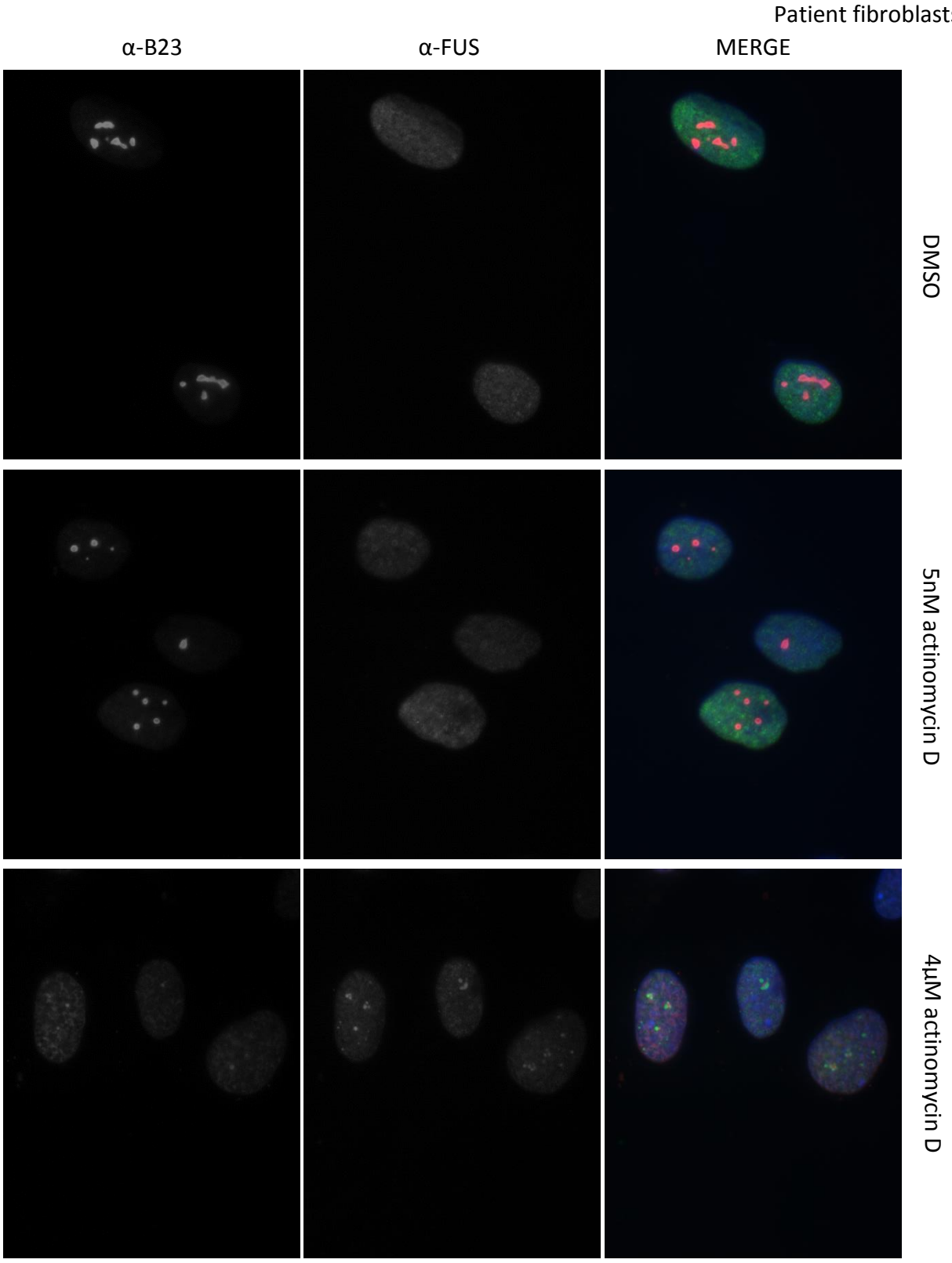


Figure 4.12b | **endogenous FUS foci form in response to ActD at high concentrations only.** FUS foci were observed to form in response to 4μM ActD, where the compound inhibits both RNAP I and RNAP II, but not at 5nM where it only inhibits RNAP I.

It was found that those cells treated with low concentration ActD (inhibiting RNAP I only) did not produce foci whereas cells treated with high concentration ActD (inhibiting both polymerases) did (Fig 4.12a) - demonstrating shrunken B23 masses with FUS foci mostly adjacent to the B23 signal, as previously seen with α-amanitin treated cells.

These data throws up a contradiction as pre-treatment of cells with RNAP I inhibitor prevents foci formation but contemporaneous inhibition of RNAP I and RNAP II still results in focus formation. The idea that FUS binds pre-rRNA and forms foci at these sites could explain this contradiction. Pre-treatment with an RNAP I inhibitor would deplete the cells of pre-rRNA and therefore prevent FUS focus formation but if both RNAP I and RNAP II were inhibited at approximately the same time then FUS could relocalise in response to the RNAP II inhibition before the pre-rRNA is degraded.

The half-life of nucleolar RNA (specifically 45S) has been shown to be equivalent between CX5461 and ActD at approximately twenty minutes (Drygin et al. 2011). It would therefore be expected that the vast majority of pre-rRNA in the cell would be depleted after three hours treatment with either CX5461 or ActD. Despite this the ActD treated cells contained FUS foci after this lengthy treatment. This may suggest that FUS forms a focus around pre-rRNA and that this focus protects the pre-rRNA from degradation.

Further experiments were planned but abandoned for lack of time to address the hypothesis that FUS binds pre-rRNA. In these experiments low concentration actinomycin D treatment would have been optimised to reduce treatment time as much as possible (to minimise turnover of rRNA) using EU labelling to test that RNAP I was inhibited and RNAP II was not. Cells would have been treated with ActD for this much reduced timeframe or for three hours and then treated with DRB.

It would be expected if the hypothesis is correct that the cells briefly treated with ActD would not have turned over their RNA and would still be capable of FUS foci formation, but that the cells treated for three hours would have depleted all their nucleolar RNA and be incapable of foci formation.

4.5 3D localisation of FUS and TDP43 in the nucleolus

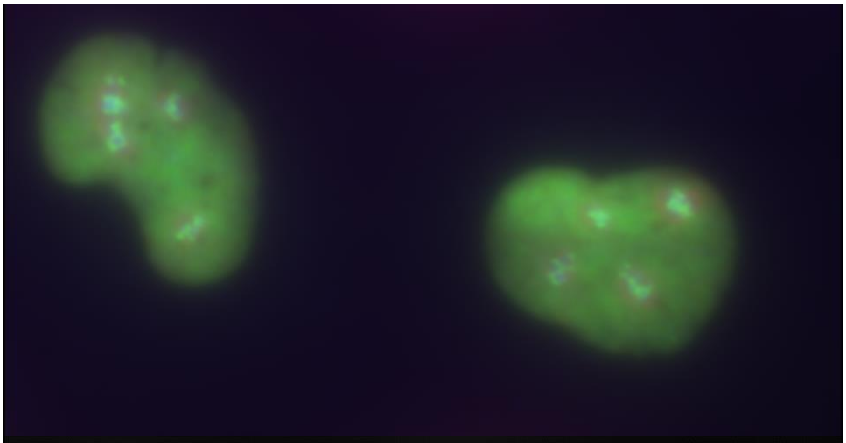
The localisation of FUS in what appears to be the nucleolar necklace after α-amanitin or high-dose actinomycin D treatment suggests FUS foci localise to the FCs/DFCs of the nucleolus and then migrate outward with the components of these nucleolar subcompartments during the process of nucleolar disruption caused by RNAP II inhibition. This in turn implies the role of FUS foci is likely to be related to rRNA transcription, early processing of rRNA or an interaction with the pre-rRNA itself as suggested in chapter 4.4.

However though LAP-tagged FUS was shown to either colocalise with or be proximal to fibrillarin in Fig 4.1b but the single plane image makes it difficult to determine which is the case. Furthermore there was no image of LAP-tagged FUS foci where both B23 and fibrillarin are marked by IF.

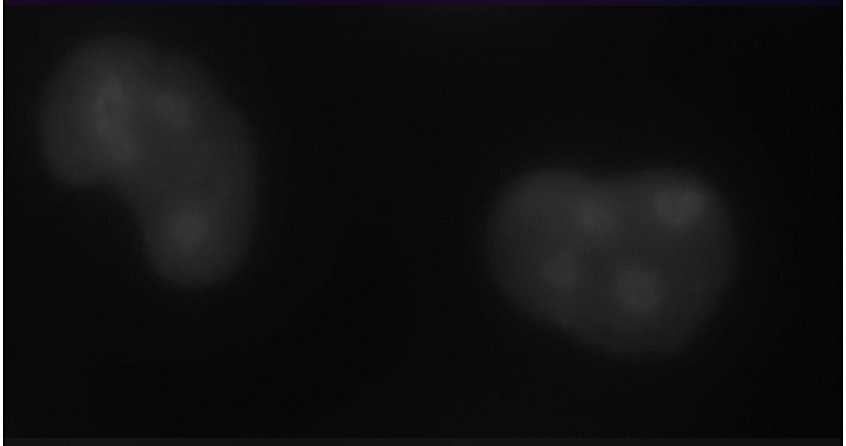
In order to verify the subnucleolar localisation of FUS a further experiment was planned where LAP-FUS Helas would be CPT treated and IF performed for both fibrillarin and B23. Images from this experiment would be taken on the Olympus IX73 microscope so images could be taken on multiple planes to reduce the ambiguity of the final image.

LAP-TDP43 Helas were available so these cells were also treated to see if TDP43, another RNA-binding ALS-causative protein, underwent a similar relocalisation to FUS. These cells were tested for LAP-TDP43 expression by western blot in the same manner as the LAP-FUS Helas had been tested for LAP-FUS expression.

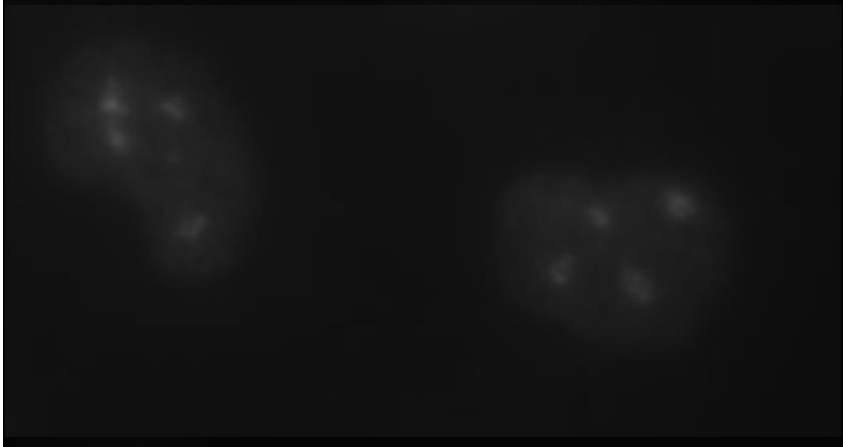
MERGE



α-B23



α-fibrillarin



GFP-FUS

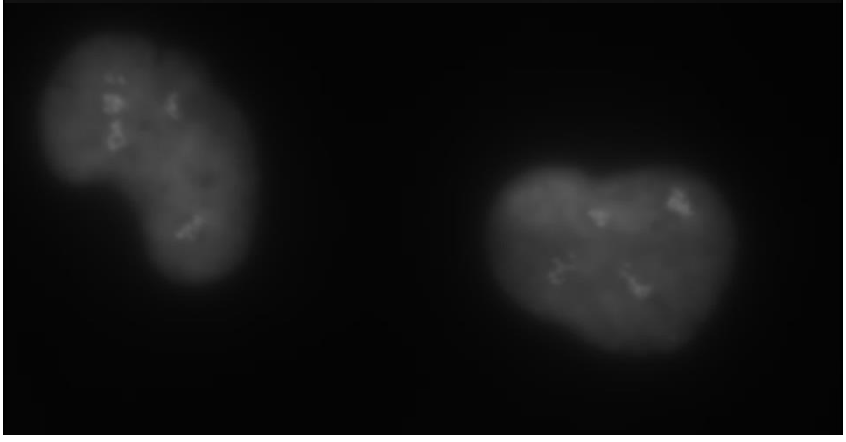


Figure 4.13 | **GFP-FUS foci localise proximal to fibrillarin.** CPT-treated LAP-FUS Helas were probed by IF for B23 and fibrillarin and Z-stacked images taken.

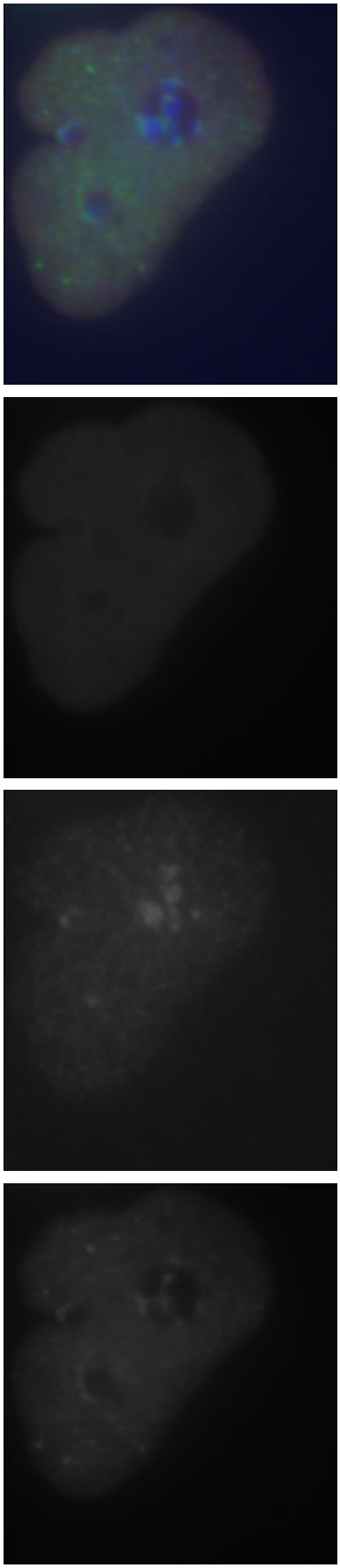


Figure 4.14a | **GFP-TDP43 foci localise proximal to fibrillarin.** CPT-treated LAP-TDP43 Helas were probed by IF for B23 and fibrillarin and Z-stacked images taken.

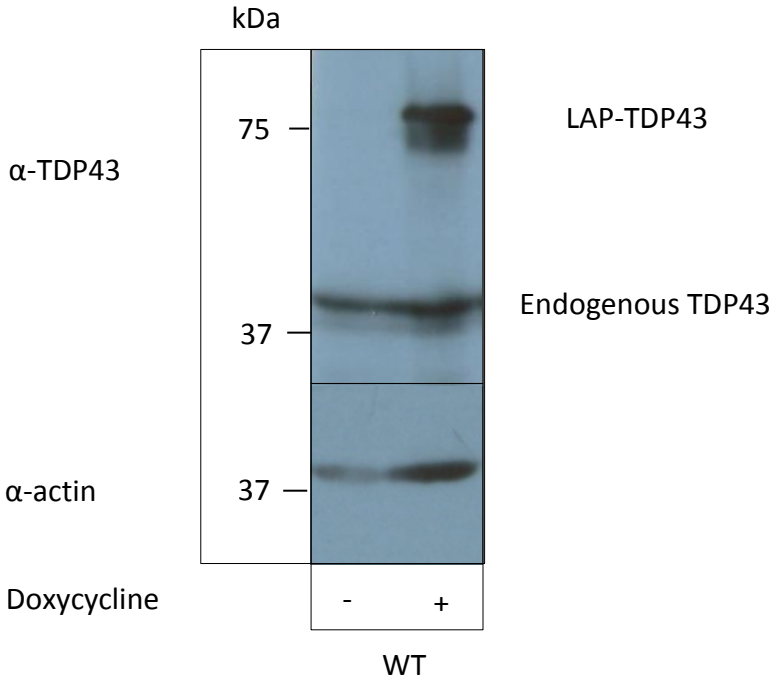


Figure 4.14b | **Expression of LAP-TDP43.** Doxycycline induction of LAP-TDP43 transcription induces moderate expression of the exogenous protein. Approximately $5x10^5$ cells per lane.

Cells were seeded and harvested as in Fig 3.3.

FUS was observed to localise to the edge of the fibrillarin signal - showing recruitment to the FCs/DFCs rather than to the GC of the nucleolus. Fibrillarin is closely associated with nucleolar transcription and early pre-rRNA processing so this is consistent with the proposal that FUS foci have a function involving pre-rRNA that has not yet been extensively processed.

These data also showed that TDP43 underwent relocalisation in response to CPT and that this closely resembled FUS' relocalisation, albeit with less protein recruited (Fig 4.14a), consistent with a similar function at the site. The LAP-TDP43 Hela cell line demonstrated modestly strong inducible exogenous protein expression (Fig 4.14b). Despite localisation of LAP-TDP43 in the nucleolus attempts at visualising endogenous TDP43 foci in 1% PFA fixed cells were not successful (data not shown).

4.6 Conclusion

It was observed that FUS foci could form in response to CPT treatment but that the foci were localised to the nucleolus rather than being pan-nuclear as XRCC1 foci are. These foci were observed both with overexpressed GFP-tagged FUS in LAP-FUS Hela cells and with endogenous FUS in multiple cell types, and were resistant to Triton extraction, suggesting association with an insoluble structure within the cell such as chromatin or RNA. Later experiments showed FUS localised proximal to the FCs/DFCs and that GFP-TDP43 foci also form after CPT treatment (potentially implying involvement in the same process at these sites). Furthermore it was observed that both mutant LAP-FUS Hela cells and the patient fibroblasts produced more FUS foci than wild-type controls - this may be a downstream consequence of the nucleolar fragmentation in these cells.

CPT is both a DNA damaging agent and a transcriptional inhibitor. In order to distinguish which one of these activities results in FUS focus formation cells were treated with two RNAP II inhibitors not thought to induce DNA damage, DRB and α-amanitin, both of which also produced FUS foci in a dose-dependent manner. That transcriptional inhibition produced nucleolar FUS foci indicates these are likely to be the same structures as the DRB-induced aggregates described by Zinszner et al. Additionally inhibition of RNAP II induces nucleolar disruption and segregation of the nucleolar compartments into a B23-containing shrunken "mass" and a nucleolar necklace containing the transcriptional and early pre-rRNA processing machinery - the treatments with CPT and DRB were too short to induce full segregation but α-amanitin treatment induced full segregation showing that FUS foci localise to the nucleolar necklace rather than the B23-containing masses. This suggests that the FUS foci are associated with early pre-rRNA processing machinery, RNAP I transcription machinery or pre-rRNA at an early stage of processing.

Treatment of cells with RNAP I inhibitors did not induce formation of FUS foci and in fact prevented FUS focus formation if the cells were subsequently treated with an RNAP II inhibitor although treatment with inhibitors such as ActD that work on both RNAP I and II still produced foci. A hypothesis to explain this could be that RNAP I pre-inhibition depletes the cell of nucleolar RNA, which has a half-life of 20 minutes after RNAP I inhibition (Drygin et al. 2011), and that FUS must bind pre-rRNA in order to form foci. Therefore pre-inhibition of RNAP I would prevent FUS binding on pre-rRNA and therefore focus formation but

inhibition of both polymerases at similar times would not prevent FUS binding pre-rRNA as it would not yet be depleted. This proposed pre-rRNA binding would be consistent with the subnucleolar localisation of FUS foci as well as the Triton extraction data and FUS foci being localised to nucleolar necklace in disrupted nucleoli. Experiments to address this hypothesis were planned and laid out in chapter 4.4 but abandoned for lack of time.

5. Initial investigation into the signalling dependence of FUS relocalisation

The relocation of FUS to the nucleolus appeared to be in response to inhibition of RNAP II. However it was unclear how this inhibition was signalled to FUS and given the varied roles of the protein in the cell there were many possibilities. Although the data did not suggest a role for DNA damage repair in the process of FUS focus formation many signalling molecules involved in this process, such as members of the PARP family (Mastrocola et al. 2013; Rulten et al. 2014) or of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of protein kinases (Gardiner et al. 2008; Deng et al. 2014; Britton et al. 2014), some of which are related to DNA damage repair, have been shown to have FUS as a target. Possible involvement of these pathways in the process of FUS relocalisation were therefore investigated first.

5.1 Testing the role of PARP on FUS focus formation using a PARP inhibitor

In multiple experiments it has been shown that FUS is recruited to sites of microirradiation and that this is dependent on PARP activity (Mastrocola et al. 2013; Britton et al. 2014), specifically PARP1 (Rulten et al. 2014). As this chemical dependence has been repeatedly reported whether or not FUS relocalisation into foci had a similar PARP-dependence was tested.

The PARP inhibitor Ku59848 was available and this allowed a simple method to investigate PARP-dependence of foci formation - pre-treating cells with the compound prior to CPT treatment to see if foci still form. Western blot controls to determine that the inhibitor was effective at preventing PARylation were set up.

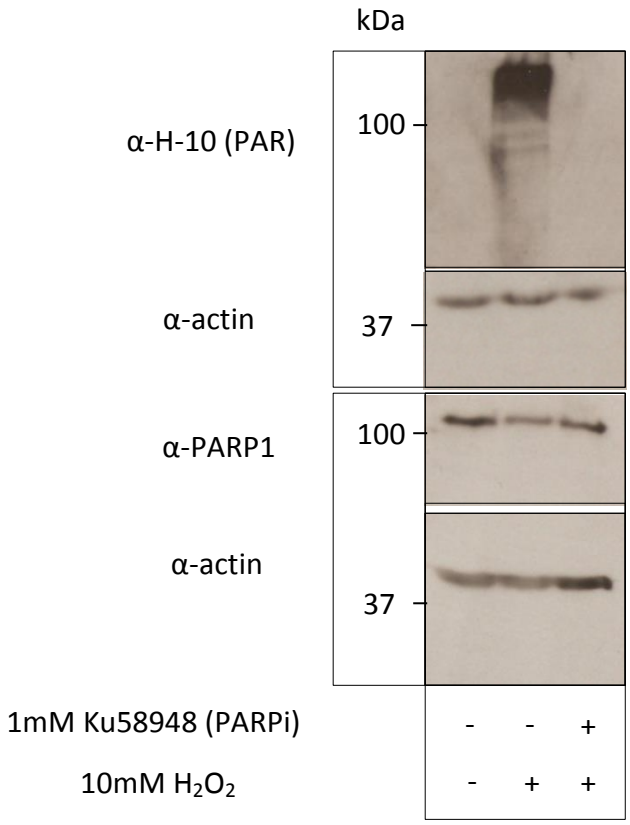


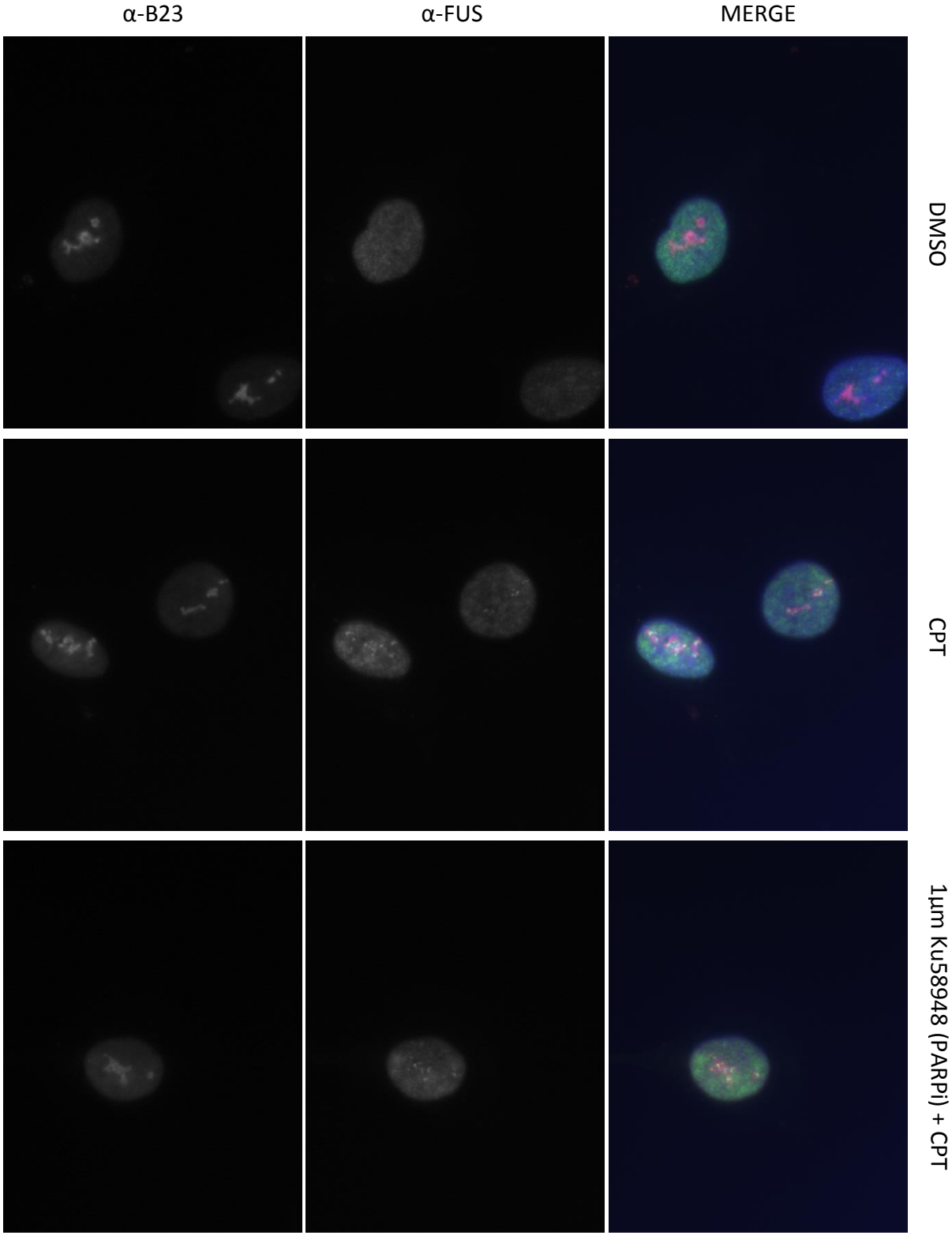
Figure 5.1a | **Western blots demonstrate that the PARP inhibitor Ku58948 is functional.** The same A549 lysates were run twice for this experiment - once probing for PAR (and actin) and the other for PARP1 (and actin). Hydrogen peroxide treated cells showed a PAR smear unless pre-treated with PARP inhibitor. Approximately $5x10^5$ cells per lane.

A549 cells were seeded and treated with Ku58948 or vehicle for an hour. Cells were then washed in PBS (to remove media) and treatmed for ten minutes in 10mM hydrogen peroxide, freshly diluted in PBS, or the equivalent volume of PBS. Due to the rapid kinetics of PARP1 activity even a brief removal of PARP inhibitor can result in PAR synthesis so to avoid false negative data all solutions of PBS and hydrogen peroxide used contained PARP inhibitor or the equivalent volume of vehicle.

Hydrogen peroxide treated lysate produced a smear of PAR on the gel (Fig 5.1a), which was absent in lysate from in cells pre-treated with PARP inhibitor prior to peroxide treatment. Protein levels of PARP1 between samples were also tested and found approximately equivalent.

It was concluded from this that Ku58948 was an effective PARP inhibitor at the concentrations and timings used. This inhibitor was therefore used for pre-inhibition of patient and control fibroblasts prior to CPT treatment.

WT fibroblasts



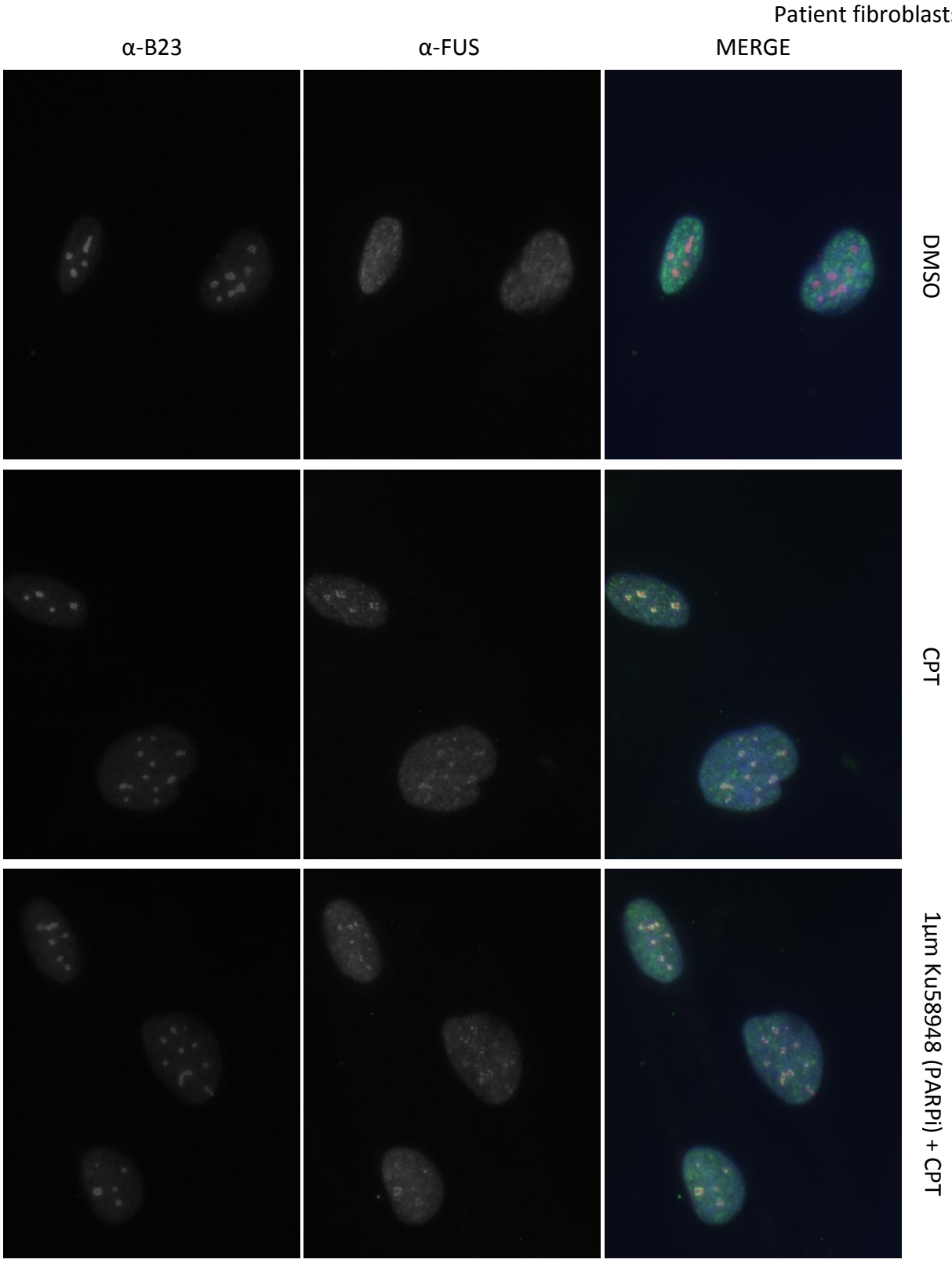


Figure 5.1b | **PARP inhibition does not affect FUS relocalisation.** Fibroblasts pre-treated with PARPi prior to CPT treatment did not show any difference to fibroblasts treated with CPT and an equivalent volume of vehicle.

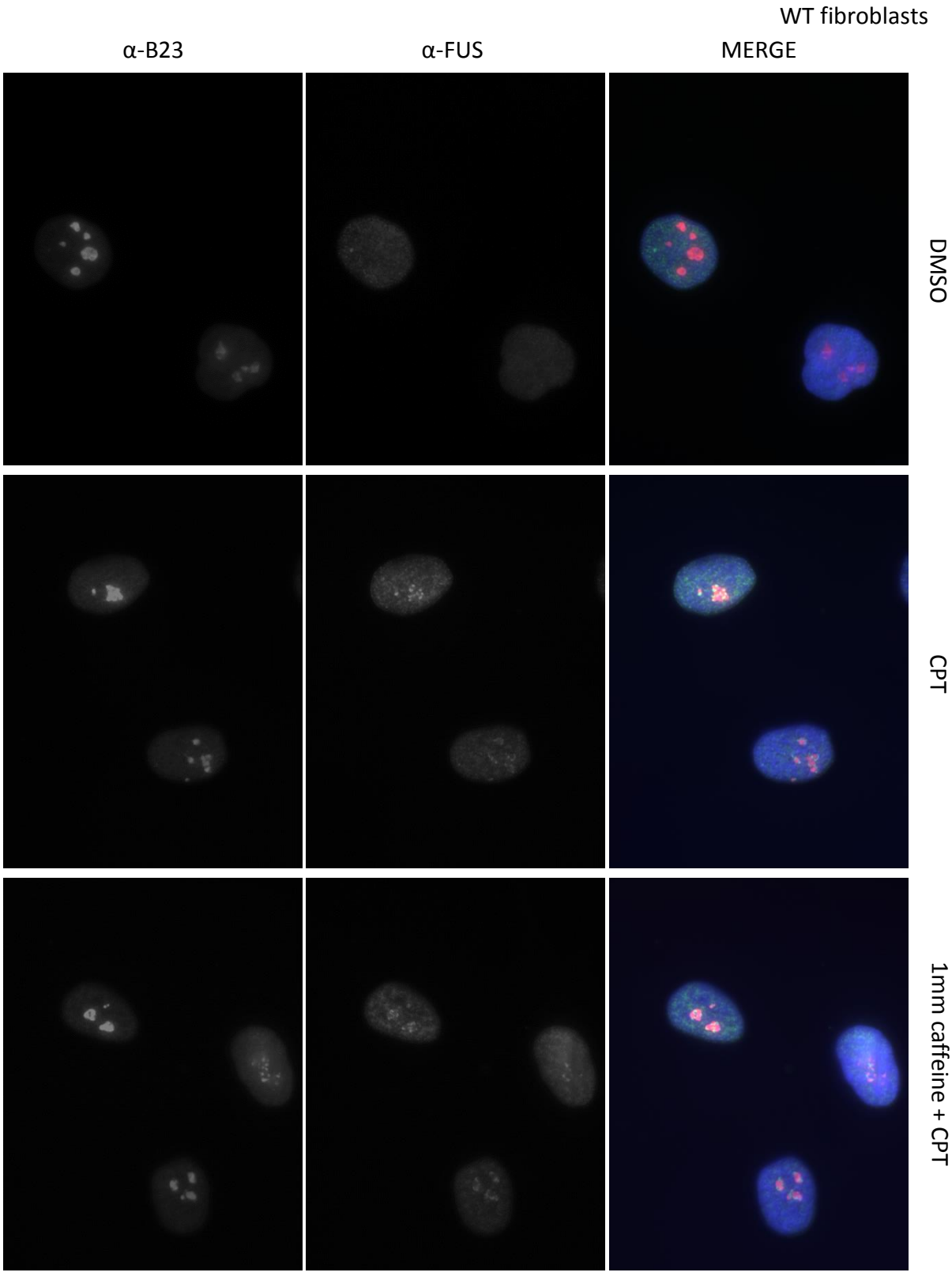
Cells were pre-treated with 1µm Ku58948 for one hour prior to treatment with CPT (with the PARP inhibitor remaining in the media), fixation and IF.

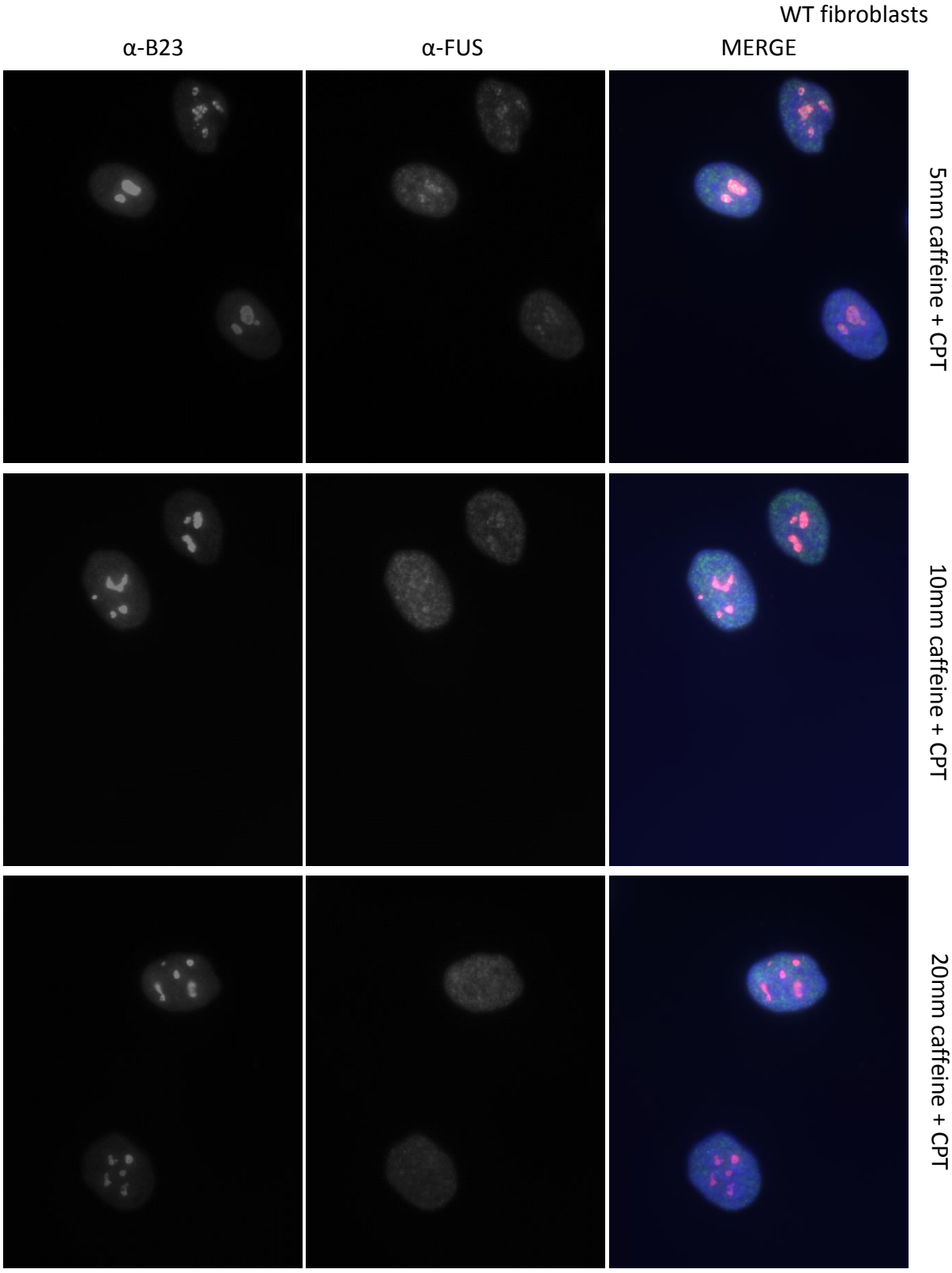
PARP inhibitor pre-treatment did not appear to induce any visible change in foci formation (Fig 5.1b) - showing that the foci do not require PARP activity to form. This lends credence to the idea that the FUS foci do not represent the same cellular event as the recruitment of FUS to laser tracks.

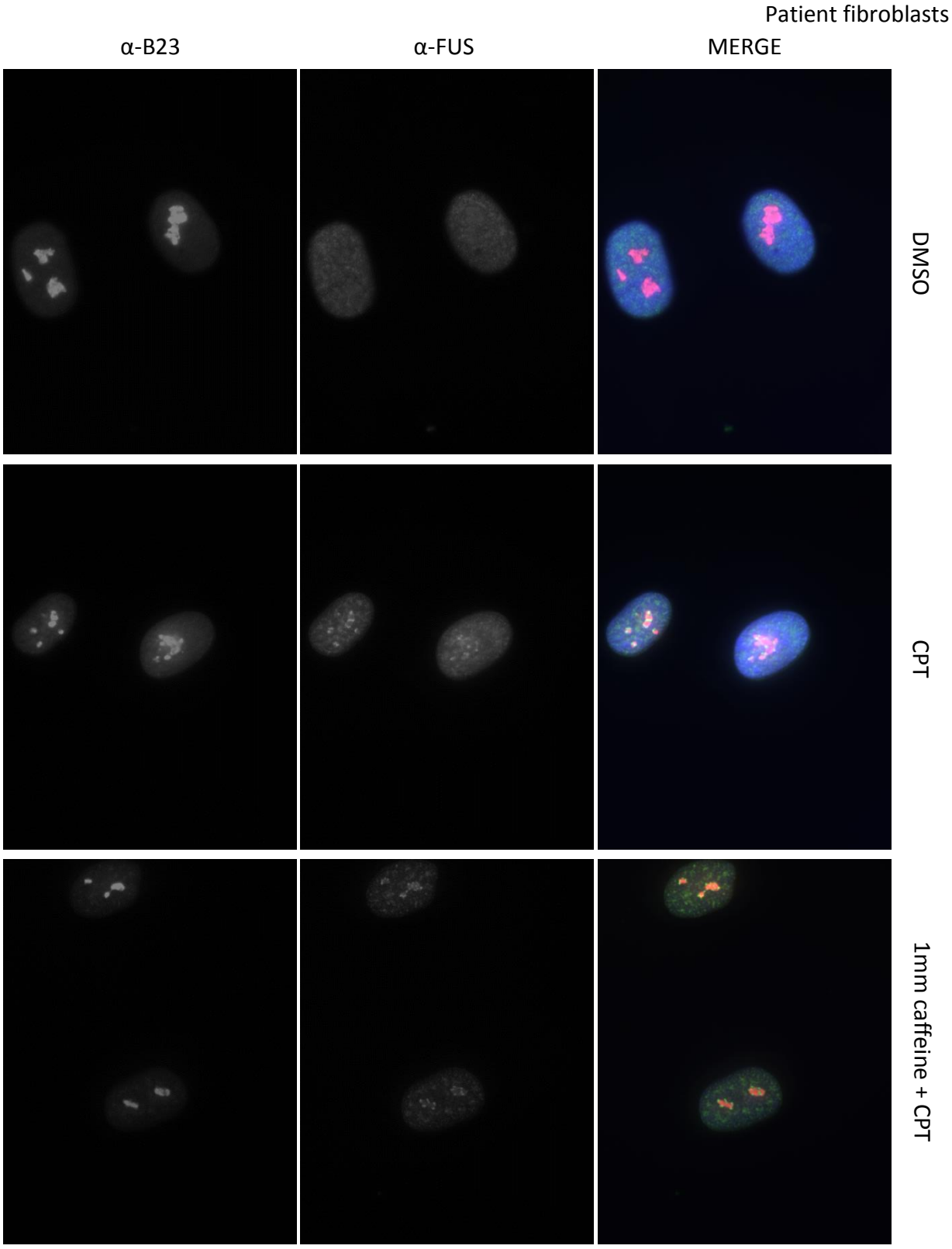
5.2 The impact of caffeine on FUS focus formation

FUS has been implicated in the past as a target for members of the PIKK such as ataxia-telangiectasia mutated (ATM) (Gardiner et al. 2008) and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (Deng et al. 2014) - and so these proteins were considered potential candidates for signalling FUS relocalisation and were considered for use in inhibitor studies.

As there are multiple members of this family a broad inhibitor of the family was desirable for use in early experiments. Caffeine inhibits members of the PIKK family in the mM range of concentrations (Blasina et al. 1999; Sarkaria et al. 1999; Block et al. 2004; Reinke et al. 2006) and so was suited for use as a pan-PIKK inhibitor.


WT fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
1mm caffeine + CPT


WT fibroblasts
α-B23
α-FUS
MERGE
5mm caffeine + CPT
10mm caffeine + CPT
20mm caffeine + CPT


Patient fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
1mm caffeine + CPT

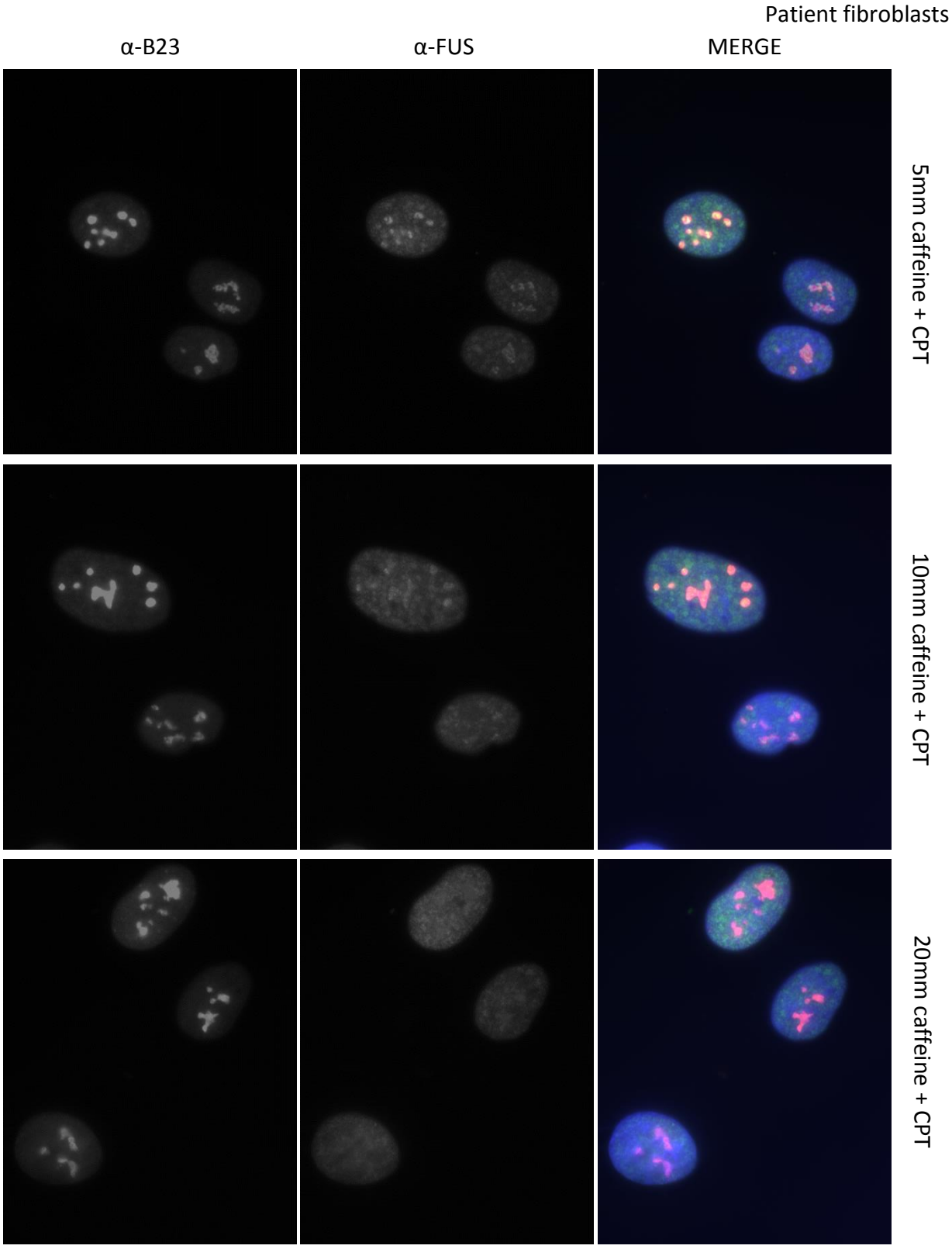


Figure 5.2 | **FUS foci formation is blocked by high concentrations of caffeine.** Pre-treatment of fibroblasts with caffeine before CPT treatment resulted in abolition of foci formation at 20mM caffeine and in smaller foci in fewer cells at 10mM caffeine.

Cells were pre-treated with caffeine for an hour before CPT treatment, with caffeine remaining in the media.

Pre-treatment of cells with caffeine showed no effect up to 5 mM caffeine, but 10mM caffeine resulted in fainter (and smaller) foci than in cells which had not been caffeine pre-treated (and abolition of FUS foci in some cells) and 20mM caffeine appeared to totally abolish FUS foci formation (Fig 5.2). This effect of 20mM caffeine was also observed in WT CD1 mouse cortical neurons (Ryan Green, personal communication).

Doxycycline-induced LAP-FUS Hela were set up for a similar experiment using only the 20mM caffeine pre-treatment concentration, pre-treatments with the chemically similar molecule theophylline were also set up. The theophylline was introduced to the cells at the same concentration and for the same length of time as caffeine. Additionally these pre-treatment experiments were repeated in the LAP-TDP43 Hela line to see if the nucleolar relocalisation of LAP-tagged TDP43 could also be blocked by caffeine pre-treatment.

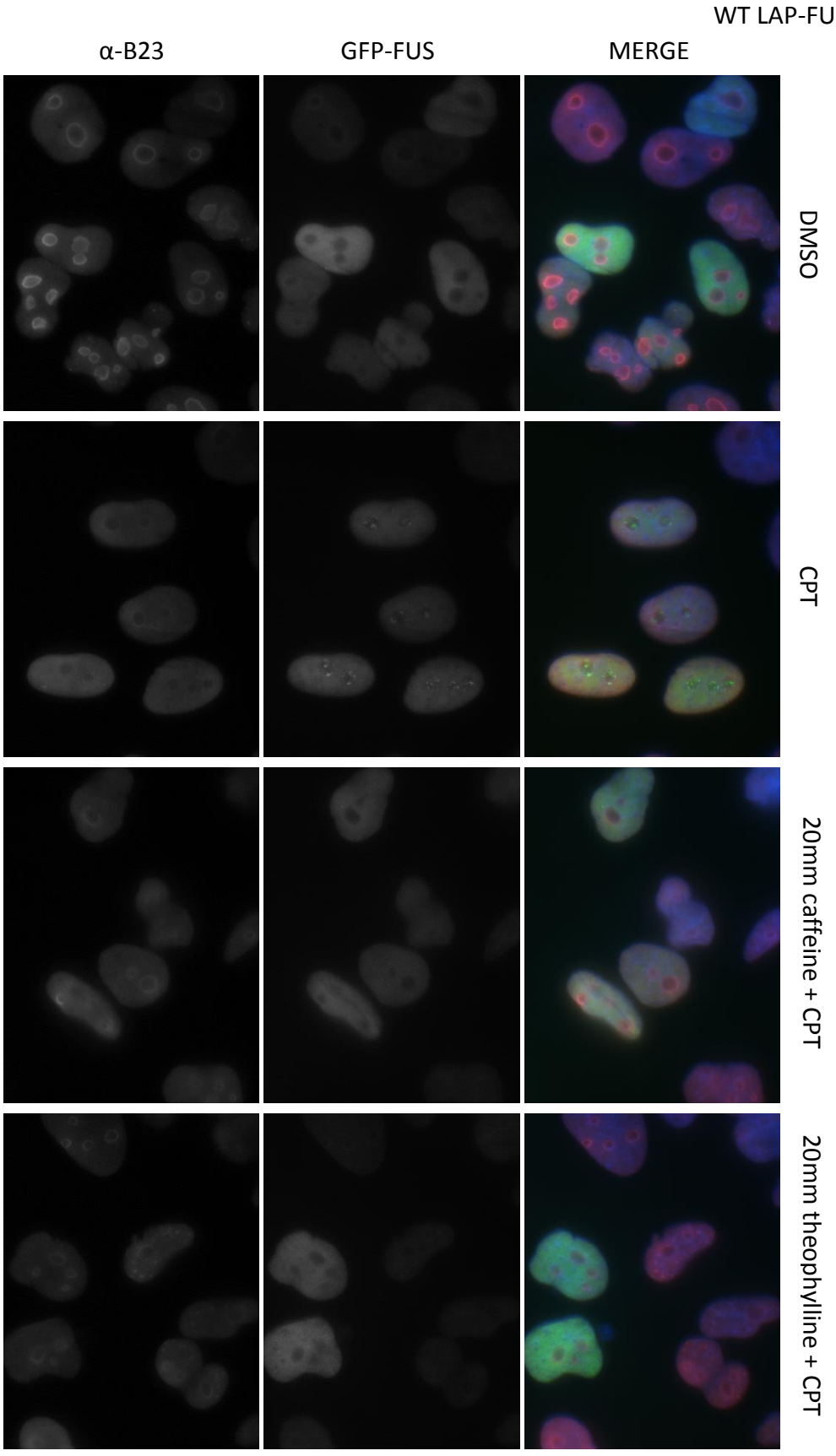


Figure 5.3 | **Caffeine and theophylline both prevent FUS foci formation in LAP-FUS Helas.** Pre-treatment of doxycycline-induced LAP-FUS Helas with either methylxanthine prevented CPT-induced FUS relocalisation.

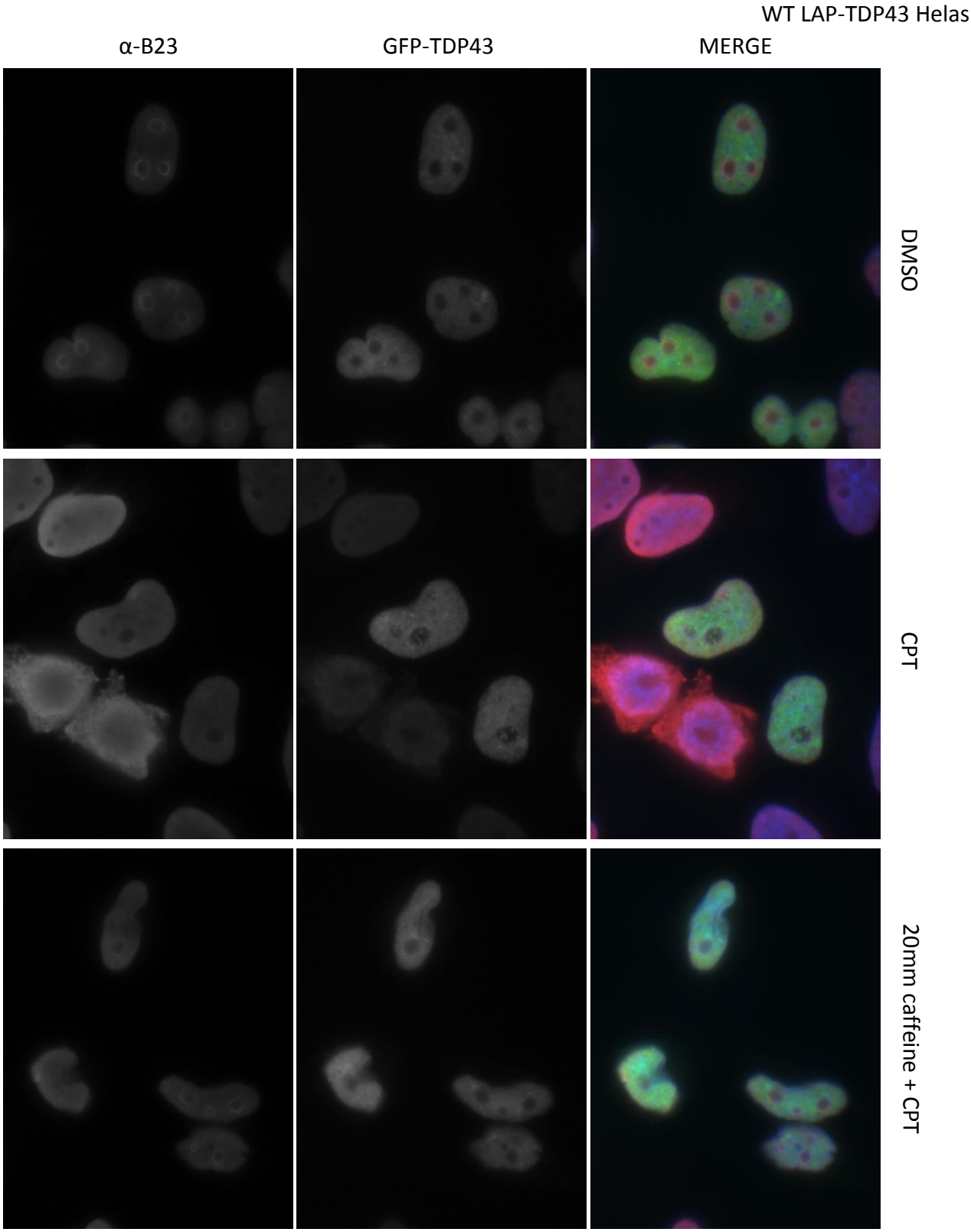


Figure 5.4a | **GFP-TDP43 foci are abolished by caffeine or theophylline pre-treatment. (caffeine)** Pre-treatment of LAP-TDP43 Helas with caffeine prevented CPT-induced TDP43 relocalisation.

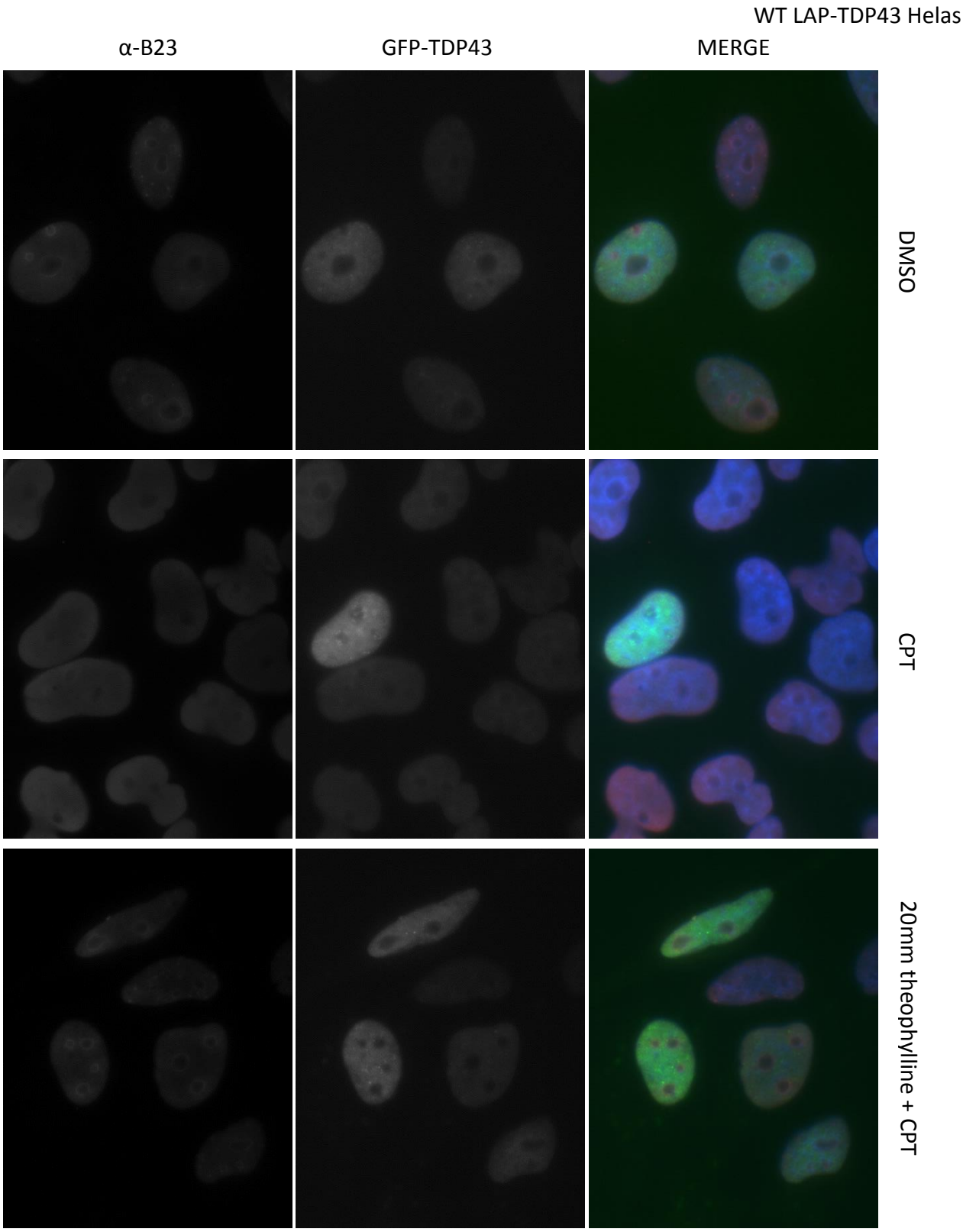


Figure 5.4b | **GFP-TDP43 foci are abolished by caffeine or theophylline pre-treatment (theophylline).** Pre-treatment of LAP-TDP43 Helas with theophylline prevented CPT-induced TDP43 relocalisation.

In both the LAP-FUS (Fig 5.3) and LAP-TDP43 Helas (Fig 5.4a) 20mM caffeine totally abolished foci formation. Treatment with theophylline produced identical results in both LAP-FUS (Fig 5.3) and LAP-TDP43 (Fig 5.4b) Helas. These data in combination suggested a possible shared signalling dependence for FUS and TDP43 foci and that this signalling may be PIKK mediated.

5.3 Investigating the role of PIKKs in caffeine-induced inhibition of FUS focus formation

The previous data established that caffeine could prevent foci formation and as caffeine was intended to act as a pan-PIKK inhibitor further experiments were designed to establish which PIKK was of relevance to this process. The PIKK family consists of six family members: ATM, ataxia- and Rad3-related (ATR), DNA-PKcs, mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia (SMG1) and transformation/transcription domain-associated protein (TRRAP). TRRAP, although in the PIKK family, does not retain any kinase activity and so was excluded from further investigation (Paull 2015).

In order to determine which PIKK was involved in the relocalisation process an array of PIKK inhibitors were utilised to pre-incubate patient and control fibroblasts (for one hour): initially 10μM Ku55933, an ATM inhibitor; 10μM ATR Kinase Inhibitor II, an ATR inhibitor; and 10μM NU7441, a DNA-PKcs inhibitor. The concerted activity of multiple PIKK family members have been associated with relocalisation of FUS from sites of DNA damage in a previous study (Britton et al. 2014), so combinatorial effects were also considered.

The functionality of these inhibitors were also tested. A549 cells were pre-incubated with the PIKK inhibitors for the same time and then subjected to treatment with the DNA damaging agents listed in Table 5.1 before being harvested. The lysates were then examined by western blot to check the phosphorylation status of known targets of the proteins - Chk2 for ATM (Matsuoka et al. 1998), Chk1 for ATR (Liu et al. 2000) and RPA32 for DNA-PKcs (Shao et al. 1999).

Inhibitor tested	Damage source	Damage time
Ku55933 (ATMi)	5 Gy IR	Cells rested for 30 minutes after IR
ATR Kinase Inhibitor II (ATRi)	2mM hydroxyurea	16 hours
NU7441 (DNA-PKi)	4μM CPT	45 minutes

Table 5.1 | **Damage sources used to test PIKK inhibitors.**

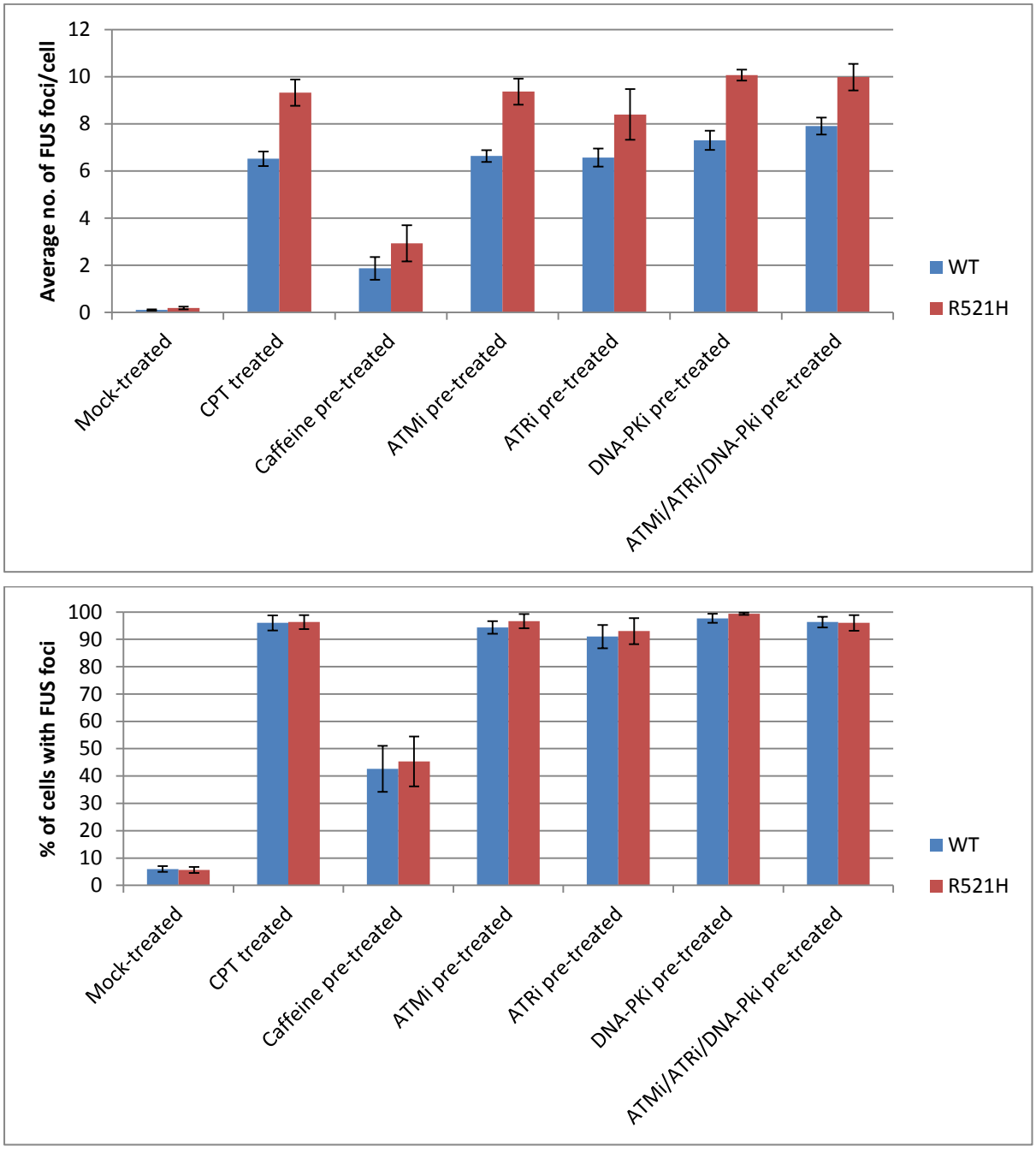
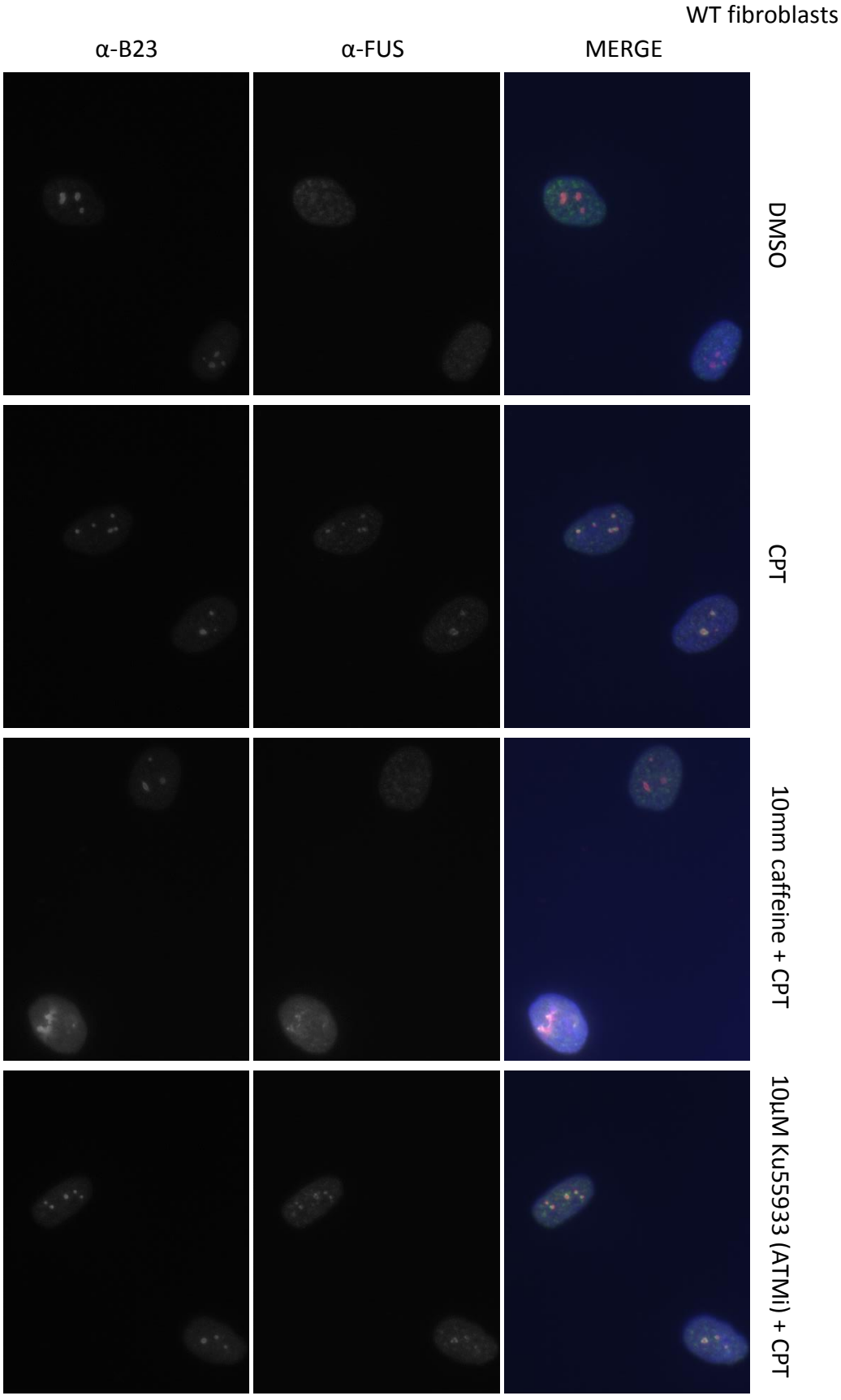
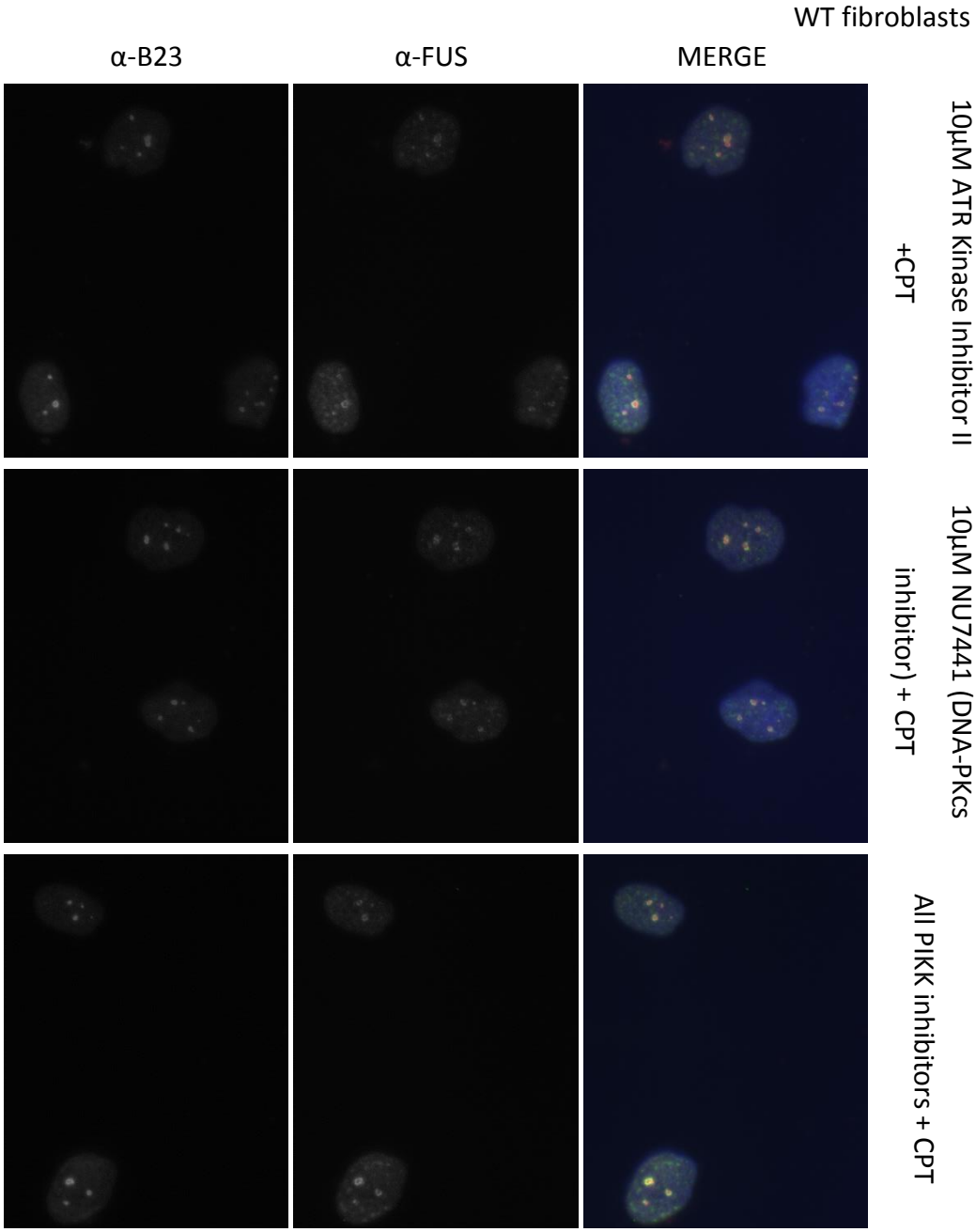
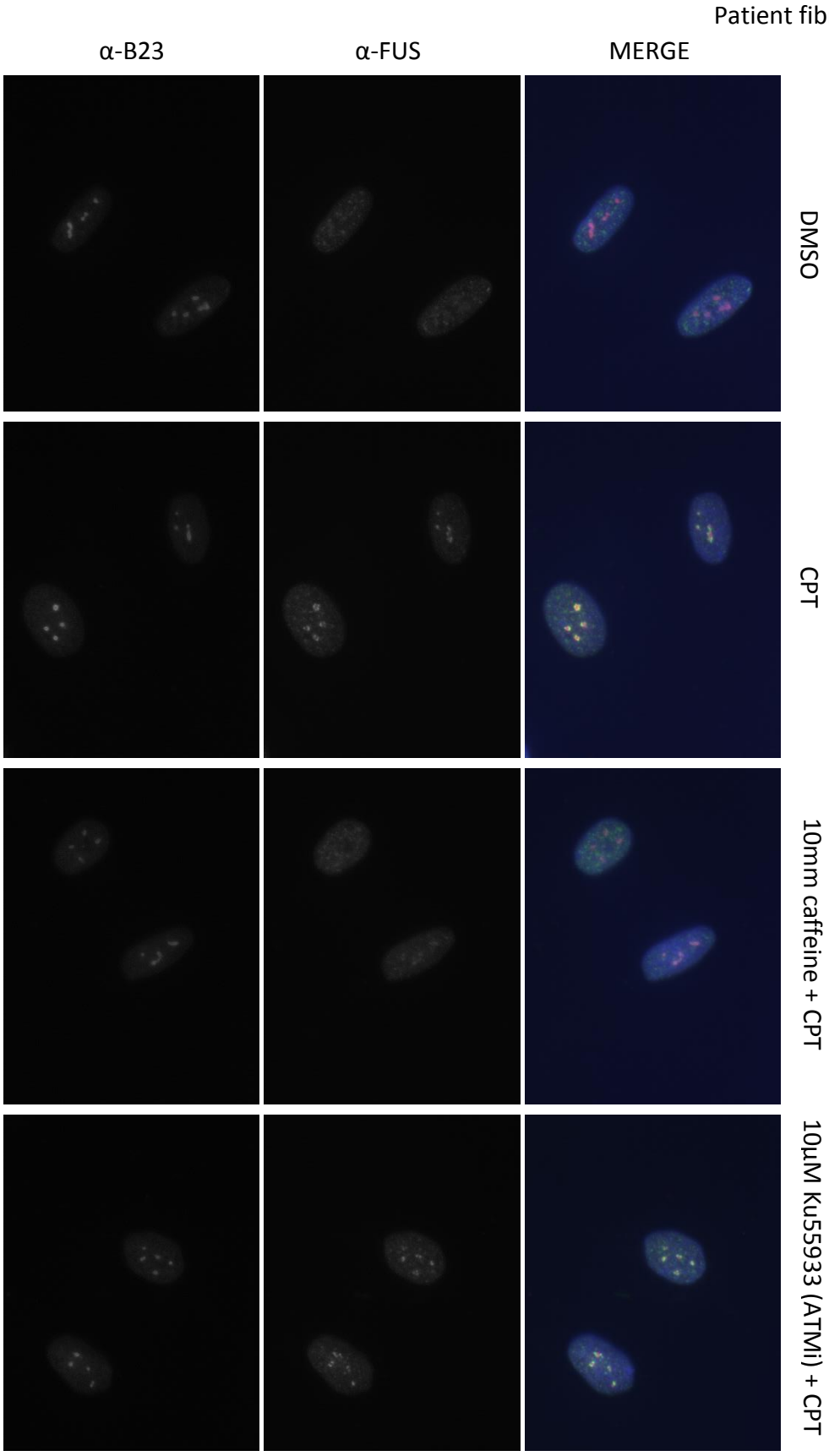


Figure 5.5a | **FUS foci still form in the presence of PIKK inhibitors.** Pre-treatment of patient or control fibroblasts with ATM, ATR or DNA-PKcs inhibitors (alone or all three in combination) fails to produce any reduction in FUS foci. n=6.


WT fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
10mm caffeine + CPT
10μM Ku55933 (ATMi) + CPT


WT fibroblasts
α-B23
α-FUS
MERGE
10μM ATR Kinase Inhibitor II +CPT
10μM NU7441 (DNA-PKcs inhibitor) + CPT
All PIKK inhibitors + CPT


Patient fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
10mm caffeine + CPT
10μM Ku55933 (ATMi) + CPT

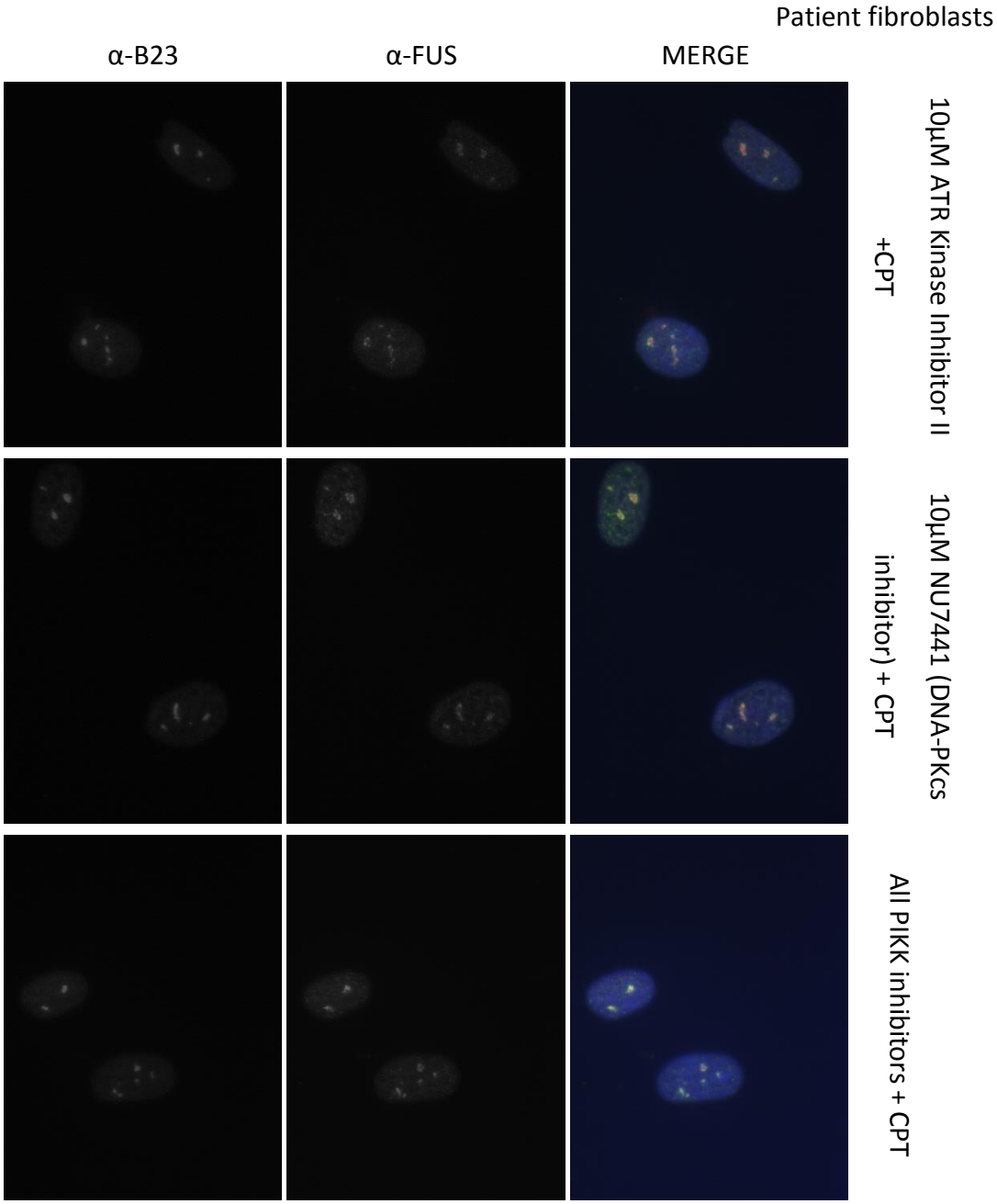


Figure 5.5b | **FUS foci still form in the presence of PIKK inhibitors.** Representative images of cells counted for figure 5.5a. "All PIKK inhibitors" refers to a combination of Ku55933, ATR kinase inhibitor II and NU7441.

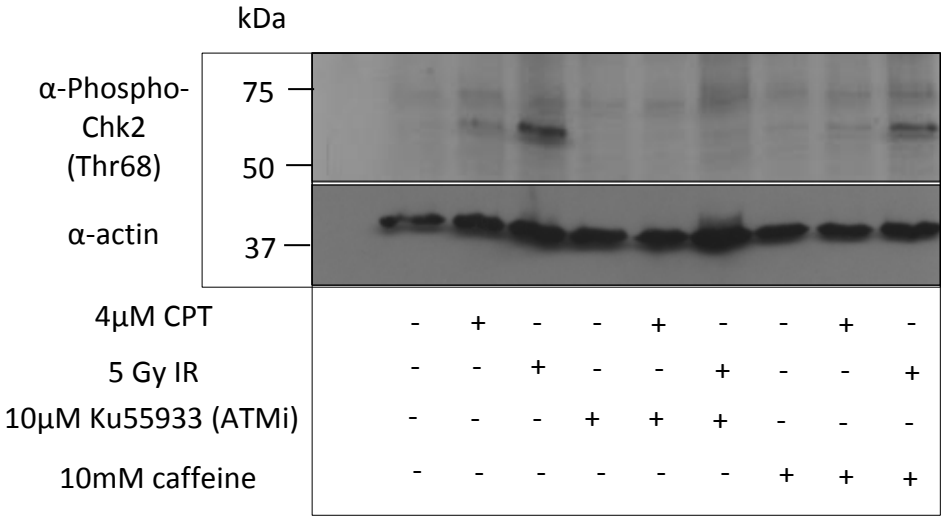


Figure 5.5c | **Western blots showing effects of ATM inhibitor Ku55933.** ATM inhibitor prevents phosphorylation of Chk2 at Thr68 and is more effective at doing so than 10mM caffeine. Approximately $1x10^6$ cells per lane.

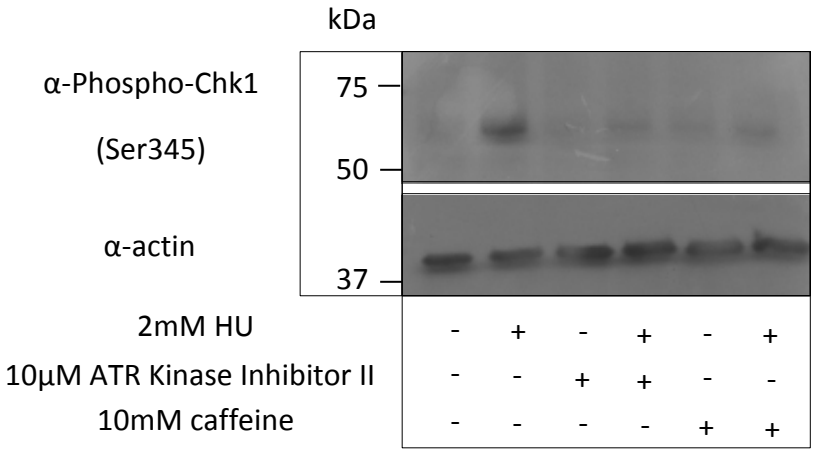


Figure 5.5d | **Western blots showing effects of ATR Kinase Inhibitor II.** ATR inhibitor prevents phosphorylation of Chk1 at Thr68 as well as 10mM caffeine does. Approximately $5x10^5$ cells per lane.

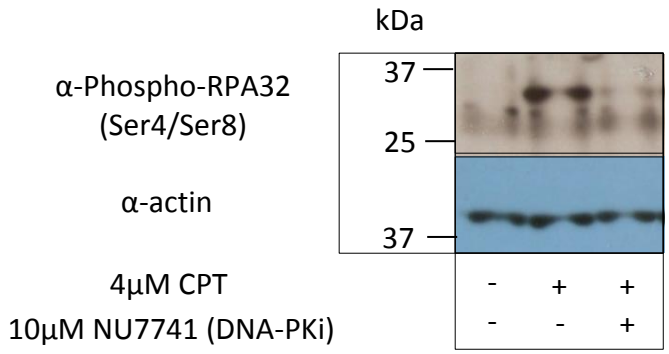


Figure 5.5e | **Western blots showing effects of NU7441 (DNA-PKi).** DNA-PKcs inhibitor prevents phosphorylation of RPA32 at Ser4 and Ser8. Approximately $5x10^5$ cells per lane.

As expected caffeine dramatically reduced the number of cells that contained FUS foci but unexpectedly none of PIKK inhibitors appeared to affect foci formation in any detectable manner even in combination with each other (Fig 5.5a). Furthermore it was also clear that the inhibitors were indeed functioning as they all prevented phosphorylation of their respective targets (Fig 5.5c-e). Therefore it was concluded that FUS focus formation is independent of these three DNA damage related PIKKs.

As the three PIKKs so far inhibited did not affect FUS focus formation it was decided to investigate the remaining two catalytially active members of the family - once again by one hours pre-treatment with inhibitors prior to CPT treatment and IF. mTOR is associated with transcriptional regulation (Yecies & Manning 2011) and SMG1 is involved in nonsense-mediated RNA decay (Yamashita et al. 2001) (amongst other cellular processes (Gewandter et al. 2011)) so their signalling to the nucleic acid binding protein FUS would not be illogical.

The specific mTOR inhibitor Ku0063794 (García-Martínez et al. 2009) was used at 1μM for pre-treatment experiments. However a specific SMG1 inhibitor could not be obtained and so wortmannin was used instead (at 200nM). Wortmannin is a broad acting drug which inhibits SMG1 but also the p110α catalytic subunit of some members of the phosphatidylinositol 3-kinase (PI3K) family of kinases (Wymann et al. 1996; Yamashita et al. 2001). As such 10μM PI103 was also used, as it is an inhibitor of p110 PI3K inhibitor subunits (Raynaud et al. 2009). Therefore should wortmannin, but not PI103 or Ku0063794, prevent foci formation then that could have indicated SMG1 involvement and future experiments would be designed to verify its role.

As before the activity of the remaining PIKK inhibitors were assayed in A549 cells. The inhibitor of mTOR was assayed for its ability to prevent phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Brunn et al. 1996); wortmannin was assayed for its ability to inhibit SMG1 by measuring phosphorylation of p53 (Gewandter et al. 2011); and PI103 was assayed for its ability to inhibit p110α by measuring the phosphorylation status of retinoblastoma protein (Rb) (Sivertsen et al. 2006). The treatments used to assay the function of these inhibitors are listed in the table below - all inhibitors were applied one hour before the treatments listed in the table:

Inhibitor tested	Treatment	Treatment time
Ku0063794 (mTORi)	400nM insulin	15 minutes
Wortmannin	2 Gy IR	Cells rested for 30 minutes
PI103	serum starvation (0.1% FcS)	Four days

Table 5.2 | Treatments used to test remaining PIKK inhibitors.

An additional control was also performed where NM720 hTERT cells, severely deficient in DNA-PKcs activity (Woodbine et al. 2013), were pre-treated with either ATM inhibitor and ATR inhibitor; or ATM inhibitor, ATR inhibitor, mTOR inhibitor, wortmannin and PI103, prior to CPT treatment. They were also subject to IF by two FUS antibodies - the Novus antibody and a Sigma antibody.

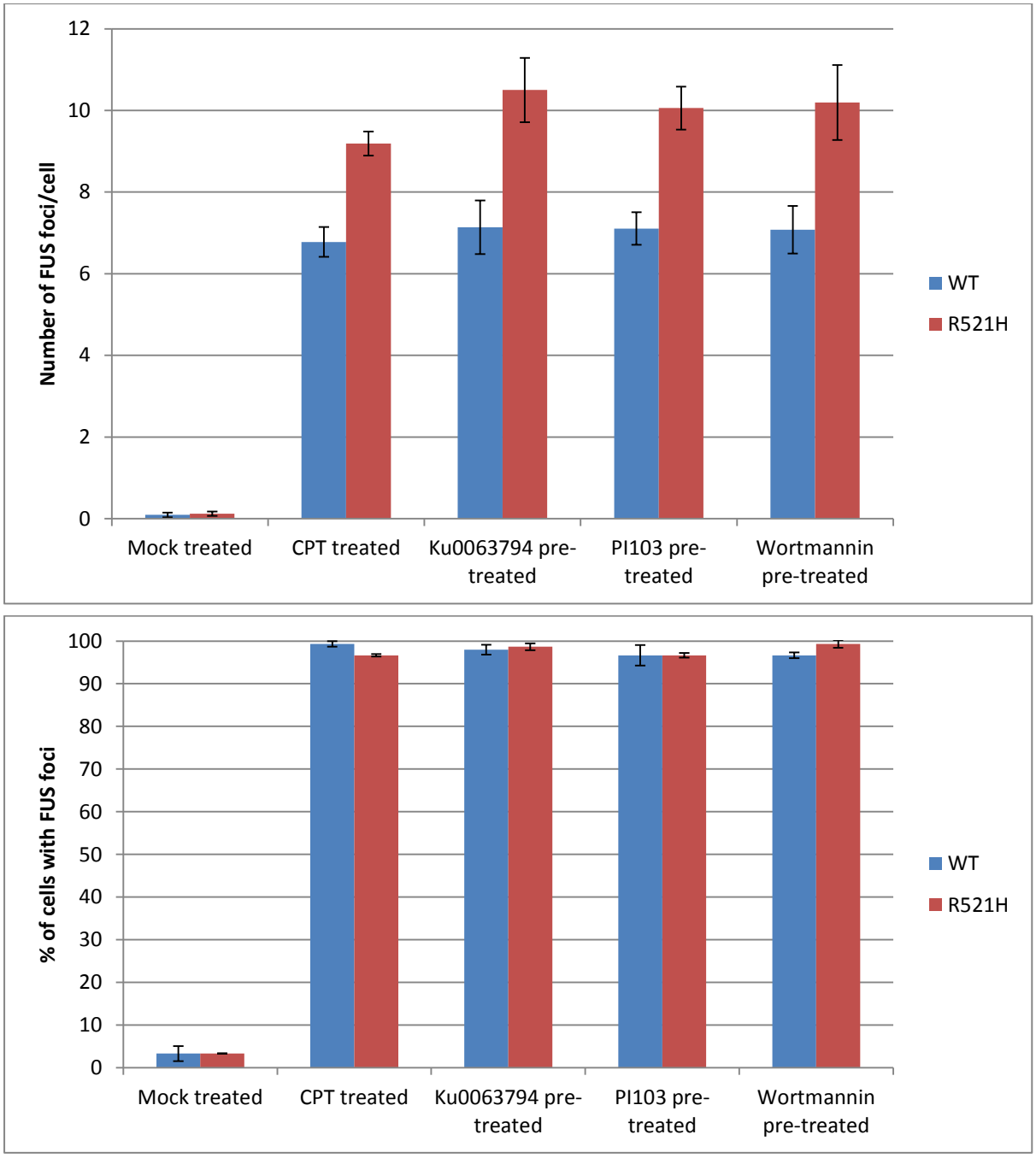
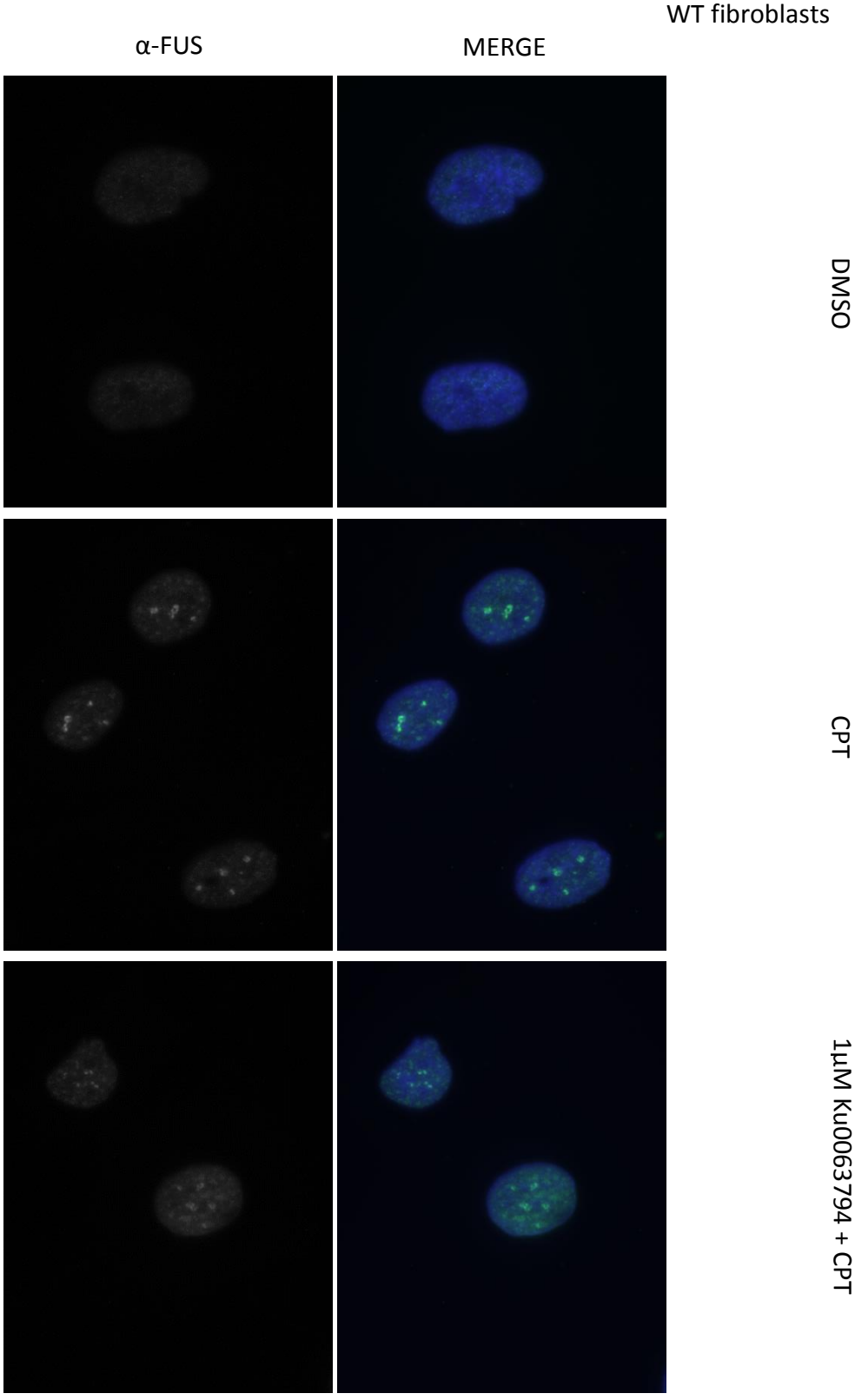


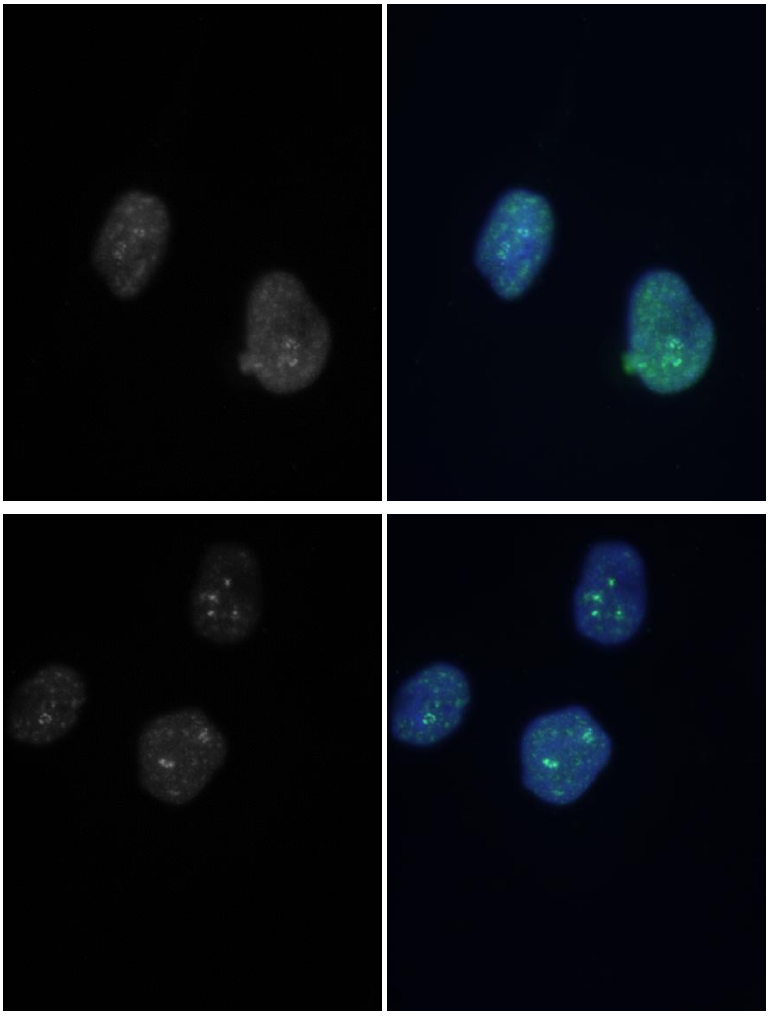
Figure 5.6a | **FUS foci still form in the presence of inhibitors of remaining PIKKs (mTOR, SMG1).** Pre-treatment of cells with inhibitors targeting mTOR and SMG1 produced no effect on foci counts. These was no statistically significant difference in foci count between pre-treated and non pre-treated cells (by one-tailed ANOVA at 95% confidence intervals) for either WT or R521H fibroblasts. n=3.


WT fibroblasts
α-FUS
MERGE
DMSO
CPT
1μM Ku0063794 + CPT

WT fibroblasts

α-FUS

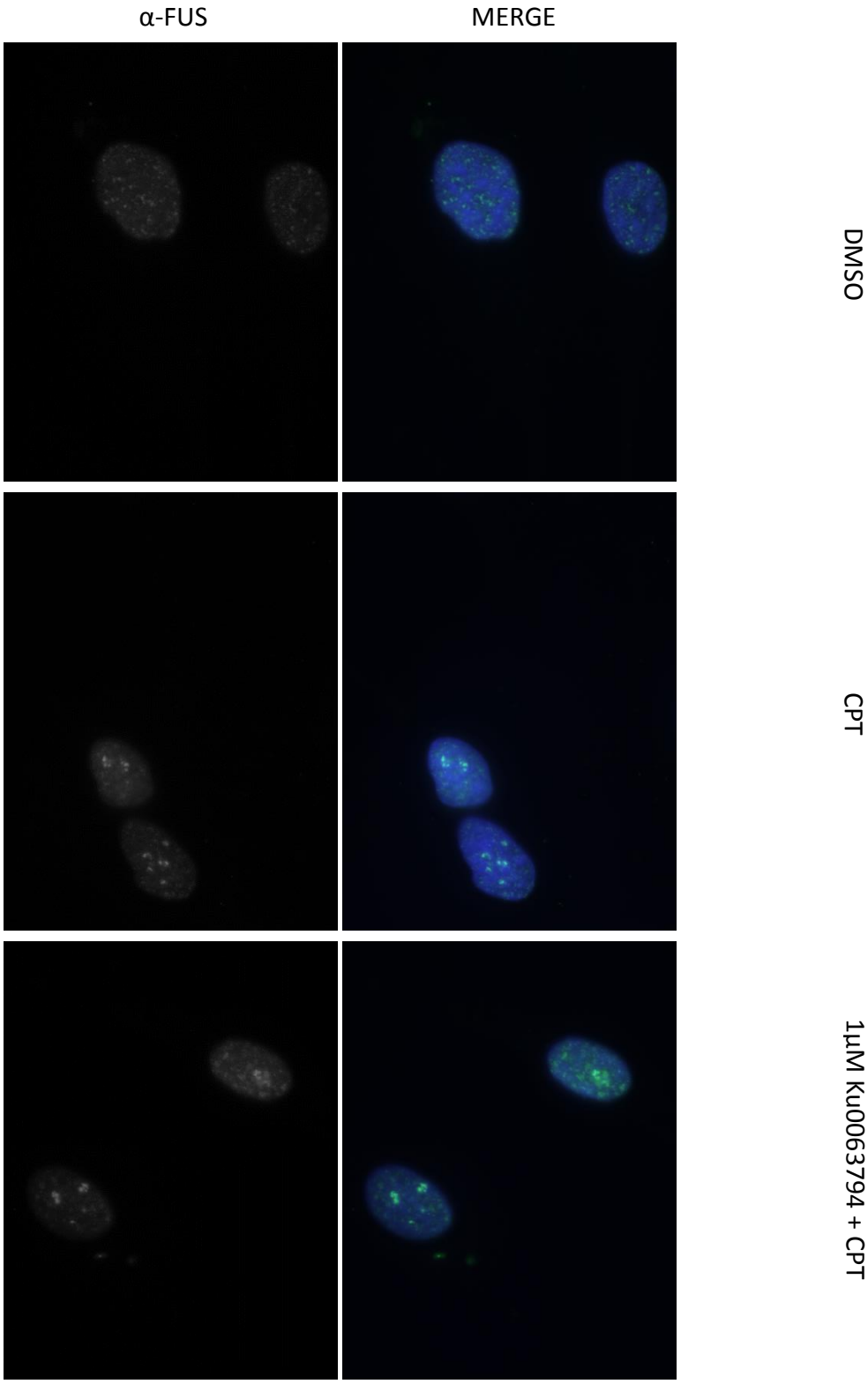
MERGE



10μM PI103 + CPT

200nM wortmannin + CPT

Patient fibroblasts



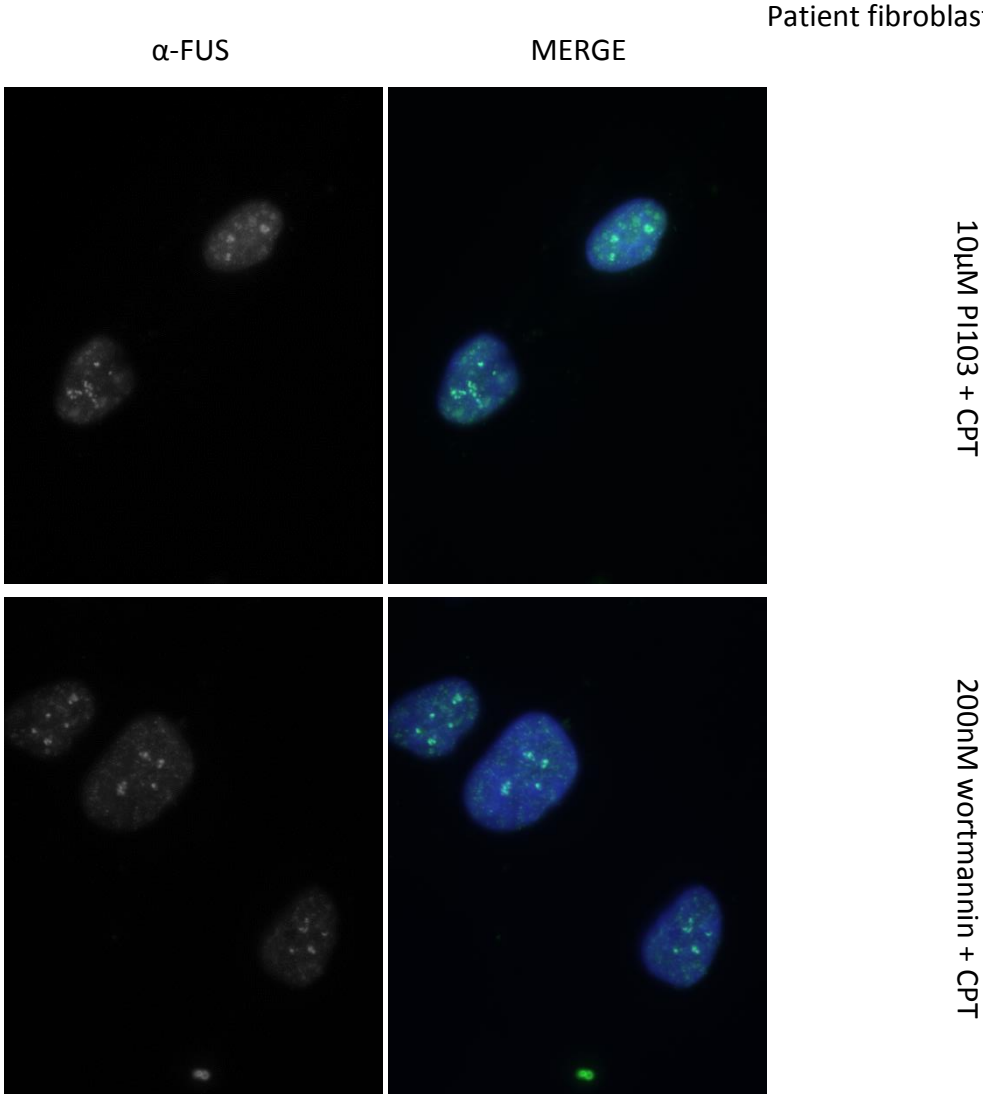


Figure 5.6b | **FUS foci still form in the presence of inhibitors of remaining PIKKs (mTOR, SMG1).** Representative images of cells used in counts for figure 5.6a.

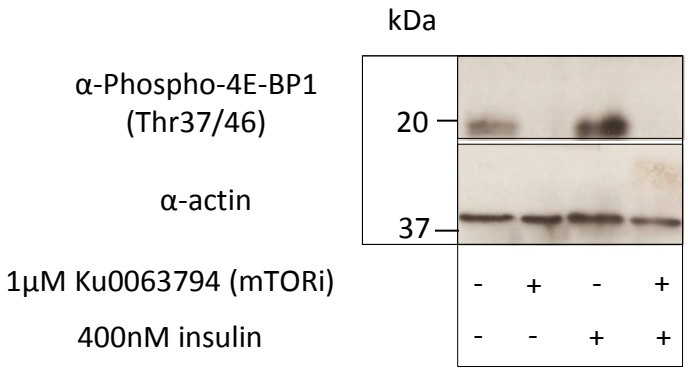


Figure 5.6c | **Western blots showing effects of Ku0063794 (mTORi).** mTOR inhibitor prevents phosphorylation of 4E-BP1 at Thr37 and Thr46. Approximately $5x10^5$ cells per lane.

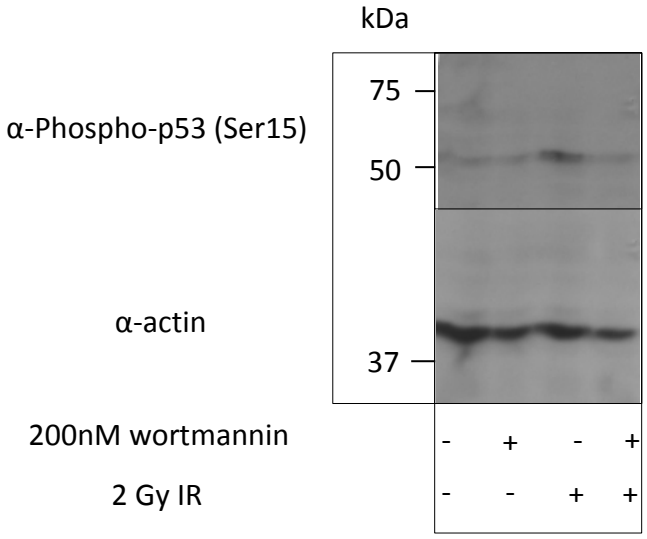


Figure 5.6d | **Western blots showing effects of wortmannin.** Wortmannin prevents phosphorylation of p53 at Ser15. This demonstrates its ability to inhibit SMG1. Approximately $5x10^5$ cells per lane.

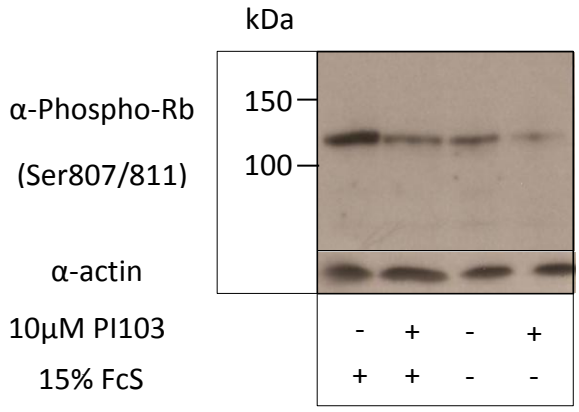
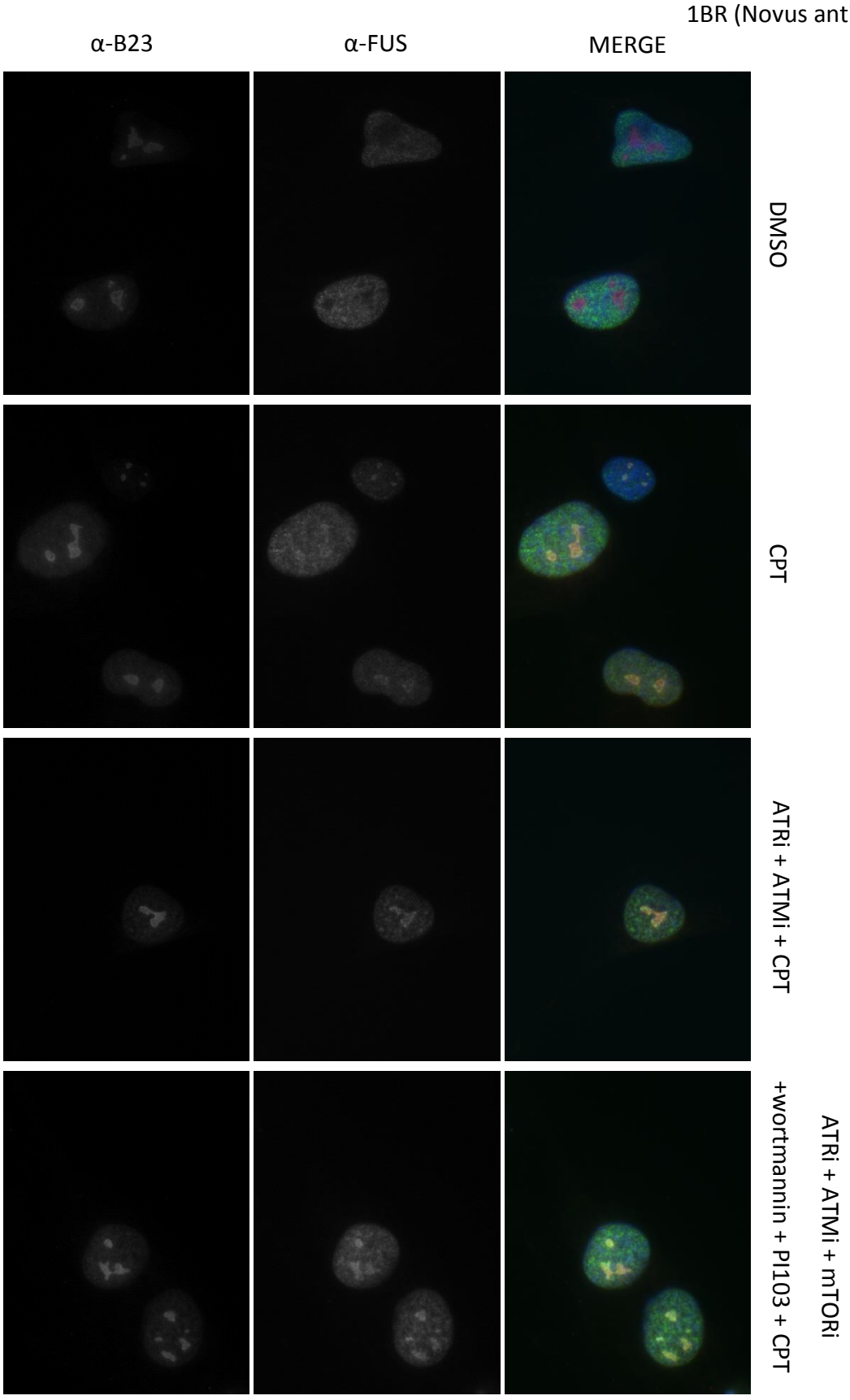
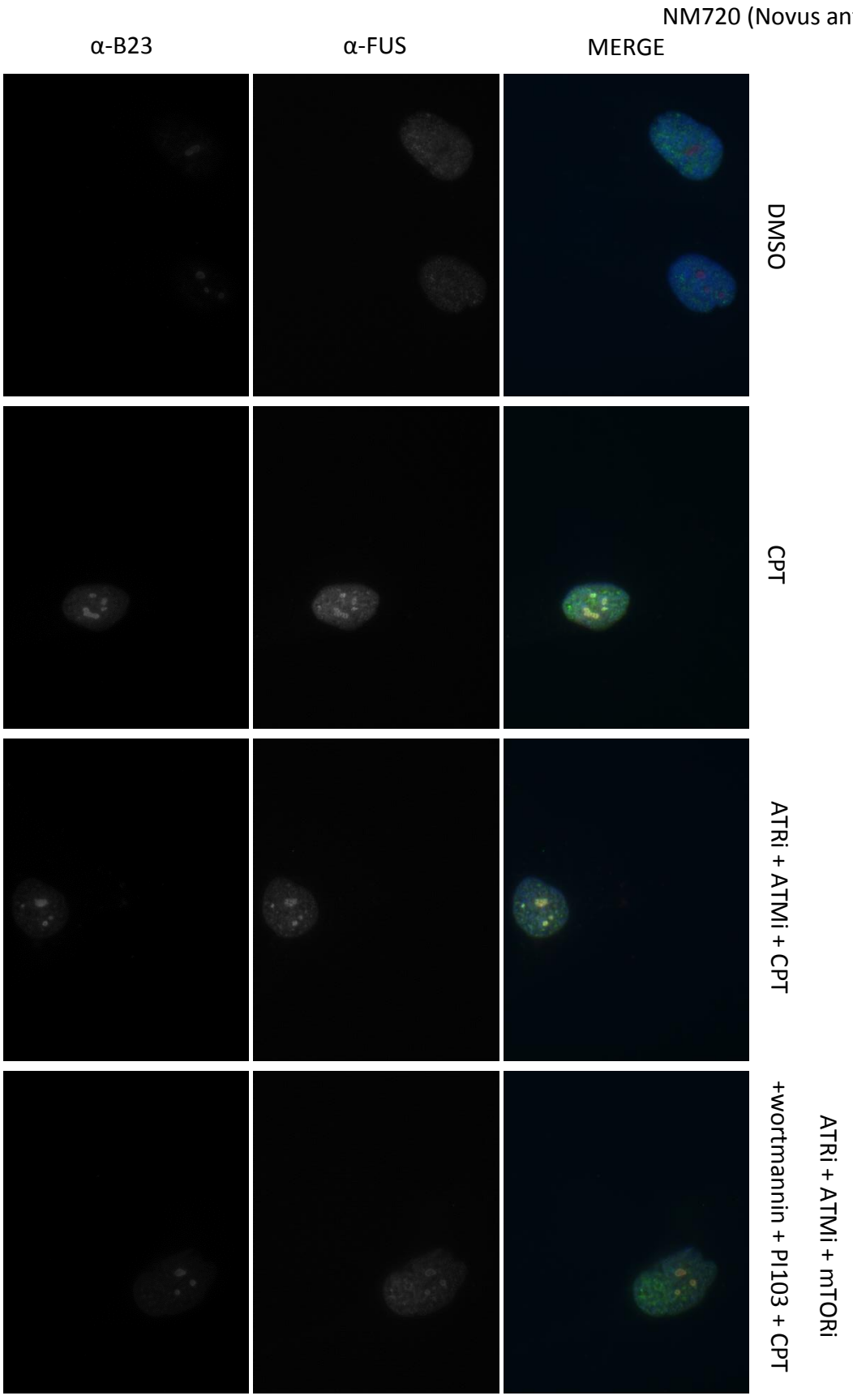
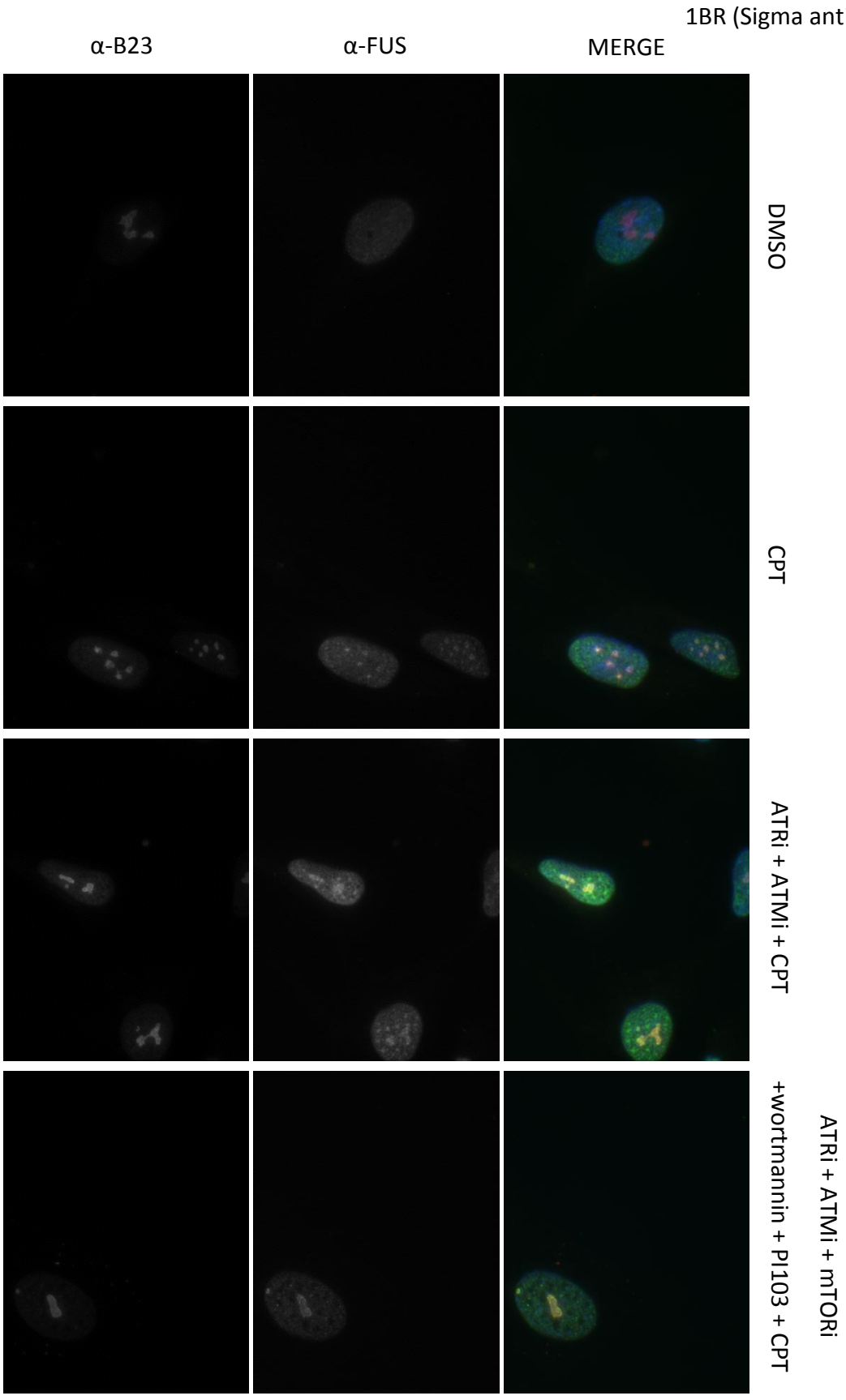


Figure 5.6e | **Western blots showing effects of PI103.** PI103 greatly reduces phosphorylation of Rb at Ser807 and Ser811, demonstrating its inhibition of PI3Ks. Approximately $5x10^5$ cells per lane.


1BR (Novus antibody)
α-B23
α-FUS
MERGE
DMSO
CPT
ATRi + ATMi + CPT
ATRi + ATMi + mTORi
+wortmannin + PI103 + CPT


NM720 (Novus antibody)
α-B23
α-FUS
MERGE
DMSO
CPT
ATRi + ATMi + CPT
ATRi + ATMi + mTORi
+wortmannin + PI103 + CPT


1BR (Sigma antibody)
α-B23
α-FUS
MERGE
DMSO
CPT
ATRi + ATMi + CPT
ATRi + ATMi + mTORi
+wortmannin + PI103 + CPT

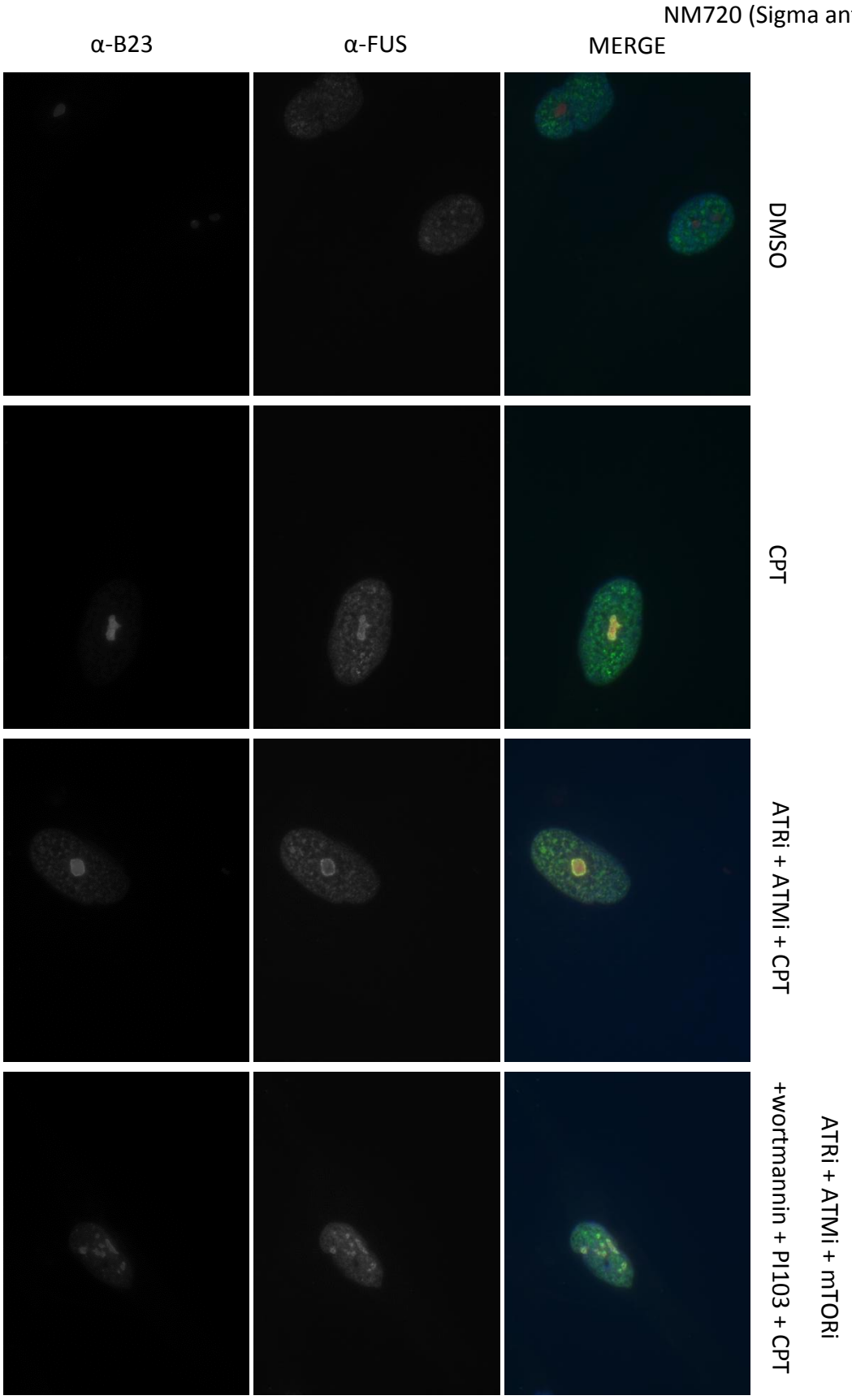
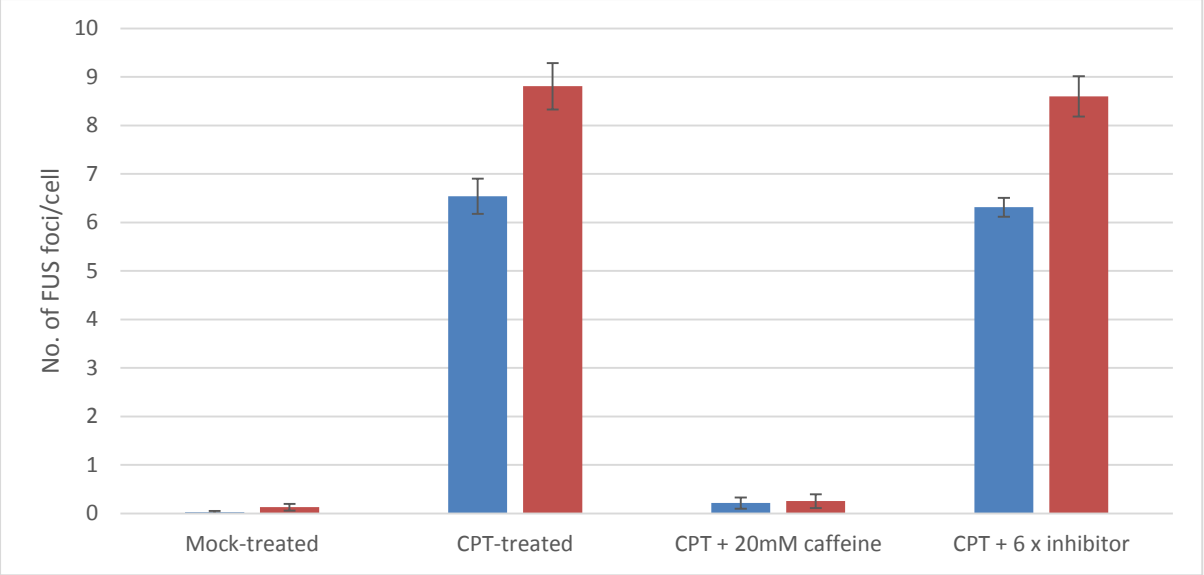


Figure 5.6f | **FUS foci form in NM720 cells (minimal PK activity) even in the presence of inhibitors for all other PIKKs.** FUS foci form similarly in both in 1BR cells and NM720 cells, with or without PIKK inhibitors. Tested with two anti-FUS antibodies.

None of these three chemicals, nor their action in combination, appeared to affect FUS relocalisation (Fig 5.6a). Once again all inhibitors were shown to be functional (Fig 5.6c-e) and the additional control experiment in the DNA-PKcs deficient NM720 cells showed that both they and the control 1BR cells produced FUS foci and that PIKK inhibitors could not prevent this.

At this stage it appeared very probable that FUS foci formation was not mediated through PIKKs at all and that abolition of focus formation in response to caffeine was due to some other aspect of the pharmacology of caffeine. A final experiment, where cells were pre-treated with 20mM caffeine or a combination of all six inhibitors used, was performed to rule out the possibility of combinatorial effects between the inhibitors used in the first experiment and in the second.



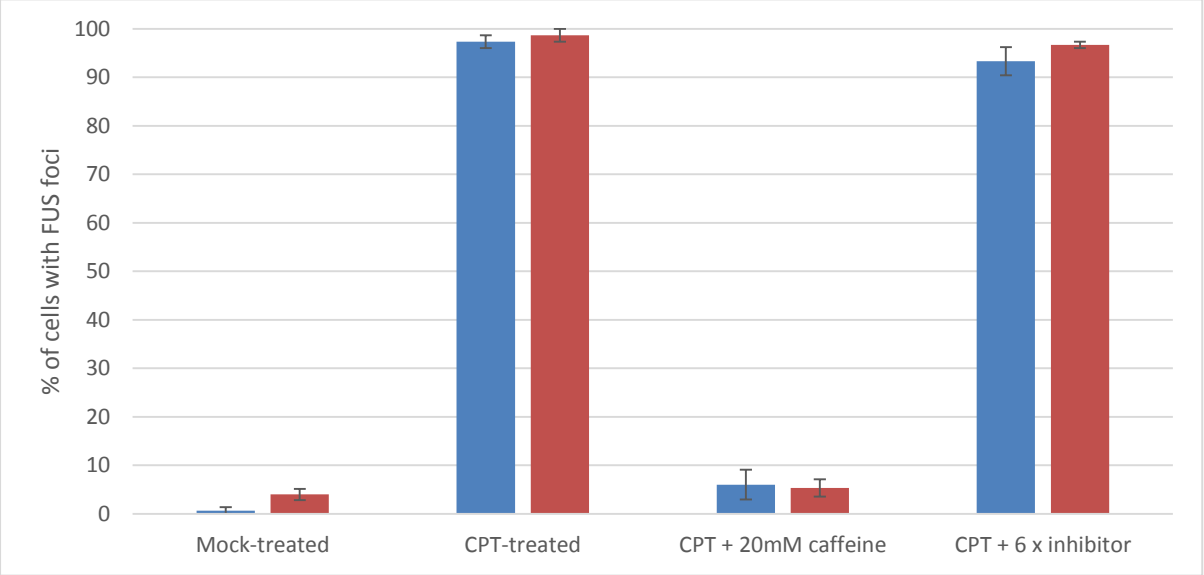
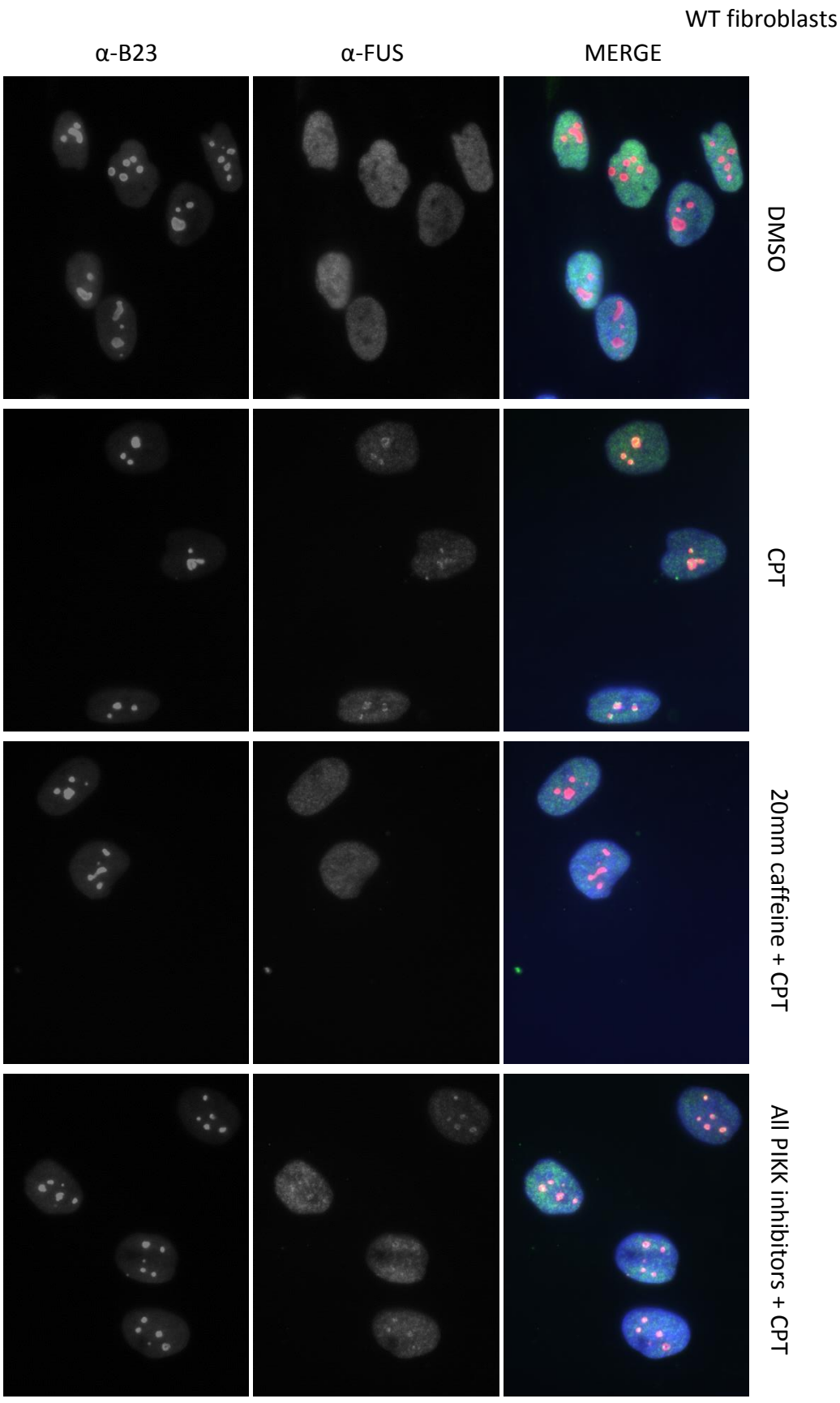


Figure 5.7a | **FUS foci still form in the presence of inhibitors of all PIKKs.** Pre-treatment with 20mM caffeine prevents FUS foci formation (the p values by Student's t-test for WT and R521H respectively are p = 0.002424 and p = 0.003683 respectively - the null hypothesis is therefore rejected) .

However pre-treatment with a combination of all PIKK inhibitors used in these experiments does not inhibit foci formation (the p values by Student's t-test for WT and R521H respectively are p = 0.367763 and p = 0.1646 - the null hypothesis is therefore accepted).


WT fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
20mm caffeine + CPT
All PIKK inhibitors + CPT

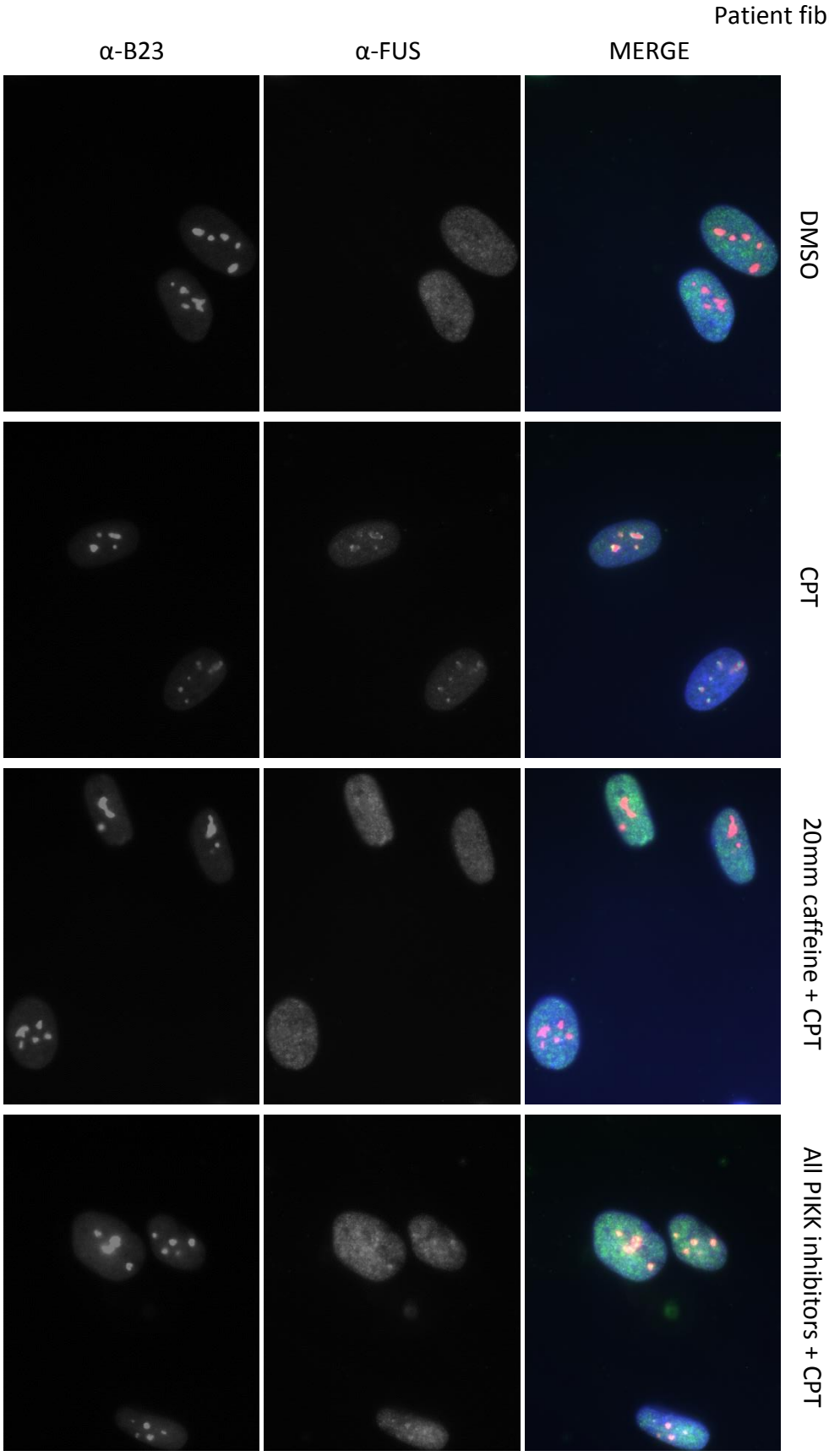


Figure 5.7b | **FUS foci still form in the presence of inhibitors of all PIKKs.** Representative images of cells counted for figure 5.7a. "All PIKK inhibitors" refers to a combination of Ku55933, ATR kinase inhibitor II, NU7441, Ku0063794, PI103 and wortmannin. Contrast set to maximum per image, rather than maximum per image set.

The combinatorial effects of all six inhibitors used in these experiments could not replicate the effects of 20mM caffeine (Fig 5.7a). As such it was concluded that FUS focus formation was not directed by any PIKK signalling but rather some other pathway which could be inhibited by caffeine.

5.4 Conclusion

Pre-treatment of cells with PARP inhibitor before RNAP II inhibition did not affect foci formation, however treatment with high doses of caffeine or theophylline did prevent formation of foci of both FUS and TDP43. This indicates that there is no role for PARP signalling in recruitment of FUS to the nucleolus after transcriptional inhibition.

The other family of interest was the PIKK family and it was tested whether pre-inhibition caffeine, a pan-PIKK inhibitor, could block FUS focus formation. Caffeine and the related molecule theophylline were capable of doing this, and also of preventing formation of nucleolar foci of LAP-tagged TDP43. However pre-inhibition of all members of the PIKK family (as well as one member of the PI3K family) alone or in combination did not prevent foci formation. As such these data show that there is no involvement of either the PARP or PIKK families in the signalling pathway leading to FUS foci formation.

6. Investigation into putative PDE involvement in FUS relocalisation

The data had excluded the involvement of the PARP and PIKK families of enzymes in FUS relocalisation but had indicated that the process could nevertheless be inhibited via caffeine treatment. Future experiments were designed from the point of view of investigating known targets of caffeine, which is well known to be a "dirty drug", acting on a broad range of biological targets. Beyond PIKK inhibition some of caffeine's reported functions in the literature are as a broad acting phosphodiesterase (PDE) inhibitor (Sutherland & Rall 1958) an antagonist of adenosine receptors (Snyder et al. 1981; Rivera-Oliver & Díaz-Ríos 2014), an activity with broad indirect effects on neurotransmitter release (Daly et al. 1994); an acetylcholinesterase inhibitor (Pohanka & Dobes 2013); a mobiliser of intracellular and possibly extracellular calcium (Thayer et al. 1988; McPherson et al. 1991); and an inhibitor of the γ-aminobutyric acid A ($GABA_A$) receptor (Daly 2000).

6.1 Testing the abilities of caffeine and theophylline to inhibit FUS relocalisation at the same concentration

Caffeine, and many related methylxanthines, are observed to very easily cross cell membranes (Garattini et al. 1979) meaning concentrations used for *in vitro* studies on inhibitors should approximate equivalent intracellular concentrations in tissue culture. This was demonstrated by a study in which the rate of entry of caffeine into individual rat myocytes was measured - within 10 seconds of application of 10mM caffeine the intracellular concentration of caffeine was found to also be 10mM (Donoso et al. 1994). Similar results were found with two other methylxanthines, theophylline and theobromine, though the rates of entry were slower with these chemicals - it took approximately one minute for the applied and intracellular concentrations to match.

It is therefore notable that *in vitro* kinetic analyses of caffeine show its partial inhibition of PDEs at concentrations where FUS foci formation was partially abolished, around 10mM (Butcher & Sutherland 1962). In this same paper it was noted that theophylline, previously observed preventing FUS (and TDP43) relocalisation (Figs 5.3 and 5.4b), is a more efficient inhibitor of cAMP catalysing PDEs at this concentration.

On this basis a simple titration of caffeine and theophylline was designed - pre-treatment with either methyxanthine at concentrations from from 5mM to 15mM prior to CPT

treatment. Percentages of cells with foci were counted rather than full foci counts as treatment with 10mM caffeine appeared to primarily affect the percentages of cells producing foci, and the size of the foci, rather than the number of foci per cell (Figs 5.2 and 5.5).

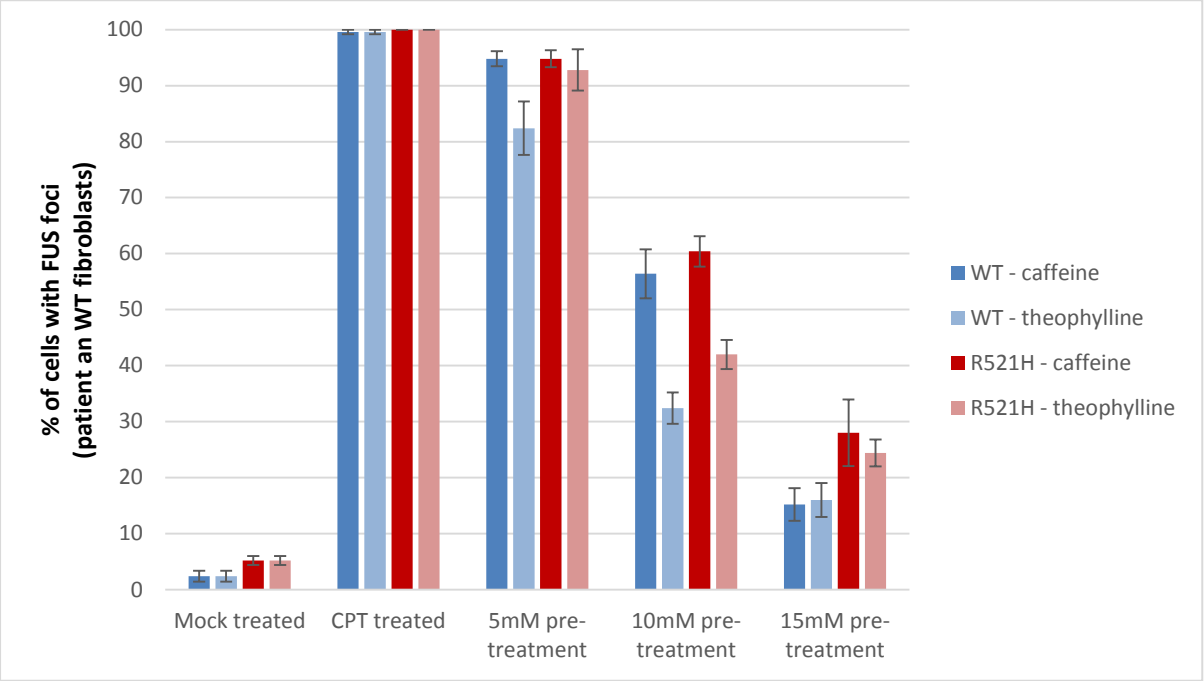
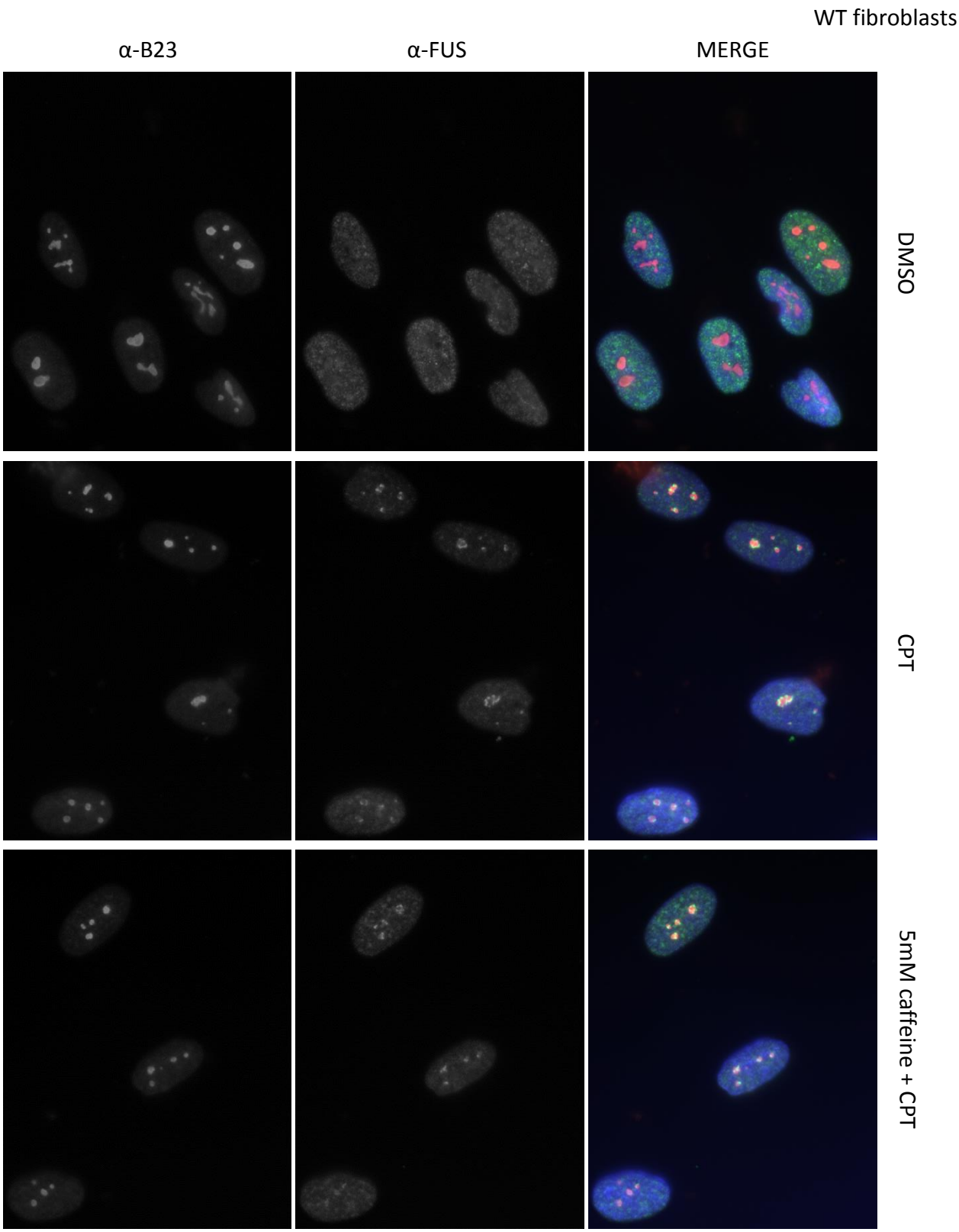
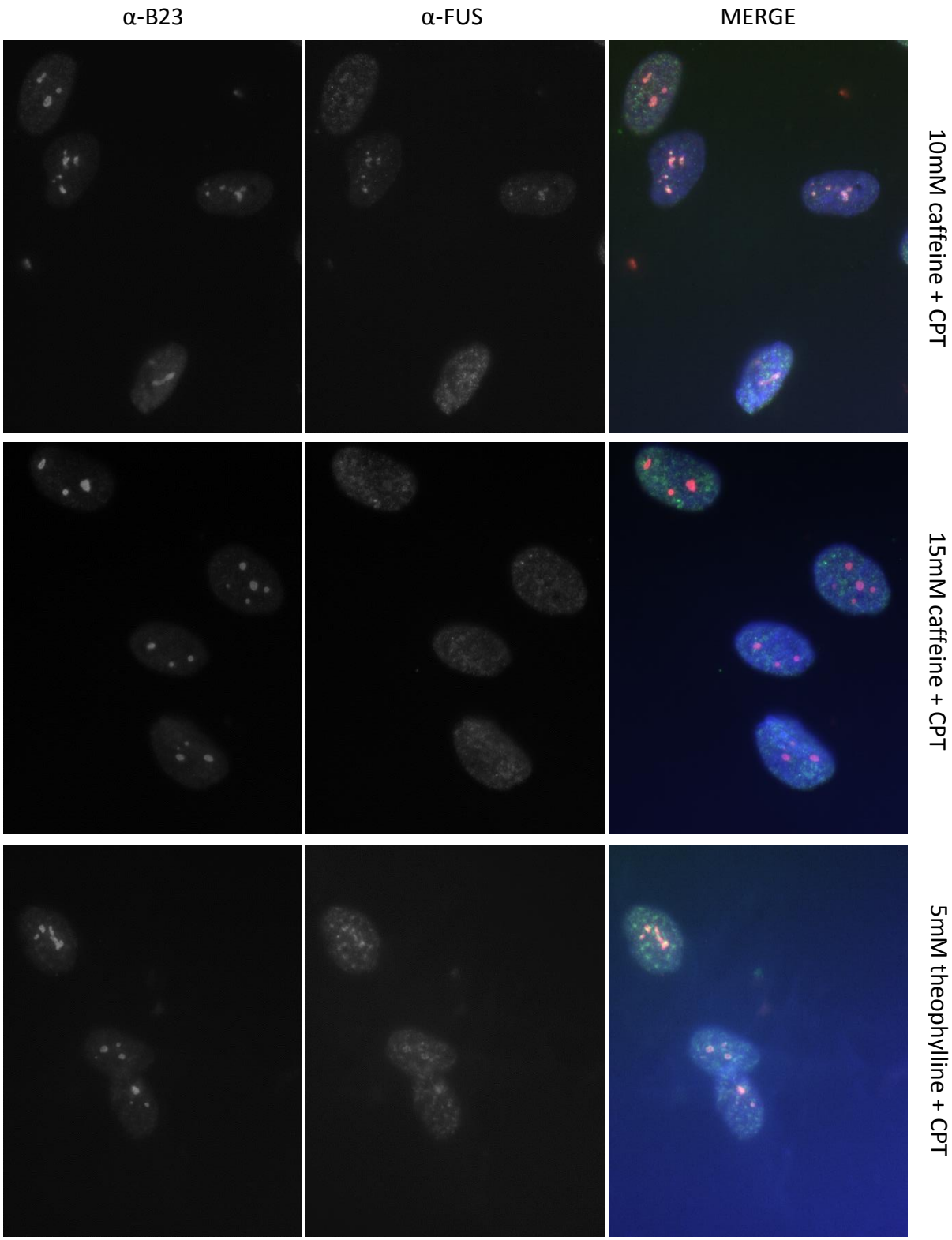


Figure 6.1a | **FUS focus formation is inhibited to varying extents by caffeine and theophylline.** 10mM caffeine pre-treatment is less effective than 10mM theophylline pre-treatment at preventing foci formation. This was also true at 5mM for WT cells only. These results were statistically significant (by Student's t-test - p values between caffeine and theophylline percentages for WT (5mM), WT (10mM), R521H (10mM) = 0.039512, 0.009511, 0.014385). Caffeine pre-treatment indicated by darker bars, theophylline with lighter bars.

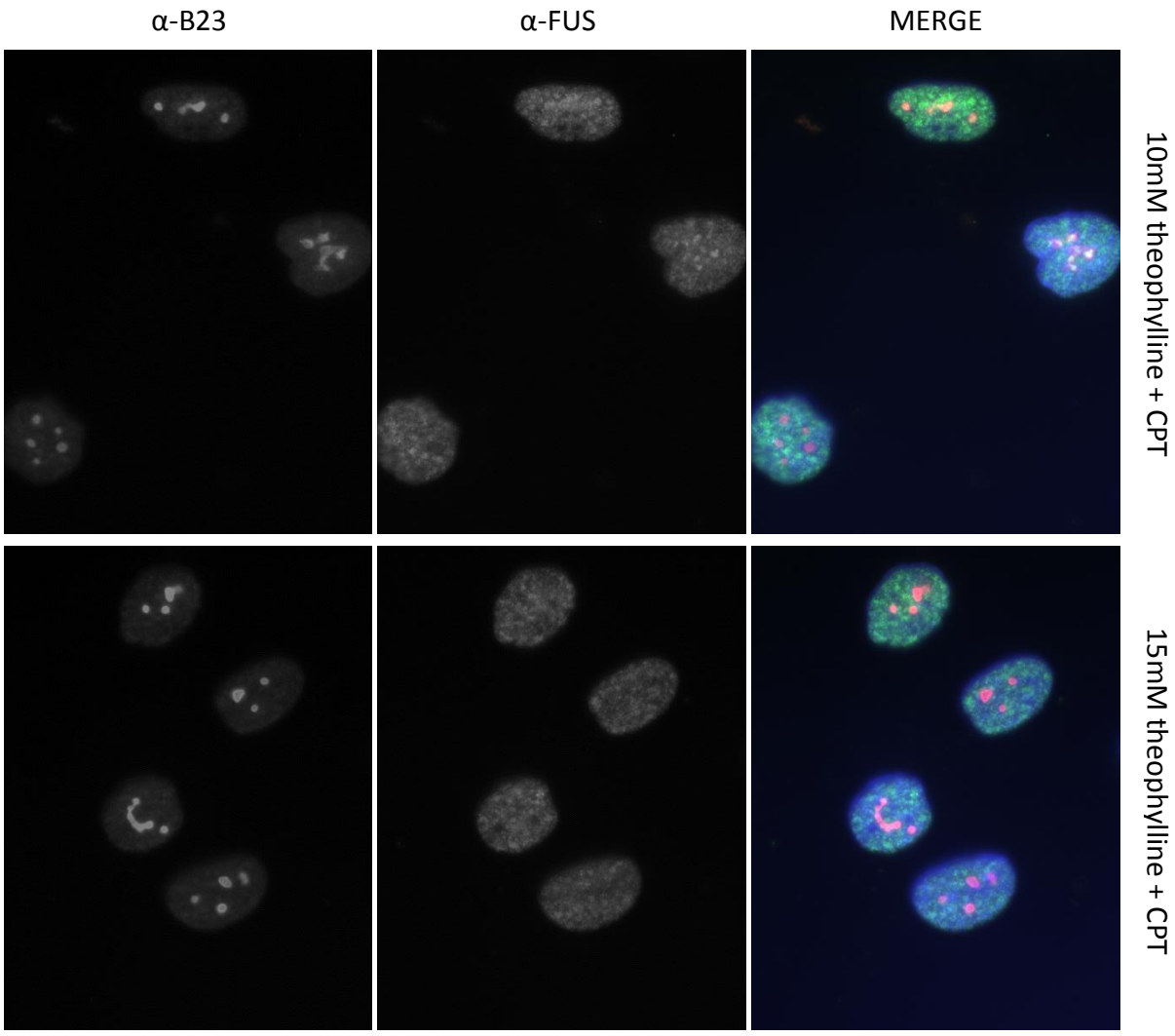
Cells were pre-incubated with either caffeine or theophylline for an hour before CPT treatment and IF. A range of methyxanthine concentrations were used for pre-incubations.


WT fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
5mM caffeine + CPT

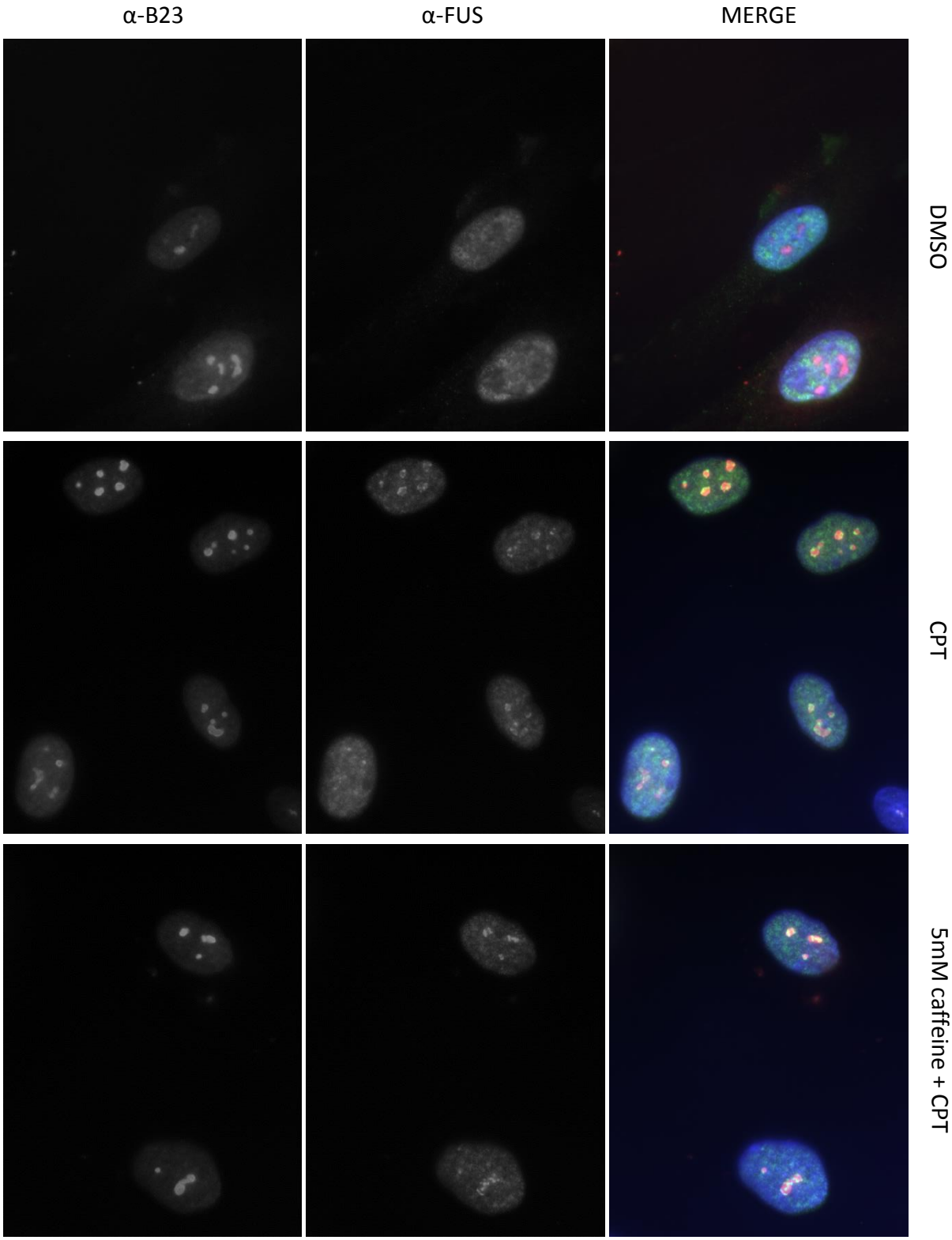
WT fibroblasts



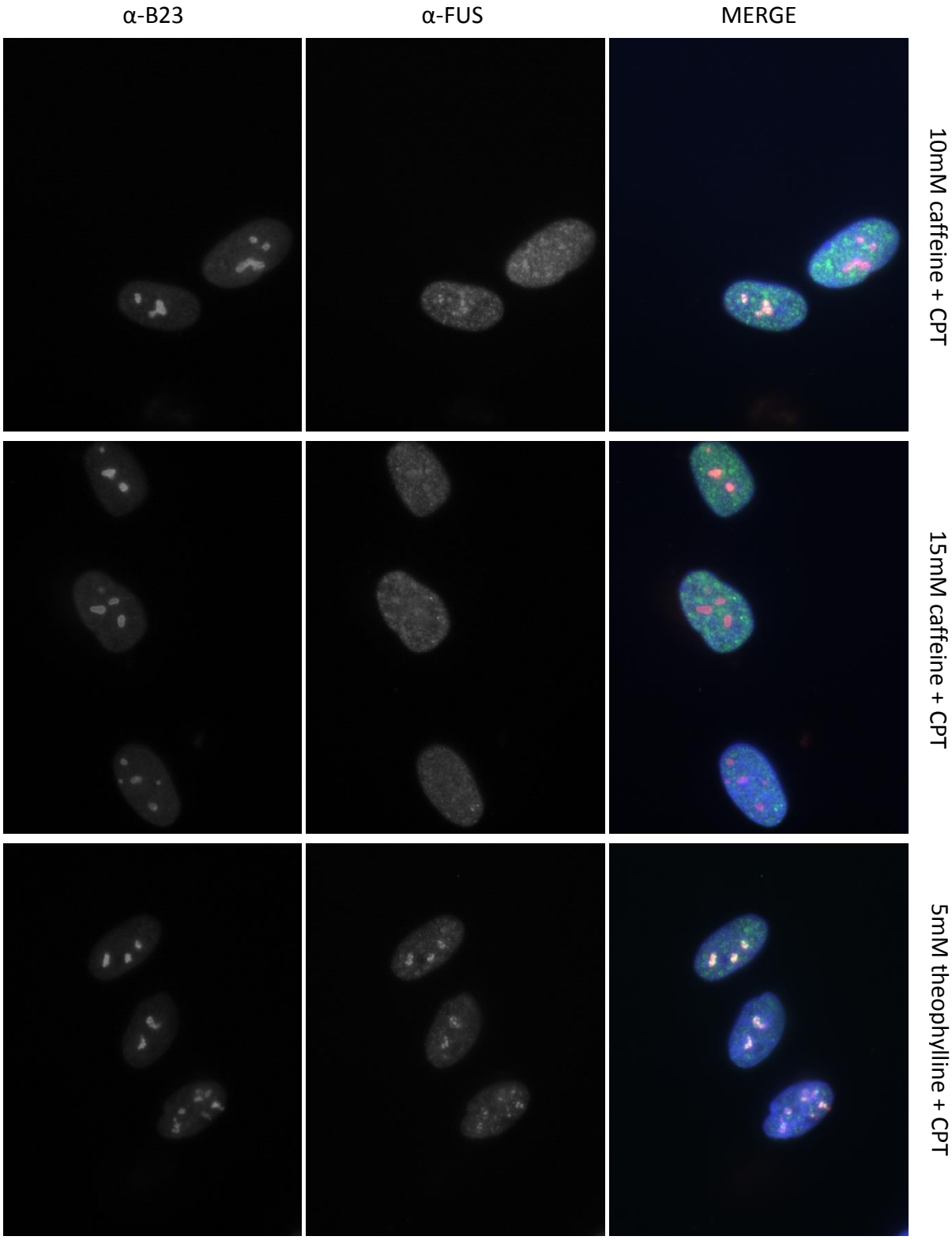
WT fibroblasts



Patient fibroblasts



Patient fibroblasts



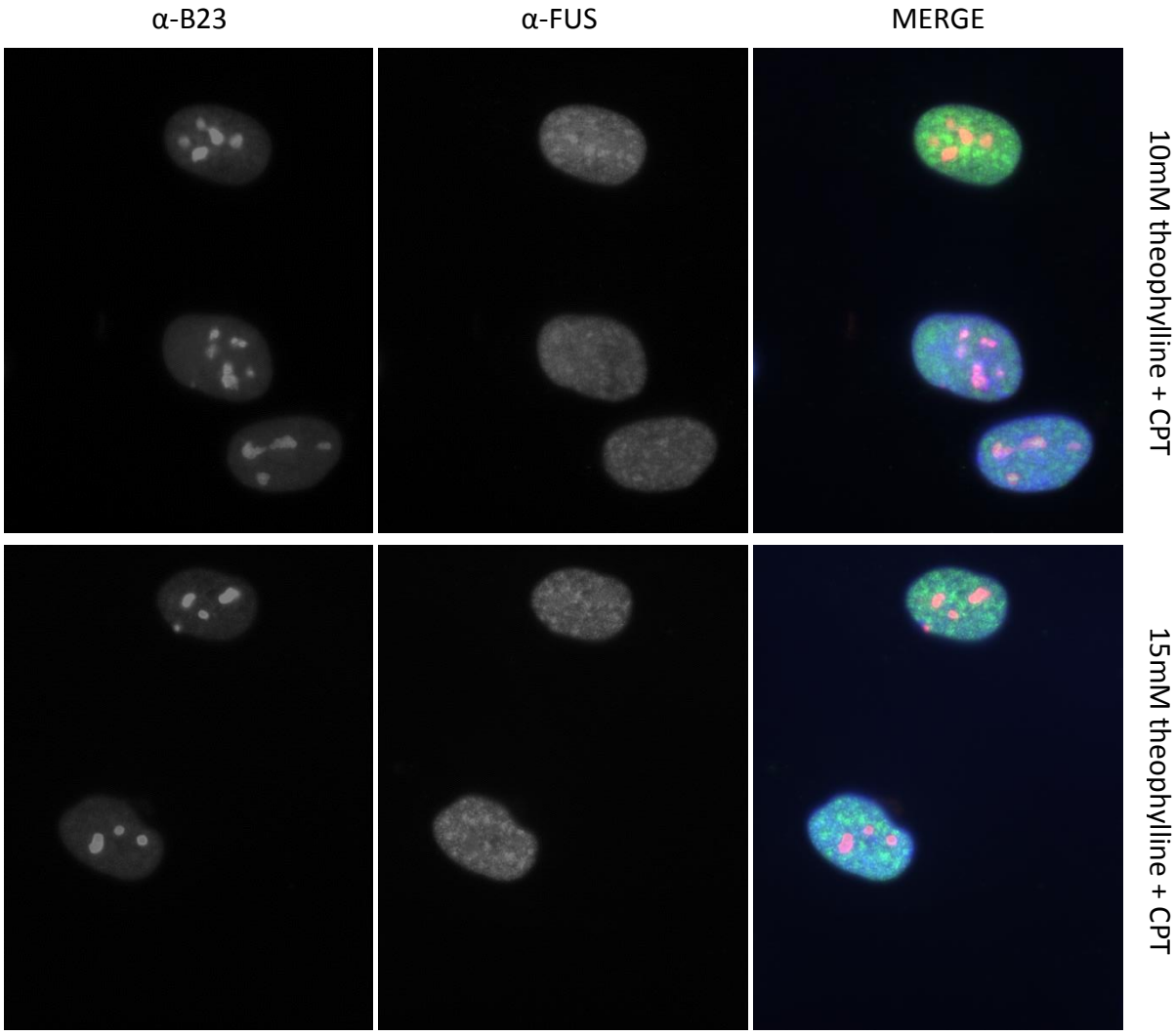


Figure 6.1b | **FUS focus formation is inhibited to varying extents by caffeine and theophylline.** Representative images of cells counted for figure 6.1a. Contrast set to the maximum per image, rather than to the maximum per image set.

The pre-treatment titration revealed that 10mM theophylline inhibited foci formation to a significantly greater extent than caffeine, indicating that the foci formation process may be mediated by cAMP catalysing PDEs (Fig 6.1).

6.2 Investigating PDE function in FUS focus formation using IBMX

As the previous experiment had implied a role for PDEs in signalling FUS relocalisation subsequent experiments were dedicated to attempting to identify which PDE family was responsible. With this in mind fibroblasts were pre-treated for one hour with 3-isobutyl-1-

methylxanthine (IBMX), a compound known to inhibit the members of all eleven PDE families save for PDE8 and PDE9 (Essayan 2001), prior to CPT treatment. Concentrations of 500nM-1mM in cells are typically used to inhibit PDEs1-7 and PDEs10-11 and IBMX also functions as an adenosine receptor antagonist at these concentrations (Daly 2000).

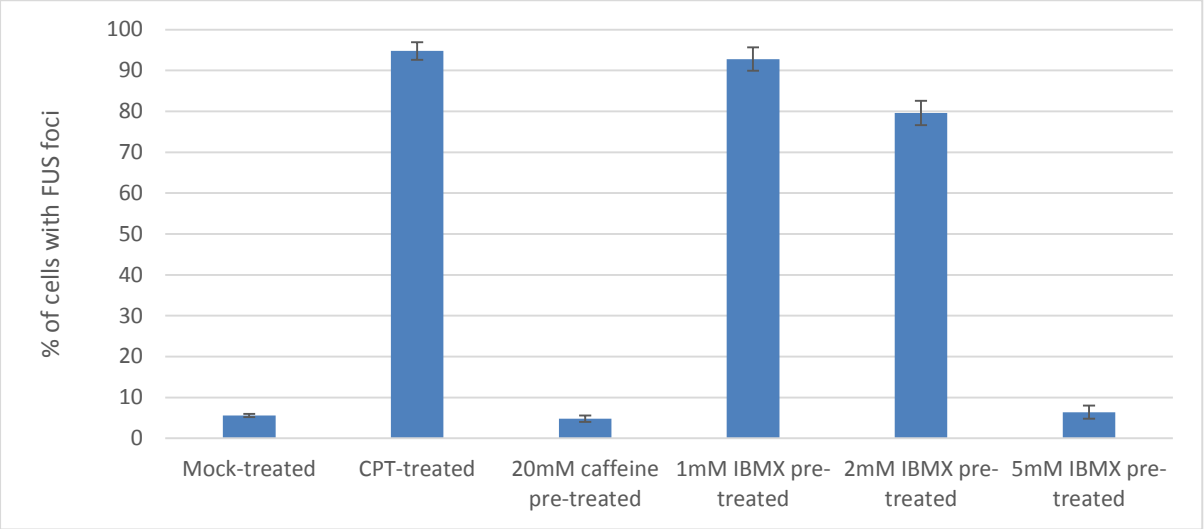
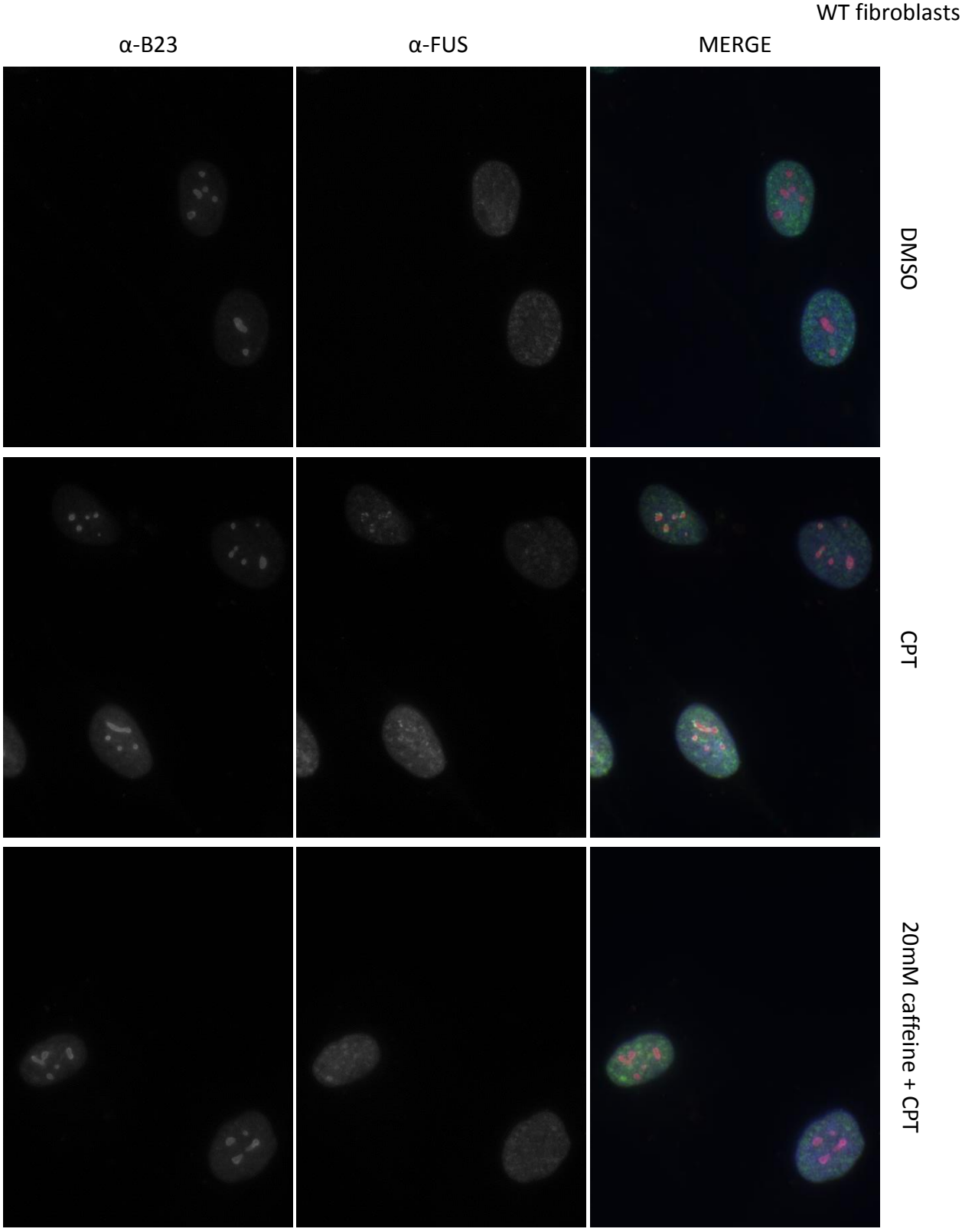


Figure 6.2a | **IBMX prevents formation of FUS foci in fibroblasts, but only at extremely high concentrations.** Pre-treatment with 5mM IBMX prevents focus formation, but lower concentrations do not. n=3.

Cells were pre-treated for an hour with either 20mM caffeine or IBMX at 1mM, 2mM or 5mM concentrations prior to CPT treatment and IF. As caffeine and theophylline treatment appeared to affect both wild-type and patient fibroblasts equally only the wild-type line was used in this experiment.


WT fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
20mM caffeine + CPT

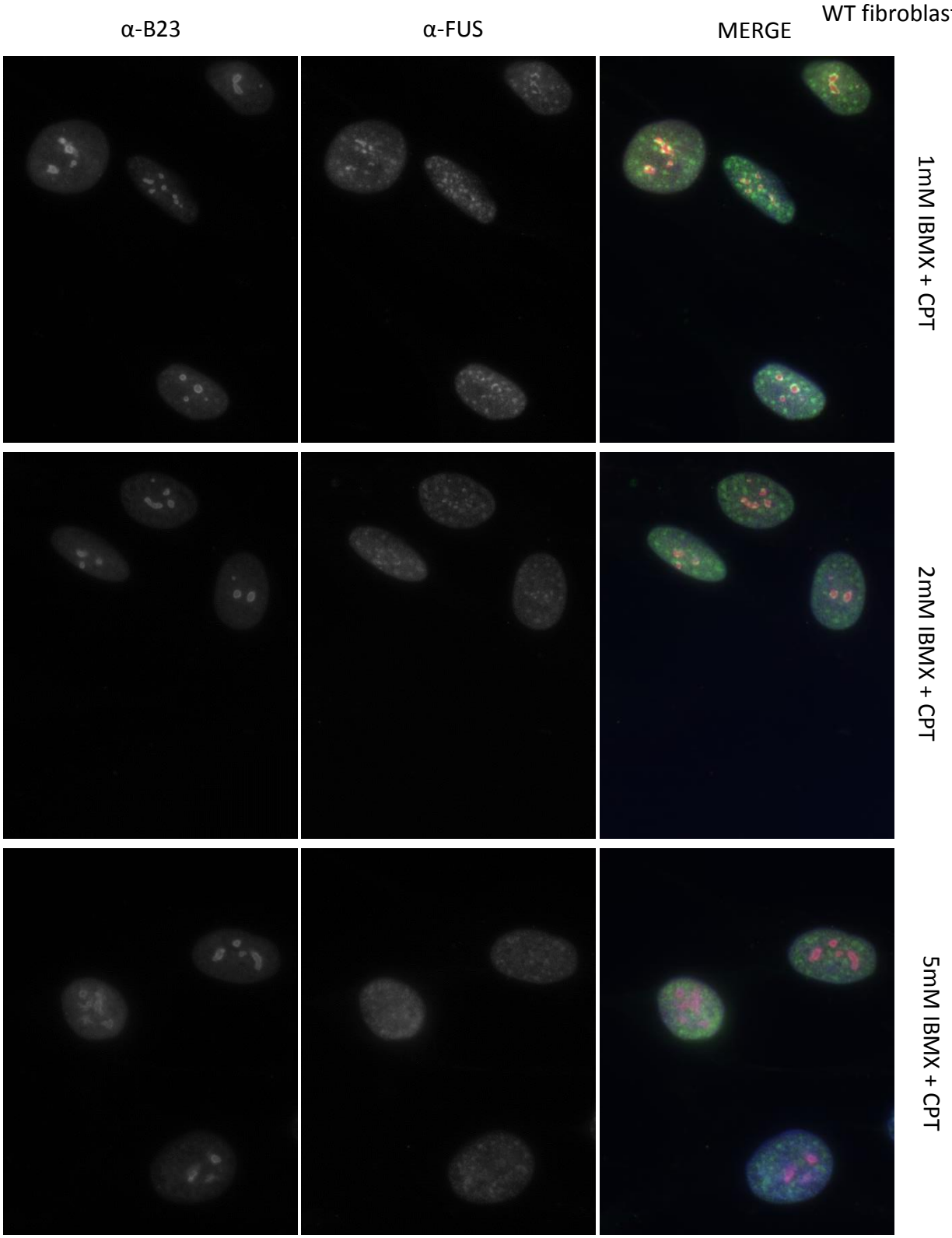


Figure 6.2b | **IBMX prevents formation of FUS foci in fibroblasts, but only at extremely high concentrations.** Representative images of cells counted in figure 6.2a.

Surprisingly IBMX was observed to prevent foci formation but not at the expected concentrations (Fig 6.2) - demonstrating only a mild inhibition of FUS relocalisation at 2mM and near total ablation of it only at 5mM. Given the latter concentration is a full order of

magnitude higher than typical concentrations used to inhibit IBMX-sensitive PDEs this suggests that this ablation may be due to unknown mechanism of action of the drug.

This experiment was also repeated in doxycycline-induced LAP-FUS Helas.

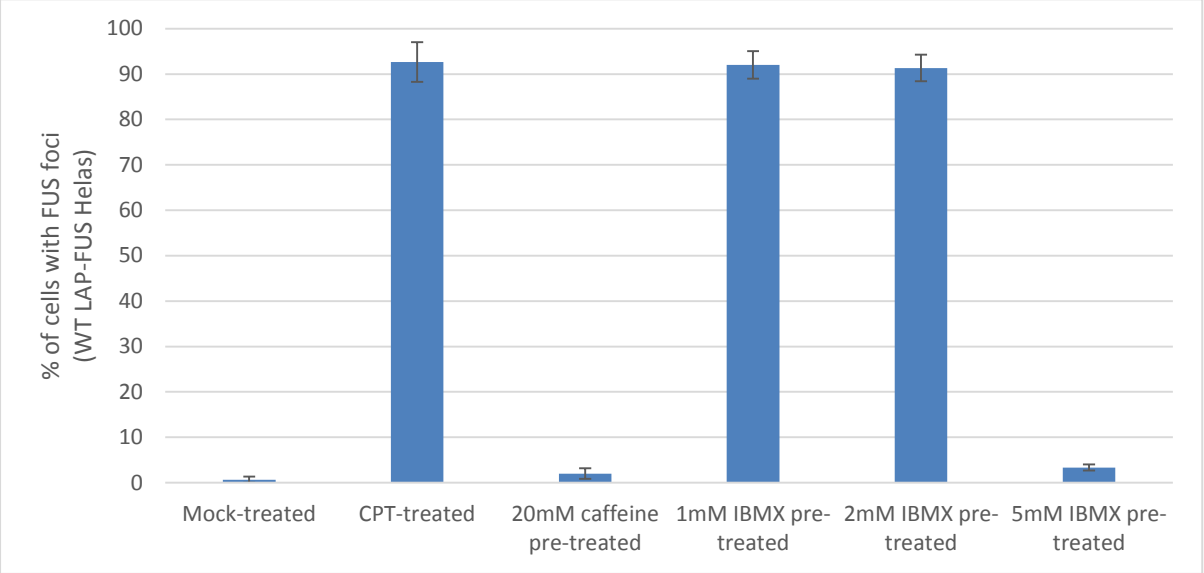
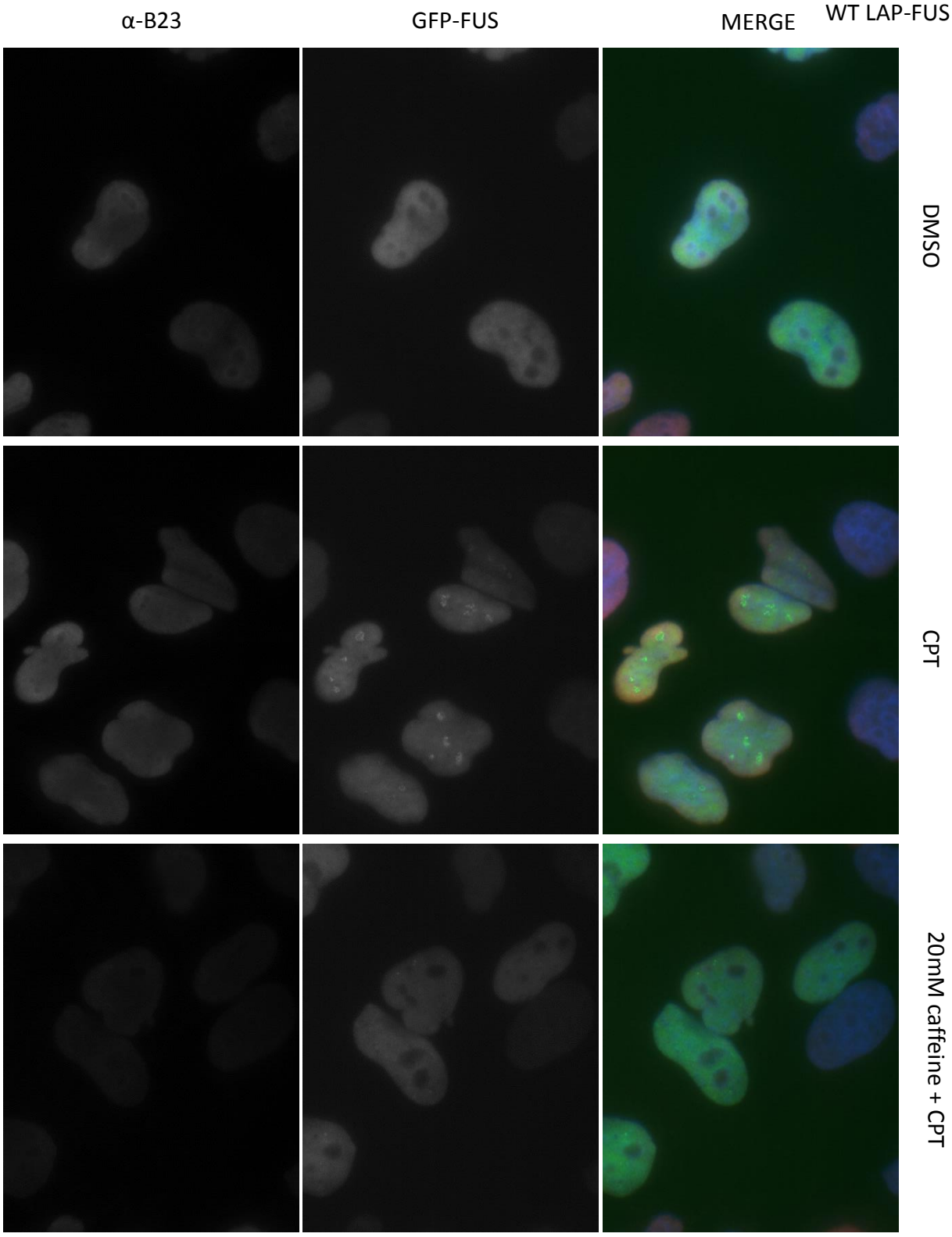


Figure 6.3a | **IBMX prevents formation of FUS foci in LAP-FUS Helas, but only at extremely high concentrations.** Pre-treatment with 5mM IBMX prevents focus formation, but lower concentrations do not. n=3.


α-B23
GFP-FUS
MERGE
WT LAP-FUS Helas
DMSO
CPT
20mM caffeine + CPT

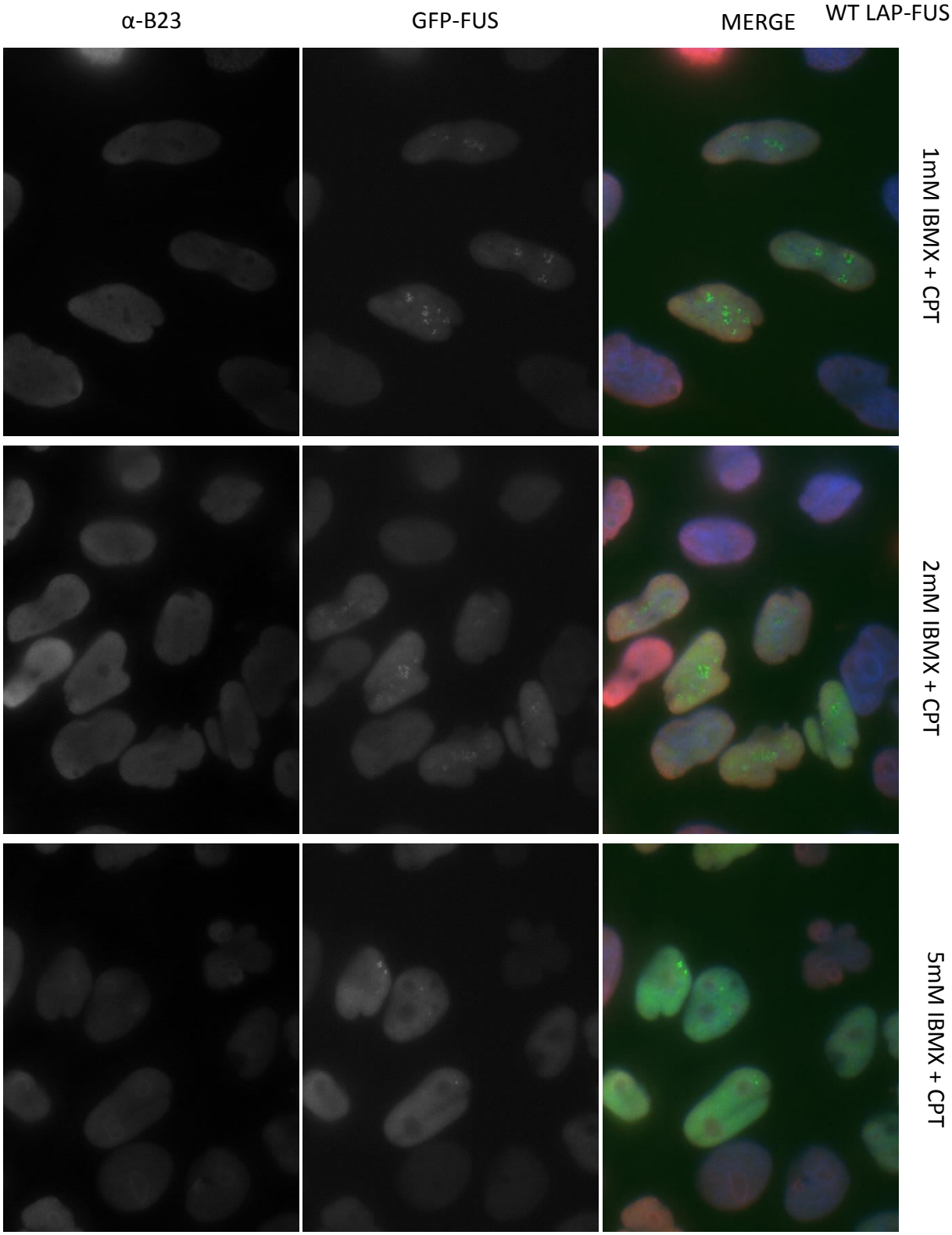


Figure 6.3b | **IBMX prevents formation of FUS foci in LAP-FUS Helas, but only at extremely high concentrations.** Representative images of cells counted in figure 6.3a.

These cells also showed a lack of FUS localisation at 5mM though 2mM IBMX did not affect foci formation (Fig 6.3).

A hypothesis for 5mM IBMX pre-treatment preventing focus formation could be that at very high concentrations IBMX can act on those members of the PDE family described as IBMX-insensitive. In studies on the (IBMX-insensitive) PDE8 family member PDE8A1 it was demonstrated that IBMX insensitivity derived from a difference in a single residue from other members of the PDE superfamily. It was also found that the IC_{50} of IBMX on the enzyme was 698µM *in vitro* relative to 65.8µM for a mutant where the point mutation was reversed (H. Wang et al. 2008) - only around one order of magnitude. The point mutation conferring IBMX insensitivity is also found in all members of the PDE8 and PDE9 families and it is therefore not implausible that IBMX could be inhibiting these members of the PDE8 or PDE9 families in cells at 5mM.

6.3 Further investigations into the role of PDEs in FUS focus formation using dipyridamole

The curious result of the IBMX experiment indicated that FUS relocalisation either involved the IBMX-insensitive PDEs (the PDE8 and PDE9 families), or was unrelated to PDEs altogether. One drug that inhibits the IBMX-insensitive PDEs is dipyridamole, and moreover it targets PDE9 at a much lower concentration than it does PDE8 - allowing differential inhibition of these two families of PDEs at different concentrations. Studies have been performed in isolated rat tissues using concentrations of the drug in the µM range to target PDE9 and using the much higher concentration of 100µM to partially inhibit PDE8 (Jackson et al. 2007).

An experiment was set up where varying concentrations of dipyridamole were used to pre-incubate cells prior to CPT treatment and IF. As the gap between these concentrations was large, and the study cited estimated the concentrations of dipyridamole to use based on *in vitro* studies, the concentrations used in the following experiment were increased approximately threefold to allow for differences in permeability of cell membranes to dipyridamole between the fibroblasts and the cells used in the Jackson study. As such the concentrations of dipyridamole used were 10µM, 200µM and 300µM.

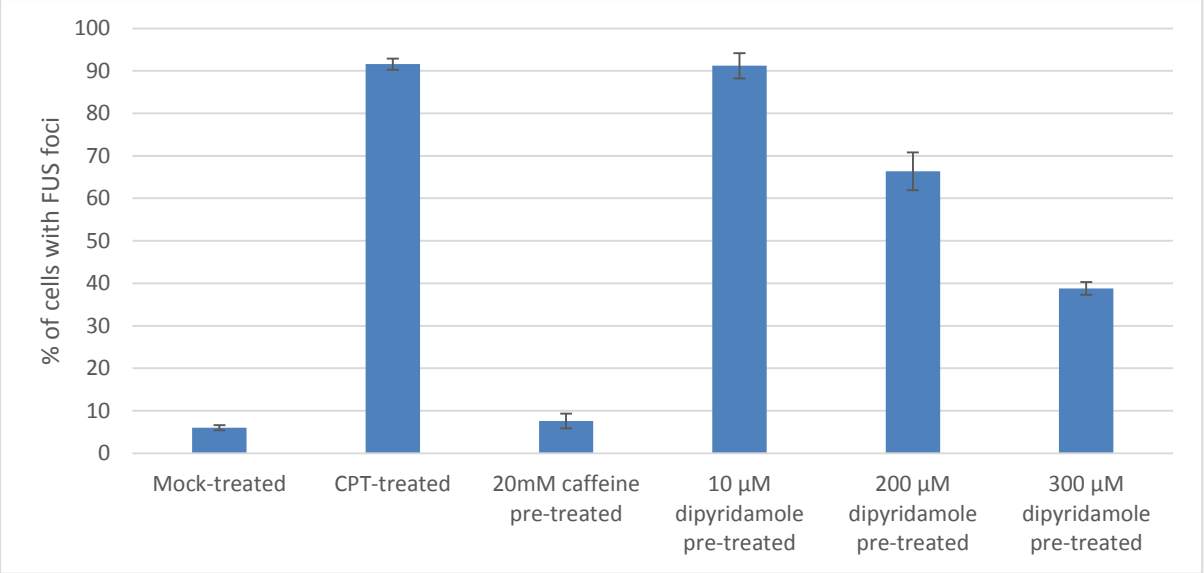
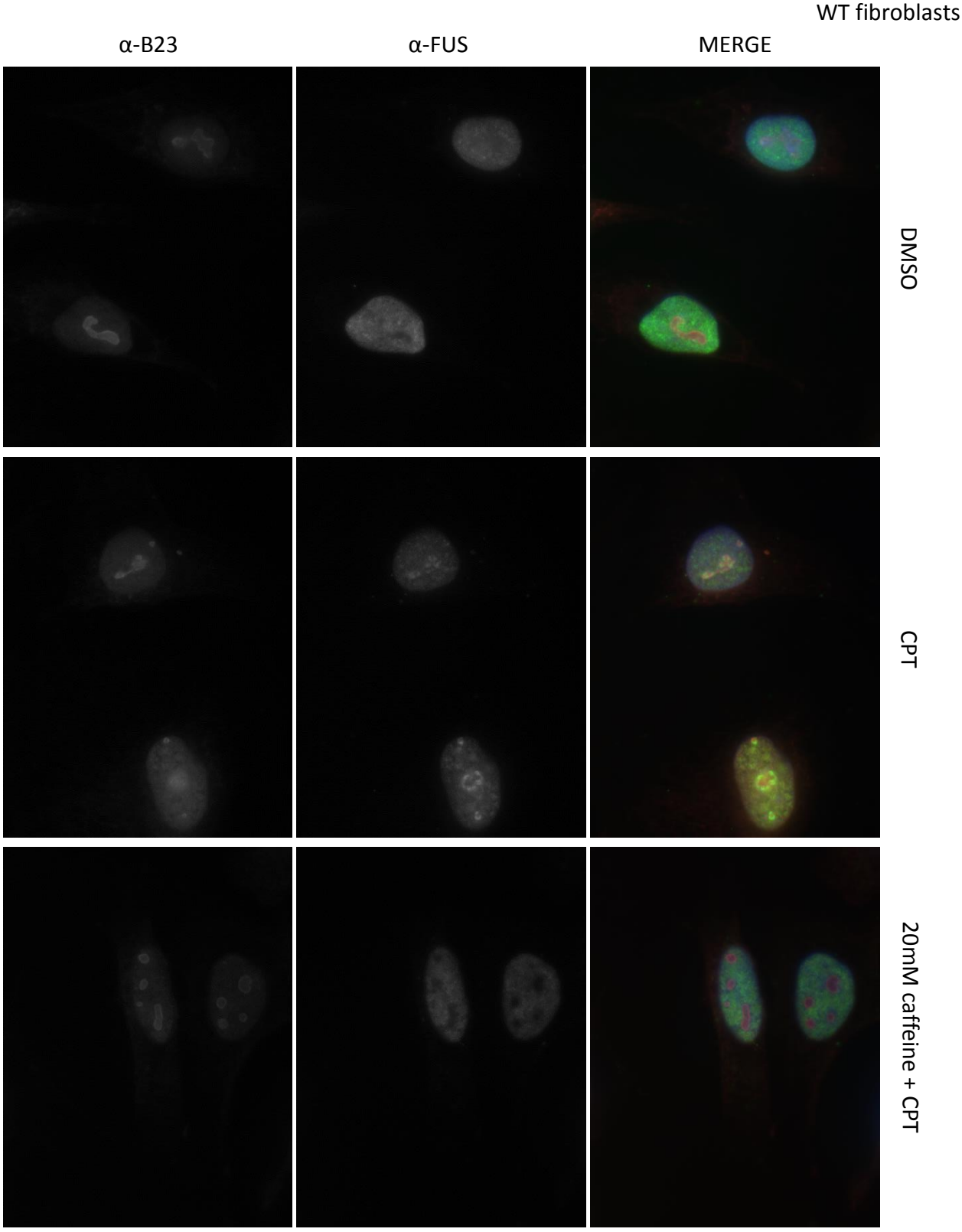


Figure 6.4a | **Partial inhibition of FUS focus formation in fibroblasts with pre-treatment of high dose dipyridamole.** Pre-treatment of Helas with high concentrations of dipyridamole, liable to inhibit PDE8 and PDE9, resulted in inhibition of FUS focus formation whereas pre-treatment with lower concentrations liable to inhibit PDE9 only did not. The change in percentage of cells with foci is statistically significant by one-way ANOVA. n=5.

Cells were pre-treated for one hour with 10µM, 200µM or 300µM dipyridamole or with 20mM caffeine before CPT treatment and IF.


WT fibroblasts
α-B23
α-FUS
MERGE
DMSO
CPT
20mM caffeine + CPT

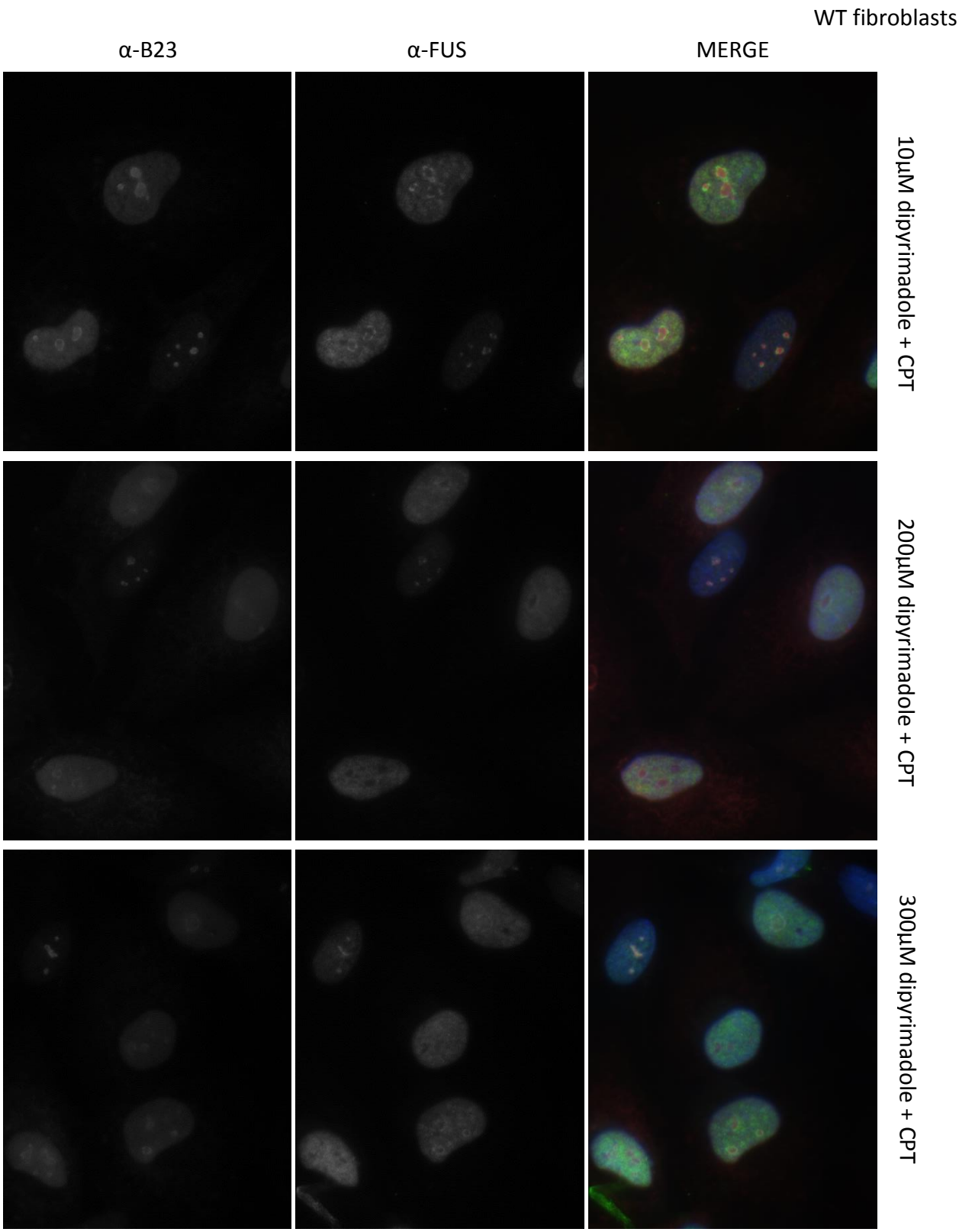


Figure 6.4b | **Partial inhibition of FUS focus formation in fibroblasts with pre-treatment of high dose dipyridamole.** Representative images of cells used for counting in figure 6.5a.

Cells treated with at 10μM dipyridamole, a concentration where PDE9 would be inhibited but PDE8 would not, reliably formed foci. However there was a reduction of cells with foci at

200µM and 300µM - these data suggested PDE8 involvement in foci formation (Fig 6.4). Higher concentrations could not be tested, as the drug appeared to be approaching its solubility limit in water and would precipitate out of solution if the concentration was raised further. The experiment was repeated in LAP-FUS Helas.

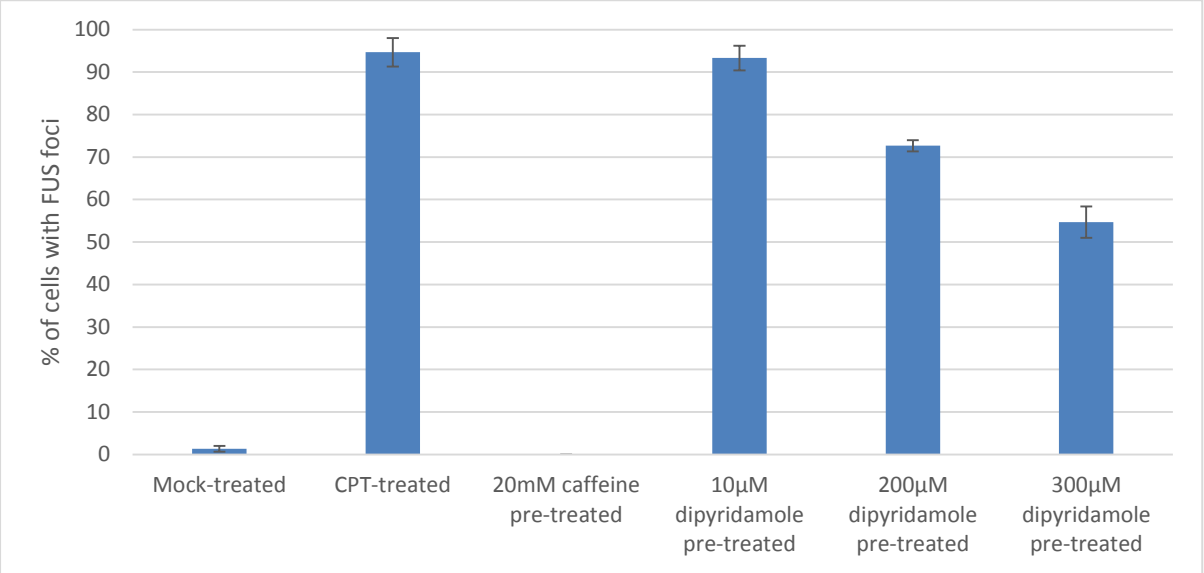
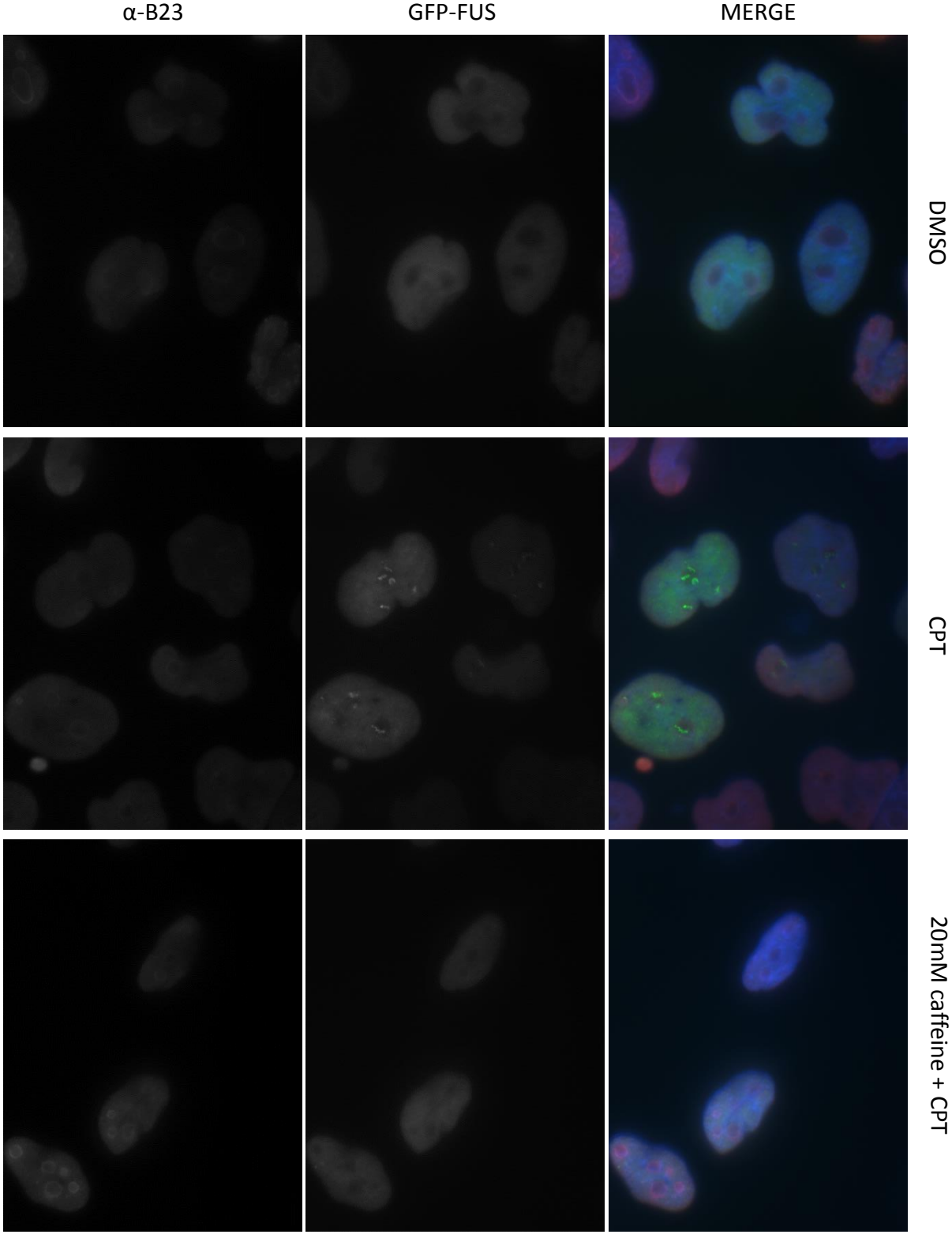


Figure 6.5a | **Partial inhibition of FUS focus formation in LAP-FUS Helas with pre-treatment of high dose dipyridamole.** Pre-treatment of Helas with high concentrations of dipyridamole, liable to inhibit PDE8 and PDE9, resulted in inhibition of FUS focus formation whereas pre-treatment with lower concentrations liable to inhibit PDE9 only did not. The change in percentage of cells with foci is statistically significant by one-way ANOVA. n=3.

WT LAP-FUS Helas



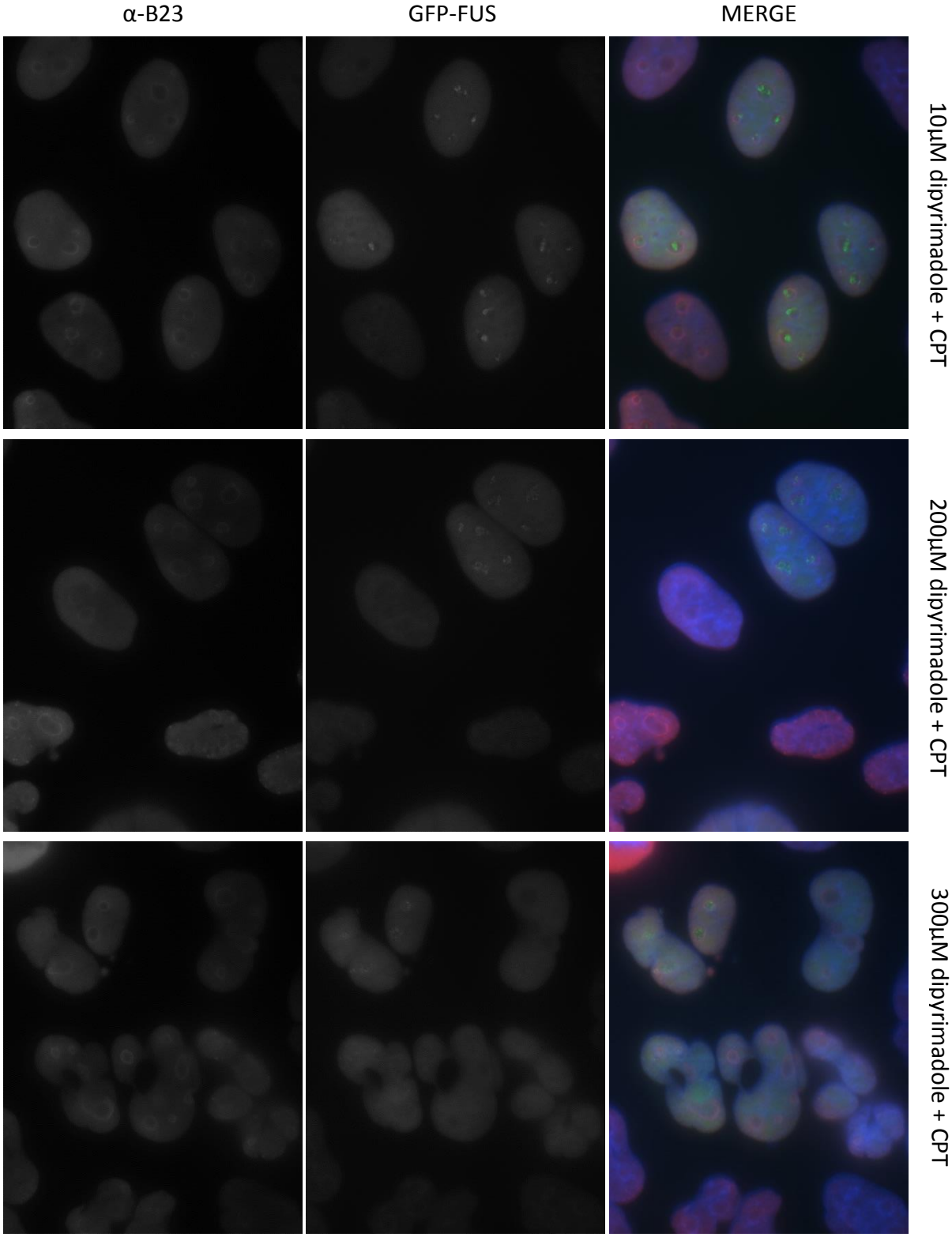


Figure 6.5b | **Partial inhibition of FUS focus formation in LAP-FUS Helas with pre-treatment of high dose dipyridamole.** Representative images of cells used for counting in figure 6.5a.

300μM dipyridamole also reduced foci formation, to a lesser extent, in LAP-FUS Helas (Fig 6.5). This was also tested on cortical neurons from WT CD1 mice and high concentration

dipyridamole was observed inhibiting foci formation in these cells (Ryan Green, personal communication). These data suggest PDE9 enzymes are not involved in FUS recruitment but that suggest that members of the PDE8 family are.

6.4 siRNA depletion of PDE8A and its effects on FUS relocalisation

The previous experiments had indicated the likelihood of PDE8 proteins having an involvement in the process of FUS relocalisation. The PDE8 family contains two members, with five splice variants each (Wang, Wu, Egan, et al. 2001; Gamanuma et al. 2003), with PDE8A being widely expressed in a range of tissues but PDE8B only being abundantly expressed in the thyroid gland, with some expression in the placenta and brain (Hayashi et al. 1998; Hayashi et al. 2002; Keravis & Lugnier 2012).

To confirm the involvement of PDE8 in foci formation U2OS cells were subjected to a reverse siRNA transfection against PDE8A. The siRNA used for this targets PDE8A1-3 (as listed in the Gene section of the NCBI website) and also PDE8A4 and PDE8A5 (the sequences for these splice variants were published in Wang, Wu, Egan, et al. 2001).

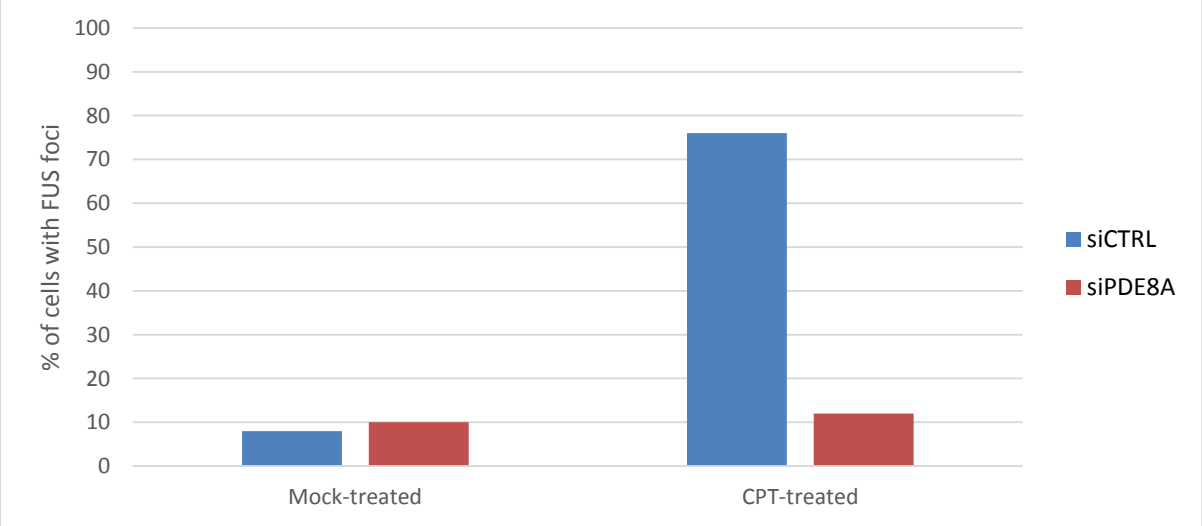


Figure 6.6a | **PDE8A siRNA knockdown in U2OS prevents FUS focus formation.** Knockdown of PDE8A appeared to reduce the amount of foci observed in CPT-treated U2OS cells to the same level as observed in mock-treated cells.

U2OS cells were reverse transfected (seeded onto transfection mix) with control or PDE8A siRNA and rested for two days before CPT-treatment and IF.

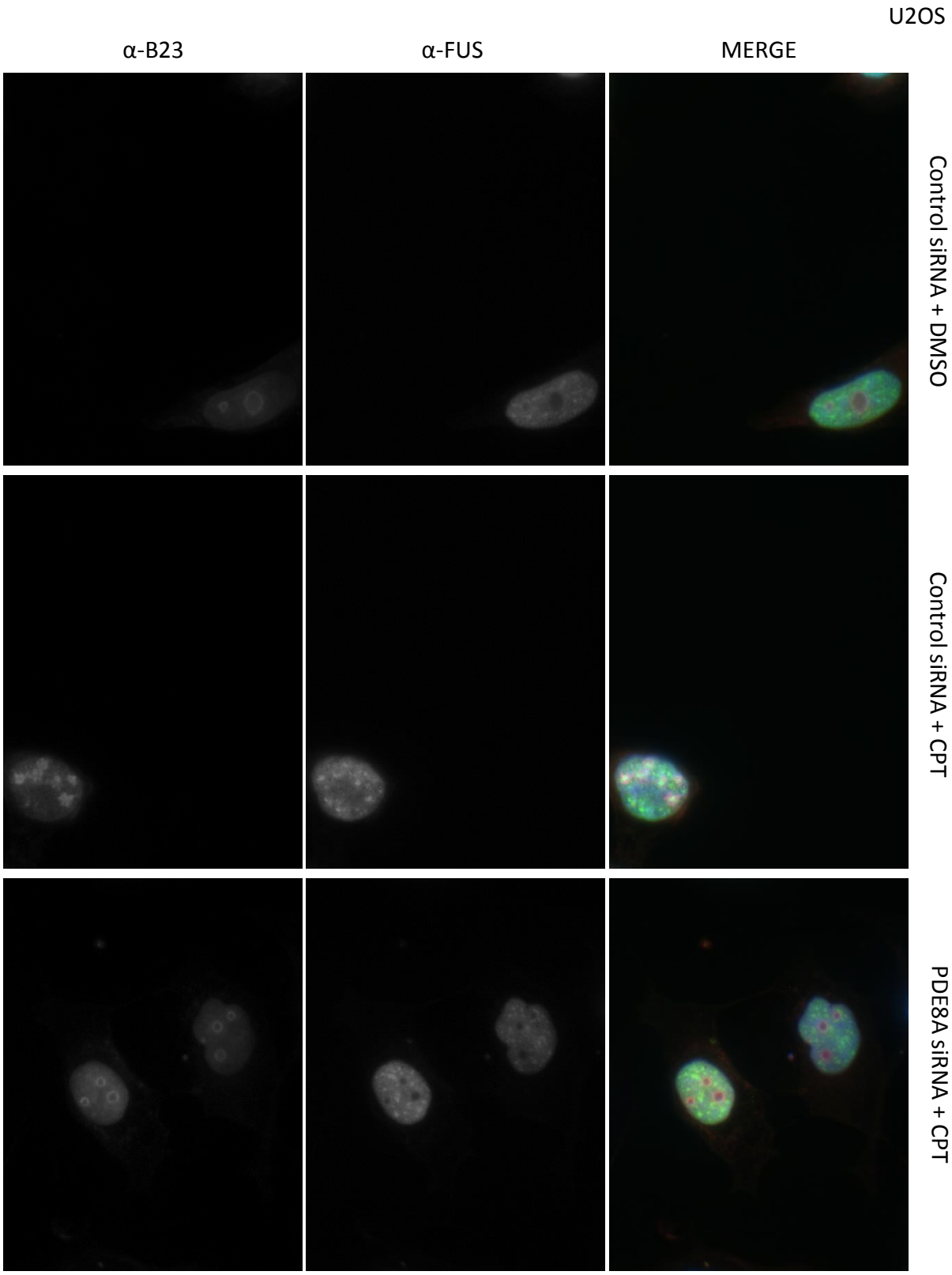


Figure 6.6b | **PDE8A siRNA knockdown in U2OS prevents FUS focus formation.** Representative images of cells used for counts in figure 6.6a.

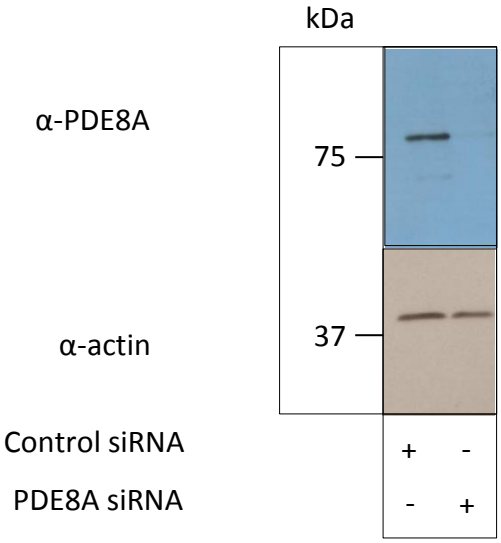


Figure 6.6c | **Western blot showing a successful PDE8A knockdown in U2OS.** U2OS cells reverse transfected with PDE8A siRNA show a large drop in PDE8A levels relative to those treated with control siRNA.

U2OS cells were reverse transfected (seeded onto transfection mixture) with control or PDE8A siRNA and rested for two days before harvesting for western blotting. Approximately $1x10^5$ cells per lane.

A strong knockdown of PDE8A was observed in the western blot (Fig 6.6c) and the PDE8A knockdown resulted in FUS focus formation being identical between CPT and mock treated cells (Fig 6.6a). It is worth noting that the antibody used to detect PDE8A targets a region of protein not found in the PDE8A3-5 splice variants, but that expression of the PDE8A2-5 mRNAs are much less abundant than that of the PDE8A1 mRNA. Moreover PDE8A1 is considered to be the predominant form of PDE8A in the cell (Wang, Wu, Egan, et al. 2001).

A siRNA knockdown of PDE8B was also performed but whether or not the knockdown was successful could not be verified by western blotting. Therefore data from the PDE8B knockdown has been excluded.

From this data it was concluded that FUS focus formation is dependent on the activity of the PDE8 family of phosphodiesterases.

6.5 Conclusion

The differential sensitivity of FUS foci formation between caffeine and theophylline implicated cAMP-catalysing PDEs in the signalling pathway that recruits FUS for focus formation. Further studies with the PDE inhibitors IBMX and dipyridamole excluded the involvement of members of all PDE families save for PDE8. This was because IBMX

prevented foci formation only at concentrations far higher than those needed to inhibit members of the PDE1-7, PDE10 and PDE11 families; and dipyridamole only reduced foci formation at concentrations where PDE8 would be (partially) inhibited rather than the lower concentrations needed to inhibit PDE9.

The involvement of PDE8 in FUS foci formation was further evidened by an siRNA knockdown - cells treated with the PDE8A siRNA were unable to form foci. Although it remains unclear how PDE8A activity is activated in response to RNAP II inhibition and whether its activity is signalled to FUS via PKA or another cAMP-associated mechanism these data implicate PDE8A in the process of FUS foci formation.

7. Conclusion

It has been established that FUS forms nucleolar foci in cells in response to inhibition of RNAP II but not of RNAP I. Moreover the localisation of FUS in these foci is consistent with it being localised to the nucleolar necklace, the site of rRNA transcription and early processing after nucleolar disruption, as the foci are located close to but are distinct from the shrunken masses of B23 that form after complete disruption. This relocalisation was observed in all cell lines tested. 3D imaging also indicated FUS localised immediately adjacent to fibrillarin, involved in early rRNA processing, suggesting that the role for FUS in these foci is somehow related to pre-rRNA at the early processing stage. Another ALS associated protein with similar and overlapping molecular functions, TDP43, was observed having identical localisation to FUS in response to transcriptional inhibition. This could suggest that both proteins are involved in the same activity at the nucleolus - however although localisation of endogenous FUS to the nucleolus has been observed TDP43 nucleolar localisation has only been observed in an overexpression system with a GFP tag.

Intruigingly RNAP I inhibition of cells not only fails to produce foci but actively prevents foci formation by subsequent RNAP II inhibition. Inhibition of both polymerases at similar times still produces FUS foci indicating that it is not RNAP I inhibition itself but some downstream result of inhibition that prevents FUS focus formation. The model proposed to explain this is that FUS binds nascent pre-rRNA in the early stages of processing and that pre-inhibition of RNAP I depletes the nucleolus of RNA - preventing FUS localising to the nucleolus. 45S rRNA has a half-life of twenty minutes when RNAP I is inhibited (Drygin et al. 2011) so the three hour CX5461 treatments used in the experiments would be expected to thoroughly deplete nucleolar RNA.

It was also observed that both the patient-derived fibroblast and GFP-Hela lines harbouring ALS-causative FUS mutations (R521H/R521G respectively) produced more foci per cell relative to wild type controls - this may be a consequence of these cells having constitutively more fragmented nucleoli relative to wild-type. In *S. cerevisiae* fragmented nucleoli are a sign of ageing (Sinclair et al. 1997) and may be formed from large, stressed nucleoli as a result of rDNA instability, in turn downstream of oxidative stress (Lewinska et al. 2014). In humans fragmented nucleoli are also associated with defects in rRNA production - notably siRNA knockdown of the ribosomal RPS19 protein resulted in nucleolar fragmentation and

also a defect in rRNA maturation (Choesmel et al. 2007). This is noteworthy as cells from patients with c9orf72 expansions (the most common cause of fALS) demonstrate enlarged, though not fragmented, nucleoli and rRNA maturation defects (Haeusler et al. 2014) and in addition a possible rRNA maturation defect was observed in FUS R521H patient fibroblasts along with fragmented nucleoli. Dysregulation of RNA metabolism is thought to be important in ALS pathogenesis so both the nucleolar fragmentation and the possible rRNA maturation defect are potentially of relevance to the process. It is unclear whether or not any role FUS may have in rRNA maturation is connected to its role in nucleolar foci.

A final defect was observed in the FUS patient fibroblast line, a survival defect in response to CPT but not IR, but no connection between this and the other defects in the line could be established. However some potential causes were excluded: the patient cell line did not demonstrate any statistically significant defects in DNA damage repair capacity relative to wild type, though the involvement of FUS in DNA damage repair had been previously reported (Baechtold et al. 1999; Mastrocola et al. 2013; Wang et al. 2013; Rulten et al. 2014), nor did the cells demonstrate any defect in their ability to recover transcription after inhibition.

The signalling dependence of FUS recruitment to the nucleolar necklace was examined and found to independent of the activities of the PARP enzymes and the PIKK enzymes, both of which have been implicated in signalling to FUS previously (Gardiner et al. 2008; Mastrocola et al. 2013; Rulten et al. 2014; Deng et al. 2014). However focus formation could be abolished by application of high doses of caffeine or the related methyxanthine theophylline, as could formation of GFP-TDP43 foci, implying FUS and TDP43 foci are regulated by the same signalling pathways. Theophylline is a more effective inhibitor of cAMP hydrolysing PDEs than caffeine at 10mM (Butcher & Sutherland 1962) and was also more effective at preventing FUS foci formation at this concentration, potentially implicating the PDE superfamily in FUS recruitment. Further studies indicated foci formation to be insensitive to IBMX treatment at 1mM but sensitive at the very high concentration of 5mM. As the IBMX-insensitive PDEs 8 and 9 (hydrolysing cAMP and cGMP respectively) are inhibited by the compound at approximately ten times the concentration as other members of the superfamily it was plausible that FUS focus formation was dependent on the activity of one or both of them. Treatment with dipyridamole at concentrations expected to inhibit

PDE9 in cell culture failed to abolish focus formation but treatment at the highest concentrations at which the compound remained soluble, where it would be expected to partially inhibit PDE8 (Jackson et al. 2007), resulted in partial abolition of focus formation. Furthermore treatment with siRNA for PDE8A abolished formation of FUS foci. These data indicate that relocalisation of FUS to the forming nucleolar necklace is dependent on the cAMP signalling pathway and implicates PDE8A in control of FUS recruitment via cAMP, though a concerted role for PDE8A and PDE8B or a non-specific role for either PDE8 isoform cannot be ruled out.

From these data a model of a FUS function at the nucleolar necklace can be proposed. Upon RNAP II inhibition PDE8A in the nucleus is activated, reducing cAMP levels and indirectly allowing recruitment of FUS to pre-rRNA. This occurs as nucleolar disruption is occuring, with the nucleolus separating into the nucleolar necklace and the B23-containing masses. Two questions are left open by this model - how PDE8A is activated and how its decreasing cAMP concentration signals to FUS.

With the first question the fact that FUS foci form during nucleolar disruption suggests that PDE8A is activated as a result of transcriptional inhibition itself rather than of nucleolar disruption, which makes pathways activated downstream of nucleolar disruption (such as p53 signalling) unlikely candidates for activation of PDE8A. Regarding the second question there are multiple pathways by which cAMP-hydrolysing PDEs can affect target proteins - some proteins are directly regulated by cAMP concentration, others can be affected via PKA signalling and/or by the Epac proteins - which are activated by cAMP and act as guanine exchange factors to Ras-related GTPases (Grandoch et al. 2010). FUS lacks any domains likely to interact directly with cAMP so it is likely that it is signalled to by PKA, indirectly via one of the Epac proteins or both.

A hypothesis on the role of FUS at the nucleolar necklace (as it forms) is that it could protect the nascent pre-rRNA from degradation in order to allow rapid continuation of rRNA processing after the stress on the cell has abated. This is evidenced by the continuing presence of FUS foci after three hour treatments with high dose actinomycin D, as it would be expected that exposed pre-rRNA would have been degraded or processed by this time, but further work would be needed to fully address this hypothesis.

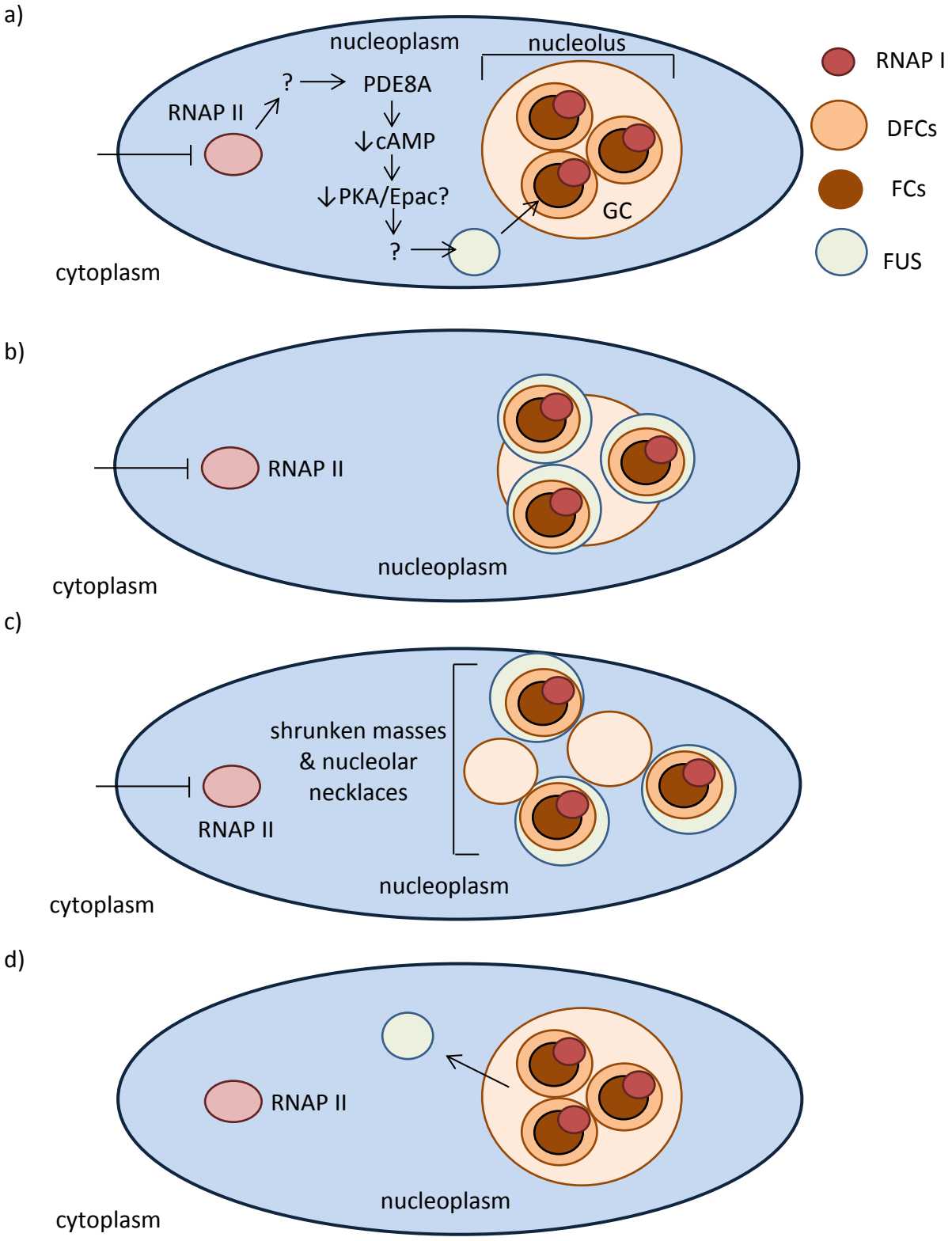


Figure 7.1 | **Illustration of FUS recruitment to the nucleolar necklace.** a) RNAP II is inhibited, signalling through an unknown intermediary to FUS via PDE8A. RNAP I transcribes at the junction between the FC and DFC. b) Nucleolar disruption begins, rDNA decompacts and FUS foci form on the pre-rRNA at the segregating DFCs and FCs. c) Nucleolar disruption continues, FUS remains associated with the pre-rRNA in the nucleolar necklace. RNAP I transcription continues but processing is impaired. d) Up to one hour after RNAP II inhibition is released the nucleolus returns to its normal morphology, FUS is released from the pre-rRNA and normal processing restarts.

Appendix A: ImageJ macros

Special thanks to Alex Herbert who was responsible for the bulk of the development of these macros.

Setting display values to the maximum contrast per image, conversion to RGB colour

```
dir1 = getDirectory("Choose Source Directory ");
dir2 = getDirectory("Choose Destination Directory ");

function processDirectory(dir) {
   list = getFileList(dir);
   for (i=0; i<list.length; i++) {
      if (endsWith(list[i], "\\") || endsWith(list[i], "/"))
         processDirectory(""+dir+list[i]);
      else
         processFile("" + dir + list[i]);
   }
}

function processFile(filename) {
   if (endsWith(filename, "tif")) {

      open(filename);

      title = getTitle();
      for (j=1; j<=nSlices; j++) {
         setSlice(j);
         resetMinAndMax();
      }
      run("Stack to RGB");
      selectWindow(title);
      close();
```

```
      basename = substring(filename, lengthOf(dir1));
      basename = replace(basename, "\\", "_");
      basename = replace(basename, "/", "_");
      basename = dir2 + basename;
      print(basename);
      saveAs("tiff", basename);
      close();

   } // end is tif
}

processDirectory(dir1);
```

Setting display values to the same values in a batch of images, conversion to RGB colour

```
dir1 = getDirectory("Choose Source Directory ");
dir2 = getDirectory("Choose Destination Directory ");

var count = 0;
var amin = newArray(3);
var amax = newArray(3);
Array.fill(amin, 65536);

function findMinAndMaxDirectory(dir) {
   list = getFileList(dir);
   for (i=0; i<list.length; i++) {
      if (endsWith(list[i], "\\") || endsWith(list[i], "/"))
         findMinAndMaxDirectory(""+dir+list[i]);
      else
         findMinAndMaxFile("" + dir + list[i]);
   }
}

function findMinAndMaxFile(filename) {
```

```
  if (endsWith(filename, "tif") || endsWith(filename, "tiff")) {

    open(filename);
    title = getTitle();
    for (j=1; j<=nSlices; j++) {
      // Resize arrays
      if (amin.length < nSlices) {
        amin2 = newArray(nSlices);
        amax2 = newArray(nSlices);
        Array.fill(amin2, 65536);
        for (k=0; k<amin.length; k++) {
          amin2[k] = amin[k];
          amax2[k] = amax[k];
        }
        amin = amin2;
        amax = amax2;
      }

      setSlice(j);
      getStatistics(area,mean,min,max);
      print(title,j,min,max);
      if (min < amin[j-1]) {
        amin[j-1] = min;
      }
      if (max > amax[j-1]) {
        amax[j-1] = max;
      }
    }
    count++;
    close();

  } // end is tif
}
```

```
function processDirectory(dir) {
   list = getFileList(dir);
   for (i=0; i<list.length; i++) {
      if (endsWith(list[i], "\\") || endsWith(list[i], "/"))
         processDirectory(""+dir+list[i]);
      else
         processFile("" + dir + list[i]);
   }
}

function processFile(filename) {
   if (endsWith(filename, "tif") || endsWith(filename, "tiff")) {

      open(filename);

      title = getTitle();
      for (j=1; j<=nSlices; j++) {
         setSlice(j);
         setMinAndMax(amin[j-1], amax[j-1]);
      }
      run("Stack to RGB");
      selectWindow(title);
      close();

      basename = substring(filename, lengthOf(dir1));
      basename = replace(basename, "\\", "_");
      basename = replace(basename, "/", "_");
      basename = dir2 + basename;
      print(basename);
      saveAs("tiff", basename);
      close();
```

```
   } // end is tif
}

// -=-=-=-=-=-

print("Finding channel min/max");

findMinAndMaxDirectory(dir1);

print("Global min/max");
for (i=0; i<amin.length; i++) {
   print(i+1, amin[i], amax[i]);
}

print("Converting to RGB");

processDirectory(dir1);
```

Converting RGB images into individual images for each channel (MicroManager output)

```
dir1 = getDirectory("Choose Source Directory ");
dir2 = getDirectory("Choose Destination Directory ");
list = getFileList(dir1);
setBatchMode(true);
for (i=0; i<list.length; i++) {
showProgress(i+1, list.length);
filename = dir1 + list[i];
shortfilename = list[i];
if (endsWith(shortfilename, "tif")) {
open(filename);
imageTitle=getTitle();
run("Split Channels");
selectWindow(imageTitle+" (blue)");
run("Blue");
```

```
saveAs("tiff", dir2+shortfilename+" (blue)");
selectWindow(imageTitle+" (red)");
run("Red");
saveAs("tiff", dir2+shortfilename+" (red)");
selectWindow(imageTitle+" (green)");
run("Green");
saveAs("tiff", dir2+shortfilename+" (green)");
}
else{}
}
```

Converting RGB images into individual images for each channel (SimplePCI output)

```
dir1 = getDirectory("Choose Source Directory ");
dir2 = getDirectory("Choose Destination Directory ");
list = getFileList(dir1);
setBatchMode(true);
for (i=0; i<list.length; i++) {
showProgress(i+1, list.length);
filename = dir1 + list[i];
shortfilename = list[i];
if (endsWith(shortfilename, "jpg")) {
open(filename);
imageTitle=getTitle();
run("Split Channels");
selectWindow(imageTitle+" (blue)");
run("Blue");
saveAs("Jpeg", dir2+shortfilename+" (blue)");
selectWindow(imageTitle+" (red)");
run("Red");
saveAs("Jpeg", dir2+shortfilename+" (red)");
selectWindow(imageTitle+" (green)");
run("Green");
saveAs("Jpeg", dir2+shortfilename+" (green)");
}
```

```
else{}
}
```

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