ROLE OF BET FAMILY OF TRANSCRIPTION REGULATORS IN DNA REPLICATION STRESS

By

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Abstract:

BET proteins function as epigenetic readers, and BET inhibition has been shown to have activity against many different cancers. BET inhibitors have been fast-tracked into first clinical trials, however the effects of these inhibitors are still poorly understood. A more detailed understanding about the physiological effects of BET inhibition is important and may lead to improved clinical applications as well as allowing safer use of BET inhibitor drugs.

In this thesis we have been able to identify that BET inhibition unexpectedly leads to an increase in RNA synthesis that is associated with conflicts between transcription and replication, leading to replication fork slowing, a sign of replication stress. We have identified BRD4 as the main target of BET inhibition in this process which is needed for normal fork progression. Interestingly, our data suggest that increased RNA synthesis requires free P-TEFb to be released from its inhibitor complex HEXIM1 to allow increased RNA polymerase II phosphorylation. HEXIM1 is required for BET inhibitor-induced replication-transcription conflicts.

We have shown that BET inhibitor-induced fork slowing does not activate the canonical ATR-Chk1 replication stress response pathway. However, it activates the homologous recombination factor RAD51, which is recruited into nuclear foci in response to BET inhibitor treatment. RAD51 depletion followed by BET inhibition prevents replication fork slowing but activates the replication stress response. HEXIM1 depletion has the same effect, preventing fork slowing and activating the replication stress response. These data suggest that i) replication fork slowing is required to prevent DNA damage formation in presence of BET inhibitors and ii) this

depends on HEXIM1, which is upstream of transcription-replication conflicts, and RAD51, which acts downstream of transcription-replication conflicts.

Our data shed light on the initial stress response during the first 8 hours of BET inhibition. They implicate HEXIM1 and RAD51, which both play potential roles in BET inhibitor resistance, in the BET inhibitor-induced replication stress pathway.

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Author Contributions:

I have performed all the work and experiments presented in this thesis except for Figures 7.1 C which was performed by Dr Ann Liza Piberger and Figure 6.6 D performed by Dr Eva Petermann. Where information has been used from other sources has been explicitly indicated in the thesis.

Abbreviations:

53BP1: p53-Binding protein 1
ALL: Acute lymphoblastic leukaemia
AML: Acute myeloid leukaemia
APS Ammonium persulfate
ARM: Arginine rich binding motif
ATM: Ataxia telangiectasia mutated
ATP Adenosine triphosphate
ATR: ATM and Rad4 related
ATRIP: ATR interacting protein
BLM: Bloom syndrome protein
Bp: Base pair
BRCA1: Breast cancer associated gene 1
BRCA2: Breast cancer associated gene 2
BSA: Bovine Serum Albumin
BIR: Break-induced replication
Cdc45 Cell division cycle 45
Cdc2: Cell division cycle 2
Cdc6: Cell division cycle 6
CDK1: Cyclin dependent kinase 1
CDK2: Cyclin dependent kinase 2
CDK5: Cyclin dependent kinase 5
CDK7: Cyclin-dependent kinase 7
CDK9: Cyclin-dependent kinase 9
CDKs: Cyclin dependent kinases
Cdt1: Chromatin licensing and DNA replication factor 1
CFS: Common fragile sites
CHK1: Checkpoint kinase 1
CHK2: Checkpoint kinase 2

CLL: Chronic lymphocytic leukaemia

CldU: 5-Chloro-2'-deoxyuridine

CMG: Cdc45/MCM/GINS complex

CPA: 3'end cleavage and polyadenylation complex

CPT: Camptothecin

CRPC: Castrate-resistant prostate cancer

Con: Control

CtIP: Ct-BP interacting protein

CTD: C-terminal Domain

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethylsulfoxide

DNA2: DNA replication helicase/nuclease 2

DNA-PKcs: DNA-dependent protein kinase catalytic subunit

dNTP: Deoxynucleotide triphosphate

DPE: Downstream Promotor Element

DSB: Double strand break

DSIF: DRB-sensitivity inducing factor

dsDNA: Double stranded DNA

E. Coli: Escherichia Coli

ERFS: Early replicating fragile sites

ES: Embryonic stem cells

ET domain: Extra terminal domain

EU: Ethynyl Uridine

EXO1 Exonuclease 1

FBH1: F-box DNA helicase 1

FBS: Fetal bovine serum

G1: Growth phase 1

G2: Growth phase 2

GFP: Green fluorescent protein

GINS: Go, Ichi, Ni, San (Sld5, Psf1, Psf2 and Psf3)

GTF: General transcription factor

HAT: Histone acetyltransferase

HDAC: Histone deacetylase
HJ: Holliday junction
HMBA: Hexamethylene bis-acetamide
HR: Homologous recombination
HU: Hydroxyurea
ICLs: Inter-strand cross-links
IdU: Iododeoxyuridine
IF: Immunofluorescence
INR: Initiator
Kb: Kilobase
LB: Luria Bertani
M: Mitosis
MCM: Minichromosome maintenance complex
MCM2-7: Minichromosome maintenance complex 2-7
Min: Minute
MLL: Mixed lineage leukaemia
MM: Multiple myeloma
m-RNA: Messenger RNA
MRN: Mre11/Rad50/Nbs1
MYC: MYC Proto-Oncogene
NELF: Negative elongation factor
NHEJ: Non-homologous end-joining
NS: Not significant
NSCLC: Non-small cell lung cancer
NT: Nucleotide
NTP: Nucleotide triphosphate
ORC: Origin recognition complex
PARP1: Poly-ADP ribose-polymerase 1
PARPi: Poly-ADP ribose-polymerase inhibitor
PBS: Phosphate buffered saline
PCNA: Proliferating cell nuclear antigen
pS2: Phosphorylated serine 2

pS5: Phosphorylated serine 5

PFA: paraformaldehyde

PR: Partial response

PIC: Preinitiation complex

Pre-RCs: Pre-replication complex

P-TEFb: Positive elongation factor

PTM: Post-translational modification

RAD51: Radiation sensitivity gene 51

RECQ1: ATP-dependent DNA helicase Q1

RFC: Replication factor C

RPA: Replication protein A

RPM: Rotations per minute

RPMI: Roswell Park Memorial Institute

RNA Pol I: RNA polymerase 1

RNA Pol II: RNA polymerase 2

RNA Pol III: RNA polymerase 3

RNase H: Ribonuclease H

SDS: Sodium Dodecyl Sulphate

S.E.M: Standard error of the mean

SiRNA: Small interfering RNA

S-phase: Synthesis phase

SMARCAL1: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1

SSB: Single strand break

ssDNA: Single stranded DNA

SEC: Super elongation complex

SEED motif: Serine-glutamine-aspartate rich motif

Ser2: Serine 2

Ser5: Serine 5

Ser7: Serine 7

SL (1-4): Stem-loops (1-4)

Sn-RNA: Small nuclear RNA

t-RNA: Transfer RNA

TAE: Tris Acetic acid EDTA buffer

TBST: TBS tween

TEMED: Tetramethylethylenediamine

TF: Transcription factor

Thr: Threonine

TOPBP1: DNA topoisomerase 2-binding protein 1

TSS: Transcription start site

UTB: Urea Tris Buffer

WRN: Werner Syndrome, RecQ-Helicase-like

XLF: XRCC4-like factor

XRCC: X-ray complementing Chinese hamster gene

ZRANB3: Zinc-finger, RAN-binding domain containing 3

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1. Introduction

1.1: Transcription and its regulation by HEXIM1

1.1.1: Transcription overview

Transcription is an important biological process in which a DNA template information can be read and lead to synthesis of various RNA species. In eukaryotic cells there are three different RNA polymerases (RNA Pols) that carry out RNA synthesis in the cell: RNA Pol I, RNA Pol II and RNA Pol III.

RNA Pol I and III transcribe the majority of non-protein coding RNA such as transfer RNA's (tRNAs), ribosomal RNA's (rRNAs) and U6 small nuclear RNA's (snRNAs) which accounts for the vast majority of RNA in the cell. In particular RNA Pol I transcribes ribosomal RNA while RNA Pol III transcribes tRNAs and snRNAs (Paule and White 2000). RNA Pol II is involved in transcribing messenger RNA (mRNAs) from thousands of genes that help encode for protein synthesis. The process of transcription can be further divided into three crucial steps: initiation, elongation and termination. The mechanism and structure of RNA Pol II has been widely studied and will be described in further detail below.

1.1.2: Transcription initiation

RNA Pol II transcription is a highly regulated and controlled process and requires many factors to allow for transitions between the three stages. Eukaryotic RNA Pol II consists of a 12 subunit complex (RPB1-12), and have a catalytic core consisting of 10 subunits (Armache, Kettenberger et al. 2003). RNA Pol II has a mass over 500kDa (Fu, Gnatt et al. 1999, Liu, Bushnell et al. 2013). The RNA Pol II represents a claw shape over a central cleft for DNA. There are two channels for incoming nucleotide triphosphates (NTPs) and the newly ongoing synthesised RNA molecule (Bernecky, Herzog et al. 2016).

The initiation step of RNA Pol II is a highly regulated process. Firstly, the DNA is highly organised and compacted into chromatin. Transcription start sites (TSS) in highly compacted chromatin are inactive (Chiang 2006). However decomposition and opening of chromatin from external signals or Transcription factor (TF) recruitment allows TSS to recruit RNA Pol II and allow for transcription (Chiang 2006).

To help recruit the RNA Pol II to the gene start site there is a promoter region just before the transcription start site. A promoter can be defined as a sequence of DNA that is required for proper initiation of an RNA polymerase (Krishnamurthy and Hampsey 2009). The positioning of the promoter is key to allow proper initiation and transcription of the downstream gene. Most promoters are approximately no more than a 100 base pairs (Bp) from the TSS (Krishnamurthy and Hampsey 2009). An example of a promoter is the TATA box which lies 25bp from the TSS (Smale and

Kadonaga 2003). A promoter may have promoter elements to help recruit specific transcriptional machinery to the TSS, for example the TATA box has an Initiator (Inr) and a downstream promoter element (DPE) (Smale and Kadonaga 2003). Although most promoters contain some of these elements it is not essential for the promoter to function.

For initiation to take place it requires the formation of the preinitiation complex (PIC) which requires the general transcription factors (GTFs). The GTFs are TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Matsui, Segall et al. 1980, Buratowski, Hahn et al. 1989, Chiang 2006). Without the GTFs RNA Pol II is unable to recognize the promoter (Chiang 2006). Another component of the PIC is the large mediator complex. In the case of TATA box promoter, the TATA binding subunit of TFIID can recognize and bind to the TATA sequence (Nikolov, Hu et al. 1992, Krishnamurthy and Hampsey 2009, Louder, He et al. 2016). If there is another promoter, binding can happen with another subunit of TFIID which can recognize the promoter sequence. TFIIB recognizes the TATA-binding protein (TBP) and DNA complex and binds to DNA to form a TFIIB-TFIID-DNA complex (Nikolov, Hu et al. 1992, Krishnamurthy and Hampsey 2009). This complex can help recruit the RNA Pol II which is helped by TFIIF. Finally TFIIE binds to complete the PIC (Krishnamurthy and Hampsey 2009) (Figure 1.1). The mediator complex helps RNA Pol II interact with regulatory signals coming from proteins bound to enhancers (Davis, Takagi et al. 2002, Lewis and Reinberg 2003). Once the PIC has been formed the RNA Pol II is ready to transition to elongation.

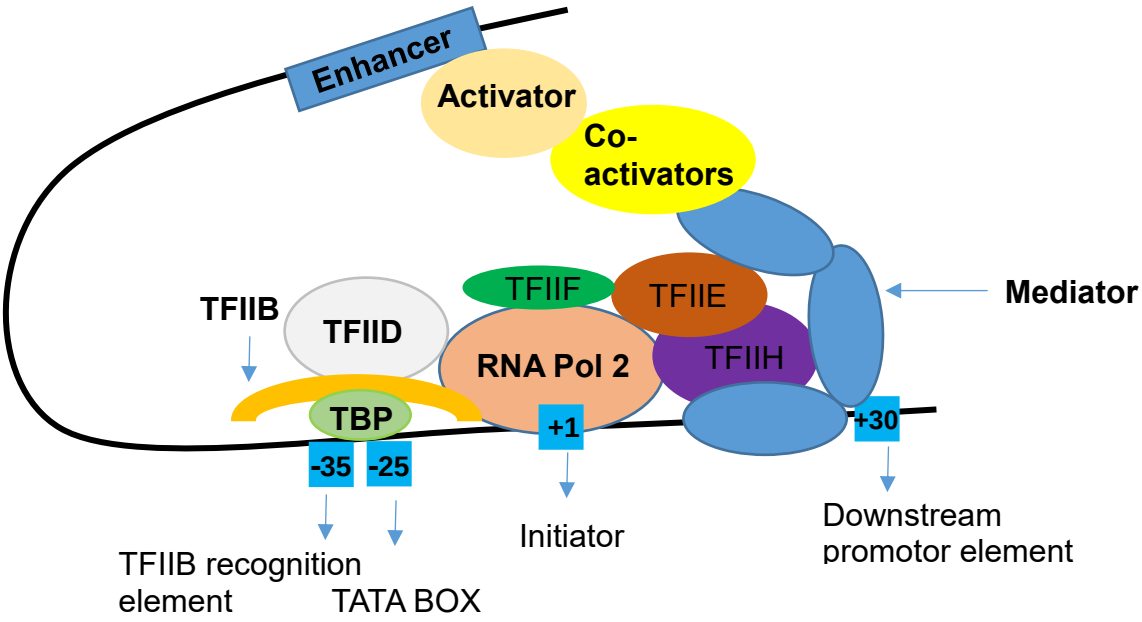


Figure 1.1: Diagram showing how RNA Pol II is recruited to the TSS via GTF's, promoter sequences (TATA Box) and enhancers.

1.1.3: Transcription elongation

To allow a transition of RNA Pol II into the elongation phase requires C-terminal domain (CTD) phosphorylation and elongation factors (Heidemann, Hintermair et al. 2013). RNA Pol II elongation requires regulation throughout the process. Regulation of transcription often happens when RNA Pol II is paused roughly around 30-100 nucleotides (Nt's) downstream of TSS in a process known as promoter-proximal pausing of RNA Pol II (Adelman and Lis 2012). This process happens in the initial stages of transcription elongation. This step is the rate limiting step in transcription which helps regulate the process and act to ensure correct modifications to RNA Pol II and 5' capping has occurred before elongation occurs. Proximal pausing of RNA Pol II occurs after initiation of transcription has occurred and requires

transcription factors negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) to induce pausing and stabilize RNA Pol II that is paused downstream of the TSS (Wada, Takagi et al. 1998, Yamaguchi, Takagi et al. 1999, Jonkers and Lis 2015). RNA Pol II pausing is an extremely dynamic mechanism which leads to continuous turnover of RNA Pol II causing premature terminations rather than stable persistent paused RNA Pol II (Krebs, Imanci et al. 2017). To allow for RNA Pol II to continue for productive elongation further signals are required.

A key factor in allowing release of paused RNA Pol II is the positive elongation factor b (P-TEFb) complex which includes the cyclin-dependent 9 kinase (CDK9). Research has shown how important the CDK9 activity is in allowing transition into productive elongation. A study showed that if P-TEFb is inhibited in the cell it affects 95% of genes actively transcribed indicating the importance of the transition between initiation and elongation as a key regulatory step in transcription (Jonkers, Kwak et al. 2014). P-TEFb is recruited to the RNA Pol II via its interactions with transcriptional co-factors. A large number of co-factors have been shown to interact with P-TEFb including BRD4 (Peterlin and Price 2006, Adelman and Lis 2012). Once recruited, P-TEFb can lead to phosphorylation of the CTD at Serine 2 (Ser2) and NELF and DSIF (Marshall and Price 1992, Wada, Takagi et al. 1998, Yamaguchi, Takagi et al. 1999, Yamada, Yamaguchi et al. 2006). P-TEFb can form activating complexes with BRD4 or the super elongation complex (SEC) (Smith, Lin et al. 2011, Itzen, Greifenberg et al. 2014). P-TEFb has an inhibitory complex HEXIM1-7SK which can help regulate RNA Pol II transcription as well (Quaresma, Bugai et al. 2016). This process allows RNA Pol II to escape the paused state and

allow productive elongation to occur (Peterlin and Price 2006, Jonkers and Lis 2015) (Figure 1.2).

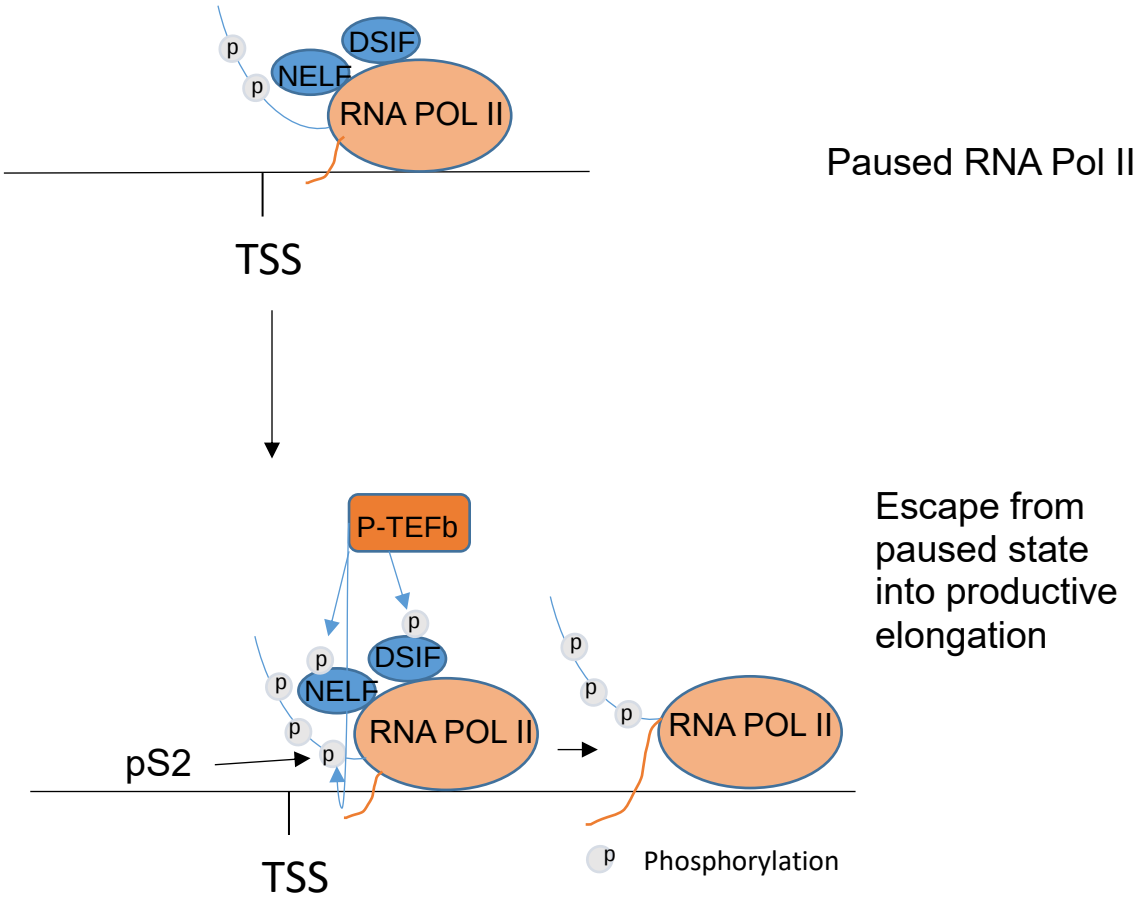


Figure 1.2: Diagram illustrating key events that lead to escape from RNA Pol II proximal pausing.

Transcription elongation can occur through diffusion of RNA Pol II through different conformational states rather than being driven by an ATP dependent process (Herbert, Greenleaf et al. 2008). The first state which occurs when a new nucleotide is added to the newly synthesised RNA molecule. This is followed by a state where

the active site is empty after the RNA Pol II has moved one nucleotide along the DNA allowing for another nucleotide to be incorporated (Bar-Nahum, Epshtein et al. 2005, Herbert, Greenleaf et al. 2008, Liu, Bushnell et al. 2013). The last state is when the polymerase moves backwards (backtrack) moving the newly incorporated nucleotide back out of the active site (Herbert, Greenleaf et al. 2008). For RNA Pol II to move forward and be stable in each state requires the binding and hydrolysis of the correct nucleotide which is to be incorporated into the growing chain (Bar-Nahum, Epshtein et al. 2005, Herbert, Greenleaf et al. 2008, Liu, Bushnell et al. 2013).

It is crucial to ensure that the correct nucleotides are incorporated into the growing RNA chain. Backtracking can help allow regulation of correct nucleotides (Selth, Sigurdsson et al. 2010). Studies have shown that when an incorrect nucleotide is incorporated then it can lead to slowing down of the incorporation of the next nucleotide, increasing its ability to be cleaved by RNA Pol II which has endonuclease activity to allow for removal of incorrect nucleotides (Sydow, Brueckner et al. 2009, Selth, Sigurdsson et al. 2010). This mechanism is aided and stimulated by elongation factor TFIIS which increases the removal of incorrect nucleotides (Selth, Sigurdsson et al. 2010).

Another mechanism to insure RNA Pol II fidelity is the trigger loop structure in RNA Pol II of the Rpb1 subunit (Kaplan, Larsson et al. 2008). The trigger loop resides below the active site. It functions by forming a plethora of different interactions with the new incoming nucleotide (Wang, Bushnell et al. 2006). These interactions serve to ensure that the incoming nucleotide correctly orientates with the trigger loop which allows for the correct chemistry to occur so that there is a formation of a

phosphodiester bond between the incoming nucleotide and the growing RNA chain. If a nucleotide is disorientated with the trigger loop it causes slowing down of the formation of phosphodiester bond allowing RNA Pol II to cleave or backtrack to ensure its removal (Wang, Bushnell et al. 2006, Kaplan, Larsson et al. 2008). Recent studies in yeast have shown that the Rpb9 subunit can also affect RNA Pol II fidelity (Nesser, Peterson et al. 2006). Further research highlighted that Rpb9 may act to slow closure of the trigger loop on the incoming nucleotide (Walmacq, Kireeva et al. 2009).

Transcription elongation does not happen uniformly but varies in elongation rate (Selth, Sigurdsson et al. 2010, Adelman and Lis 2012, Jonkers, Kwak et al. 2014). This may be due to a number of factors including elongating through chromatin or DNA sequences that are hard to transcribe and perform barriers to elongation such as G-quadruplexes or R-loops (Jonkers and Lis 2015). Factors such as histone chaperones or chromatin re-modellers can help loosen DNA to help facilitate transcription (Chiang 2006, Jonkers and Lis 2015). Modifications to histones can also signal for changes to chromatin structure (Jonkers and Lis 2015). Overall elongation is subject to significant pausing while transcribing with average elongation rates usually are around 20-30 nucleotides/second, however there have been rates of 70 nucleotides/second measured when elongation is not encountering significant pausing (Darzacq, Shav-Tal et al. 2007).

1.1.4: Transcription termination

Although many details of transcription have been widely studied, transcription termination is just only now being defined. Although the complicated mechanism of termination is clearer now it is still poorly understood. Firstly the process of transcription termination can occur at any point within the gene (Proudfoot 2016). The process of termination of RNA Pol II also linked with mRNA processing which occurs co-transcriptionally (Proudfoot 2016). Genes are regions of genome that make up one transcription unit from the promoter to the terminator region of the gene (Proudfoot 2016). When the RNA Pol II transcribes over the terminator the rate of RNA Pol II movement slows down. The terminator is a sequence of DNA that provides signals in the new RNA transcript (poly-A) that trigger processes such as the releases of mRNA and RNA Pol II (Porrua and Libri 2015, Proudfoot 2016). This is due to the recognition of the poly-A signal appearing in the transcript which is recognised by 3'end cleavage and polyadenylation complex (CPA) (Takagaki, Ryner et al. 1989, McCracken, Fong et al. 1997, Proudfoot 2004). When the mRNA transcript is formed, R-loops are more readily formed due to the terminator sequence transcribed which is more likely to form R-loops. The transcript will then invade the antisense DNA strand which has been transcribed to further slowdown RNA Pol II (Skourti-Stathaki, Kamieniarz-Gdula et al. 2014). CPA can lead to cleavage of the mRNA at the poly-A signal releasing the mRNA from the RNA Pol II (Xiang, Tong et al. 2014, Proudfoot 2016). RNA Pol II will still transcribe despite the release of the mRNA and requires further action to dissociate. At the same time Xrn2 exonuclease can attack the transcribing RNA from the 5' end and degrade the

RNA transcript one nucleotide at a time (Proudfoot 2004, Porrua and Libri 2015). When the Xrn2 reaches the RNA Pol II it causes conformational changes to the RNA Pol II and its active site leading to RNA Pol II being released from the DNA (West, Gromak et al. 2004, Porrua and Libri 2015, Proudfoot 2016).

Termination is important for gene regulation, firstly genes can generate a multitude of different mRNAs varying in length caused by alternative poly-A sites which lead to termination at various points (Tian and Manley 2013). This produces mRNAs with different 3'UTR sequences which can encode for various functions (Tian and Manley 2013, Proudfoot 2016). Termination can also serve to rid the cell of transcription errors, as mis-synthesised RNA can cause early termination before the gene end and allows for degradation of the RNA (Proudfoot 2016). Aberrant termination can lead to prolonged transcription through the downstream gene leading to downregulation of gene expression of this gene (Shearwin, Callen et al. 2005). Once mRNA is transcribed it can be processed, spliced and is ready for exportation.

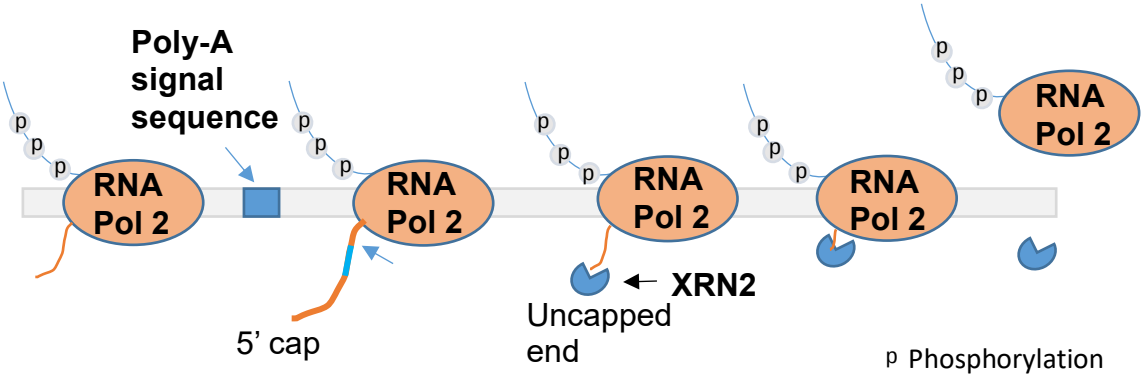


Figure 1.3: Diagram illustrating mechanism of transcription termination.

1.1.5: RNA Pol II CTD code

RNA Pol II subunit Rpb1 contains CTD which contains multiple heptad repeats consisting of Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 which is repeated 26 times in yeast and 52 times in vertebrates (Corden 1990). The CTD is heavily modified, mostly via phosphorylation, at each position which gives rise to different patterns during the transcription processes and helps regulate and modulate this process (Phatnani and Greenleaf 2006, Hsin and Manley 2012). This modification of the CTD can lead to recruitment of various transcription factors associated with initiation, elongation and termination allowing for regulation of these processes and for transition between the stages.

A general model of transcription highlights that at the beginning of transcription as one of the first events of initiation serine 5 (Ser5) is phosphorylated by Cyclin dependent kinase 7 (CDK7) kinase of the initiation factor TFIIH (Guidi, Bjornsdottir et al. 2004, Phatnani and Greenleaf 2006, Harlen and Churchman 2017). During elongation Ser2 is increasingly modified by phosphorylation via the P-TEFb's CDK9 kinase, as Ser5 is gradually removed by phosphatases (Peterlin and Price 2006, Phatnani and Greenleaf 2006, Harlen and Churchman 2017). These phosphorylation's can broadly act as markers for different stages of transcription with Ser2 for elongation/termination and Ser5 through initiation.

The CTD via modification can serve to recruit histone modifiers and chromatin re-modellers to the RNA Pol II to help regulate transcription processes through initiation to termination (Spain and Govind 2011). CTD modifications also have key

regulatory roles in pre-mRNA processing roles such as capping, splicing, and 3' end processing (Proudfoot, Furger et al. 2002, Hsin and Manley 2012). Phosphorylation at Ser5 can lead to 5' capping enzymes being brought to the RNA Pol II and within vicinity of the mRNA ongoing transcript being produced (Burley and Sonenberg 2011). Research has recently shown Ser5 having a role causing chromatin remodelling and histone modification (Krogan, Keogh et al. 2003, Ng, Robert et al. 2003). Studies have shown that serine 7 (Ser7) is normally phosphorylated during transcription initiation as well. Ser7 is phosphorylated by CDK7 too, meaning both are phosphorylated by the same kinase during transcription initiation (Glover-Cutter, Larochelle et al. 2009). Studies in yeast cells indicate that Ser7 remains phosphorylated throughout elongation before being removed at termination (Hajheidari, Koncz et al. 2013). Ser7 acts to recruit RPAP2 and integrator complex to snRNA which is important in 3' processing of the mRNA, which can only happen once Ser5 is dephosphorylated (Egloff, Zaborowska et al. 2012).

Ser5 phosphorylation is removed by RPA2 and Ssu72 phosphatase in aid with PIN1 (Egloff and Murphy 2008, Krishnamurthy, Ghazy et al. 2009). Ssu72 is also involved in removing Ser7 phosphorylation as well (Zhang, Mosley et al. 2012). Removal of Ser5 is crucial to allow pre-mRNA processing mechanisms to function.

As mentioned earlier Ser2 phosphorylation is key in allowing processive elongation to occur after proximal pausing. This occurs via the activity of P-TEFb CDK9 kinase. The phosphorylation of Ser2 lasts through termination and functions by recruiting both elongation and termination transcription factors to the RNA Pol II (Meinhart and Cramer 2004, Yoh, Cho et al. 2007, Lunde, Reichow et al. 2010). Ser2 phosphorylation is also involved in recruiting splicing factors which helps define

where the splice sites are and allows for spliceosome assembly (Hsin and Manley 2012). It also serves a function is 3' end processing by recruiting Pcf11 (Meinhart and Cramer 2004). Ser2 phosphorylation is removed by phosphatase Fcp1. Thr4 is phosphorylated by CDK9 kinase during transcription elongation as well (Cho, Kobor et al. 2001, Ghosh, Shuman et al. 2008).

During transcription termination the CTD has some key roles. A protein dimer of p54/PS4 can bind to a phosphorylated Ser2 on the CTD. This dimer can facilitate termination by recruiting Xrn2 nuclease (Kaneko, Rozenblatt-Rosen et al. 2007, Hsin and Manley 2012). When p54 was depleted there were termination defects seen in HeLa cells (Kaneko, Rozenblatt-Rosen et al. 2007).

1.1.6: RNA polymerase I and III

As mentioned earlier there are three different RNA polymerases that transcribe RNA molecules. The previous chapter has been focusing on the role of RNA Pol II. There are some key differences between the three RNA Pol's in how they transcribe DNA.

Firstly, both RNA Pol I and RNA Pol III lack the heptad repeat on CTD which as described above is crucial for RNA Pol II function and regulation (Hsin and Manley 2012). RNA Pol I has the same mechanisms of transcription initiation, elongation and termination as RNA Pol II but has different transcription factors to allow transcription. The PIC of RNA Pol I requires specific transcription factors. SL1 acts to recognize specific RNA Pol I promoters, and UBF and RRN3 mediate promoter binding and RNA Pol I recruitment (Cavanaugh, Hirschler-Laszkiewicz et al. 2002,

Friedrich, Panov et al. 2005, Lin, Comai et al. 2006, Goodfellow and Zomerdijk 2012). Proximal pausing is relieved by phosphorylation of RRN3 allowing its release from RNA Pol I (Hirschler-Laszkiewicz, Cavanaugh et al. 2003, Bierhoff, Dundr et al. 2008). Both UBF and SL1 help mediate elongation with combination with other RNA Pol I specific elongation factors (Stefanovsky, Langlois et al. 2006, Denissov, Lessard et al. 2011). Termination employs a similar method to RNA Pol II but uses endonuclease RNT1 to cleave nascent pre-RNA followed by Xrn2 exonuclease to release RNA Pol I (Goodfellow and Zomerdijk 2012).

Less is known about the RNA Pol III mechanism. Again initiation requires three transcription factors: TFIIIA, TFIIIB and TFIIIC (Acker, Conesa et al. 2013). TFIIIB is composed of Brf1, Bdp1 and TBP. TFIIIC is a large complex that functions to recognize the promoter (Sentenac and Riva 2013, Turowski and Tollervey 2016). TFIIIC can lead to the recruitment of TFIIIB which can lead to RNA Pol III recruitment to DNA (Deprez, Arrebola et al. 1999). Events following initiation are less clear. Both elongation and termination occur in similar mechanisms to RNA Pol II, while having specific RNA Pol III transcription factors to allow for these processes to occur (Turowski and Tollervey 2016).

1.1.7: Role of 7SK-snRP and HEXIM1

As mentioned previously, P-TEFb has a vital role in regulating RNA Pol II transcription and allowing its release from a paused state. It is therefore crucial that the P-TEFb is tightly regulated. This actually requires the work of RNA Pol III, which produces a non-coding small nuclear RNA 7SK (Sinha, Leinisch et al. 2014). 7SK is

able to bind to P-TEFb and inactivate its kinase ability (Nguyen, Kiss et al. 2001). More recent research has actually shown that 7SK functions as a scaffold for other proteins. This scaffold forms a base for which a complex known as the 7SK-snRNP can be formed which enables inhibition of P-TEFb and enhances 7SK stability (Quaresma, Bugai et al. 2016). The 7SK-snRNP comprises of four proteins: 7SK, LARP7, MePCE as well as the P-TEFb inhibitor HEXIM1 (Yik, Chen et al. 2003, Jeronimo, Forget et al. 2007, He, Jahchan et al. 2008). The majority of P-TEFb is found sequestered in this complex and can only be activated by disruption of the complex and release of free P-TEFb (Yik, Chen et al. 2003) (Figure 1.4).

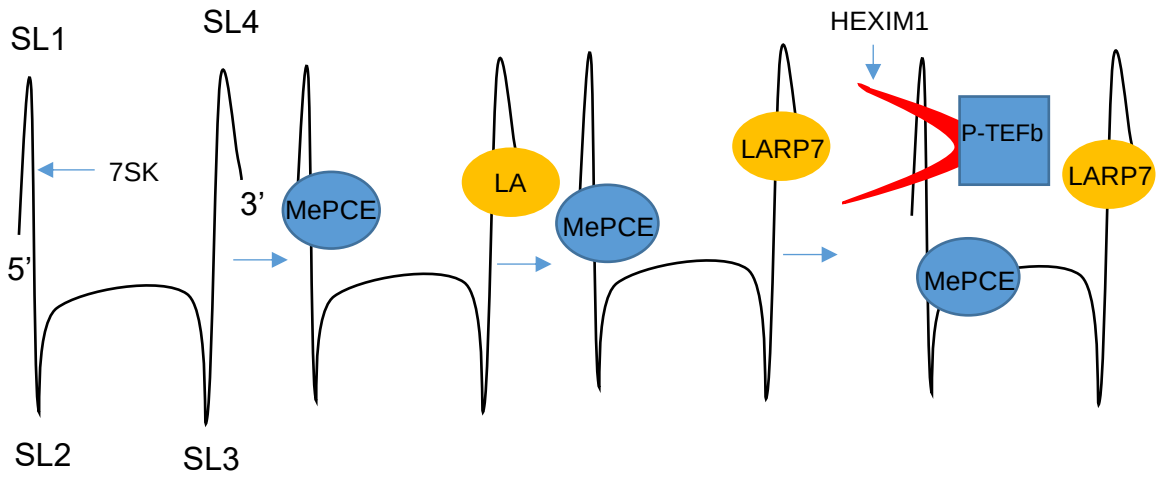


Figure 1.4: Diagram illustrating the formation of the 7SK-snRNP complex to sequester P-TEFb.

To form the 7SK-snRNP complex, firstly 7SK is folded and forms a structure that consists of four stem loops (SL1-4) (Quaresma, Bugai et al. 2016, Brogie and Price 2017). This is followed by binding of LA and MePCE that associate with basal end

of SL1 and the 7SK 3'terminal U-rich region (Chambers, Kurilla et al. 1983, Muniz, Egloff et al. 2013). This helps prevent 7SK degradation and caps the 5'end of the 7SK which triggers LA being replaced by LARP7 which associated with both the U-rich region and SL4 providing 7SK stability (Chen, Xue et al. 2009). HEXIM1 contains an N-terminal regulatory domain, an arginine rich binding motif (ARM), an acidic and a coil-coil region which mediates HEXIM1 dimerization (Zhou and Yik 2006). The last part involves HEXIM1 binding to the 7SK SL1 via its ARMS which acts as a 7SK binding domain (Michels, Fraldi et al. 2004). This allows the dimeric coil-coil region of HEXIM1 to bind to P-TEFb which has been phosphorylated at Threonine (Thr) 186 by CDK7 to complete the 7SK-snRNP complex (Blazek, Barboric et al. 2005). This phosphorylation on P-TEFb has been shown to be crucial for the 7SK-P-TEFb interaction. This is the crucial step, and HEXIM1 is suggested to inhibit P-TEFb by stopping ATP association with the CDK9 kinase (Li, Price et al. 2005). The acidic domain interacts with the dimerised basic region in the ARM to prevent early binding of P-TEFb to HEXIM1 without interaction with the 7SK (Zhou and Yik 2006). When P-TEFb is released, the 7SK-snRNP still remains stable and can rebind free P-TEFb (Bartholomeeusen, Xiang et al. 2012, Quaresma, Bugai et al. 2016) .

7SK-snRNP complexes have been shown to be anchored to chromatin near promoters and enhancers by chromatin adaptor factors (Flynn, Do et al. 2016, Quaresma, Bugai et al. 2016). This allows the inactive pool of P-TEFb to be in close proximity to RNA Pol II allowing activation of P-TEFb and release of RNA Pol II from proximal pausing to occur in quick time (Quaresma, Bugai et al. 2016).

P-TEFb is released from 7SK via a series of post-translational modifications and proteolysis. Firstly, HEXIM1 is phosphorylated along a number of residues just before its coil-coil domain which hinders its ability to bind to P-TEFb (Contreras, Barboric et al. 2007). P-TEFb phosphorylation at Thr 186 is dephosphorylated (Cho, Schroeder et al. 2009). These changes allow P-TEFb to be released. While this process is reversible as 7SK core remains intact, there are a few changes that cause P-TEFb activation to be irreversible by destabilizing the 7SK core as shown in a recent study in megakaryopoiesis (Elagib, Rubinstein et al. 2013). This involves the MePCE proteolysis and demethylation of 7SK. In addition LARP7 was also downregulated, possibly by proteolytic pathways and transcriptional and posttranscriptional repression, which lead to 7SK core being completely destabilized (Elagib, Rubinstein et al. 2013). The post translational modifications (PTMs) involved are normally a result of a cell signalling cascade. For example, hexamethylene bis-acetamide (HMBA) treatment lead to phosphorylation of HEXIM1 via the PI3K/AKT pathway which lead to P-TEFb activation (Contreras, Barboric et al. 2007). HIV 1-Tat virus can cause global release of P-TEFb or can prevent its sequestration to allow for continued viral transcription (Matija Peterlin, Yik et al. 2007). Various transcription factors can allow for P-TEFb release from 7SK-snRNP to allow for transcription elongation. These include BRD4, SRSF2 and DDX21 (Ji, Zhou et al. 2013, Liu, Ma et al. 2013, Calo, Flynn et al. 2014).

1.2: BET proteins and their role in transcription

1.2.1: BET proteins overview

BET proteins are at the cutting edge of new experimental targets for cancer treatment. Pre-clinical data as well as latest research show that targeting BET proteins can potentially be used and developed as an effective anticancer therapeutic in the future. They have been seen to play a role in the maintenance of DNA replication as well (Da Costa, Agathanggelou et al. 2013). A more detailed understanding of these proteins and their function is a necessity to further understand and develop future clinical applications.

There are approximately 61 bromodomains discovered in humans coming from over 46 human proteins (Filippakopoulos and Knapp 2012). Human bromodomain proteins are further sub-classified; one particular subfamily of bromodomains is known as the BET family (Figure 1.5). The BET subfamily of bromodomain proteins contain four different members; BRD2, BRD3, BRD4 and BRDT (Wu and Chiang 2007).

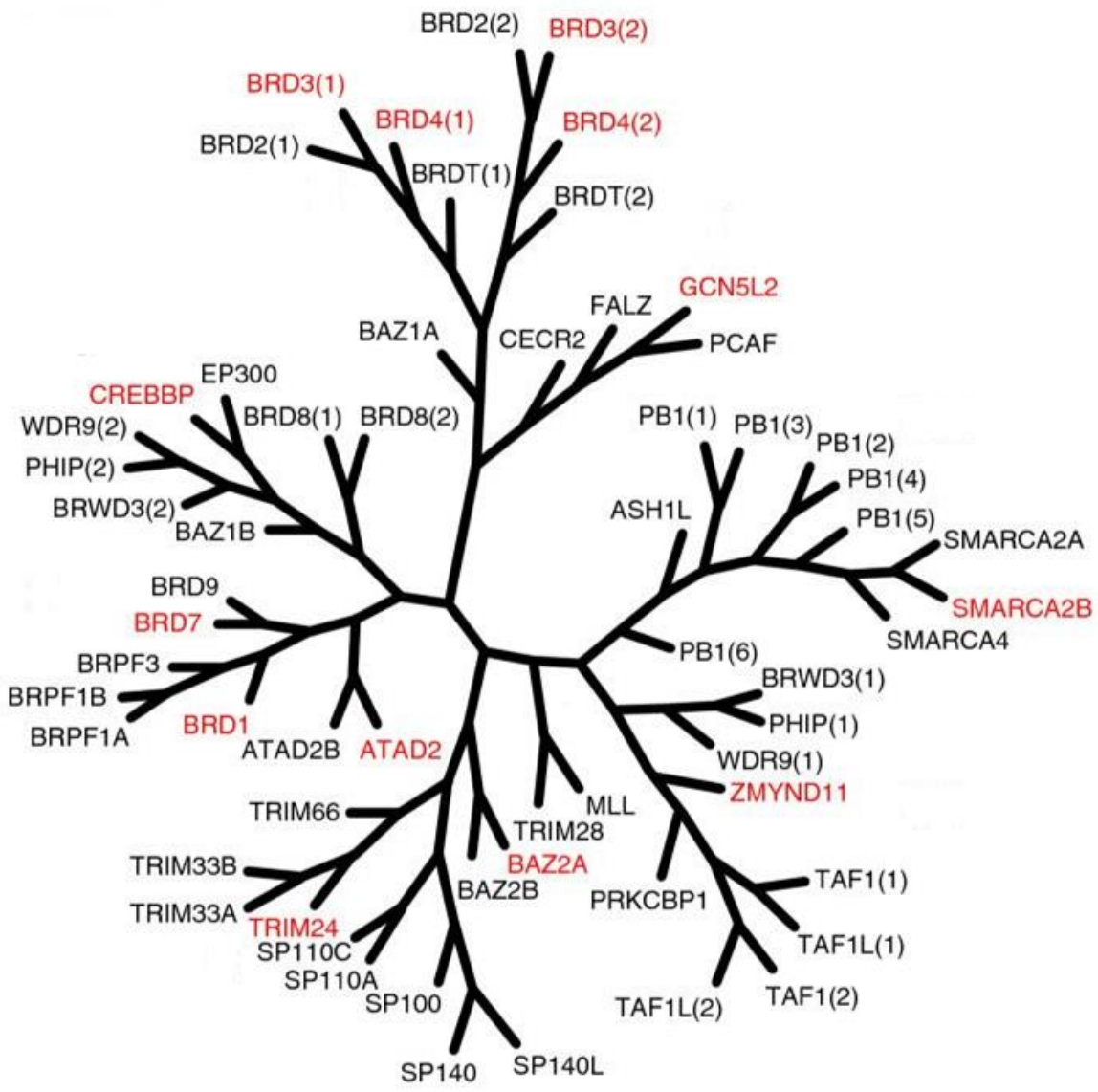


Figure 1.5: Phylogenetic tree depicting bromodomains based on structure. Retrieved from (Philpott, Rogers et al. 2014).

1.2.2: BET protein architecture

Each BET protein has a conserved architecture, consisting of two N-terminal tandem bromodomains and contain an extra terminal (ET) domain which is involved in facilitating protein-protein interactions (Florence and Faller 2001). BET proteins

also contain other motifs such as A, B or the serine-glutamine-aspartate rich region (SEED motif). BRD4 and BRDT also contain a C-terminal motif (Filippakopoulos, Picaud et al. 2012) (Figure 1.6).

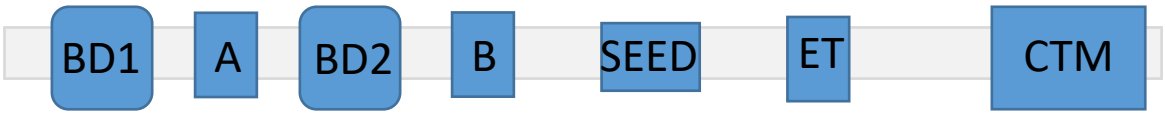


Figure 1.6: BET family protein domain organisation comprising of two tandem bromodomains, extra-terminal domain, a SEED motif and A and B motifs. BRD4 long isoform and BRDT also comprise of a C-terminal Motif (CTM).

The conserved bromodomains consists of a highly conserved motif of 110 amino acids, and all have the same conserved fold forming a left-handed bundle of four alpha-helices: αz, αa, αb, αc. These alpha helices are linked together by highly variable loop regions called ZA and BC loops (Dhalluin, Carlson et al. 1999, Zeng and Zhou 2002) (Figure 1.7).

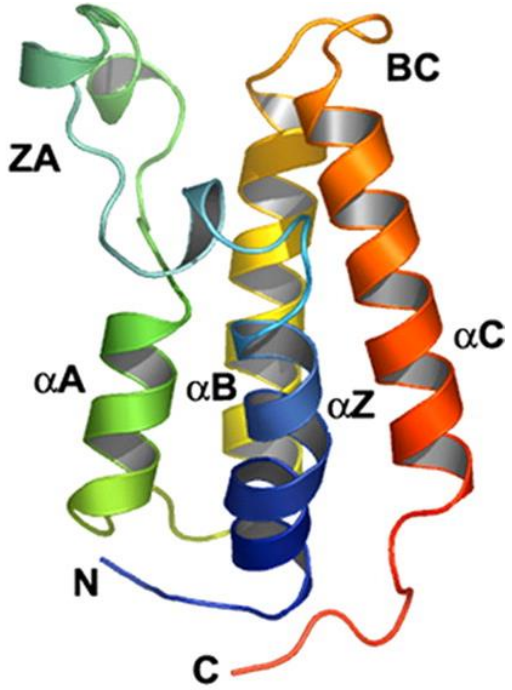


Figure 1.7: Diagram of the structure of BET-family bromodomains, illustrating a left handed bundle of four α-helices and two loop regions ZA and BC. Retrieved from (Nakamura, Umehara et al. 2007).

In between the two tandem bromodomains in BET proteins is a conserved 12 amino acid stretch (KGVKRKADTTTP). This stretch of amino acids serves to localise BET proteins to the nucleus. A study using HEK293T cells showed that deletion of this region led to mis-localisation of BET proteins (Fukazawa and Masumi 2012). A study resolved the extra-terminal domain structure, showing that is also an α-helical protein consisting of three α-helices (Lin, Umehara et al. 2008).

1.2.3: BET protein interactions

Chromatin may be modified in numerous ways; one modification of chromatin is the N-terminal acetylation of ε-lysine residues on histone tails. This modification is commonly associated with increased transcriptional activation and with open chromatin structure (Marushige 1976).

The diverse loop regions of the BET protein contain some conserved residues including a PxY motif at the C-terminal of the ZA loop region. A tyrosine residue is situated in the αB loop allowing a salt bridge to form to another residue on the αB loop which allows for fold stabilisation (Fujisawa and Filippakopoulos 2017).

High resolution crystal structures have shown that binding of BET protein bromodomains to acetylated lysine's on histones requires the conserved asparagine residue at the beginning of the BC loop (Owen, Ornaghi et al. 2000). By acetylating the lysine side chains, the lysine charge is neutralized. A stable hydrogen bond between the acetylated lysine and asparagine can promote the interaction (Filippakopoulos, Qi et al. 2010). A small hydrophobic binding pocket to allow binding of the acetylated lysine side chain is formed though the four α-helices (Dhalluin, Carlson et al. 1999, Fujisawa and Filippakopoulos 2017).

Once the conserved asparagine has initiated binding for the acetylated lysine's, the peptide backbone of the acetylated lysine forms interactions with the charged surface around the acetylated lysine binding pocket of BET proteins (Fujisawa and Filippakopoulos 2017).

Not only can the bromodomains interact with acetylated lysine's on histones, but they can also interact with a large number of other acetylated proteins such as transcription factors GATA1 and TWIST (Gamsjaeger, Webb et al. 2011, Shi, Wang et al. 2014).

BET proteins can form interactions with a myriad of diverse proteins via their ET domains. The ET domains consists of approximately 80 amino acids. The ET domain can regulate different BET protein functions by recruiting specific effector proteins such as NSD3. (Rahman, Sowa et al. 2011).

1.2.4: BET proteins role in transcription

BET proteins have been shown to play a multitude of roles in transcription. BET proteins have the ability to act as a scaffold to help recruit and build larger protein complexes including transcription factors via their ET domain (Rahman, Sowa et al. 2011). Binding of BET protein complexes to acetylated histone tails can help to increase transcription activation by increasing the effective concentration of transcription activators around the promoter region.

The BET protein BRD2 is a known Serine/Threonine kinase and its activity is upregulated with cellular proliferation (Denis and Green 1996). BRD2 functions as an important protein in cell-cycle progression. Research using 3T3 fibroblast showed that BRD2 promotes the activity of transcription factor E2F (Denis, Vaziri et al. 2000). E2F functions as a cell cycle transcription factor, which leads to the increased synthesis of proteins required to regulate the cell cycle progression

through the G1/S transition (Lee, Chang et al. 2002). BRD2 has also been shown to recruit TBP to the E2F-1 protein complex at promoters (Peng, Dong et al. 2007). In addition to E2F proteins, BRD2 can recruit histone deacetylases (HDACs) and histone acetyltransferases (HATs) which are involved in chromatin remodelling to regulate transcription (Houzelstein, Bullock et al. 2002). Genome wide associated studies (GWAS studies) showed that BRD2 regulates the expression of 1450 different proteins in the cell. It was also shown in HeLa cells that BRD2 could promote alternative splicing of genes while not changing the ability of RNA Pol 2 processivity around the alternative spliced elements in the gene (Hnilicová, Hozeifi et al. 2013). Other findings have shown that insufficient amounts of BRD2 protein in mice can lead to reduced number of neuronal cells hence BRD2 plays an important role in neuronal development as well (Shang, Wang et al. 2009, Tsume, Kimura-Yoshida et al. 2012).

Recent studies using an *in vitro* transcription assay in HeLa cells have shown that both BRD2 and BRD3 contain nucleosome chaperone activity, which can help RNAPII to elongate along the DNA and produce transcripts through regions that have heavily hyperacetylated nucleosomes (LeRoy, Rickards et al. 2008). A similar study using transcription assays confirmed that BRD4 also plays a similar role in helping the elongation of RNA Pol II through hyperacetylated nucleosomes (Kanno, Kanno et al. 2014).

BRD3 plays another role in transcription regulation in cells. BRD3 has been shown to directly interact with transcription factor GATA1 via GATA1's acetylated lysine residues (Gamsjaeger, Webb et al. 2011, Lamonica, Deng et al. 2011). GATA1 functions by regulating expression of genes specific for erythropoiesis

(Katsumura, DeVilbiss et al. 2013). Erythropoiesis is the process which leads to the formation of red blood cells (Hattangadi, Wong et al. 2011). By disrupting BRD3's ability to bind to chromatin, transcriptional repression of GATA1 mediated transcription can occur. ChIP-sequencing data showed that all four BET proteins occupy GATA-1 bound loci, however the amount of BRD3 increase at these loci following GATA1 activation is the most significant (Stonestrom, Hsu et al. 2015). Interestingly, BRD3 depletion in G1E-ER cells showed very little marked effect on erythroid transcription after GATA1 activation. BRD2 and BRD4 depletion lead to a more marked effect leading to less erythroid gene activation and lower levels of GATA1 transcript levels (Stonestrom, Hsu et al. 2015). However, when BRD3 is depleted in cells already depleted of BRD2 the effect on erythroid gene activation is greatly exacerbated (Stonestrom, Hsu et al. 2015). This points to maybe a synergistic effect of these proteins suggesting that BET protein functions may overlap with each other.

In addition to the roles shown above BRD2 is thought to promote transcription activation of HOXA11 and D11 shown in HEK293 cells (LeRoy, Rickards et al. 2008). BRD2 can also interact with LANA of KSHV which leads to episomal replication and persistence of viral genomes (Platt, Simpson et al. 1999, Viejo-Borbolla, Ottinger et al. 2005). BRD3 can promote the transcriptional activation of HOXB3-B6, C8-C10 and A3,A5-7 as shown in HEK293 cells (LeRoy, Rickards et al. 2008).

BET proteins BRD4 and BRDT are key regulators of transcriptional elongation in the cell. These proteins can actively recruit positive transcription elongation factor P-TEFb via their ET domain to chromatin (Jang, Mochizuki et al. 2005, Yang, Yik et al.

2005). P-TEFb is activated once free of its inhibitory complex HEXIM1 and can act as a protein kinase (Schroder, Cho et al. 2012). To allow for recruitment of P-TEFb, BRD4 interacts with JMJD6 protein via its JmjC domain. This causes demethylation of 7SK and H4K3me2, allowing release of P-TEFb from HEXIM1 (Liu, Ma et al. 2013). P-TEFb is recruited by BRD4 to chromatin where it can freely phosphorylate RNA Pol II on its CTD predominately at serine 2 (Itzen, Greifenberg et al. 2014). This phosphorylation allows RNAPII, which is paused at the transcription start site, to be activated and allow normal transcription elongation to occur (Phatnani and Greenleaf 2006) (Figure 1.8). Research has shown that BRD4 occupation can be widespread throughout the genome allowing it to stimulate transcription of both protein-coding and noncoding enhancer RNA's (Kanno, Kanno et al. 2014).

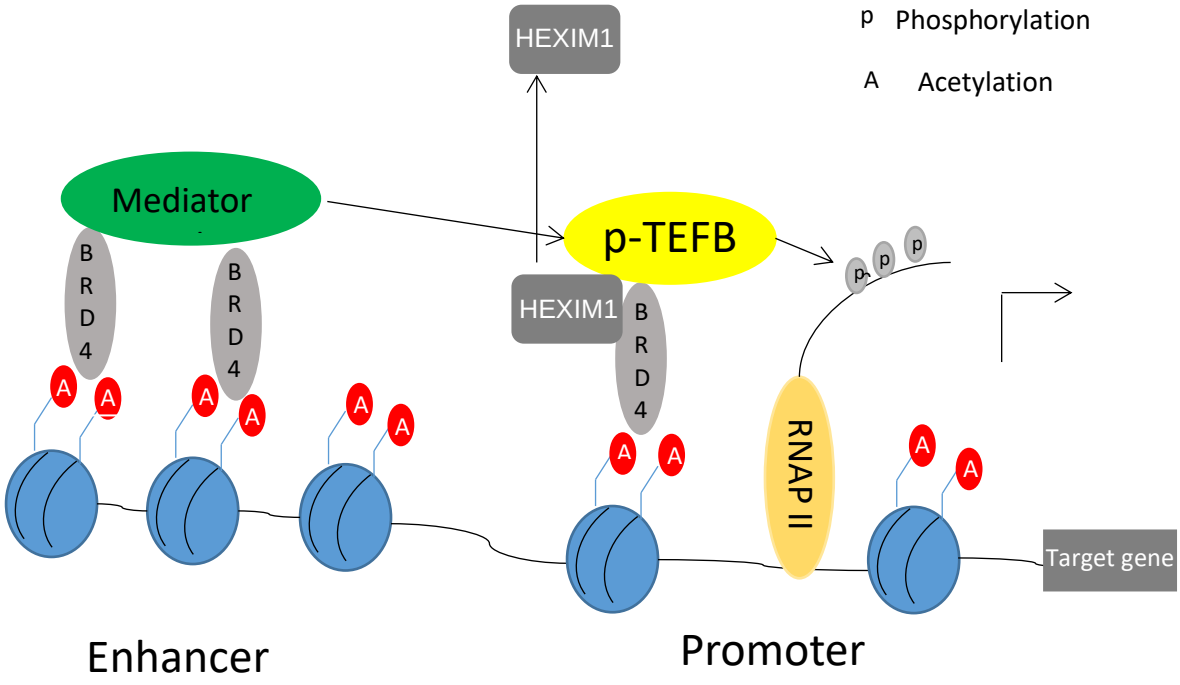


Figure 1.8: Diagram illustrating the role of BRD4 in recruiting higher order complexes such as P-TEFb to the RNAPII leading to increased transcription downstream.

BRD4 can also play a role in transcription regulation via the ET domain interactions. These interactions lead to changes of chromatin environments around BET protein-targeted genes. This was shown when NSD3 interacts with BRD4 via its ET domain. NSD3 can change and regulate levels of histone H3 K36 methylation which is a modification leading to more active transcription (Rahman, Sowa et al. 2011). BRD4 can also lead to transcriptional activation of Nanog which helps maintain pluripotency of embryonic stem cells (Liu, Stein et al. 2014). BRD4 can stimulate transcriptional activation of HOXB2- B8, A4, and C5 in HEK293 cells nucleosomes (LeRoy, Rickards et al. 2008). BRD4 can also regulate transcription factor E2 which

controls episomal maintenance and DNA replication of the human papillomavirus (HPV) genome (Schweiger, You et al. 2006).

BRD4 also has several other functions that it can perform in the cell. BRD4 plays an important role in the cell cycle and loss of BRD4 can lead to aberrant mitosis leading to genome instability. BRD4 associates with late mitotic/early G1 genes during mitosis acting as a transcriptional bookmark, which lead to quick activation of these genes after mitosis in the daughter cells (Dey, Nishiyama et al. 2009). However, BRD4's predicted role as a mitotic bookmark has been recently challenged by work done in the Blobel lab, who have observed that loss of BRD4 from the chromatin did not impair post-mitotic activation of transcription. They suggest that BRD4 is not functioning as a mitotic bookmark, but that BRD4 is probably a mitotic passenger gene. Additionally, their work suggests that BRD4's binding may be associated with restoring lineage-specific transcription patterns post mitosis (Behera, Stonestrom et al. 2019). BRD4 plays a role in cell differentiation and development. BRD4 is not a general transcription factor but actually regulates genes that either control cell differentiation or are involved in cell cycle (Whyte, Orlando et al. 2013). BRD4 is important in maintaining ESC identity and maintenance of other cellular differentiation programs such as B/T cells differentiation in the lymph node (Bolden, Tasdemir et al. 2014, Di Micco, Fontanals-Cirera et al. 2014).

The protein BRDT is expressed only in the testis (Jones, Numata et al. 1997, Shang, Salazar et al. 2004). BRDT is essential for the process of spermatogenesis (Shang, Nickerson et al. 2007). BRDT is expressed firstly at early spermatocyte stage during meiosis and is expressed though the post-

meiotic stage of spermatogenesis (Gaucher, Boussouar et al. 2012). Studies using transcriptome analysed have shown that BRDT regulates over 3000 genes (Gaucher, Boussouar et al. 2012). One such gene is Cyclin A1, which is vital to allow spermatocytes to enter the first meiotic division (Liu, Matzuk et al. 1998). BRDT regulates expression involved in allowing meiotic progression through the process of spermatogenesis. BRDT can also modulate chromatin remodelling during spermatogenesis (Berkovits and Wolgemuth 2011).

BRDT can interact and recruit P-TEFb complex to chromatin to help it function to regulate transcription during spermatogenesis and suggested that BRDT is a testis tissue specific paralogue of BRD4 (Gaucher, Boussouar et al. 2012). BRDT can interact with Smarce1 which is a member of the SWI/SNF family. Smarce1 is part of the multimeric chromatin remodelling complexes which act to regulate transcriptional activation during spermatogenesis (Dhar, Thota et al. 2012).

1.2.5: BET proteins role in DNA replication and DNA damage response

Recent research into the role of BET proteins have indicated a role for these proteins in the biological processes of DNA replication and the DNA damage response, both of these processes will be described in more depth later in the introduction.

Research done by the Stankovic group showed that inhibiting BET proteins using BET inhibitor JQ1 in acute lymphoblastic leukaemia (ALL) cell line Nalm 6 could lead to rapid slowing and stalling of replication fork seen using the DNA fibre assay

(Da Costa, Agathanggelou et al. 2013). This suggested BET proteins may play a role in replication. Further studies have highlighted that BET proteins BRD2 and BRD4 bind to TICRR/TRESLIN protein. This protein is needed for DNA replication initiation and loss of this interaction with BET proteins causes abnormal S-phase progression (Sansam, Pietrzak et al. 2018). In addition, loss of this interaction lead to slower euchromatin replication in U20S cells. The findings in this paper suggest that BET proteins are directly involved in regulating DNA replication via the interaction of BET-TICRR by being able to recruit replication factors to chromatin. DNA replication initiation is regulated by BET proteins. BET protein BRD4 also interact with replication protein RFC, which is part of the DNA replication machinery and is essential for DNA replication and cell cycle progression. Deletion of this interaction lead to dysregulation of cell cycle progression into S-phase indicating the importance of this interaction on cell cycle regulation (Maruyama, Farina et al. 2002). BRD4 has also be known to interact with and regulate cell division cycle 6 (CDC6), a replication pre-factor ((a replication factor required for formation of the pre-replicative complex (pre-RC) at a replication origin)) which functions in DNA replication checkpoint signalling. Loss of this interaction led to erroneous replication re-initiation (Zhang, Dulak et al. 2018). These results suggest that BET proteins play a role in DNA replication from regulating its timing to regulating DNA replication initiation and progression.

Research done by Floyd et al. pointed to a role of BET protein BRD4 in the DNA damage response. They observed that BRD4 depletion led to no visible increase in replication stress damage marker γH2AX. They also observed that BRD4 loss modulates chromatin structure, leading to a more open chromatin and increased

damage signalling and survival in response to irradiation (Floyd, Pacold et al. 2013). This suggested a role of BRD4 in modulating DNA damage signalling in response to damage that induces double strand breaks (DSB's). More recent studies have also shown that inhibiting BET proteins or BRD4 depletion led to no changes in amount of DNA damage signalling seen even in the presence of replication stress (Zhang, Dulak et al. 2018). However, notably factors involved in HR such as Radiation sensitivity gene 51 (RAD51) were downregulated in response to BET inhibitor treatment (Yang, Zhang et al. 2017, Pawar, Gollavilli et al. 2018). Cells that had acquired resistance to BET inhibition showed increase DNA damage and repressed expression of DNA damage repair and signalling factors (Pawar, Gollavilli et al. 2018). Taken together this suggests that BET proteins have important roles in DNA damage signalling in response to specific DNA damage and play a role in regulating expression of DNA damage response factors.

1.3: BET inhibitors to target BET protein roles in cancer

1.3.1: Role of BET proteins in cancer

BET proteins function as transcription regulators and epigenetic readers that allow the regulation of a myriad of different transcriptional programs including spermatogenesis and erythropoiesis. As key transcriptional regulators, any dysfunction can potentially lead to disease and cancer formation.

Recent research suggests that BET family bromodomains are potential targets for the use in cancer therapy. Research has shown that BET family proteins are involved in oncogenic functions, such as affecting cell proliferation and viability, of many different cancers ranging from haematological malignancies to solid tumours which will be described in more detail later in this chapter.

MYC is a useful example to illustrate how BET family bromodomains can be involved in cancer pathology. C-MYC oncoprotein is a crucial regulatory factor of cell proliferation. C-MYC oncoprotein pathogenesis in human cancers works by causing a coordinated upregulation of transcriptional programmes which help promote cell division and cell survival (Dang 2009). C-MYC oncoprotein is involved in the pathogenesis of the majority of human cancers, including many cancers where BET family proteins play a role (Mertz, Conery et al. 2011). The transcription of the *MYC* gene will usually be accompanied with increases in histone lysine acetylation on the chromatin (Frank, Parisi et al. 2003). This increase of histone lysine acetylation will allow the recruitment of higher order transcription complexes

to that area of chromatin (LeRoy, Rickards et al. 2008). BET proteins can recognise histone acetylation and bind to the transcriptional start sites of the *MYC* gene (Figure 1.9). BRD4 is bound to the transcriptional start sites when the cell is undergoing the M/G1 transition marking them for transcriptional memory, which helps allow BRD4 to influence the initiation of transcription for these genes when the cell exits mitosis (Dey, Nishiyama et al. 2009). BRD4 promotes increases in transcription by regulating transcription elongation. It will serve this by allowing recruitment of P-TEFb (Jang, Mochizuki et al. 2005, Yang, Yik et al. 2005). The recruitment of P- TEFb to BRD4 on the chromosome during mitosis will lead to the increased transcriptional activation of the *MYC* gene, which will lead to an increase in the expression of growth promoting and cell survival *MYC* dependent target genes (Yang, He et al. 2008).

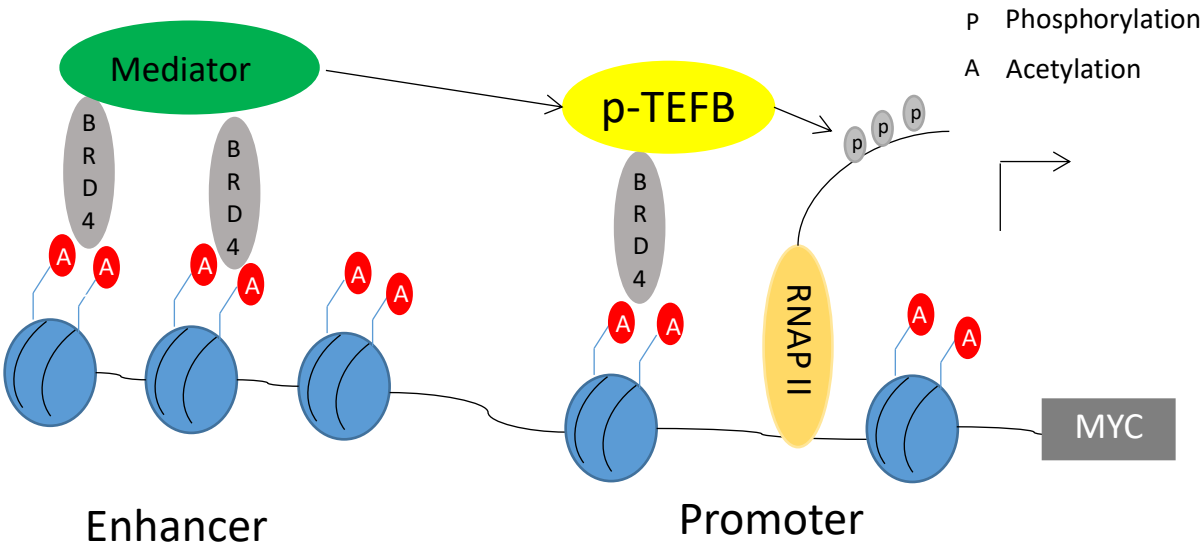


Figure 1.9: The role of BET proteins on MYC driven cancers. BRD4 transcription factor helps increase transcription of the downstream MYC gene.

Another example is the role BRD2 plays in induced lung adenocarcinoma. BRD2 can form a complex with RUNX3 transcription factor dependent on K-RAS oncogene. This led to increased expression of p21 a cell cycle inhibitor allowing cells to freely proliferate (Lee, Lee et al. 2013).

Another cancer where BET proteins play a role is the Nut midline carcinoma, which is an aggressive human squamous carcinoma (French, Miyoshi et al. 2001). BRD4 has been identified in this carcinoma which consists of a t (15; 19) chromosomal translocation, and this translocation has both the tandem bromodomains of BRD4 and the NUT protein fused together and expressed forming the NUT midline carcinoma (NMC) protein (French, Miyoshi et al. 2003). Research undertaken has shown that BRD4-NUT oncoprotein mediates characteristics such as proliferation advantage for this malignancy (French, Ramirez et al. 2008). It has been shown that this fusion acts upstream of the *MYC* promoter allowing constant *MYC* overexpression (Grayson, Walsh et al. 2014). Conversely, using RNA silencing of the BRD4-NUT oncoprotein arrests proliferation (Filippakopoulos, Qi et al. 2010).

With this information we can see that there is a wide-ranging therapeutic use for a molecule that can act as a specific inhibitor of human bromodomain proteins.

1.3.2: BET inhibitor JQ1

As BET proteins are implicated in diseases such as cancer, it opened a potentially new therapeutic way to treat cancer by targeting BET proteins with small-molecule inhibitors. Targeting the acetylated lysine and bromodomain interaction was

possible due to the hydrophobic nature of the binding pocket in bromodomains which potentially allowed for the development of small-molecule compounds. Recently in 2010, there was the discovery of two small-molecules that could inhibit BET bromodomains, one of which was JQ1 and the other being I-BET (Filippakopoulos, Qi et al. 2010, Nicodeme, Jeffrey et al. 2010). JQ1 is a cell-permeable small molecule that binds competitively to bromodomains. JQ1 is a novel thieno-triazolo-1, 4-diazepine. JQ1 contains a t-butyl ester functional group at C6. JQ1 has a stereo centre at C6 and hence consists of two stereoisomers: (+)-JQ1 and (-) - JQ1 (Filippakopoulos, Qi et al. 2010).

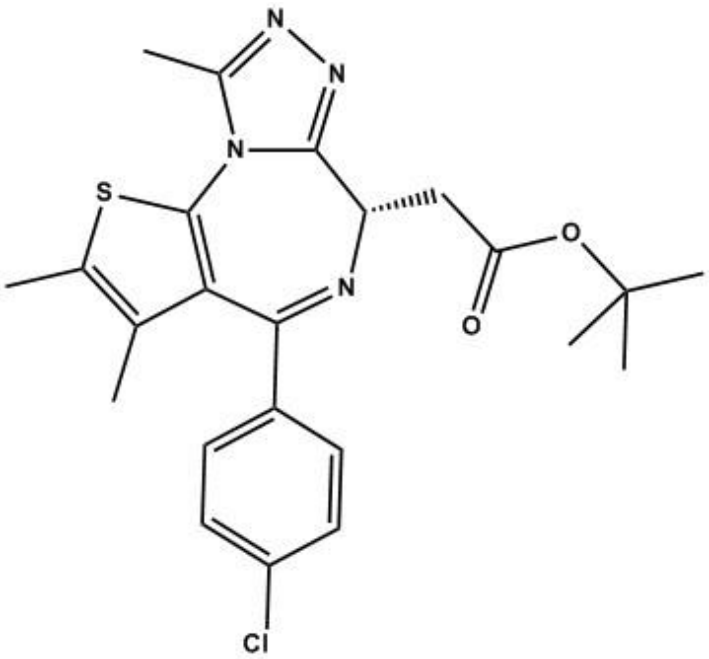


Figure 1.10: Chemical structure of small molecule inhibitor (+)-JQ1.

JQ1 was known as an inhibitor of bromodomains; how selective it was for inhibiting BET family bromodomains was not known and needed to be established. Research

screening all human bromodomains was done by sub-cloning all human bromodomains into bacterial expression vectors, which yielded soluble protein to screen all bromodomain subfamilies. A differential scanning fluorimeter screen (thermal shift assay) highlighted the selectivity of JQ1 for BET family bromodomains exclusively, indicating that JQ1 is a highly specific inhibitor of BET family bromodomains (Filippakopoulos, Qi et al. 2010). It also further highlighted that (+)-JQ1 was a highly selective ligand for BET proteins while (-)-JQ1 showed no discernible interaction with BET protein indicating (+)-JQ1 is an active ligand against BET proteins. Further research using a luminescence proximity homogenous assay (alpha-screen) that was adapted to BET bromodomains, showed that JQ1 was competitively binding with acetylated lysine's that were found on histone tails (Filippakopoulos, Qi et al. 2010).

This was further evidenced using co-crystal structures of JQ1 bound to BRD4. Here (+)- JQ1 was bound to the bromodomain of BET proteins that would normally be interacting and binding with acetylated histones, hence competitively inhibiting binding of BET proteins to chromatin (Filippakopoulos, Qi et al. 2010). The crystal structure also showed JQ1 has shape complementarity with the acetylated lysine binding site. JQ1 when bound to BRD4 will occupy the whole binding pocket, and its bound state is usually being stabilised by hydrophobic interactions with BET residues from the ZA and BC loop regions as well as a hydrogen bond with the conserved asparagine (Filippakopoulos, Qi et al. 2010). BRD2 was later shown to bind JQ1 in the same way as BRD4 (Filippakopoulos, Qi et al. 2010). Therefore, JQ1 is now known as a specific and competitive inhibitor of BET family bromodomains.

1.3.3: JQ1 and cancer treatment

JQ1 has potential as a cancer therapeutic and this has been followed up with various studies carried out in mouse models. When JQ1 was first reported, it was shown that its treatment in midline carcinoma cell lines can cause the release of BRD4-NUT from chromatin leading to terminal squamous cell differentiation and apoptosis (Figure 1.11). The report also showed that JQ1 treatment helped decrease tumour size and extend the survival in mice models of NMC. This was the first test that showed JQ1 as a potential cancer therapeutic (Filippakopoulos, Qi et al. 2010).

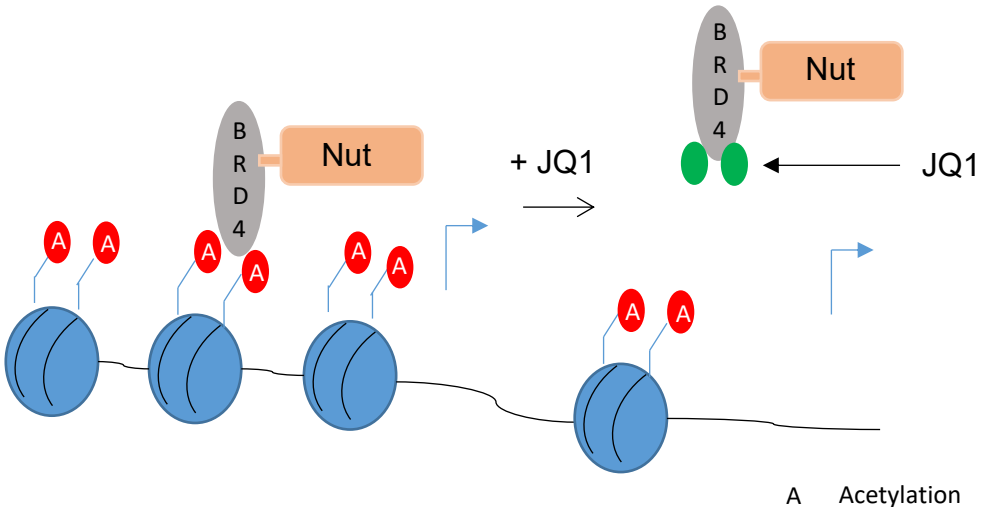


Figure 1.11: JQ1's mechanism in inhibiting proliferation of tumour cells. Here acetylated lysine's can be bound by epigenetic readers in BRD4 hence increasing the oncogenic activity of the BRD4-NUT oncoprotein. Once JQ1 bound to BRD4 inhibits the binding to acetylated lysine's and hence inhibit proliferation of these tumour cells expressing the BRD4-NUT protein. Adapted from (Chung 2012).

Studies from several laboratories, including at Birmingham, have identified therapeutic effects for BET inhibitors in mouse models of haematological malignancies, such as acute myeloid leukaemia, multiple myeloma, Burkitt's lymphoma and B-cell lymphoma (Delmore, Issa et al. 2011, Mertz, Conery et al. 2011, Ott, Kopp et al. 2012, Da Costa, Agathanggelou et al. 2013). MYC oncoprotein regulates the transcription and cell cycle in all the above mentioned malignancies (Dang 2009). As of yet, current cancer treatments i.e. cytotoxic treatments, are often non-specific and can have side effects. A new way was hypothesised to target C-MYC transcriptional function, by potentially disrupting the chromatin-dependent signal transduction. BET proteins are required for the cancer-promoting transcriptional activities of oncogenes such as MYC, as discussed earlier. Studies have shown that *MYC* transcription can be regulated by bromodomains. Recent research was undertaken using JQ1, to understand the role of BET proteins in MYC-dependent transcription and to find out the potential of BET proteins having a role as cancer promoters. Multiple myeloma (MM) cell lines were used as a model system, which is an incurable hematologic malignancy and features dysregulation of MYC (Shou, Martelli et al. 2000, Wu and Chiang 2007). MYC rearrangement or translocation is one of the most common events in MM and more than 60% of patient MM cells have MYC pathway activation, which presents MYC dysregulation as a commonly observed molecular feature present in MM (Chng, Huang et al. 2011). Results for this experiment showed that BET inhibition by JQ1 can hinder BRD4 binding to the MYC gene enhancers and hence leading to the inhibition of MYC transcription. With the downregulation of MYC transcription the cells can be driven to cell cycle arrest and senescence (Delmore, Issa et al.

2011). Reports have stated that the inhibition of the MYC transcription may represent a major mechanism of the cellular killing caused by BET inhibition in tumour cells (Delmore, Issa et al. 2011). JQ1 leads to the suppression of C-MYC driven malignancies such as testis- midline carcinoma, acute myeloid leukaemia (AML) and multiple myeloma, by displacement of BET family proteins from the MYC promoter/enhancer (Dawson, Prinjha et al. 2011, Delmore, Issa et al. 2011, Cheng, Gong et al. 2013, Da Costa, Agathanggelou et al. 2013) (Figure 1.12). In summary, JQ1 produces a powerful antiproliferative effect showcased by cell cycle arrest and leading to cellular senescence.

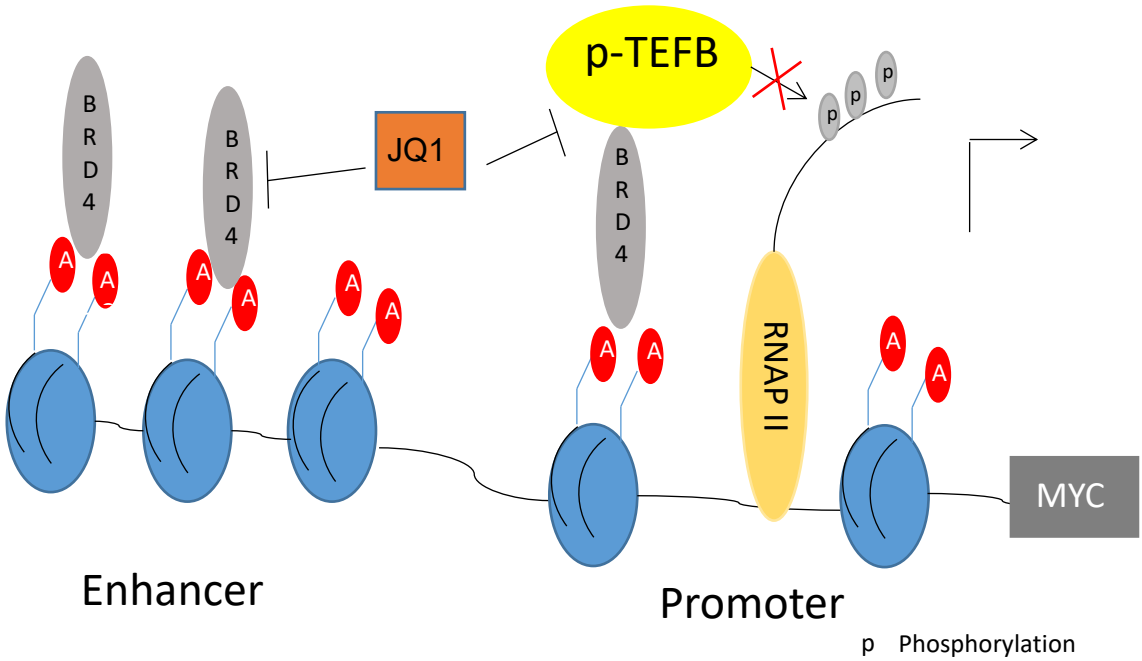


Figure 1.12: Diagram illustrating how JQ1 dirsupts BRD4 from binding to the promoter or enhancer upstream of MYC, disrupting transcription of the MYC gene downstream**.**

JQ1 could also play a role as a therapeutic in solid tumours as well. Non-small cell lung cancer are sensitive to JQ1 as are some neuroblastomas that contain *NMYC* amplifications (Lockwood, Zejnullahu et al. 2012, Puissant, Frumm et al. 2013). Other solid tumours that have shown sensitivity to JQ1 are glioblastoma, medulloblastoma, hepatocellular carcinoma, colon cancer, pancreatic cancer, prostate cancer, and breast cancer (Cheng, Gong et al. 2013, Asangani, Dommeti et al. 2014, Bandopadhayay, Bergthold et al. 2014, Kumar, Raza et al. 2015, Sengupta, Biarnes et al. 2015, Zhang, Tong et al. 2015, Li, Guo et al. 2016, Shu, Lin et al. 2016). Here, we can understand the role of JQ1 could have against a widespread set of malignancies.

Downregulation of MYC is not the only effect of BET inhibition. Other mechanisms are also affected such as in ALL where there is reduction in interleukin 7 receptor gene and in large B cell lymphoma where there is reduction in other transcription proteins such as *E2F1, POU2AF1, BCL6, IRF8,* and *PAX5* (Ott, Kopp et al. 2012, Chapuy, McKeown et al. 2013). BET inhibitor transcriptional effects are normally very specific to each cell type. When leukaemia cells are treated with JQ1 there is a significant reduction in MYC transcript levels, which is not the same as when JQ1 is treated to fibroblast cells, where MYC levels are barley affected (Zuber, Shi et al. 2011).

1.3.4: I-BET151 and Cancer

Another BET inhibitor used is I-BET151 (GSK1210151A) which was first reported in 2011 (Dawson, Prinjha et al. 2011). I-BET151 has shown to be effective in mixed-lineage leukaemia (MLL) where treatment led to apoptosis and cell cycle arrest in MLL cell lines. Inhibition of BET proteins in particular BRD3 and BRD4 by I-BET151 caused decreased amounts in the *BCL-2* (regulates apoptosis), *MYC*, and Cyclin dependent kinase 6 (*CDK6)* (regulates cell cycle progression) genes causing cell death (Dawson, Prinjha et al. 2011). It works similarly to JQ1 by competitively inhibiting binding of BET proteins to acetylated histones and has been shown to have potent anti-myeloma activity through repression of MYC (Chaidos, Caputo et al. 2014). I-BET151 has also shown to have activity against myeloproliferative neoplasms, glioblastoma, nucleophosmin 1 (*NPM1)*-mutated acute myeloid leukaemia seen in both cell lines and murine models (Dawson, Prinjha et al. 2011, Pastori, Daniel et al. 2014, Wyspianska, Bannister et al. 2014).

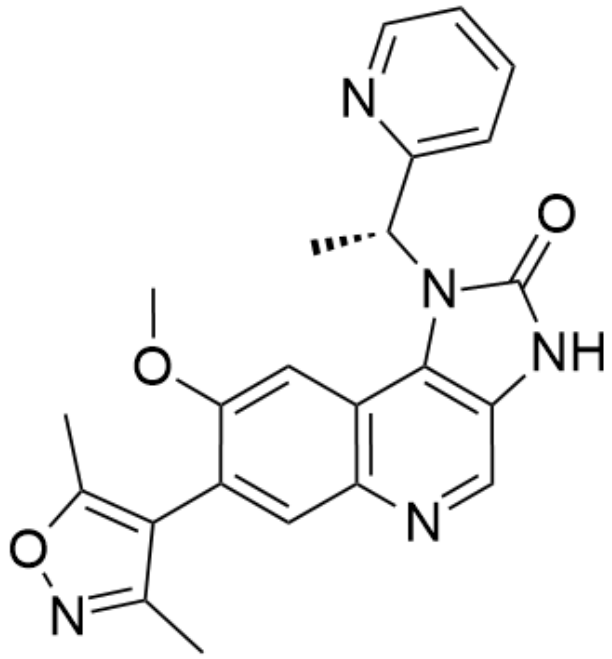


Figure 1.13: I-BET151 chemical structure**.**

1.3.5: Current status of BET inhibitors as therapeutics

The table below shows the current pre-clinical studies of BET inhibitors in cancer. Thirteen BET inhibitors have since been taken into early-phase clinical studies to be used as potential therapeutic agents as shown in table 1.1 (Doroshow, Eder et al. 2017).

Agent	Target	Tumour type	Phase of development
ABBV-075	BRD2/3/4/T	Solid tumours, multiple myeloma, breast cancer, lung cancer	I
BAY1238097	Undisclosed	Solid tumours and lymphoma	I (terminated)
BI 894999	Undisclosed	Solid tumours and non-Hodgkin lymphoma (NHL),	I
BMS-986158	Undisclosed	Solid tumours	I/II
CPI-0610	BRD4	Lymphoma, multiple myeloma, myelodysplastic syndrome (MDS), acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myeloid leukaemia (CML)	I
FT-1101	BRD2/3/4/T	AML/MDS	I
GS-5829	Undisclosed	Large B-cell lymphoma (LBCL), T-cell lymphoma, solid tumours, metastatic castrate-resistant prostate cancer (CRPC)	I/II
GSK525762/I-BET762	BRD2/3/4/T	Hematologic malignancies, AML, NHL, multiple myeloma, colorectal cancer, lung cancer, neuroblastoma, ER+ breast cancer, CRPC	I
GSK2820151/I-BET151	BRD2/3/4	Solid tumours	I

INCB054329	Undisclosed	Haematological malignancies, solid tumours	I/II
OTX015/MK-8628	BRD2/3/4	AML, DLBCL, solid tumours	I
PLX51107	BRD4	AML, MDS, Solid tumours, lymphoma	I
RO6870810/TEN-010		AML, MDS, Solid tumours	I
ZEN003694	BRD2/3/4	Metastatic CRPC	I

Table 1.1: Table depicting BET inhibitors that are in pre-clinical trials, their targets and which tumours they are looking to target. Adapted from (Doroshow, Eder et al. 2017).

These phase1 trials for BET inhibitors have shown mixed results so far. CPI-0610 was trialled in hematologic malignancies in patients with refractory/respiratory lymphoma, where out of 44 patients there were 2 complete responses, 1 partial response and 5 with responses not qualifying as a partial response (Abramson, Blum et al. 2015). MK-8628/OTX015 inhibitor was used in hematologic malignancies. 33 patients were treated for lymphoma and 12 for myeloma. 3 patients with diffuse large B-cell lymphoma showed a partial response while no effect was seen with those with myeloma (Amorim, Stathis et al. 2016). Patients presented with diarrhoea and fatigue at the dose limiting toxicity for this drug, in addition to which nausea and skin changes were also seen (Amorim, Stathis et al. 2016). Clinical trials have also investigated the effect in solid tumours. OTX015 was tested in 47 patients with either NMC, castrate-resistant prostate cancer (CRPC) or non-small cell lung cancer (NSCLC) (Massard, Soria et al. 2016). Four partial responses (PR's) were observed with 3 being in NMC. Side-effects included anaemia, fatigue and thrombocytopenia (Massard, Soria et al. 2016). A study

testing BAY1238097 in patients with solid tumors was halted due to side-effects such as vomiting, migraines and lower back pain when the dose was below therapeutic levels (Postel-Vinay, Herbschleb et al. 2016). These are just a couple examples of the results seen, and a fully detailed list of all the BET inhibitors and responses in clinical trials can be found in (Doroshow, Eder et al. 2017).

Overall these preliminary results have been mixed and the anti-cancer response effects have been short-lived so far. However, more pre-clinical data has shown that BET inhibitors may be more therapeutic when used in combination therapy (Doroshow, Eder et al. 2017). For example, JQ1 was shown to reduce tumor volumes and weights when combined with trametanib in ovarian cancer cell lines, while JQ1 treatment alone led to only cell cycle arrest (Jing, Zhang et al. 2016). Other potential combinations are with immune checkpoint inhibitors. JQ1 treatment combined with a PD1 (immune checkpoint regulator) inhibitor led to decreased tumor volume and size in a KRAS-mutant NSCLC xenograft (Adeegbe, Liu et al. 2017). Combination with epigenetic therapies has been shown to have significant effects in pre-clinical studies, including synergistic effects with HDAC inhibitors (Borbely, Haldosen et al. 2015, Heinemann, Cullinane et al. 2015, Shahbazi, Liu et al. 2016). Combination treatments may be the future for BET inhibitors as therapeutic tools.

Nevertheless, a major concern for the continuation of BET inhibitors as therapy drugs are their toxicity profile, which can cause patients with unwanted side-effects. Despite moving BET inhibitors forward in clinical trials, there are concerns regarding whether we know enough about their mechanisms of action to use them as safe therapeutics.

Most BET inhibitors as of yet are not selective for a specific BET protein and as mentioned before BET proteins regulate numerous transcription pathways. BET protein have crucial roles in numerous processes including insulin production, cytokine gene regulation and T cell differentiation (Wang, Liu et al. 2009, Belkina, Nikolajczyk et al. 2013). There is also research indicating that BET inhibitors can lead to the re-activation of HIV in human cells (Banerjee, Archin et al. 2012). BET inhibitors also work as transcriptional repressors and activators to a host of different genes. There could be serious safety issues if BET inhibitors currently in clinical trials were to be used as therapeutics. How BET inhibitors work in transcriptional regulation of different pathways affecting metabolism, differentiation and secretory pathways is still barley understood. These are reasons as to why BET inhibitors should be studied more intensely with regard to their biological roles and functions before using them as a cancer therapy tool. This is further backed with the results seen with clinical-trials which show moderate effects coupled with a high toxicity profile.

1.4: DNA replication and replication stress

1.4.1: Overview of DNA replication

DNA replication is the biological process in which two identical copies of DNA are produced from one DNA molecule. Allowing the genome to be faithfully replicated is vital for allowing successful transmission of genetic information and preserving genome stability. In order for this to be accomplished, it necessitates the navigation of different endogenous and exogenous sources of DNA modifications, which can lead to replication stress locally or globally (Branzei and Foiani 2010). The process of DNA synthesis mostly occurs in the S-phase stage of the cell cycle (Leman and Noguchi 2013). Replication occurs in three distinct stages: initiation, elongation and termination all of which need to be tightly regulated. In this section I will describe the process of replication and what happens when it encounters blocks on the DNA.

1.4.2: Initiation of DNA replication

DNA replication in eukaryotes starts from multiple points on the DNA termed replication origins, which when fired lead to the formation of bi-directional replication forks leading to new double strand DNA synthesis (Fu, Yardimci et al. 2011, Fragkos, Ganier et al. 2015). DNA replication in eukaryotic cells needs to start from thousands of different replication origins all across the genome (Masai, Matsumoto

et al. 2010). Before replication can be initiated each origin is required to be licensed before it is fired.

Origin licencing requires the Origin recognition complex (ORC) being recruited to a potential origin on the DNA (Bell and Stillman 1992). How ORC is recruited to the origin still remains unknown, however evidence suggests that more chromatin compaction and epigenetic marks play a role (MacAlpine and Almouzni 2013). CDC6 bound with an ATP will associate with ORC (Wang, Feng et al. 1999). Chromatin licensing and DNA replication factor 1 (CDT1) is also recruited to the ORC followed by the minichromosome maintenance complex (MCM) which is a hexamer. MCM 2-7 is loaded as a double hexamer onto the DNA. The MCM consists of six subunits (MCM 2-7). ORC, CD6 and MCM2-7 are AAA+ ATPase's (Evrin, Clarke et al. 2009, Remus, Beuron et al. 2009). This forms the pre-RC. Licencing starts in G1 phase when the pre-replicative complex is assembled at each origin. Origin firing needs the pre-RC to be activated which can only occur in S phase (Pacek and Walter 2004, Sun, Fernandez-Cid et al. 2014).

Origin firing involves the formation of the pre-initiation complex (pre-IC) and the activation of the MCM helicase complex. The pre-IC is formed via the activities of CDKs and DBF4 dependent kinases (DDKs), which are activated at the G1-S phase transition. DDKs phosphorylate MCM 2-7, which leads to the recruitment of Treslin/TICCR and CDC45. CDKs phosphorylate Treslin/TICRR which leads to the recruitment of TOBP1 and GINS allowing for the formation of the CMG helicase (Sheu and Stillman 2006, Francis, Randell et al. 2009, Boos, Sanchez-Pulido et al. 2011, Heller, Kang et al. 2011, Kumagai, Shevchenko et al. 2011, Boos, Yekezare et al. 2013, Kang, Warner et al. 2014, Fragkos, Ganier et al. 2015, Deegan, Yeeles

et al. 2016). The MCM2-7 double hexamer splits into two different hexamers allowing for bi-directional replication forks to be established. Activated helicases can recruit RFC, proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) and other DNA polymerases such as DNA polymerase α to convert the pre-IC into a replication fork (Gambus, Jones et al. 2006, Moyer, Lewis et al. 2006). The replication fork (replisome) consists of a CMG complex (CDC45, MCM helicases and GINS complex) with PCNA, DNA Ligase1 and FEN1 and the DNA polymerases δ and ε (Gambus, Jones et al. 2006, Moyer, Lewis et al. 2006). Only about a third of origins are activated in a replication unit. This means that the initiation of these origins must be tightly controlled, and they are inhibited by ATM and Rad4 related (ATR) and Ataxia telangiectasia mutated (ATM kinases) which activate checkpoint 1 kinase (CHK1) or checkpoint 2 kinase (CHK2) (Fragkos, Ganier et al. 2015).

After firing; replication needs to replicate DNA faithfully at a fast rate bi-directionally. During replication not every origin is activated initially, but additional origins can be fired later on, if needed to help complete replication if previously established replication forks have stalled (Branzei and Foiani 2010, Fragkos, Ganier et al. 2015). Thus, replication origins are not all activated at the same time and are controlled in a specific manner which is normally regulated by chromatin structure, transcriptional programmes and rate-limiting replication factors (Fragkos, Ganier et al. 2015).

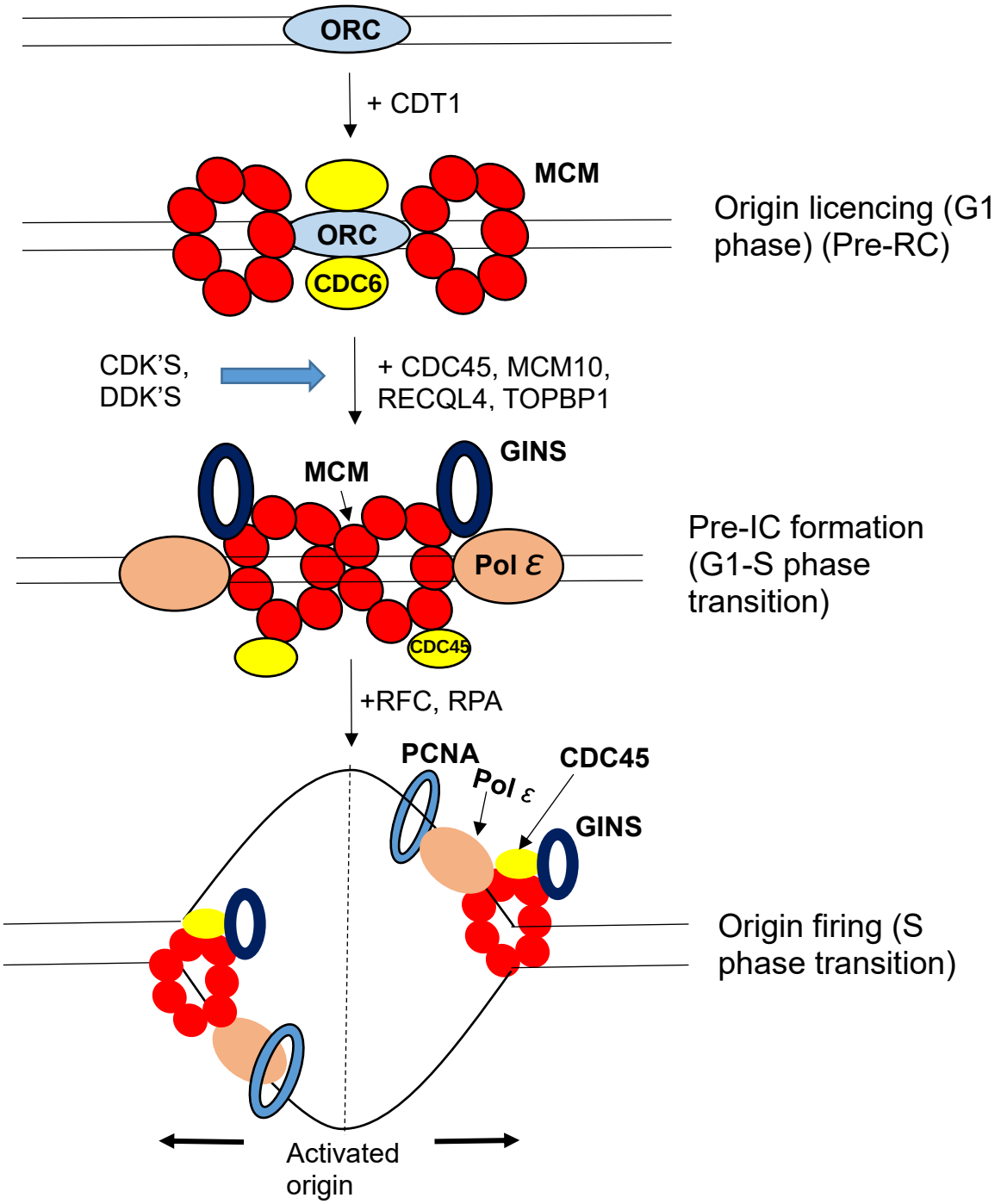


Figure 1.14: Diagram illustrating the steps and mechanism of replication initiation as detailed in the chapter above. Adapted from (Fragkos, Ganier et al. 2015).

1.4.3: Replication progression

A replication fork is formed to allow the process of replication elongation. In a replication fork structure double stranded DNA is unwound by an activated CMG helicase which hydrolyses ATP to unwind dsDNA. This leaves two strands of single stranded DNA (ssDNA) which allows access to unpaired DNA nucleotides (Gambus, Jones et al. 2006, Moyer, Lewis et al. 2006, Sengupta, van Deursen et al. 2013). CMG is a 3′-5′ helicase and translocates on the leading strand (Moyer, Lewis et al. 2006). Thus, nucleotides can be read, allowing for complementary base pairing of free nucleotides to form two identical double-stranded DNA molecules.

The formation of complementary base pairing to allow the formation of the daughter DNA molecule, are catalysed by DNA polymerases. There are three different replicative DNA polymerases involved in replication elongation: α, δ, and ε, all of which are members of the B family of DNA polymerases (Leman and Noguchi 2013, Burgers and Kunkel 2017). DNA polymerases can move along ssDNA and elongate the new DNA strand being formed by reading the template (original) strand. They catalyse the incorporation of new bases which can either be adenine, guanine, thymidine or cytosine hence extending the 3’ end of the newly synthesised DNA (Leman and Noguchi 2013). They also require the replication clamp PCNA loaded onto the DNA by clamp loader RFC to allow DNA polymerases to elongate further without falling off (Mailand, Gibbs-Seymour et al. 2013).

Free deoxyribonucleotides used for DNA synthesis need to contain three phosphate groups, making them deoxyribonucleotide triphosphates (dNTPs) (Alberts, Johnson

et al. 2002). DNA polymerases catalyse the reaction of these nucleotides with the free 3'-hydroxyl group on the previous nucleotide that has be incorporated. This reaction leads to two phosphates being released (pyrophosphate) which provides the energy for the polymerization reaction allowing the remaining 5' monophosphate to covalently bond to the 3' oxygen forming a phosphodiester bond (Alberts, Johnson et al. 2002, Leman and Noguchi 2013). Some DNA polymerases also have the ability to proofread to ensure the right nucleotide is being incorporating into the DNA (Alberts, Johnson et al. 2002).

There are two strands of DNA that are replicated at the same time after being unwound. These are known as the leading and lagging strands (Figure 1.15). Both strands require Pol α–RNA primase complex to allow for DNA synthesis to occur (Leman and Noguchi 2013). To allow DNA synthesis an RNA primer needs to be laid down first (Leman and Noguchi 2013, Burgers and Kunkel 2017). Pol α has two activities, one being RNA primase activity and the other being DNA polymerase activity. Primase normally elongates for approximately 10 nucleotides long before Pol α can synthesise further (Bullock, Seo et al. 1991, Nethanel, Zlotkin et al. 1992). This leads to it leaving a RNA-DNA primer in a 5’-3’ direction allowing other more processive polymerases to continue DNA synthesis after (Pellegrini and Costa 2016). Pol α has high error rate as it lacks exonuclease activity and has low processivity (McCulloch and Kunkel 2008).

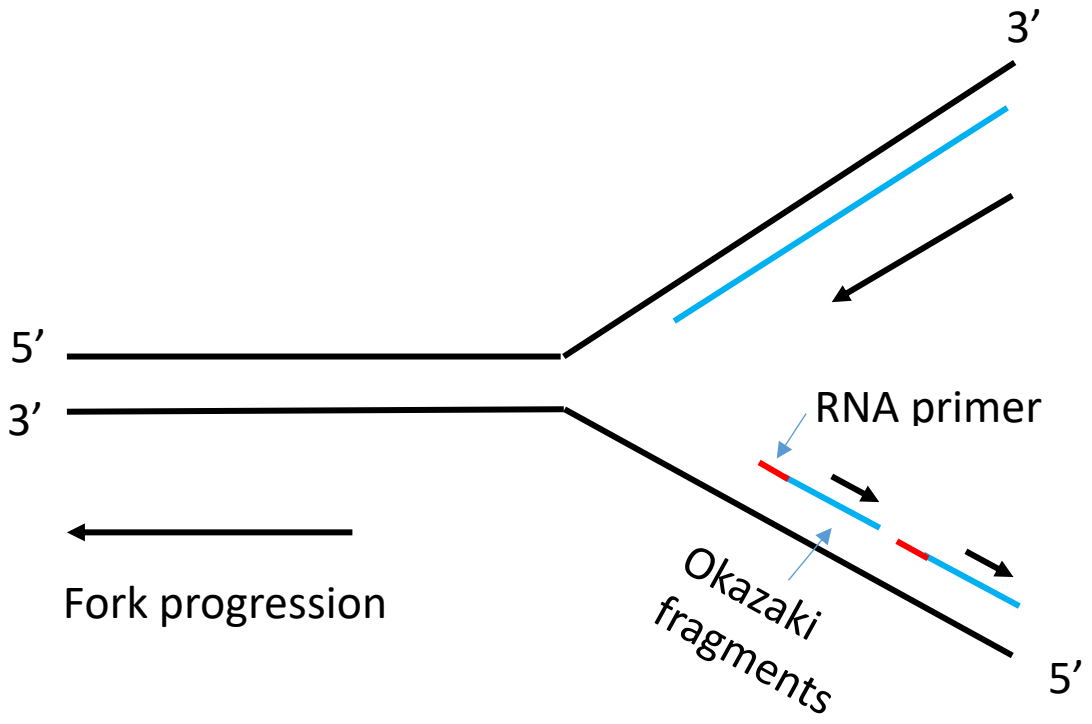


Figure 1.15: The replication fork consisting of both the leading and laggings strands being synthesised.

The leading strand elongates DNA using the CMG helicase and Pol ε (Pursell, Isoz et al. 2007, Muramatsu, Hirai et al. 2010, Sengupta, van Deursen et al. 2013). Pol ε (epsilon) synthesizes DNA continuously in the same direction that the DNA is being unwound in. CMG is a 3′-5′ helicase so the DNA is being unwound in this direction (Moyer, Lewis et al. 2006). All polymerases including Pol ε synthesise DNA from 5’-3’ direction (Leman and Noguchi 2013). This means that DNA synthesis can occur in a continuous fashion on the leading strand. Pol ε has both DNA polymerization and 3′-exonucleolytic activity, and is a very processive polymerase (Hogg,

Osterman et al. 2013). Pol ε contains a small cleft in the catalytic domain allowing Pol ε to encircle newly formed double stranded DNA (dsDNA), which is likely why Pol ε has high processivity and hence can replicate continuously on the leading strand (McCulloch and Kunkel 2008). The 3′-exonuclease activity of Pol ε allows it to check the newly synthesised DNA and correct any replication errors that may have occurred (Pursell, Isoz et al. 2007, Burgers and Kunkel 2017).

The other stand is known as the lagging strand. On the lagging strand, Pol α DNA synthesis can only synthesise DNA for approximately 20–30 NT's (Bullock, Seo et al. 1991, Nethanel, Zlotkin et al. 1992). After the primer is laid down, DNA synthesis can continue further with DNA Pol δ. The lagging strand cannot be synthesised continuously as it is being unwound due to it having opposite directionality to the DNA helicase. When DNA synthesis occurs of the lagging strand, it consist of small stretches of discontinuous DNA replication known as Okazaki fragments which are approximately 100 to 200 bases long (Ogawa and Okazaki 1980). As DNA synthesis occurs in stretches and not continuously like on the leading strand, it causes a much longer stretch of ssDNA. To protect the ssDNA from forming secondary structures or activating any other responses due to exposed ssDNA, the ssDNA is coated by single-stranded binding proteins. These proteins help to stabilize ssDNA stretches and help prevent secondary structure formation. This ssDNA binding protein is known as RPA which is a heterotrimeric complex (Wold and Kelly 1988, Alani, Thresher et al. 1992).

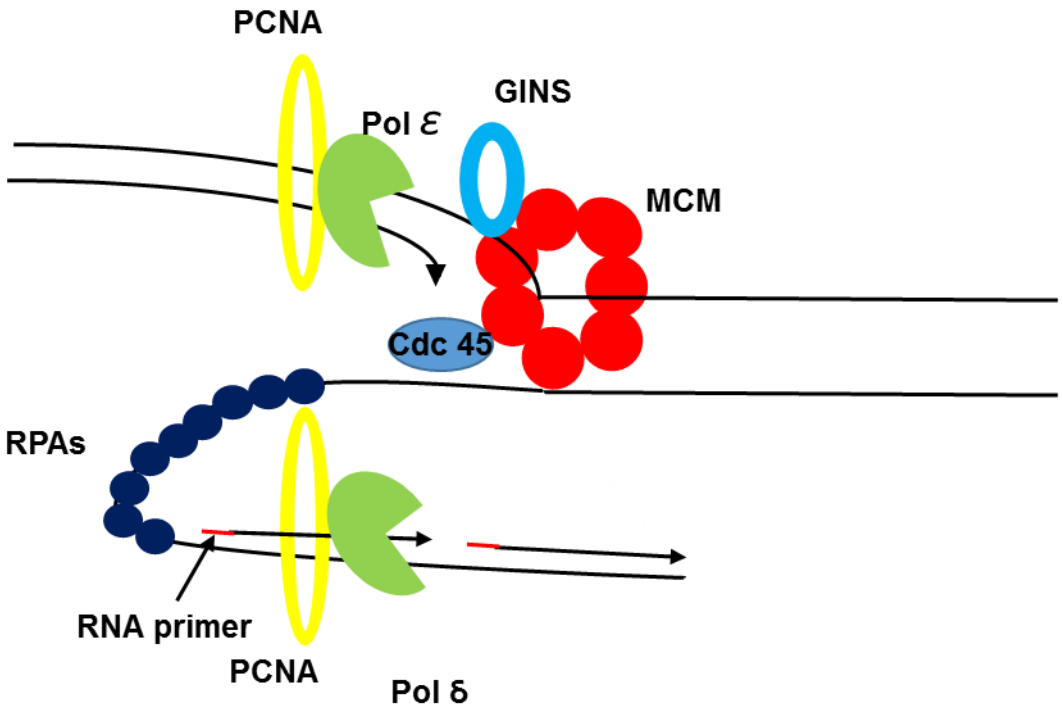


Figure 1.16: The replisome with Pol δ and Pol ε leading DNA synthesis with the CMG helicase complex unwinding DNA. Adapted from (Leman and Noguchi 2013).

As each Okazaki fragment is synthesised it will run into the previous Okazaki fragment synthesised. When this occurs DNA pol δ can displace the RNA primer. A small segment (usually not more than 1 nucleotide) of DNA upstream of the RNA primer is also displaced leaving a flap like structure (Bae, Bae et al. 2001, Leman and Noguchi 2013, Burgers and Kunkel 2017). This flap acts as a substrate for FEN1 endonuclease which can cleave the flap one nucleotide at a time (Balakrishnan and Bambara 2013, Stodola and Burgers 2016). Sometimes due to loss of regulation of either DNA Pol δ or FEN1 long flaps can be produced. These flaps are too long for FEN1 to cleave so DNA replication helicase/nuclease 2 (DNA2), a nuclease/helicase, can act to cleave this flap using its 5'-3' endonuclease activity (Bae, Bae et al. 2001, Kang, Lee et al. 2010, Wanrooij and Burgers 2015).

The cleavage of the flap results in a small nick in DNA which can be ligated by the enzyme DNA ligase1 (Bambara 1993) (Leman and Noguchi 2013). DNA ligase I can bind to the clamp loader PCNA that helps the stabilization of DNA ligase onto DNA substrate (Vijayakumar, Chapados et al. 2007). Due to the discontinuous nature of DNA replication synthesis on the lagging strand, it is often less efficient and requires more time than leading-strand synthesis. Hence, there are mechanisms in place that help coordinate both leading and lagging strand synthesis. Research done in T4 bacteriophages showed that the lagging strand needed to be looped back through the replisome as shown in Figure 1.16 in a model termed ‘The trombone model’ (Alberts, Barry et al. 1983). This was further shown in E.coli systems as well (O'Donnell 1987). How coordination between the two strands occur in eukaryotic systems is less well understood, however a study in yeast showed that Ctf4 protein is able to bind and interact with both DNA pol α and ε and linking these two machineries together suggesting a potential role in coordination between these two (Simon, Zhou et al. 2014).

1.4.4: Replication termination

The final step of DNA replication is replication termination. Previous steps of replication; initiation and elongation, have been extensively researched and studied however, replication termination has notably been studied much less.

There are approximately about as many replication termination events occurring as there are replication initiation events during a normal S phase of mammalian cells (McGuffee, Smith et al. 2013, Dewar and Walter 2017).

There are no defined termination sites for replication. Most termination events will occur wherever two replication forks will converge into each other. As initiation events at origins are changing due to which origins are activated, this causes termination sites to change as well depending on where origins are being activated (McGuffee, Smith et al. 2013, Petryk, Kahli et al. 2016). The lack of defined termination sites is adapted to the ever-changing activation of origin firing that exists in cells.

When replication is almost complete two replication forks will approach each other which promotes formation of supercoils in the regions of DNA yet to be replicated (Dewar and Walter 2017). During this step topological stress formed previously can be relieved by topoisomerase 1 and topoisomerase 2 enzymes (Keszthelyi, Minchell et al. 2016, Dewar and Walter 2017). However as forks converge closer, it is harder for these enzymes to act so forks are rotated to form catenanes (Keszthelyi, Minchell et al. 2016).

When replication forks converge, the meeting of two different CMG's does not lead to any detectable replication stress. This is because the two converging CMGs can bypass each other as they both are bound to leading strands (Fu, Yardimci et al. 2011, Dewar, Budzowska et al. 2015).

Once the CMG helicases have bypassed each other, they continue to synthesise downstream to at least a few bases away (Dewar, Budzowska et al. 2015). How the last Okazaki fragment is matured and synthesised is still unclear.

The replisome machinery (CMG) is stably bound to the DNA throughout DNA synthesis. A key step to allow replication termination is to be able to disassemble this machinery once DNA synthesis is complete. During replication termination, it can be covalently modified by undergoing polyubiquitylation on its MCM7 subunit (Maric, Maculins et al. 2014, Priego Moreno, Bailey et al. 2014). These polyubiquitin chains can be made either by SCFDIA2 (Skp, Cullin, F-box-containing complex associated with DIA2) or CRL2LRR1 (Cullin RING ligase 2 associated with LRR1) (Maric, Maculins et al. 2014, Dewar, Low et al. 2017, Sonneville, Moreno et al. 2017). After the MCM7 has been poly-ubiquitylated it is recognized by p97/VCP/Cdc48 protein complex that can lead to CMG being removed from chromatin (Maric, Maculins et al. 2014, Priego Moreno, Bailey et al. 2014).

The catenanes are removed by topoisomerase type 2 which resolves the two daughter molecules (Baxter and Diffley 2008).

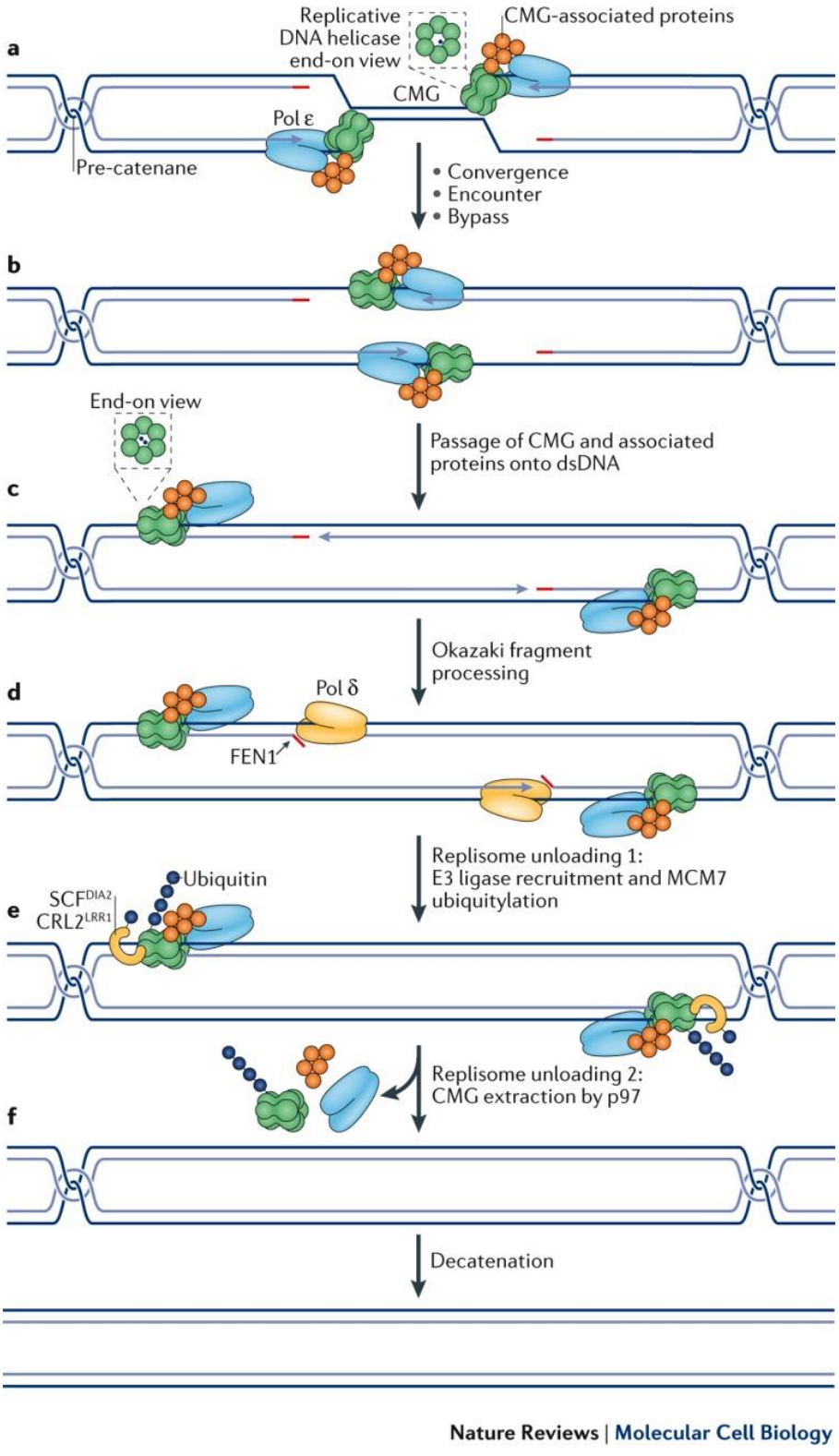


Figure 1.17: Model of the mechanism of replication termination in eukaryotic cells. Taken from (Dewar and Walter 2017).

1.4.5: Replication stress

Replication stress is defined as the slowing or stalling of replication fork progression, leading to incomplete DNA synthesis, and can be induced by a variety of different hindrances (Zeman and Cimprich 2014). Replication stress frequently involves the increased formation of single stranded DNA due to slowing or stalling of the DNA polymerase. Replication stress causes genome instability and decreases cell survival (Ciccia and Elledge 2010).

1.4.6: Sources of replication stress

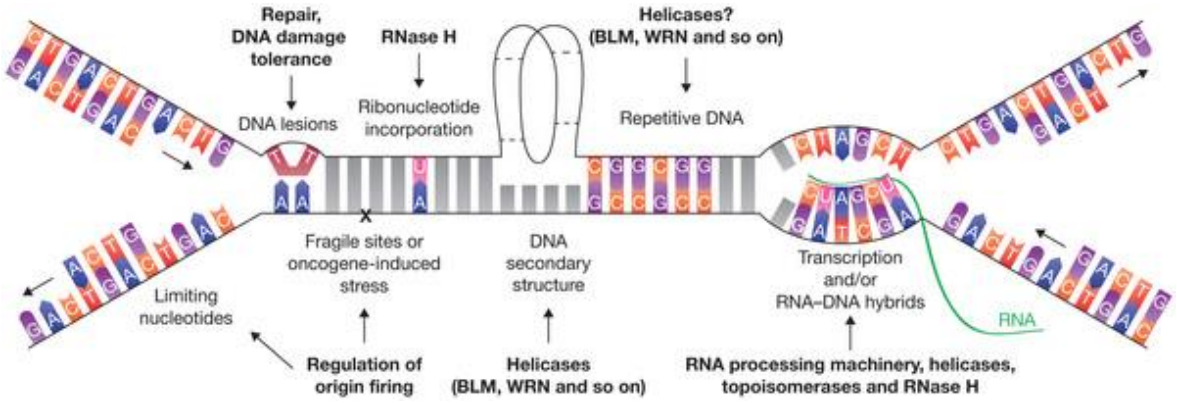


Figure 1.18: Diagram illustrating the various sources that can lead to replication stress in the cell. Retrieved from (Zeman and Cimprich 2014).

Replication stress occurs through blockages and obstacles formed either endogenously or exogenously to the replication fork that slow its progression (Zeman and Cimprich 2014) (Figure 1.8). There are many different sources of these

blockages and obstacles, which will in turn lead to increased amounts of replication stress. These sources usually provide barriers that prevent the DNA polymerases from synthesising the DNA at its normal rate. Replication stress usually leads to the formation of physical structures, namely the formation of large stretches of ssDNA which are coated by RPA (Pacek and Walter 2004). ssDNA is formed when the replicative polymerase is stalled by a blockage to replication, but the helicase continues to unwind. This uncoupling leads to formation of large stretches of ssDNA (Pacek and Walter 2004). There are however some blockages that can lead to impediment of both helicases and replicative polymerases such as interstrand cross links (ICL's) or DNA-protein complexes (Lambert and Carr 2013).

Nicks and gaps in the DNA as well as ssDNA can act as sources of replication stress. In a lot of recombination and repair pathways, nicks and gaps in the DNA act as intermediates in the process, and if the replisome comes into contact with these nicks/gaps in the DNA this can lead to the conversion into double stranded breaks (Zeman and Cimprich 2014).

Unrepaired DNA damage lesions act as physical block to replication fork progression which can lead to replication stress (Zeman and Cimprich 2014). There are a lot of endogenous and exogenous causes of DNA damage which lead to the formation of lesions on the DNA.

Exogenous damages include exposure to toxic agents such as Ultra-violet (UV) or organic compounds found in tobacco smoke (benzo-a-pyrene) or cooked foods that can react with DNA to form bulky lesions. UV causes the formation of bulky adducts such as a host of pyrimidine dimers, for example 6-4 pyrimidine-pyrimidone

photoproducts (6-4 PPs) (Chatterjee and Walker 2017). These bulky lesions cause blockages to the DNA polymerase but are bypassed by the helicase leading to replication stress (Yajima, Lee et al. 2009). Other DNA lesions such as ICL's and DNA- protein cross-links can form potent blockages to both the helicase and DNA polymerase (Lambert and Carr 2013). Endogenous damages that can lead to the formation of single stranded breaks (SSB's) include oxidative base damages or the misincorporation of bases by DNA polymerases all of which can cause a barrier that impedes DNA polymerases from normal replication (Ciccia and Elledge 2010). These are usually dealt with by a host of different DNA repair mechanisms but any fault or errors in the DNA repair pathway can lead to increased replication stress.

Nucleotide depletion is another mechanism of replication stress. These depletions can either be caused through endogenous or exogenous sources. Increased firing of origins can lead to nucleotide depletion for example in the case of oncogene activation due to deregulation of normal replication firing (Sørensen and Syljuåsen 2012). HU a genotoxic compound can cause nucleotide depletion exogenously (Koç, Wheeler et al. 2004)

Incorrect ribonucleotide incorporation has been shown to be another source of replication stress. Replicative polymerases have high processivity and fidelity when carrying out base pairing, however incorporate ribonucleotides instead of deoxyribonucleotides at a high frequency. This is due to ribonucleotides, being many folds more abundant in the cell than deoxyribonucleotides, anywhere from 36 to 190 fold higher in *S. cerevisiae* depending on which base (Dalgaard 2012). Research has shown that if ribonucleotides have been mis incorporated into the

DNA it can lead to replication fork progression to slow and lead to genome instability (McEllin, Camacho et al. 2010).

Overexpression of oncogenes can be a major driving force leading to replication stress (Bartkova, Rezaei et al. 2006). Oncogenes such as cyclin E and MYC have been shown to cause replication stress when overexpressed (Jones, Mortusewicz et al. 2013, Srinivasan, Dominguez-Sola et al. 2013). Cyclin E overexpression for example is associated with impaired replication fork progression and DNA damage. Cyclin E-induced replication slowing, and DNA damage has been shown to be due to the consequence of excessive origin firing as well as due to interference between replication and transcription. An increase in origin firing and replication-transcription conflicts are both sources for increased replication stress (Jones, Mortusewicz et al. 2013).

There are a lot of DNA sequences that can be challenging for the progression of replication, examples include secondary DNA structures or trinucleotide repeats. These secondary DNA structures such as hairpins basically block fork progression. These sequences not only provide obstacle for replication but also promotes replication slippage (McMurray 2010, Kim and Mirkin 2013). The replication stress response is crucial for stabilising these sequences. G-quadruplexes, which commonly occur in regions of DNA that are rich in guanine and cytosine (GC), are also secondary structures that act as a block to replication and slow replication speeds and lead to increased DSB's (Bochman, Paeschke et al. 2012). Stabilising these structures chemically slows replication fork speeds and increases amounts of DSBs (Bochman, Paeschke et al. 2012, Paeschke, Bochman et al. 2013).

There are certain regions in a genome that are hard for polymerases to replicate; these are known as fragile sites. These sites can be divided into two classes, common fragile site (CFS) or early replicating fragile sites (ERFS) (Barlow, Faryabi et al. 2013). Fragile sites are prone to replication stress and increased double strand breaks (Mazouzi, Velimezi et al. 2014). It has been suggested that these fragile sites may lead to increase stress and DSB formation as there are no replication origins in these regions, making it harder to rescue stalled forks (Debatisse, Le Tallec et al. 2012). Recent research has linked these fragile sites with transcription. These sites overlapped with extremely long genes which means that transcription will take a long time to fully transcribe one of these genes. A study done in CSF's showed that transcription was happening at the same time as replication, indicating that the increased breakages may be due to increased transcription-replication interference (Helmrich, Ballarino et al. 2011). Additional research showed that these CSF's were enriched for long genes and indicates that the reason for increased fragility is linked to transcription (Le Tallec, Millot et al. 2013). Similar findings were found with ERFS sites where these sites overlap with transcribed genes that are highly expressed again linking the fragility of these sites with transcription (Barlow, Faryabi et al. 2013). I will describe transcription-replication conflicts in more detail below.

1.4.7: Transcription-replication conflicts.

A major cause of replication stress in cells is the conflicts that occur between the two processes of replication and transcription (Bermejo, Lai et al. 2012). Cells have tried to develop a variety of mechanisms to reduce and prevent these collisions including co-orientating these processes or genome organization (Merrikh, Machon et al. 2011, García-Muse and Aguilera 2016). Replication fork collisions with transcription will occur because both processes act on DNA, and as transcription is needed for cell survival and viability while replication is required for cell proliferation (Helmrich, Ballarino et al. 2013). The interference between both mechanisms is particularly common in eukaryotic cells as eukaryotic cells fire multiple origins (Bermejo, Lai et al. 2012). DNA replication only requires a single replisome on the DNA to be replicated, however when transcribing a gene multiple RNA Pol are present to ensure a gene is fully transcribed. Hence when a replisome encounters the first RNA Pol it can't replicate further as there are multiple RNA Pol's present behind the first RNA Pol hence replication is impeded (Azvolinsky, Giresi et al. 2009, Merrikh, Machon et al. 2011).

Conflicts between transcription and replication can occur in two orientations (figure 1.19) (García-Muse and Aguilera 2016). A head-on orientation conflict means the replisome and RNA Pol are moving in opposite directions occurs on the lagging strand, hence both the replisome and the RNA Pol ll clash against each other on the DNA, which will lead to fork stalling and replication stress (Prado and Aguilera 2005, Poveda, Le Clech et al. 2010). This will also lead to the dissociation of the replisome machinery from the DNA and formation of recombinogenic reversed or

collapsed fork (Prado and Aguilera 2005, Poveda, Le Clech et al. 2010). Further studies have shown that transcription can enhance recombination in a process called transcription associated recombination (TAR). Increased TAR requires both increased amounts of transcription and the cell being in S-phase (Gottipati, Cassel et al. 2008). As I will describe later in this chapter, recombination is an important factor in dealing with stressed forks and DSB's, indicating that head-on collisions lead to DNA damage, DSB's and genomic instability.

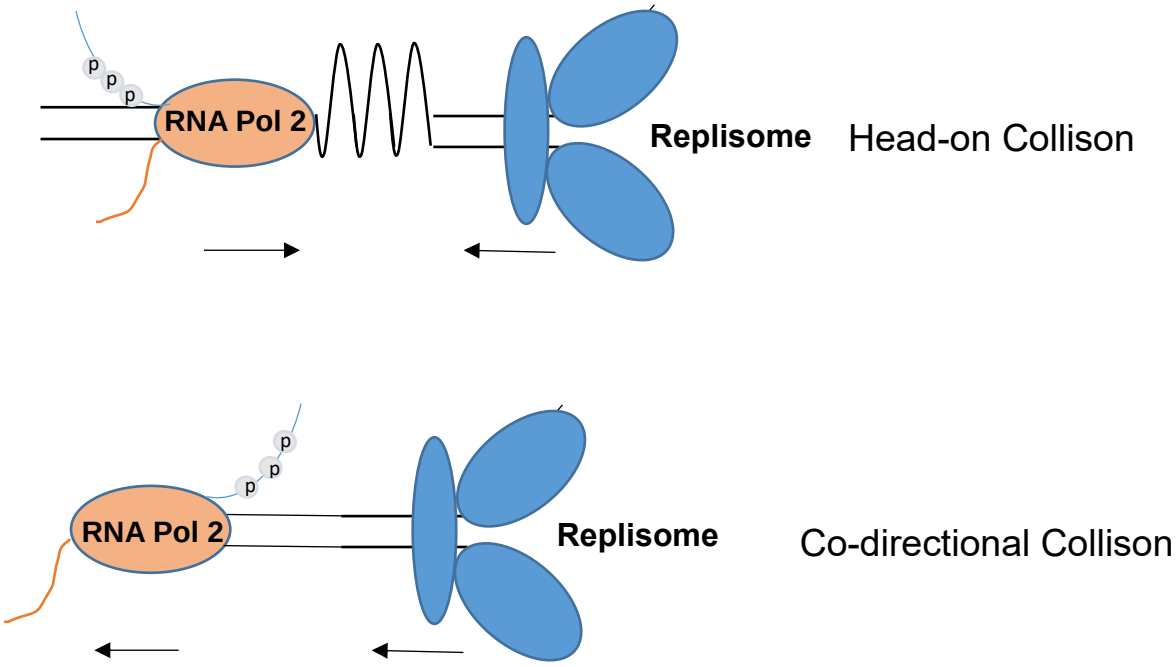


Figure 1.19: Diagram illustrating how transcription-replication can occur in cells. These collisions can either happen in two orientations: head on and co-directional.

The other orientation these conflicts can occur is co-directional orientation occurring on the leading strand. Both the RNA Pol and replisome occur in the same direction and if the replisome moves faster than RNA Pol it can cause conflicts with the RNA

Pol (Mirkin and Mirkin 2007, Soultanas 2011). These conflicts can be resolved by the removal of the RNA Pol from the DNA. Co-directional conflicts can also lead to fork stalling and induce DNA breaks (Merrikh, Machon et al. 2011). Research has shown that conflicts in the head-on orientation can have a much more dramatic effect on replication and genome stability (Prado and Aguilera 2005, Srivatsan, Tehranchi et al. 2010).

There is also evidence that when these two processes come together this leads to replication stress in an indirect mechanism before the replication and transcription machineries even meet. This may include R-loop formation, DNA secondary structures such as G quadraplexes and or topological stress from supercoiling (Huertas and Aguilera 2003, Tuduri, Crabbe et al. 2009, Aguilera and Garcia-Muse 2012, Alzu, Bermejo et al. 2012, García-Muse and Aguilera 2016).

When replication and transcription converge in a head-on orientation it can lead to the formation of torsional stress via supercoiling (García-Muse and Aguilera 2016). Research has shown that enzymes such as helicases and topoisomerases relieve stress and prevents these collisions (Bermejo, Doksani et al. 2007, Tuduri, Crabbe et al. 2009, Alzu, Bermejo et al. 2012). This shows that the torsional stress generated is involved in conflicts between replication and transcription and cause replication stress.

1.4.8: R-loops

RNA: DNA hybrids form naturally during transcription and replication. These formed hybrids are usually short, however longer forms can be generated. These longer forms are generated when the nascent RNA can be hybridised with the template DNA during transcription leaving a displaced ssDNA (Huertas and Aguilera 2003). These forms are known as R-loops and form when the nascent RNA exits the RNA Pol (Huertas and Aguilera 2003). There are different situations where-in R-loop formation can be favoured such as DNA supercoiling, high G content or whether there are nicks in the DNA (Huertas and Aguilera 2003).

R-loops have been shown to have a number of roles in cells including: regulating gene expression, DNA replication and DNA repair (Costantino and Koshland 2015, Sollier and Cimprich 2015). R-loops have been linked to causing DNA damage (Bermejo, Capra et al. 2011, Aguilera and Garcia-Muse 2012, Sollier, Stork et al. 2014). An experiment overexpressing RNaseH1, which helps resolve R-loops, in cells with high levels of R-loops showed a decrease in DSB marker γH2AX (Chernikova, Razorenova et al. 2012). This implies R-loops as a source of DNA damage in the cell. Further studies have strengthened the link between R-loops and DNA damage, including genome wide screens that show DNA damage being supressed in a ribonuclease H (RNase H) dependent manner (Paulsen, Soni et al. 2009). A question remained in how R-loops and DNA damage are linked.

Studies have indicated that R-loop formation can hamper on-going replication fork progression and induce DNA breakage (Aguilera and Garcia-Muse 2012, Sollier, Stork et al. 2014). As mentioned earlier, research has shown that the fragility of CFSs and ERFs can be due to the increased number of collision between replication and transcription (Helmrich, Ballarino et al. 2011, Barlow, Faryabi et al. 2013). Indeed studies have shown that inhibiting replication in either yeast or human cells can prevent the DSB DNA damage caused by R-loop formation (Wellinger, Prado et al. 2006, Tuduri, Crabbe et al. 2009, Gan, Guan et al. 2011). This indicates that the interference between replication and R-loops is leading to R-loop associated DNA damage.

Overall, R-loops are an emerging source of induced DNA damage and genomic instability in the cell. Research has shown that R-loops are directly involved in transcription-replication interference and cause fork stalling and could be a major source of DNA breaks in the cell.

As transcription replication conflicts and R-loops can provide a major problem for the cell, pathways have evolved to try and prevent these collisions and to resolve R-loops. These factors include RNA Pol backtracking using GreA and GreB to help allow reactivation of transcription (Pomerantz and O'Donnell 2010, Tehranchi, Blankschien et al. 2010, Dutta, Shatalin et al. 2011). Replication fork barrier which prevents collisions in budding yeast which requires RFP protein FOB1 (Torres, Bessler et al. 2004, Merrikh, Brewer et al. 2015). FACT which remodels chromatin to allow both processes to occur without collisions (Abe, Sugimura et al. 2011, Herrera-Moyano, Mergui et al. 2014).

There are many different ways in which cells have now adapted to try and prevent excessive R-loop formation. As mentioned earlier R-loops can be resolved by RNase H enzymes of which there are two types 1 and 2 (Wahba, Amon et al. 2011). These enzymes act by degrading the RNA in the RNA: DNA hybrids. Helicases are also known to help prevent R-loop formation. Some examples of helicases that can prevent R-loops are the DHX9 RNA helicase and the SETX RNA-DNA helicase. The SETX helicase has functions that remove R-loops and resolving transcription-replication conflicts (Skourti-Stathaki, Proudfoot et al. 2011). DHX9 helicase preferably unwinds R-loops and G-quadraplexes and is involved in resolving transcription-replication conflicts (Chakraborty and Grosse 2011). The presence of RECQL5 helicases which recent studies show that can help prevent excessive R-loop formation (Li, Xu et al. 2011, Li, Pokharel et al. 2015). Topoisomerase enzymes help supress excessive R-loop formation by relieving torsional stress behind the RNA Pol (Tuduri, Crabbé et al. 2009, Yang, McBride et al. 2014).

1.4.9: Increased transcription causing replication stress

Replication-transcription interference has now been identified as a main source of replication blockages to ongoing replication (Kotsantis, Silva et al. 2016, Stork, Bocek et al. 2016, Gorthi, Romero et al. 2018, Lavado, Park et al. 2018, Nojima, Tellier et al. 2018). Recently, there have been a growing number of studies illustrating that an increase in transcription activity from steady state levels can cause increased replication stress in cells.

In a recent project in our lab, increasing levels of oncogenic HRAS was shown to lead to increased RNA synthesis in the cell (Kotsantis, Silva et al. 2016). Further experiments using DNA fibre analysis illustrated that ongoing DNA replication was being stalled and slowed down at the same time. By inhibiting transcription in conjunction with HRAS induction it was shown that this replication stress phenotype could be rescued towards control levels (Kotsantis, Silva et al. 2016).

Overexpression of transcription factor TBP further illustrated that increased RNA synthesis in a cell could directly lead to increased replication stress. Further work done in this project also illustrated the importance of increased R-loop formation. Cells overexpressing HRAS were shown to lead to increased R-loop formation. Using DNA fibre analysis, they showed a rescue of normal fork speeds in cells with overexpressed HRAS which were treated with RNase H1 to suppress R-loops. This showed that increased R-loop formation in cells can lead to increased replication stress (Kotsantis, Silva et al. 2016).

More studies have highlighted similar findings. A recent study showed that loss of SPT6 could lead to increased transcription and R-loop formation. This led to increased replication stress and DNA damage in the cell (Nojima, Tellier et al. 2018). The hormone estrogen (E2) induces increased transcription and R-loops in cells leading to replication stress, which can be alleviated by RNase H1 (Stork, Bocek et al. 2016). A recent study saw that activation of transcription co-activators YAP/TAZ, regulated by the Hippo pathway, leads to increased transcription in the cell. This increased transcription led to increased replication stress, DNA damage and apoptosis (Lavado, Park et al. 2018). This increased transcription in cells has also been shown in Ewing Sarcoma where the fusion of EWSR1 protein and FLI1

protein caused increased transcription levels and R-loops in the cells, leading to increased replication stress and impaired homologous recombination (HR) (Gorthi, Romero et al. 2018).

These findings illustrate the importance of increased transcription in the cell in terms of replication stress. These studies show evidence that when increased transcription is induced in cells that it can directly lead to replication stress. Alleviating this increase in transcription can restore normal replication progression and loss of replication stress.

1.4.10: Replication stress and genomic Instability

Genomic instability is one of the hallmarks of most cancer cells. Cancer is a broad term to describe a range of conditions that have abnormal cell growth and proliferation or an inability to regulate these processes. Cells have a myriad of signalling pathways to control cell division and growth which when dysregulated can give rise to uncontrollable cell growth and lead to tumour formation, For the onset of tumorigenesis to happen there must be a cascade of numerous events for the onset to begin. One major event that often occurs is genomic instability.

Genomic stability is described as either the loss or gain of genetic material either through mutations or chromosomal rearrangements including translocations, deletions and inversions (Hanahan and Weinberg 2000, Hoeijmakers 2001). Genomic instability can arise most commonly from faulty DNA repair or DNA replication (Gaillard, Garcia-Muse et al. 2015). Genomic instability often occurs after

mutation in genes encoding for DNA repair or mutations in genes controlling cell division or tumour suppressor genes. Defects in processes such as the DDR, cell cycle checkpoints or cellular senescence/cell death pathways can help result in accumulation or transmission of DNA damage leading to genome instability and onset of tumorigenesis.

Replication stress can lead to breakage, chromosomal rearrangement or mis-segregation of chromosomes (Hills and Diffley 2014). Increased DNA damage will lead to increased replication stress which can be defined as a source of genomic instability and is often seen in cancerous cells (Gaillard, Garcia-Muse et al. 2015). There are numerous pathways that can deal with replication stress to try and protect genome stability by protecting stressed replication forks and allowing faithful replication. Further repair mechanisms help cells deal with consequences of replication stress such as DSB's to prevent increased genomic instability. Overall increased replication stress leads to persistent stalling and either ssDNA gaps or breakage which can in turn generate genomic instability in the cell (Hills and Diffley 2014, Gaillard, Garcia-Muse et al. 2015).

1.5: Cell response to replication stress

1.5.1: DNA damage overview

To allow DNA to be replicated each cell cycle faithfully, the cell has a series of DNA replication stress and DNA damage response pathways to help facilitate cell cycle arrest and DNA repair and ensure DNA replication restart.

To allow for these pathways to be activated, signalling is required which in the case of the DNA damage response involves phosphorylation by specific kinases. The three kinases that control the signalling pathway are DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ATR and ATM (Ciccia and Elledge 2010). These three kinase respond to specific types of damages, in the case of ATM and DNA-PK this is mainly DSBs and in the case of ATR it is persistent ssDNA (although there is crosstalk between the three kinases), and set in motion the response to deal with the damage by phosphorylating a myriad of different downstream proteins (Ciccia and Elledge 2010, Blackford and Jackson 2017).

1.5.2: Replication stress response overview

If replication stress is persistent it can cause fork collapse and formation of DSBs, or under-replicated DNA can lead to the development of DNA damage, mutations and disease. Ultimately replication stress can lead to cell death via collapsed forks forming DSBs, or cells which have unfinished replicated DNA undergoing mitotic

catastrophe. Understanding how BET inhibition can lead to tumour cell death and senescence would be crucial for eventual future clinical applications.

1.5.3: Replication stress response

Replication fork stalling has the potential to lead to the generation and persistence of ssDNA at the replication fork (Cimprich and Cortez 2008). This ssDNA occurs as the replicative helicase (MCM helicase) still continues to unwind DNA, while the replicative polymerase has stalled (Pacek and Walter 2004). The continuing presence of ssDNA leads to it being bound by RPA which acts as a signal for the activation of the replication response pathway (Byun, Pacek et al. 2005). The presence of RPA-bound ssDNA will signal to recruit and activate a number of replication stress response proteins, including the protein kinase ATR. The ATR kinase is a serine/threonine kinase and a member of the PI3K like protein kinase family. ATR will bind to the RPA coated ssDNA via its partner protein ATR interacting protein (ATRIP) (Zou and Elledge 2003). ATR-ATRIP recruitment does not lead to full activation of ATR. In addition, the RPA-coated ssDNA allows the binding of the RAD17-RFC2-5 clamp loader. The RAD17-RFC2-5 clamp loader can then load PCNA-related, RAD9-HUS1-RAD1 (9-1-1) heterotrimer leading to the activation of TOPBP1 which can stimulate ATR kinase activity leading to optimal activation of ATR (Cimprich and Cortez 2008).

ATR is crucial for the survival of proliferating cells and in allowing cells to deal with and survive replication stress. It is understood that if ATR and its downstream

pathway is not present, there is an increase of genome instability during S phase of cell cycle indicating the importance of the ATR pathway in keeping the genome stable during DNA replication (Flynn and Zou 2011). ATR activation leads to phosphorylation of factors that can help stabilise forks that have encountered stress, factors that help fork repair, fork recovery and forks restart to counter replication stress. One such factor is histone H2AX, which is phosphorylated by ATR to form γH2AX. Activation of ATR substrates allow the cell to complete DNA replication faithfully and promotes cell survival (Buisson, Boisvert et al. 2015). A major kinase activated by ATR is CHK1 via Claspin phosphorylation (Cortez, Guntuku et al. 2001). CHK1 is phosphorylated specifically on its serine residues 317 and 345 by the ATR kinase (Liu, Guntuku et al. 2000). Further factors that increase CHK1 activation have been discovered such as DONSON, Timeless, TIPIN and RAD17 (Bao, Tibbetts et al. 2001, Unsal-Kacmaz, Chastain et al. 2007, Reynolds, Bicknell et al. 2017). CHK1 leads to fork stabilisation and inhibits cell cycle progression by activating cell cycle checkpoints which allows time for the replication stress to be dealt with before going through another cell cycle (Gonzalez Besteiro and Gottifredi 2015). CHK1 also inhibits firing of new origins to prevent additional replication forks from encountering blocks and give the cell more time to repair the damage (Yekezare, Gómez-González et al. 2013). ATR pathways can lead to fork stabilisation and if the replication stress source can be eradicated then replication forks can be restarted and replication can be completed (Petermann and Helleday 2010).

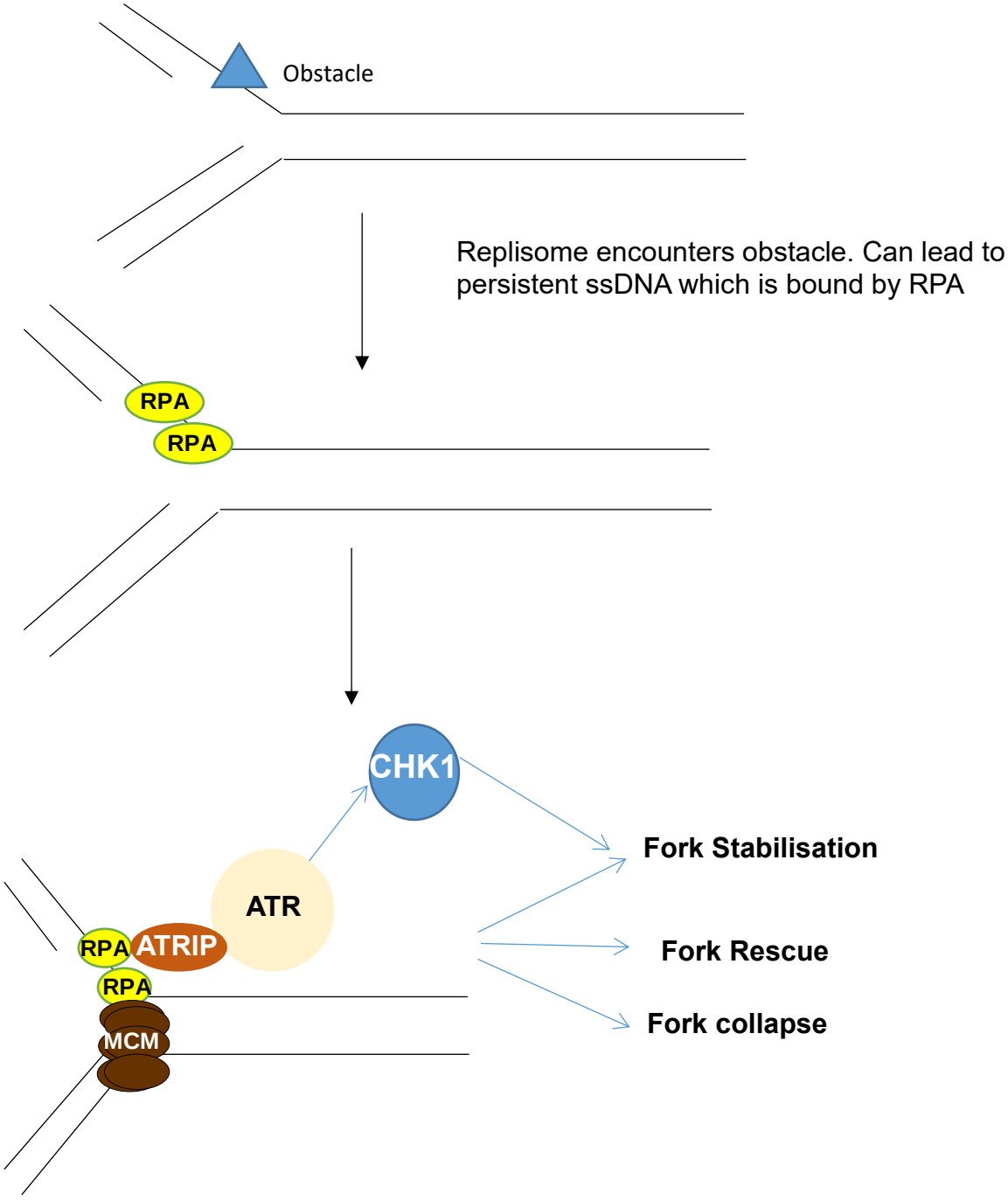


Figure 1.20: Diagram illustrating the process of the replication stress response in response to persistent replication stress.

There are many mechanisms that may lead to the restart of replication which include: firing of dormant origins, repriming at the replication fork, reversing the stalled fork which will be discussed later, or activation of DNA damage tolerance pathways which help bypass lesions (Elvers, Johansson et al. 2011, McIntosh and Blow 2012, Mailand, Gibbs-Seymour et al. 2013). If the replication stress response does not stabilise the replication fork and the replication stress is persistent, the fork is susceptible to collapse which can lead to the formation of a DSB at the fork (Sirbu, Couch et al. 2011). The mechanism that leads to the formation of DSB is still under investigation. There is mounting evidence that persistent stalled forks can be subject to recombination pathways which can form structures that are vulnerable to endonucleoytic attack by endonucleases such as Mus81 (Hanada, Budzowska et al. 2007, Petermann, Orta et al. 2010, Schlacher, Christ et al. 2011). I will discuss this in much more detail later in this chapter. In addition, persistent ssDNA or stalled forks can also be subject to endonucleolytic attack or even subject to passive breakage leading to break formation (Lopes, Cotta-Ramusino et al. 2001, Sogo, Lopes et al. 2002, Lopes, Foiani et al. 2006).

1.5.4: Double strand break repair overview

DSBs are toxic lesions for cells, which have evolved a variety of different repair mechanisms to deal with their formation. There are four major mechanisms to allow for DSB repair: Non-homologous end joining (NHEJ), HR, alternative-NHEJ (alt-NHEJ) and single-strand annealing (SSA) (Ciccia and Elledge 2010). DSBs can be

recognised and sensed by different proteins depending on context. This can lead to a signalling pathway that requires recruitment of a myriad of different signalling and effector proteins. This initial sensor can set up a pathway to help trigger DSB repair in the cell. DSB repair also requires a host of different PTMs to the chromatin and repair proteins around the lesion to facilitate fast and accurate repair (Bekker-Jensen, Lukas et al. 2006, Bekker-Jensen and Mailand 2010, Ciccia and Elledge 2010).

1.5.5: Non-homologous end joining

NHEJ is a DSB repair pathway that can occur throughout the cell cycle. NHEJ allows a rapid but more error-prone repair of DSBs involving re-ligation of the broken ends of the DNA. Here DSB are sensed by KU70/80 which bind to DSB ends (Walker, Corpina et al. 2001, Mahaney, Meek et al. 2009). KU70/80 can recruit and activate DNA-PKcs, forming DNA-PK which initiates NHEJ. DNA PK stabilizes DSB ends by preventing DNA end resection (Mahaney, Meek et al. 2009). DNA-PK can then auto-phosphorylate on its ABCDE cluster, allowing dissociation from DSB ends. This allows ARTEMIS, an end processing enzyme, to access DSB ends (Meek, Dang et al. 2008). XLF and XRCC4/DNA Ligase 4 is recruited by DNA-PK and functions to re-ligate the broken ends (Koch, Agyei et al. 2004, Mahaney, Meek et al. 2009).

1.5.6: Homologous recombination

Homologous recombination is a DSB repair mechanism that occurs during the S/G2 phases of the cell cycle and uses identical sister chromatids as a template for repair (Rothkamm, Kruger et al. 2003). Unlike NHEJ, HR is an error-free type of repair (You, Chahwan et al. 2005). The MRE11-NBS1-RAD50 (MRN) complex can be recruited to DSBs that are required to be repaired by HR and set of a signal cascade to allow for HR-mediated repair (Williams, Williams et al. 2007). MRN is composed of three components RAD50, MRE11 and NBS1, all of which have different roles in HR repair. MRE11 is crucial to enable the first steps of DNA resection, which is a crucial process for HR repair, and contains both exonuclease and endonuclease activity to help it carry out this function (Williams, Williams et al. 2007). RAD50 functions both to interact with MRE11 via its ATPase domains and to bind to DSB ends (Williams, Williams et al. 2007). NBS1 interacts and binds to ATM through its C-terminal end, allowing for MRN to be able to recruit ATM to the DSB (de Jager, van Noort et al. 2001, Paull and Lee 2005, Jazayeri, Falck et al. 2006). ATM recruitment is critical to help regulate the process of DNA end resection and functions to recruit Ct-BP interacting protein (CtIP), which is a nuclease, to the DSB (Sartori, Lukas et al. 2007, You, Shi et al. 2009). CtIP interacts with both MRN and also with Breast cancer type 1 susceptibility protein (BRCA1), allowing for the formation of the BRCA1-C complex (Sy, Huen et al. 2009). The BRCA1-C complex functions to regulate the initial stages of resection at the DSB. Before this initial resection takes place, there are inhibitory factors to the DNA resection process such

as P53 Binding protein 1 (53BP1) which helps promote NHEJ by stopping resection of DSB ends (Bunting, Callen et al. 2010). Studies have shown that the removal of 53BP1 to allow for DSB end resection can be mediated by the BRCA1-C complex (Bunting, Callen et al. 2010, Densham, Garvin et al. 2016). Another complex that is vital in the resection process is the BRCA1-BARD1 E3 ligase enzymatic activity (Densham, Garvin et al. 2016). The BRACA1-BARD1 complex help promotes extended resection by helping reposition 53BP1 by SMCARCAD1 chromatin remodelling (Densham, Garvin et al. 2016). After the initial DNA resection has been completed, extensive resection at the DSB needs to occur. This extended resection of the DSB can be mediated by Exonuclease 1 (EXO1), DNA2 and Bloom syndrome RecQ like helicase (BLM). Their activation is mediated via ATM phosphorylation which also allows for their recruitment to the DSB and therefore facilitates for extended resection to take place (Cejka, Cannavo et al. 2010). Resection of the DSB leads to the formation of a 3'ssDNA overhang. Exposed ssDNA after resection is recognized and bound by RPA. RPA can thereby act to protect and stabilize the exposed ssDNA (Wold 1997). RAD51 homolog 1 (RAD51) can then replace the RPA bound to ssDNA (West 2003). Breast cancer type 2 susceptibility protein (BRCA2) is important for HR to take place. BRACA2 can recruit and interact with RAD51 and helps facilitate with the loading RAD51 to ssDNA (Davies and Pellegrini 2007, Esashi, Galkin et al. 2007). The requirement of phosphorylation of RAD51 by CHK1 allows for increased regulation of RAD51 recruitment to the DSB. Further regulation of RAD51 can also occur by phosphorylation by both DSS1 and RAD52 (Sugiyama and Kowalczykowski 2002, Sorensen, Hansen et al. 2005, Gudmundsdottir, Lord et al. 2007). Loading of nine

RAD51 molecules allows for strand invasion into the homologous sequences of the template sister chromatid, causing D-loop formation (West 2003, San Filippo, Sung et al. 2008). The invading strand can be extended at the 3’ end by DNA polymerases until the DNA missing at the break is newly synthesised. The newly synthesised DNA can then be reannealed to the processed other end of the break (West 2003). This process can be performed by synthesis-dependent strand annealing (SDSA) or by second-end capture. Holiday Junctions (HJ) are formed after strand invasion and D-loop formation, as well as after second-end capture (West 2003, San Filippo, Sung et al. 2008). BLM helicase and Topo IIIα are able to dissolve the HJ structure avoiding the need for endonuclease cleavage (Wu and Hickson 2002). HJ can also be resolved by the action of endonucleases such as MUS81/MMS4, GEN1 or SLX1/SLX4 which by cleaving DNA can either lead to genetic information being crossed over between the two sister chromatids, or to the resolution of the sister chromatids as they were previously (Hartlerode and Scully 2009).

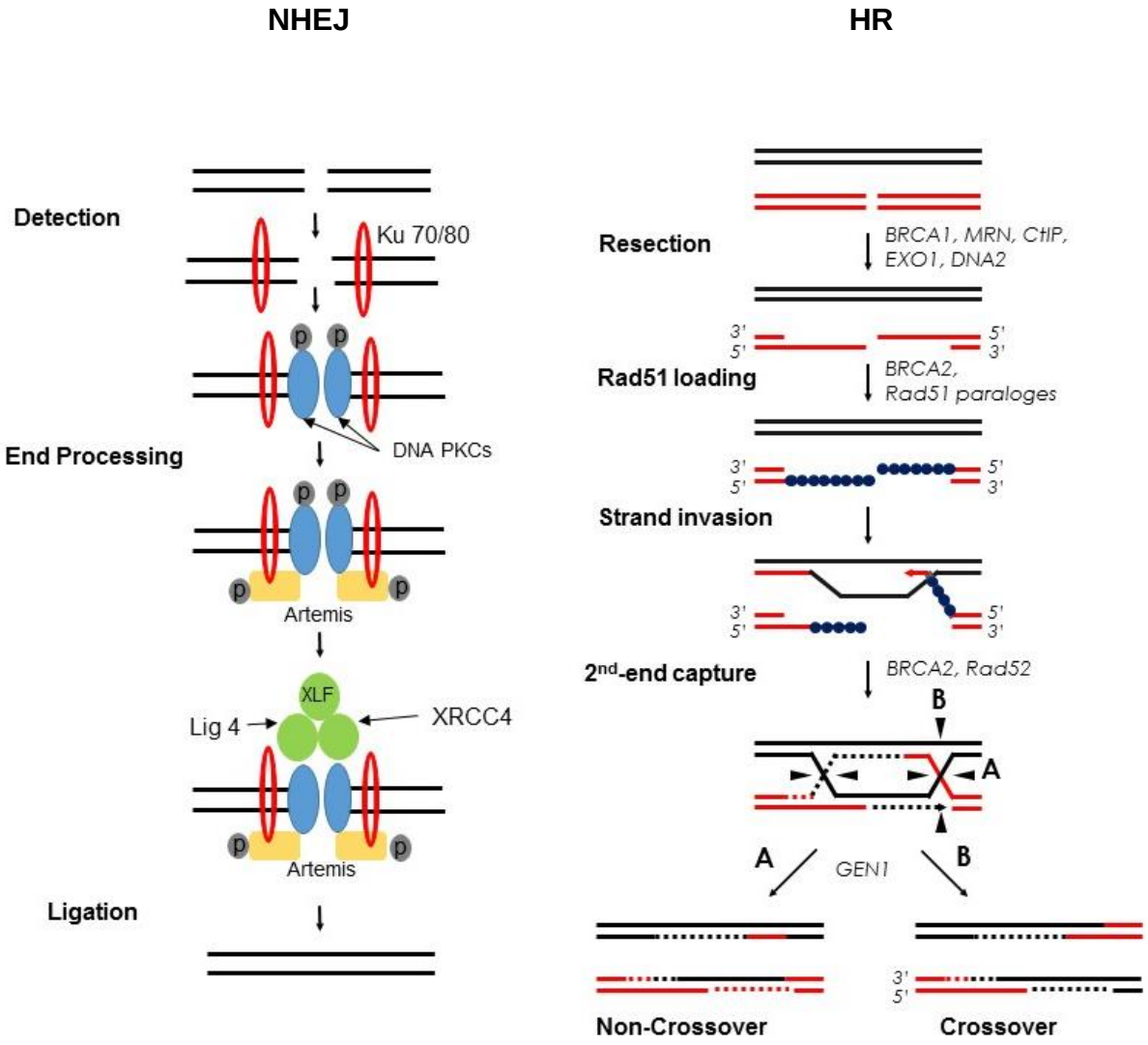


Figure 1.21: Diagram illustrating the mechanism of DSB pathways NHEJ and HR in cells.

1.5.7: HR at replication forks

Recently, HR and its factors have been shown to play roles at replication fork processes such as replication stress in addition to DSB repair. HR has been shown to be activated in response to replication stress inducing compounds such as HU or thymidine. Here HR proteins such as RAD51 or BRCA2 can be recruited to stalled forks (Petermann, Orta et al. 2010, Kolinjivadi, Sannino et al. 2017). When these factors bind they can induce recombination and HR at these forks leading to prevention of fork collapse and potentially fork restart (Arnaudeau, Lundin et al. 2001, Lundin, Schultz et al. 2003, Carr and Lambert 2013, Willis, Chandramouly et al. 2014).

Stalled forks can be restarted either in the presence or absence of DSB formation. When the replication fork encounters stress through a blockage a one-ended DSB can be formed through specific fork cleavage. One-ended DSB can only be repaired through an HR-mediated fork restart mechanism called break-induced replication (BIR) (Hanada, Budzowska et al. 2007, Pepe and West 2014). RAD51 is crucial in this process as it is loaded onto a resected ssDNA end at the one-ended DSB and can stimulate strand invasion and homology searching (Chung, Zhu et al. 2010). This leads to D-loop formation, as seen in HR before, which allows for DNA replication to start re-synthesising, hence restarting the replication fork (Lydeard, Jain et al. 2007, Hashimoto, Puddu et al. 2012, Costantino, Sotiriou et al. 2014). There are two models that have been described to how DNA synthesis is continued: either by continuous or discontinuous DNA synthesis (Lydeard, Lipkin-Moore et al.

2010, Donnianni and Symington 2013). The end result of BIR is complete DNA replication of the dsDNA.

Stalled forks do not necessarily require the formation of DSBs to allow fork restart via HR. Recent studies have indicated a role for replication restart requiring HR without the formation of a DSB (Lambert, Mizuno et al. 2010, Petermann, Orta et al. 2010). This suggests a novel role of RAD51 in restarting forks in a DSB independent manner, differing from its role in BIR mediated restart. This model required RAD51 to bind to ssDNA and lead to formation of a HJ (Petermann, Orta et al. 2010). The exact mechanism will be described in more detail below.

Recently a study showed that replication fork reversal happened in response to a variety of different genotoxic sources causing replication stress. Replication stress lead to the persistence of ssDNA accumulation which acted as a precursor for fork reversal upon different types of genotoxic stress (Zellweger, Dalcher et al. 2015). This study identified HR factor RAD51 as a cellular factor helping cells deal with replication stress by inducing the replication fork reversal process. This study also showed an important role of RAD51 in slowing down forks in response to genotoxic compounds before replication fork reversal took place (Zellweger, Dalcher et al. 2015).

1.5.8: Replication fork reversal

As previously mentioned, fork reversal can occur in order to help prevent formation of DSBs and restart replication forks after they have encountered replication stress (Ciccia and Elledge 2010).

Replication fork reversal describes the process of the conversion of a normal replication fork (three-way junction) into a four-way junction (chicken foot structure). This can occur via the annealing of the newly synthesised strands with the re-annealing of the parental strands (Neelsen and Lopes 2015). Reversed forks were first discovered in *E. coli* in 1976 (Higgins, Kato et al. 1976).

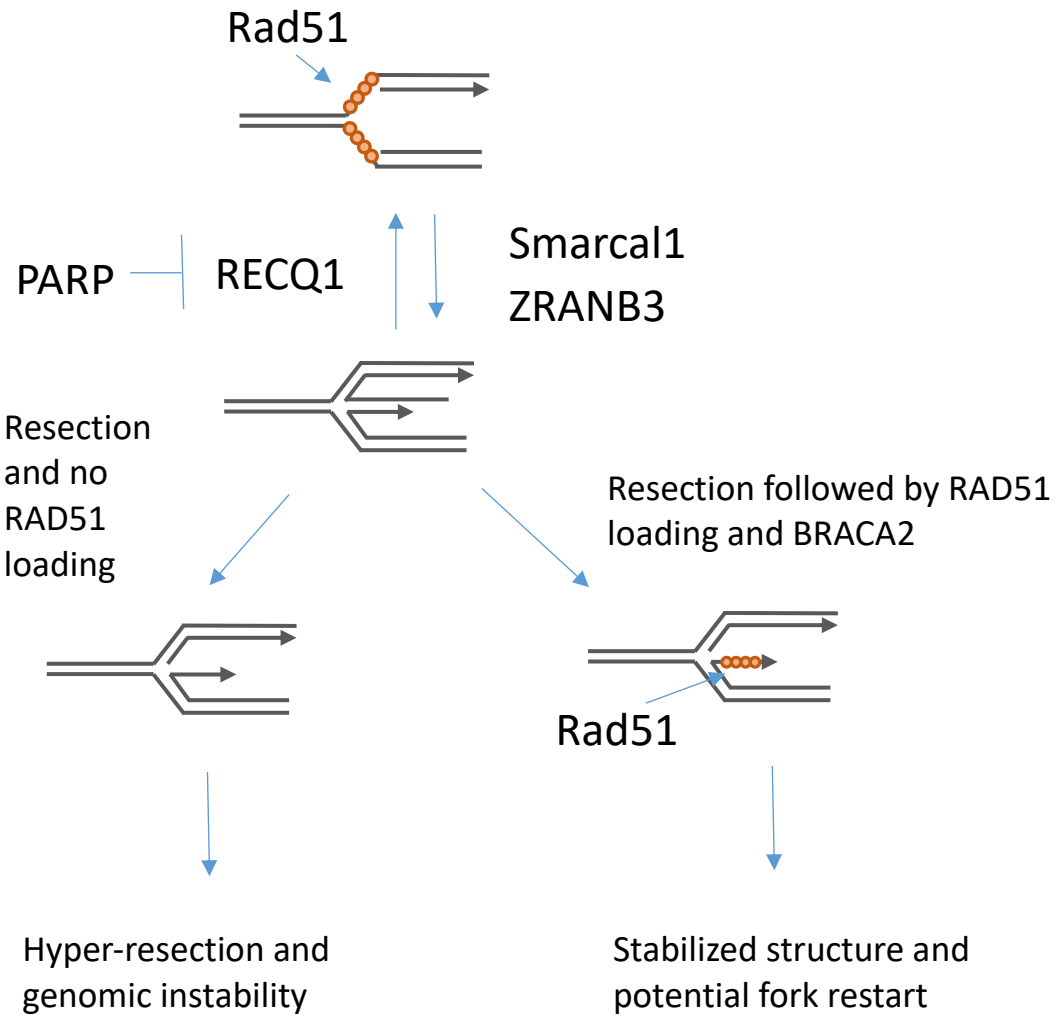


Figure 1.22: Model of fork reversal, regression and protection.

By allowing replication fork reversal, cells prevent increased genomic instability as these structures are stable thereby allowing more time for the block to be removed or DNA to be repaired (Branzei and Foiani 2010). Fork reversal keeps these DNA replication forks not only stable but also in a paused structure which helps to prevent further fork progression which would result in increasing amounts of ssDNA

to be formed. This helps the cell to either repair or bypass the lesion without having to deal with more complex and toxic lesions such as DSBs (Neelsen and Lopes 2015).

The frequency of reversed forks can be dependent on cell type, for instance in embryonic stem (ES) cells the frequency of fork reversal is 30% compared to 8% seen in somatic cells (Ray Chaudhuri, Ahuja et al. 2015). These higher reversal rates seen in ES cells may be due to the cells having increased amounts of ssDNA gaps as well as increased expression levels of two key proteins involved in fork reversal RAD51 and RPA (Ray Chaudhuri, Ahuja et al. 2015).

Recent research has identified a number of different proteins that are involved in the fork reversal process. One such protein mentioned before is the HR factor recombinase RAD51. RAD51 has been elucidated to be able to convert uncoupled forks (with extended ssDNA stretches) to reversed forks after treatment with a variety of different genotoxic sources causing various DNA damage as mentioned earlier (Zellweger, Dalcher et al. 2015). How RAD51 promotes reversed forks is still being further investigated, but it may act in combination with other enzymatic activities. Reports have stated that potentially RAD51 and RAD54 may work in combination to promote fork regression (Bugreev, Rossi et al. 2011). F-box DNA helicase 1 (FBH1 helicase), Poly-ADP ribose-polymerase 1 (PARP1), SMARCAL1 and ZRNAB3 are other proteins that have been identified to play a role in replication fork reversal (Berti, Ray Chaudhuri et al. 2013, Couch, Bansbach et al. 2013, Fugger, Chu et al. 2013). Studies have shown that FBH1 promotes fork reversal in a helicase dependent manner (Masuda-Ozawa, Hoang et al. 2013, Fugger, Mistrik et al. 2015). PARP1 can help fork reversal by firstly inhibiting the activity of ATP-

dependent DNA helicase Q1 (RECQ1) helicase which functions to resolve reversed fork structures as will be discussed later on as well as by promoting branch migration which describes a process where homologous strands successively exchange their base pairs at a HJ (Berti, Ray Chaudhuri et al. 2013). SMARCAL1 can bind to both three-way and four-way junctions and reverse forks by fork regression and branch migration (Betous, Mason et al. 2012). Studies have shown that also zinc finger RNA-binding domain containing 3 (ZRANB3) is involved in promoting reversed forks (Couch, Bansbach et al. 2013). ZRANB3 can recognize forks encountering replication stress by interacting with polyubiquitinated PCNA and remodel forks by its DNA translocase activity (Vujanovic, Krietsch et al. 2017).

Although fork reversal can act as a protective process to prevent more toxic lesions, they can also be vulnerable to hyper-resection by a number of nucleases which can lead to increased genomic instability (Schlacher, Christ et al. 2011, Schlacher, Wu et al. 2012). When reversed forks are formed, the nascent DNA has to be protected from unregulated degradation by nucleases to allow for DNA repair and fork restart.

BRCA2 is a protein whose involvement is critical in helping stabilize the nascent DNA and prevent hyper resection at persistently stalled forks (Schlacher, Christ et al. 2011). Research showed that cells deficient in BRACA2, were subject to MRE11-dependent hyper-resection leading to nascent DNA degradation and increased genomic instability (Schlacher, Christ et al. 2011).

A plethora of studies have highlighted the importance of RAD51 in protecting stalled replication forks from hyper-resection. Firstly, RAD51 was shown to protect nascent DNA from MRE11 hyper-resection in *Xenopus laevis* egg extract (Hashimoto,

Chaudhuri et al. 2010). A study conducted with mammalian cells deficient of RAD51 paralog RAD51C were again subject to increased hyper-resection (Somyajit, Subramanya et al. 2012). Further studies again showed the importance of RAD51 as well as identifying additional HR factors such as FANCD2 and BRCA1 in protecting newly synthesised DNA from hyper-resection (Schlacher, Wu et al. 2012). If cells were deficient in any of the three proteins it lead to increased degradation of newly synthesised DNA via hyper resection (Schlacher, Wu et al. 2012).

More factors recently have been identified involved in protecting stalled forks and preventing genomic instability. These include BOD1L which functions to stabilise RAD51 binding at the reversed fork as well as preventing DNA2 hyper-resection. Cells lacking BOD1L have increased degradation of nascent DNA and increased genomic instability (Higgs, Reynolds et al. 2015). Another factor is WRNIP which protects nascent DNA by again stabilizing RAD51 binding as well as being involved in allowing replication fork restart of stalled forks (Leuzzi, Marabitti et al. 2016).

Interestingly, PARP1 also has a role in stabilising stalled replication forks (Ding, Ray Chaudhuri et al. 2016). PARP1 inhibition or depletion reduced MRE11 hyper-resection in cells without BRCA2, and hence rescued cell viability (Ding, Ray Chaudhuri et al. 2016). Overall the research shows the importance of factors BRCA1, BRCA2, RAD51, SMARCAL1, ZRANB3 and PARP1 in replication fork reversal and fork protection.

1.5.9: Fork restart

Once a fork is reversed it needs to be able to restart which requires the four-way junction to revert back to the three-way junction. As previously mentioned, RECQ1, an ATP-dependent DNA helicase, is the main factor involved in restoring reversed forks. Research showed that RECQ1 restarted reversed forks after Top 1 inhibition either by its ATPase dependent mechanism or by promoting branch migration (Berti, Ray Chaudhuri et al. 2013).

PARP1 can suppress fork restart as PARP1 can inhibit RECQ1 (Berti, Ray Chaudhuri et al. 2013). Further research identified additional factors DNA2 nuclease and Werner syndrome ATP-dependent helicase (WRN) which can help promote fork restart after genotoxic prolonged stalling (Thangavel, Berti et al. 2015). Both of these factors help restart stalled replication forks via their regulated ATPase mediation degradation of the regressed arm (Thangavel, Berti et al. 2015).

After reversed forks have been resected and processed, RAD51 and XRCC3 can be recruited and fork restart can occur where the replication fork is returned back to a three-way junction (Petermann, Orta et al. 2010, Carr and Lambert 2013). The mechanism of how regressed forks are formed, controlled and reversed requires more research.

1.6: Aims

Previous work from the lab had shown a potential role for BET proteins in DNA replication. Treatment with BET inhibitor JQ1 in Nalm6 cells led to replication fork slowing, indicating replication stress. Understanding of the roles of BET proteins in processes such as DNA replication and DNA damage is vital for clinical applications. As such, the aims of this study were as follows:

- To investigate whether BET inhibitors slow replication fork speeds across a number of cell lines.
- To investigate the mechanism by which loss of BET activity causes replication fork slowing.
- To investigate which specific BET protein is required to maintain replication fork speeds.
- To investigate the DNA damage response to BET inhibitor-induced replication fork slowing.

2 Materials and methods

2.1: Buffers and chemicals

2.1.1: General buffers

PBS 1X:

1 Phosphate buffered saline (PBS) tablet (Sigma) in 200ml H_2O

LB broth:

10 g Luria Bertani (LB) Broth powder (Thermo Fisher) in 400 ml H_2O

LB agar:

400 g LB agar powder (Thermo Fisher) in 400 ml H_2O

2.1.2: Inhibitors, drug treatments and chemicals

Name	Final Concentration	Activity	Supplier
JQ1	1 µM	BET inhibitor	Tocris
IBET-151	1 µM	BET inhibitor	Tocris
DRB	100 µM	RNA Pol 2 inhibitor, weak RNA Pol 1 inhibitor	Sigma
α-amanitin	10 µg/ml	Strong RNA Pol 2 inhibitor, weak RNA Pol 3 inhibitor	Sigma
Triptolide	1 µM	RNA Pol 2 and RNA Pol 1 inhibitor	Tocris

Roscovotine	25 µM	Cdc2, CDK2 and CDK5 inhibitor	Sigma
RO-3006	10 µM	CDK 1 inhibitor	Sigma
PHA-767491	10 µM	Cdc7/CDK9 inhibitor	Tocris
HYDROXYUREA (HU)	2 mM	Ribonucleotide reductase	Sigma
Camptothecin	1 µM	Topoisomerase I inhibitor	Sigma
Etoposide	25µM	Topoisomerase II inhibitor	Sigma
HMBA	5-10 mM	HEXIM1 inducer	Sigma
CldU	25 µM	Thymidine analogue	Sigma
IdU	250 µM	Thymidine analogue	Sigma
Olaparib	5 µM	PARP inhibitor	Selleckchem
CX-5461	100 µM	RNA Pol 1 inhibitor	Medchem Express
ML-60218	100 µM/ 25 µM	RNA Pol 3 inhibitor	Cayman Chemical
AZ20	2.4 µM	ATR inhibitor	Tocris
ML216	1.4 µM	BLM inhibitor	Sigma
PD-407824	300nM	CHK1 inhibitor	Tocris

***Table 2.1*:** Drugs, inhibitors and treatments.

2.2: Bacterial work

2.2.1: Bacterial transformation

100 ng of the required plasmid was transferred to thawed One Shot TOP10 chemically competent *E. coli* High copy Plasmid (Fisher) of around 25 µl and incubated on ice for around 20 mins. Bacteria were than heat shocked for exactly 30 seconds at 42°C before being transferred back onto ice for another 2 mins. This was followed by addition of 250 µl of pre-warmed S.O.C media (Fisher) to the bacterial cells before being placed in a 37°C shaker for around 1 hour of recovery. LB agar plates either containing ampicillin (Sigma, 50 µg/ml) or kanamycin (Alfa Aesar, 50 µg/ml) depending on which antibiotic resistance the transformed plasmin contained were pre-made up-to a month before. Cells were then spread on these plates and incubated overnight at 37°C incubator.

2.2.2: Maxi-prep plasmid preparation

Bacterial colonies were selected from the plates and were grown in 200-400 ml of Luria Bertani (LB) media overnight at 37°C and shaking at 200 rotations per minute (rpm). Once the bacterial cultures were grown, they were spun down for 10 mins at 4000 rpm to form a bacterial pellet. The supernatant was removed and extracting the DNA was carried out using the PureLink HiPure Plasmid Filter Maxi-prep kit (Fisher) following manufacturer's protocol.

2.2.3: DNA quantification

Following DNA purification, the DNA was quantified using a Labtech Spectrophotometer with ND-1000 software. Blank measurements were taken using 1 µl of TE buffer 1x which the plasmids were re-suspended in. 1 µl of the DNA sample was used to measure the concentration.

2.3: Cell culture methods

2.3.1: Cell culture

MEC1 and C2 suspension cell lines were grown in Roswell Park Memorial institute (RPMI) 1640 medium (Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 1% Penicillin streptomycin (Sigma). Cells were grown in a humidified atmosphere at 37°C containing 5% CO_2. Cells were cultured in T-75 flasks and passaged twice a week to prevent cells growing too confluent. Passaging involved discarding some cells in media from the flask before adding more fresh media to rest of cells left in the flask. Cells were passaged once every three to five days depending on confluency.

Adherent U2OS and human BJ-hTERT (ATCC) cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma) with 10% FBS supplemented with 1% l-glutamine (Sigma) and 1% Penicillin streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Cells were cultured in T-75 flasks and passaged to prevent cells growing too confluent. Passaging involved washing the

cells with 1x PBS before trypsinizing the cells by adding 1x Trypsin before incubating at 37°C until all cells have fully detached. Cells were passaged once every two to three days depending on confluency.

2.3.2: siRNA transfection

Small interfering RNA (SiRNA) targeting BRD4, BRD3, BRD2, HEXIM1, RAD51, SMARCAL1 and ZranB3 and control siRNA was used in this project.

SiRNA	5'-3' sequence or Cat. No.
siControl (Ntsi)	Qiagen Allstars Negative control siRNA SI03650318
BRD2	Dharmacon ON-TARGETplus SMARTpool L-004935-00
BRD3	Dharmacon ON-TARGETplus SMARTpool L-004936-00
BRD4	Dharmacon ON-TARGETplus SMARTpool L-004937-00
Hexim1	Dharmacon ON-TARGETplus SMARTpool L-012225-01
RAD51	GAGCUUGACAAACUACUUC
Smarcal1	Dharmacon ON-TARGETplus SMARTpool L-013058-00
ZranB3	Dharmacon individual siGenome siRNA D-010025-03

Table 2.2: siRNA sequence or catalogue number.

U2OS cells were transfected using Dharmafect 1 reagent (Dharmacon). Cells were plated in a 6 well plate a day before. 6 µl of 20 µM siRNA was added to 282 µl of OptiMEM (Gibco by Life Technologies) and Dharmafect 1 was incubated with OptiMEM for 5 mins at room temperature. 288 µl of the Dharmafect 1 and OptiMEM reagent incubated previously was added to siRNA mixture and mixed well before

being incubated for 20 mins. 480 µl of mixture was added to the 1.5 ml of DMEM media on the cells before being grown for 48 hours. After 24 hours media on cells was disposed and fresh DMEM media was added to the cells. After 48 hours cells were detached by trypsin and either harvested for Western blotting, fibre labelling, immunofluorescence (IF) or EU (Ethynyl Uridine) assay.

2.3.3: Plasmid transfection

U2OS cells were transfected using Transit-2020 transfection reagent (Mirius bio). Cells were plated in a 6 well plate a day beforehand. 250 µl of pre-warmed OptiMEM was added with 2.5 µg of the required plasmid. The OptiMEM and plasmid were mixed thoroughly before 7.5 µl of Transit-2020 was added. The solution was mixed thoroughly again before incubated at room temperature for 20 mins. The solution is then added dropwise around the well of 2.5 ml fresh media before being grown for 24-48 hours.

2.3.4: Simultaneous plasmid and siRNA transfection

U2OS cells were transfected using the TransIT-X2 transfection reagent (Mirius bio). U2OS cells were seeded into a 6 well plate and grown overnight. 250 µl of pre-warmed OptiMEM was added to 2.5 µg of plasmid and mixed thoroughly. This was followed by adding 3.4 µl of 20 µM siRNA stock and mixed thoroughly. 7.5 µl of TransIT-X2 was added to the solution and mixed thoroughly before incubated at room temperature for 20 mins. The solution is then added dropwise around the well of 2.5 ml fresh media before being grown for 24-48 hours.

2.3.5: Plasmids

Recombinant DNA	Source
pcDNA6.2/N-EmGFP-BRD4(long)-DEST	Philpott et al., 2014 Kind gift from Dr Catherine Rogers
pCDNA5-3HA-BRD4(short)-DEST	Kind gift from Prof Panagis Fillipakopoulos
pcDNA3.1/V5/His-TOPO-RAD51	Sorensen et al., 2005
pcDNA3.1(+)	ThermoFisher
pEGFP-C2	Clontech

Table 2.3: Plasmids.

2.4: DNA replication and repair assays

2.4.1: Flow cytometry

Cells were seeded into a 10 cm dish and left to grow overnight. After cells were treated, they were harvested and spun down for 5 mins at 3000 rpm. Cells were then re-suspended in ice cold PBS before being spun down once again. This was repeated once more, and cells were re-suspended in 3 ml of ice-cold PBS. To fix these cells, ice cold 100% ethanol was added slowly to the cells while being vortexed to try and avoid cell clumping. Samples could be stored overnight in the fridge.

Samples were spun at 2000 rpm for 4 mins. The supernatant was removed and 10 ml of PBS was added again to sample and was spun again. This procedure was repeated twice before cells were re-suspended in 500 µl of propidium iodide

solution ((2 µl of 2 mg/ml propidium iodide (Fisher Scientific) in 500 µl of PBS)) and 0.3 µl of RNaseA (Sigma, 50 mg/ml). Cells could be stored in the fridge up to a week before analysing data.

Cell cycle profiles were than gathered from the samples using the BD LSR Fortessa X20 and analysed using the BD FacsDiva software.

2.4.2: Labelling of adherent cells for DNA fibres

Cells are plated into a 6 well plate and grown overnight. The cells were than pulse-labelled with 25 µM thymidine analogue 5-chloro-2'-deoxuridine (CldU) for 20 mins at 37°C. This is followed by pulse-labelling with 250 µM thymidine analogue 5-Iodo-2'-deoxyuridine (IdU) for 20 mins at 37°C. Labelled cells were than harvested in cold PBS and spun down. Cells were re-suspended to a concentration of $2x10^5$ cells/ml. Each sample were used to spread 5 microscope slides. 2 µl of a sample was placed on top of a microscope slide. The drop was dried for up to 5 mins until it was sticky. This was followed by addition of 7 µl of spreading buffer (200 mM Tris pH7.4, 50 mM EDTA, 0.5% SDS) which was gently stirred with cell suspension with a pipette tip. This was to help lyse the cells to ensure spread of DNA. The mixture was incubated for a further 2 mins before the microscope slide was tilted until the drop at the top of the slide has spread down the entire length of the slide leaving spread DNA on the slide. The slides were than air-dried at room temperature before being fixed in 3:1 mixture of methanol and acetic acid for 10 mins. Slides were than dried and stored at 4°C.

2.4.3: DNA fibre labelling in suspension cells

Cells are plated in 6 well plates with 1 ml of cells added to 1 ml of fresh RPMI media and can be left upwards of an hour to settle. As described above the cells are than provided with two pulses of 20 mins of thymidine analogues CldU and IdU. After cells have been labelled the cells are treated with 1 mM thymidine (Fisher Scientific) for 5 mins at 37°C to stop further incorporation of the analogues. Cells were then spun down at 1000 rotations per minute (rpm) for 5 mins. Cells were re-suspended in ice cold PBS 1x and then spun down again. This was repeated once more before cells were re-suspended in 1 ml of ice-cold PBS. Cells were re-suspended to a concentration of $7.5*10^5$ cells/ml. Further fibre spreading using these cells followed the previous protocol described for adherent cells above.

2.4.4: Staining fibres

Slides were washed twice for 5 mins with distilled H_2O. Slides were then rinsed with 2.5 M HCl before being incubated with 2.5 M Hydrochloric acid (HCl) for 75-80 mins. Slides were than washed with PBS twice before being washed twice with blocking solution (PBS, 1% BSA, 0.1% Tween20) for 10 minutes before a longer incubation of 30 mins. Primary antibodies were made up in blocking solution with Rat anti-BrdU (see Table 2.5) diluted at 1:1000 for the CldU and mouse anti-BrdU (see Table 2.5) diluted at 1:500 recognising the IdU. After blocking is finished 115 µl of antibody solution was added to the slide and then covered with a glass coverslip and were incubated for an hour at room temperature. After antibody incubation the

slides were washed three times with PBS and then slides were fixed with 1 ml of 4% paraformaldehyde (PFA). Slides were subsequently washed three times with PBS and then washed with blocking solution three times. After being washed with blocking solution the slides were incubated with secondary antibody. The secondary antibody is diluted in blocking solution with α-mouse Alexa Fluor 488 (see Table 2.6) diluted at 1:500 and α-rat Alexa Flour 555 (see Table 2.6) diluted at 1:500. 115µl of antibody solution was added to the slide and covered with glass coverslip and was incubated for 90 mins at room temperature. Slides were then washed twice with PBS and washed three times with blocking solution. Slides subsequently washed twice more with PBS. Slides were then mounted with a glass coverslip with flouroshield mounting medium (Sigma). Slides were than stored at -20°C.

DNA fibres were visualised on a Nikon E600 microscope using a Nikon Plan Apo x 60 oil lens. Images were taken from two slides per sample and imaged acquired using a Hamamatsu digital camera and Velocity acquisition software. Images were analysed using the Image J software (http://rsbweb.nih.gov/ij/). Each experimented was repeated at least three times with at least 100 DNA fibres measured for replication fork speeds, or when analysing replication structures.

2.4.5: Immunofluorescence

Immunofluorescence was used to identify and observe formation of 53BP1, γH2AX, RPA and RAD51 foci. Cells were grown in 24 well plates with a coverslip. Cells were than treated before being fixed in 4% PFA (Thermo Fisher) for 10 mins at room temperature. Cells were than washed three times with PBS before being

permeabilised with 0.25% Triton X-100 (Thermo Fisher) solution for 5 minutes at room temperature. Cells were washed twice with PBS and were blocked with 4% FBS for one hour. Cells were then treated with a specific primary antibody (see Table 2.5) in 4% FBS and incubated at 4°C overnight. Cells were than rinsed twice with PBS and washed three times with blocking solution for 5 mins. Cells were incubated with specific secondary antibody raised against species of primary antibody (see Table 2.6) in 4% FBS and incubated at room temperature protected from light for 2 hours. Cells were then washed twice with PBS before being washed three times with blocking solution. The coverslips were transferred from the 24 well plate and placed on the microscope slides cells facing downwards into flouroshield mounting media containing DAPI (Sigma). Immunofluorescence microscopy was done using the Nikon E600 microscope. Cells were identified using DAPI and cells with more than a certain pre-determined threshold were deemed positive cells. This was >5 foci for RAD51, >8 for γH2AX and >8 for 53BP1 foci. Images were taken using Velocity software and images were adjusted in ImageJ.

2.4.6: Micronuclei

Cells were seeded into a 24 well plate with a coverslip and grown overnight. Cells were treated with 2 µg/ml of cytochalasin-B (Sigma) and incubated for 24 hours at 37°C with or without JQ1 treatment. After 24 hours cells were treated with ice cold methanol for fixation for 20 mins before being permeabilised with 0.5% Triton X-100 for 10 mins. Cells were blocked for 20 mins. The coverslips were transferred from the 24 well plate and placed on microscope slides facing downwards into

flouroshield mounting media containing DAPI. Microscopy was done using the Nikon E600 microscope. Images were taken using Velocity software and images were adjusted in ImageJ.

2.5: Protein methods

2.5.1: Buffers

Urea Tris Buffer (UTB):

50mM Tris pH 7.5

150mM β-mercaptoethanol

8M urea

Running buffer 10x:

30.3g Tris Base

187.7g Glycine

10g Sodium Dodecyl Sulphate (SDS)

1l of H_2O

Transfer buffer 1x:

10% Methanol

10% Towbin 10x

80% H_2O

Loading buffer 5x:

5ml 1M Tris pH 6.7

2g SDS

100mg Bromophenol Blue

1.544g DTT

5ml H_2O

10ml glycerol

TBS 10X:

12.1g Tris Base

81.8g NaCl

pH to 7.9 with HCl

In 1l of H_2O

TOWBIN 10x:

24g Tris Base

112.6g Glycine

In 1 L of H_2O

TBST (TBS-Tween):

10% TBS 10x

0.05% Tween 20

1.5 M Tris pH 8.8:

18.15g Tris Base

80ml H_2O

Adjust pH to 8.8 with either concentrated NaOH or HCl – top up to 100ml once at desired pH

1M Tris pH 6.7:

6.06g Tris Base

80ml H_2O

Adjust pH to 6.7 with either concentrated NaOH or HCl - – top up to 100ml once at desired pH

2.5.2: Western blotting

Cells were grown and treated in 6 cm dishes before harvesting. Cells were typsinised and re suspended in 1 ml of cold PBS. Cells were counted using a haemocytometer and cells were spun down at 7500 rpm for 2 mins. PBS was removed, and pellets were re-suspended in UTB buffer to a concentration of $2x10^6$ cells/ml.

Samples were then put on ice and sonicated for 10 seconds before being cooled for 20 seconds before being sonicated again at between 4-5 input powers. Once sonicated samples were put on ice and spun down at full speed at 4 for 10 mins. Supernatant was transferred to new tubes and 5x loading buffer was added at a ratio 1:4. Samples were mixed by pipetting before being spun and boiled at 80°C for five mins. Samples were either run afterwards or stored at -20°C. SDS PAGE was carried out using a Bio-Rad Mini-PROTEAN 3 cell apparatus. Gel were cast at different percentages (see Table 2.4).

	Resolving (ml)			Stacking(ml)
	12%	9%	6%	5%
H_20	6.6	8.8	10.3	6
30% acrylamide	8	6	4	1.34
1.5 M Tris pH8.8	5	5	5	-
1 M Tris pH 6.7	-	-	-	2.5
10% SDS	0.2	0.2	0.16	0.1
10% APS	0.2	0.2	0.16	0.1
TEMED	0.018	0.018	0.016	0.01

Table 2.4: SDS polyacrylamide gel solutions.

The resolving gel was coated with isopropanol to remove bubbles. Upon polymerisation, the isopropanol was washed off and the stacking gel was poured on top with a comb. Once set the comb was removed. Samples were run next to a protein ladder (Himark, Thermo Fisher, or Blue Prestained Protein Standard, Broad Range, New England Biolabs). 20 µl of protein sample was loaded onto the gel and run in running buffer 1x diluted from 10x stock running buffer. After separation, the gel was transferred onto a nitrocellulose membrane (Fisher Scientific). Transfer sponges, blotting paper and membrane was pre-soaked in transfer buffer beforehand. The gel was placed in cassette in the following order: Clear side, sponge, blotting paper, membrane, gel, blotting paper, sponge, black side. Place

the cassette in a cassette holder and top tank with transfer buffer with an ice block. Transfer at 100 V for 100 mins. The membrane was placed in Ponceau S solution (Sigma) for 1 min to stain proteins and subsequently washed. If needed the membrane was cut into strips with relevant size bands. The membrane was placed in 5% milk and blocked on the rocker for 1 hour. The membranes were then incubated in primary antibodies (see Table 2.5) in 5% milk overnight at 4°C on a rotator.

After primary antibody incubation the membranes were washed three times with TBST on the rocker. The membrane was then incubated with secondary antibodies (see Table 2.6) in 5% milk at room temperature on a rotator for 90 mins. The membranes were then washed another three times with TBST on the rocker. Excess TBST was washed off the membrane with distilled H_20 before addition of Pierce ECL Plus Western blotting substrate (Fisher) made with equal parts of Substrate A and B. ECL was incubated for 1 min before being placed in film cassette and developed with X-ray film (SLS) in a dark room. Membranes were exposed for different exposures depending on signal strength.

2.5.3: Quantification of Western blot

Western blots were scanned and opened with ImageJ. The file is converted into 8-bit. A rectangle is drawn around the first lane of interest. After the rectangle is drawn, the analyse tab is selected on ImageJ, followed by gels and select first lane. The 2nd lane is selected by copying the first rectangle and dragging it over the 2nd lane. Again, the analyse tab is selected, followed by gels and select next lane.

Repeat this until all the lanes have been selected. Once all the lanes are selected, plot lanes are selected in the gels tab. Once the peak for the lane appears, a line can be drawn to close off the peak for accurate measurements and to get rid of any background. The area for this peak is calculated by clicking the wand tool on the peak. To accurately compare the quantified area against other lanes, the loading control must be analysed as well. To quantify the relative density of each lane the area of the band of interest must be divided by the area of the loading control.

2.5.4: Primary antibodies

Antigen	Dilution	Supplier	Species	Method
RAD51	1:1000	Abcam	Rabbit	WB/IF
BRD4	1:1000	Abcam	Rabbit	WB
BRD2	1:1000	Abcam	Rabbit	WB
BRD3	1:500	Abcam	Rabbit	WB
Hexim1	1:50000	Abcam	Rabbit	WB
ZranB3	1:500	Proteintech	Rabbit	WB
Smarcal1	1:150000	Santa Cruz	Mouse	WB
BrdU	1:1000	Abcam	Rat	Fibres
BrdU	1:500	Becton Dickinson	Mouse	Fibres
Actin	1:5000	Cell Signalling	Rabbit	WB
Tubulin	1:10000	Sigma	Mouse	WB
p-Chk1(S317)	1:1000	Cell Signalling	Rabbit	WB
p-RPA(S4/8)	1:1000	Bethyl	Rabbit	WB
RNAPOL2S2	1:500	Abcam	Rabbit	WB/IF
RNAPOL2S5	1:5000		Mouse	WB
YH2AX	1:1000	Millipore	Mouse	IF
53BP1	1:300	Bethyl	Rabbit	IF
RPA	1:500	Merck	Mouse	IF

Table 2.5: Antibodies including species raised in, dilution, conditions and protocols.

2.5.5: Secondary antibodies

Antigen	Dilution	Supplier	Species	Method
Goat anti-mouse HRP	1:500	Dako	Mouse	WB
Goat anti-rabbit HRP	1:500	Dako	Rabbit	WB
Anti-rat AlexaFluor 555	1:500	Rat	Thermo Fisher	Fibres/IF
Anti-rabbit AlexaFluor 555	1:500	Rabbit	Thermo Fisher	IF
Anti-mouse AlexaFluor 488	1:500	Mouse	Thermo Fisher	Fibres /IF

Table 2.6: Antibodies including species raised in, dilution, conditions and protocols.

2.6: RNA methods

2.6.1: EU assay

EU incorporation was performed using the Click-It RNA Alexa Flour 594 Imaging kit (Thermo Scientific). Cells were seeded in a 24 well plate with a coverslip inside. Cells were treated before being incubated with 1 mM EU for 1 hour. Cells were fixed at room temperature using 4% PFA for 10 mins at room temperature. Cells were washed three times with PBS before being permeabilised for 15 mins with 0.5% Triton X-100. Cells are then treated with 100 µl of Click-It reaction cocktail (see Table 2.7) protected from light at room temperature for 30 mins. Before the cocktail is made, the reaction buffer additive is diluted ten times. After incubation the reaction cocktail is removed, and cells are washed with Click-IT reaction wash buffer for 5 mins before being washed with PBS. The coverslips are removed from

the 24 well plate and placed on the microscope slides cells facing downwards into Flouroshield mounting media containing DAPI. The slides are then stored at 4°C and can be analysed within 24 hours. EU microscopy was performed on the Nikon e600 microscope using Velocity software. Images of DAPI staining for nuclei and Alexa Fluor 594 intensity were taken. Images were analysed on Image J where DAPI was used to generate a nuclear mask and mean Alexa Fluor 594 intensity per pixel was quantified for every nucleus.

2.6.2: EU assay in suspension cells

Cells were seeded in a 24 well plate with 1 ml of cells and 1 ml of fresh RPMI media with a circular poly-l-lysine coated glass 12 mm coverslips (Becton-Dickinson Biosciences) for 1 hour to allow cells to attach properly. The rest of the EU assay using these cells is the same as the previous protocol described for adherent cells above.

Reaction components:	1 coverslip(100 ml)	5 coverslips(500 ml)
Click-iT RNA Reaction buffer	85.6 µl	428 µl
$CuSO_4$	4 µl	20 µl
Alexa-Fluor 594 azide	0.36 µl	1.8 µl
Click-iT reaction buffer additive 1x	10 µl	50 µl

Table 2.7: Components and amounts to make reaction cocktail.

2.6.3: RNA purification

Cells were seeded into a T75 flask and grown overnight. Cells were washed twice with PBS before being typsinised. Cells were re-suspended in 10 ml of media. 10 µl of cells was mixed with trypan blue stain before being counted using a Countess II cell counter (Thermo Fisher). Cells were then spun down and re-suspended to achieve a concentration of $3x10^6$ cells/ml. Cells were than pelleted and the supernatant was removed. The rest of the RNA purification was carried out following the manufacturer's instructions from the Qiagen RNeasy Mini Kit (cat no: 74104).

2.6.4: RNA quantification

Following RNA purification, the RNA was quantified using a Labtech Spectrophotometer using ND-1000 software. Blank measurements were taken using 1 µl of RNase free water which the purified RNA was eluted in 1 µl of the RNA sample was used to measure the concentration of RNA in the cells.

3. BET inhibition induces replication-transcription conflicts

3.1: Introduction

BET proteins have been at the cutting edge of new targets for cancer treatment, with multiple BET inhibitors now undergoing clinical trials. However, a more detailed understanding of BET proteins and how they function is necessary for development of possible future therapeutic applications.

Previously the lab showed that BET proteins may play a role in maintenance of DNA replication, using BET protein inhibitor JQ1 (Da Costa, Agathanggelou et al. 2013). These data showed that JQ1 treatment can cause replication fork slowing in NALM-6 leukaemia cells. As previously detailed, replication stress can have severe effects on the cells, leading to promotion of DNA damage such as DSBs. Many cancer therapies use the induction of replication stress to lead to the formation DSBs (Kotsantis, Jones et al. 2015). These results may indicate a new role of BET proteins in the maintenance of DNA replication. To understand how BET inhibition leads to replication stress is therefore crucial for potential basis of future clinical applications.

As mentioned previously, there are numerous sources which can cause replication stress in cells (Zeman and Cimprich 2014). The main aim in this chapter is to investigate the underlying mechanism of BET inhibitor-induced replication stress.

As cells normally undergo replication stress response which can either lead to cell survival, cell death or increased genomic instability, another major aim is to test the effect BET inhibitors have on the cell, downstream of fork slowing.

Hence by understanding this, we will have a much better understanding of BET proteins and their role in replication, which will be important for any new potential therapies using BET inhibitors down the line.

3.2: BET inhibitors induce replication stress

As mentioned earlier, previous data showed JQ1 leads to the onset of replication stress in NALM-6 cells. Replication stress is often characterised by replication fork slowing (Ciccia and Elledge 2010). We wanted to confirm the effects JQ1 has on replication fork progression in U2OS cells. U2OS cells were used as the effects of replication stress and DNA damage have been well studied and documented in this cell line (Bryant and Helleday 2006, Oplustilova, Lukas et al. 2009, Giunta, Belotserkovskaya et al. 2010, Jones, Mortusewicz et al. 2013). To analyse the progression of fork speeds we used the DNA fibre technique. This produces long unbroken fibres of labelled DNA, which allows us to measure fork speeds or origin firing. 1 µM JQ1 was added for 1 hour, and then cells were labelled for 20 mins each with thymidine analogues CldU and then IdU, before fibres are spread and stained (Figure 3.1 A-B). We decieded to treat cells using a JQ1 concentration of 1 µM, as previous preliminary data had shown that 1 µM JQ1 induces replication fork slowing in Nalm 6 cells (Da Costa, Agathanggelou et al. 2013). In accordance

to what had been shown earlier we saw that 1 µM JQ1 led to a significant decrease in fork progression speeds (Figure 3.1 D-E). To compare replication fork speeds between the treatments we calculated the average median fork speeds. In each repeat the median of the distribution of every fibre analysed was calculated and averaged over the number of repeats carried out. Control treated cells had an average median fork speed of 1 kilobase (kb)/min, while JQ1 treated cells showed an average median fork speed of 0.6 kb/min (Figure 3.1 D). These results indicate that JQ1 induced replication stress in U2OS cell lines after only 1 hour of treatment.

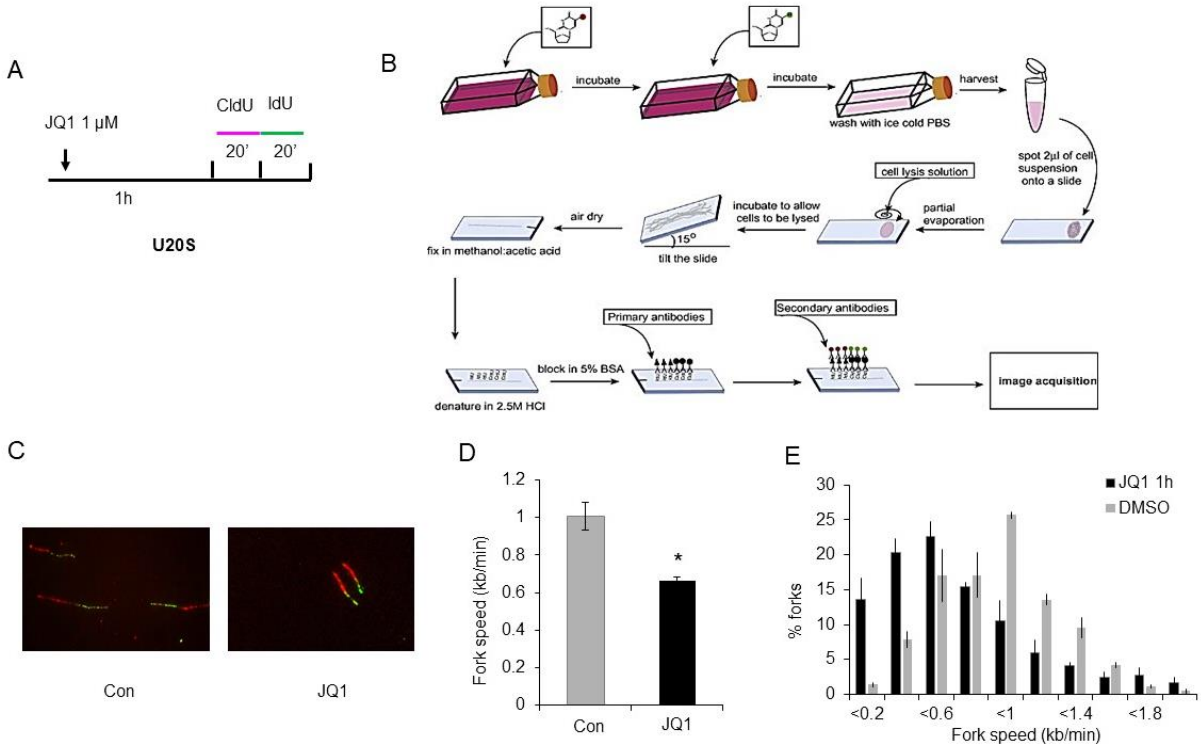


Figure 3.1: JQ1 leads to increased replication stress.

A) DNA fibre labelling performed in U2OS cells after 1 µM JQ1 treatment for 1 hour. B) Diagram illustrating the DNA fibre protocol from labelling the DNA to visualising the DNA under the microscope. C) Representative images of DNA fibres after 1 µM JQ1 addition for 1 hour. D) The average median speed was calculated from all ongoing replication forks measured after 1 µM JQ1 treatment for 1 hour. E) Distribution of replication fork speeds in cells treated with 1 µM JQ1 for 1 hour or control treated cells. (N=3, error bars: Standard error of the mean (S.E.M), Statistical analysis: Student's t-test. * P value <0.05)

The previous figure showed that JQ1 induced replication fork slowing after JQ1 treatment. The concentration used for this was 1 µM. A variety of previous studies and research using JQ1 as a cancer therapy drug had used lower concentrations of JQ1 to look into the effects it has on cells (Herrmann, Blatt et al. 2012, Kumar, Raza et al. 2015). To ensure that the results we had seen in the previous experiment

were a result of JQ1 effects on replication and not due to an artefact of using high concentrations of JQ1 we decided to repeat the experiments using a range of lower concentrations of JQ1. Previous research had shown JQ1 to be effective in inhibiting growth of cancer cells from concetrations of 250 nM, so I decided to test concentrations of 250 nM upwards to 750 nM of JQ1 (Filippakopoulos, Qi et al. 2010, Herrmann, Blatt et al. 2012, Kumar, Raza et al. 2015). I treated cells with the desired JQ1 concentrations before using the same protocol for DNA fibres as before (Figure 3.2 A). Cells treated with lower concentrations of JQ1 showed the same effects as 1 µM treatment, with all showing significantly reduced average median fork speeds compared to control treated samples (Figure 3.2 B-C). Even the lowest concentration of 250 nM JQ1 showed a significant drop in average median replication fork speed from 1 kb/min to around 0.77 kb/min (Figure 3.2 B). The data show that replication fork speeds are reduced over a range of JQ1 concentrations and the most prenounced fork slowing of the concentrations analysed was seen with 1 µM JQ1. As 1 µM JQ1 seemed to have the most pronounced effect on replication speeds (Figure 3.2 C), we decided to carry on using this concentration of JQ1 for all further experiments carried out in this thesis.

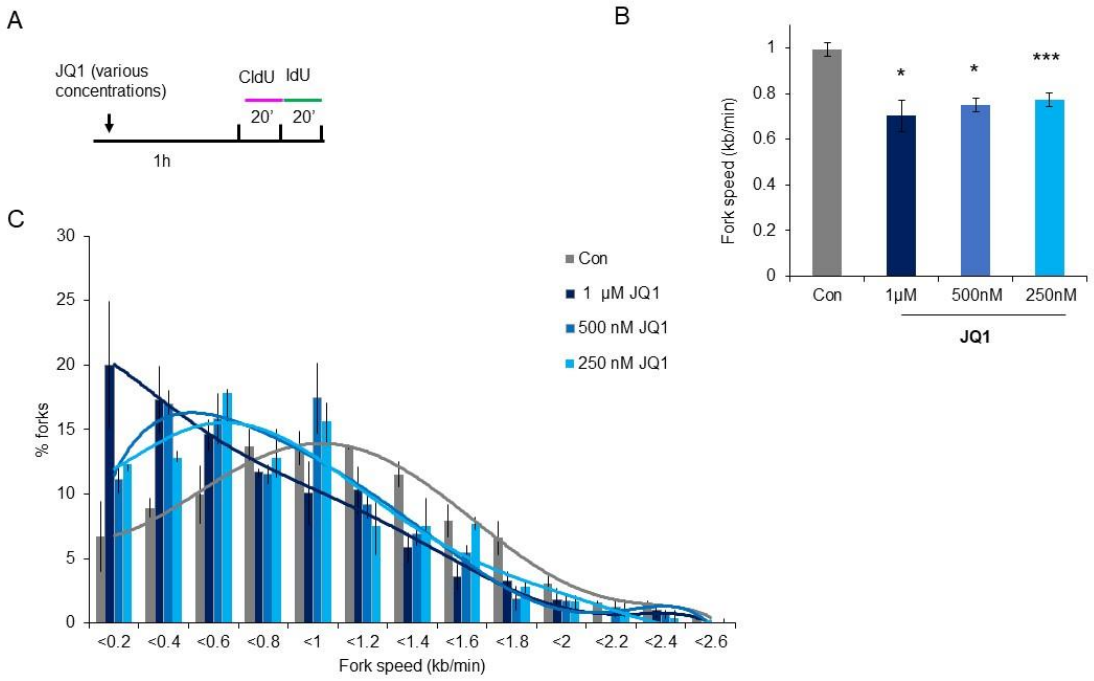


Figure 3.2: **A titration of lower JQ1 concentrations also show induced replication stress.**

A) DNA fibre labelling performed in U2OS cells with either 1 µM, 500 nM or 250 nM JQ1 treatment for 1 hour. B) Average median replication fork speeds for cells treated with 1 µM or 500 nM or 250 nM JQ1 for 1 hour. C) Distribution of replication fork speeds in cells treated 1 µM or 500 nM or 250 nM JQ1 for 1 hour. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01, NS=Not significant.)

The previous results showed the impact of JQ1 on replication fork speeds after 1 hour of treatment. To understand the long-term effects of JQ1 on replication fork speeds, we looked at a range of different time points after JQ1 addition. To test this we used the DNA fibre technique as stated previously, with labelling after the stated 1 μM JQ1 treatment time (Figure 3.3 A).

Fork speeds between 1 and 8 hours after continuous JQ1 addition were significantly slowed down, compared to control treated cells. The average median fork speeds for these times were all around 0.6 kb/min compared to 0.9 kb/min in control treated samples. After 24 hours, fork speeds were rescued to control treated levels (Figure 3.3 B). To test if this was due to JQ1 being degraded and loss of JQ1 activity, we added fresh JQ1 to cells after 23 hour treatment for further 1 hour before fibre labelling. Fork speeds were unaffected by the addidtion of fresh JQ1 after 23 hours, indicating JQ1 activity was not lost (Figure 3.3 B). Anlaysis of fork progression was also conducted for further timepoints after 48 and 72 hours of JQ1. Fork speeds remained unperturbed for these timepoints, with fork speeds mirroring those seen in control-treated samples (Figure 3.3 C). These results indicate that JQ1 leads to replication fork slowing after 1-8 hours but the replication forks are able to overcome this and return to normal speeds after 24 hours of treatment.

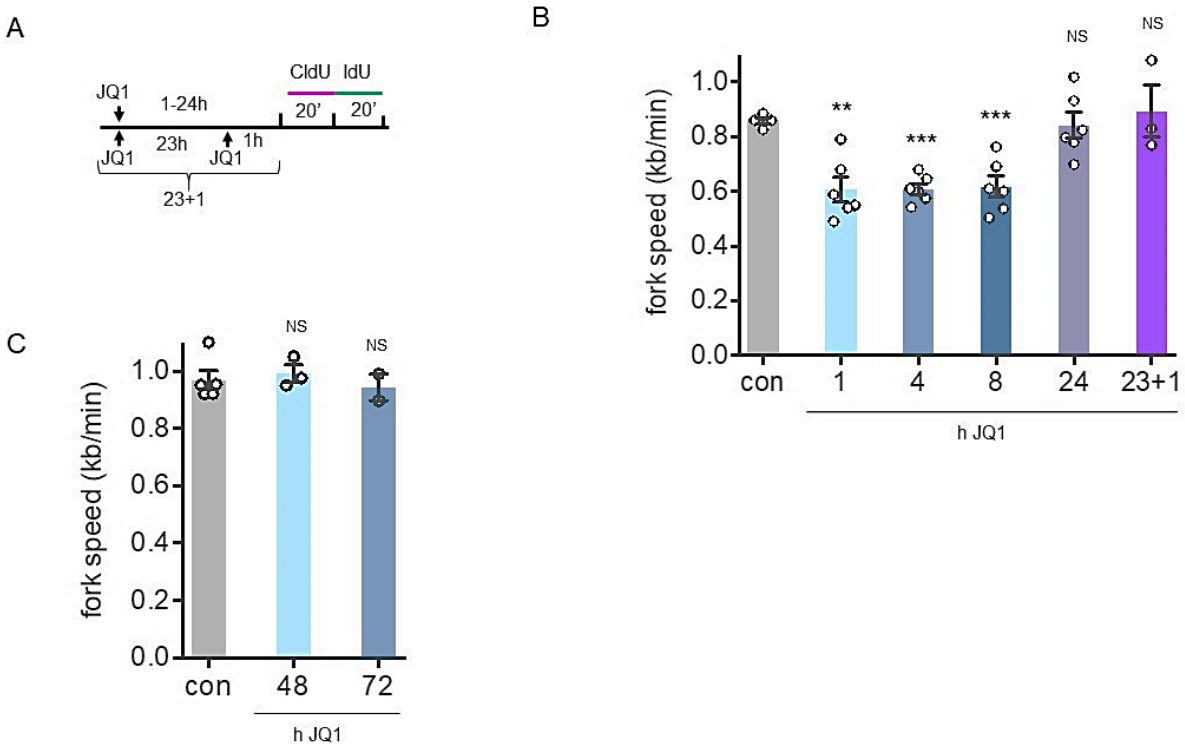


Figure 3.3: Time course of fork speeds after JQ1 treatment.

A) DNA fibre labelling performed in U2OS cells for 24, 8, 4, 1 and 23+1 hour 1 μM JQ1 treatments. B) Average median replication fork speeds in cells treated for 24, 8, 4, 1 and 23+1 hour 1 μM JQ1 treatments. C) Average median replication fork speeds in cells treated for 48 and 72 hour 1 μM JQ1 treatments. (N=5, error bars: S.E.M, Statistical analysis: Student's t-test. ** P value <0.01, *** P value < 0.001, NS=Not significant.)

Proliferating cells undergo cell cycle which has four main parts, G1, S, M, G2. Each phase is controlled by checkpoints. Replication occurs during S phase. During replication stress an S phase checkpoint is activated to stop replication progression until the blockage causing replication stalling or slowing is removed (Heffernan, Unsal-Kacmaz et al. 2007). Cell cycle effects leading to pertubed S phase or dergulate S phase entry, can lead to replication problems such as replication stress (Petermann and Caldecott 2006, Petermann, Maya-Mendoza et al. 2006). To test this was not causing BET inhibitor induced fork slowing we checked cell cycle profiles of JQ1 treated cells.

To analyse the cell cycle profiles of cells treated with JQ1 between 1 and 24 hours we used flow cytometry. After treatment of the cells they were fixed and stained with PI before being analysed to measure the percentage of cells in each stage of the cell cycle (Figure 3.4 A). JQ1 treatment between 1 and 8 hours showed no changes in cell cycle profile compared to control cells, with S phase percentages being almost identical (Figure 3.4 B). Only after 24 hours, JQ1 lead to accumulation of cells in G1 with the percentage going up form 54% to 78%, indicating a G1 arrest (Figure 3.4 B). This data suggests that fork stalling is due to a direct effect of JQ1 on replication forks and not due to JQ1 causing changes to the cell cycle.

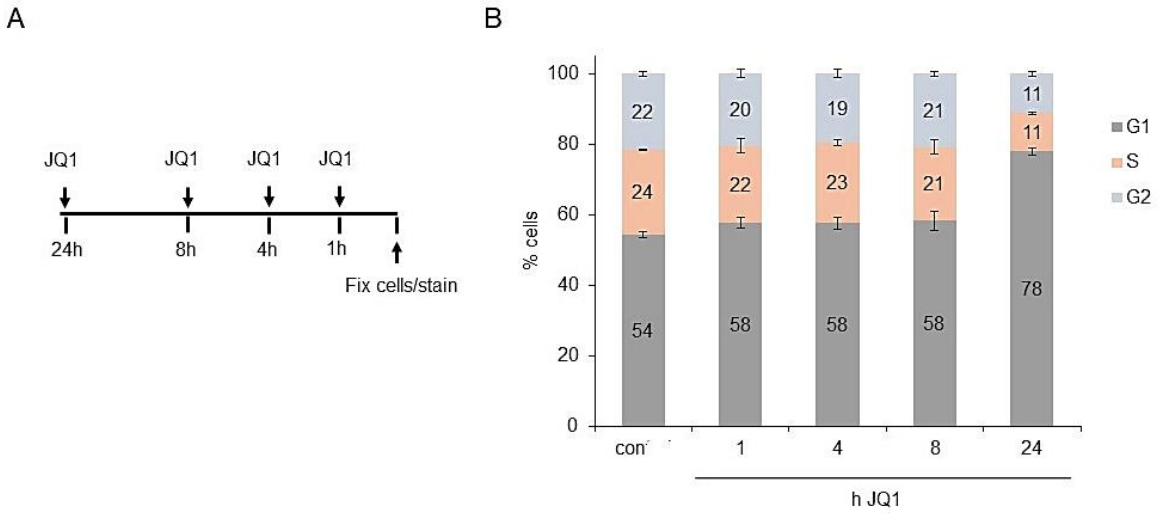


Figure 3.4: JQ1 treatment shows no change in cell cycle profile up to 8 hours before cells undergo G1 arrest.

A) Cells were harvested, fixed and stained for cell cycle analysis after 1 μM JQ1 treatment for 24, 8, 4 and 1 hour. B) Cell cycle distribution measured by flow cytometry of U2OS cells treated with 1 μM JQ1 for 24, 8, 4- and 1-hour time points. (N=3, error bars: S.E.M.)

3.3: BET inhibitors induce increase in RNA synthesis

As previously stated, there are a myriad of different obstacles for replication that can result in the induction of replication stress. Transcription has been shown recently to be a problem for replication and interference between the two machineries can lead to replication stress (French 1992, Poveda, Le Clech et al. 2010, García-Muse and Aguilera 2016). The lab had previously reported transcription as a mechanism of replication stress in both Cyclin E and H-RAS^{V12} overexpression in cells (Jones, Mortusewicz et al. 2013, Kotsantis, Silva et al.

2016). We decided to look into BET inhibitors effect on RNA synthesis. To analyse nascent RNA synthesis we used an assay that measures incorporation of the RNA-specific modified nucleoside 5-ethynyluridine (EU). 1 μM JQ1 was added to U2OS cells for 1, 4, 8 and 24 hours, matching the time points we used in the DNA fibre assay. EU was added for the last hour before cells were fixed, stained and quantified (Figure 3.5 A). As shown by Figure 3.5 B, staining cells for EU gives a red signal under the microscope, the brightness of which can be quantified to give a read-out of total nascent RNA synthesis in the cells. EU incorperation after JQ1 went up around 35% after just 1 hour after addition (Figure 3.5 C). This level of EU incorperation remained upwards after 24 hours with an increase of around 25% (Figure 3.5 C). To look at long term effects of JQ1 and transcription, we looked at longer time points of 48 and 72 hours. EU incorporation remained steadily increased for these timepoints, with a 50% increase after 48 hours and 60% after 72 hours (Figure 3.5 D). To confirm these results an alternative method was used, isolating total RNA from cells followed by RNA quantification and normalising RNA yields to cell numbers to accuratley compare (Figure 3.5 E) (Lin, Loven et al. 2012, Nie, Hu et al. 2012). After 8 hours of JQ1 treatment there was an increase of total RNA, almost double the yield compared to the control treated cells (Figure 3.5 F).

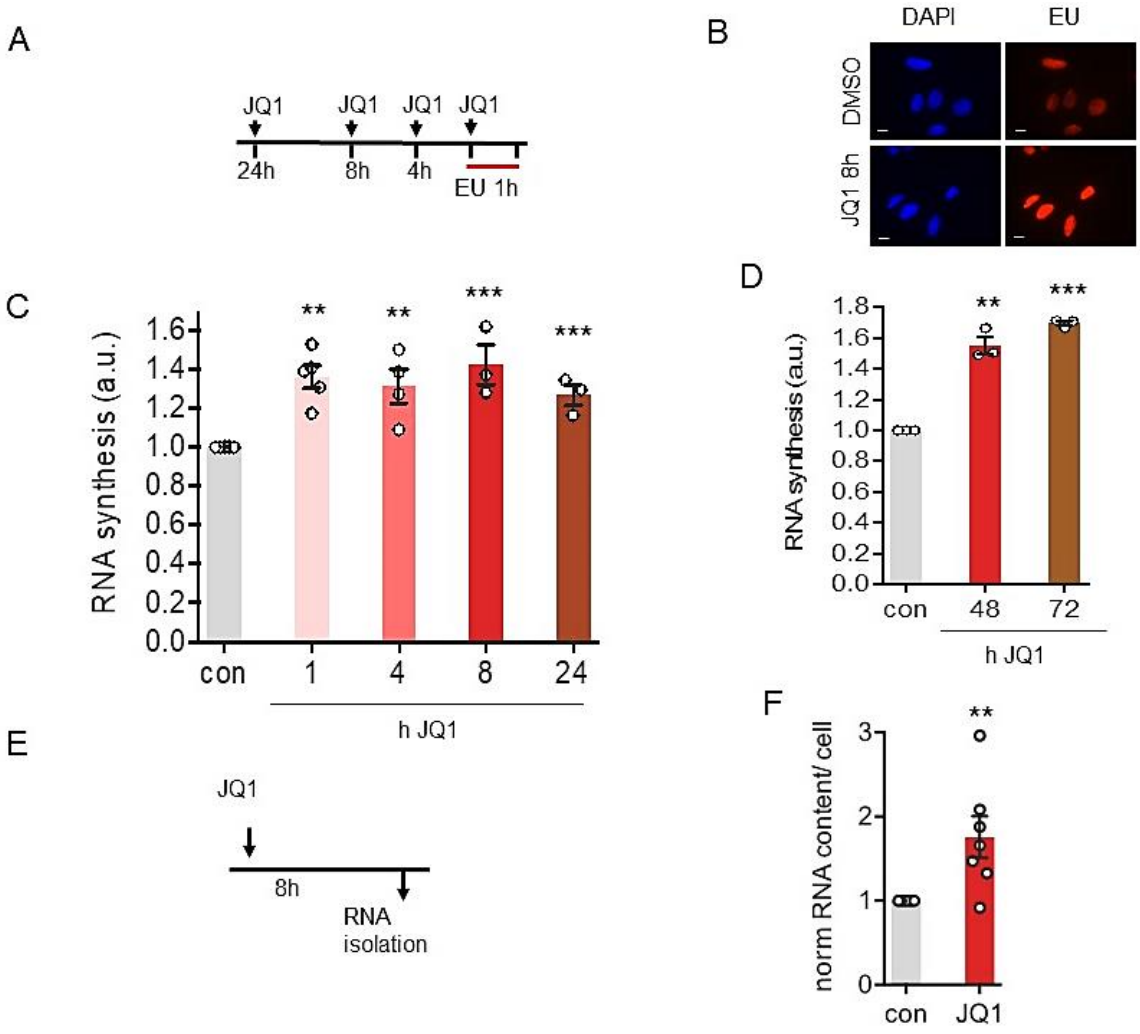


Figure 3.5: JQ1 treatment leads to prolonged increase in total RNA synthesis.

A) U2OS cells were treated with JQ1 for 1, 4, 8 and 24 hours and nascent RNA synthesis was quantified by incorporation of a nucleoside analogue of uridine, 5-Ethynyl Uridine (EU) into the cells. B) Representative images of click-stained with Alexa-Flour 594 EU cells and DAPI +/- 8 hours 1 µM JQ1 treatment. Scale bar 10 µm. C) Nuclear EU intensities after 24, 8, 4 and 1 hour 1 µM JQ1 treatment. (n=4). D) Nuclear EU intensities after 72 and 48 hour 1 µM JQ1 treatment. (n=3). E) Total RNA was extracted after 8 hours 1 µM JQ1 treatment. F) Fold change in total RNA expression between DMSO and 1 µM JQ1 after treatment for 8 hours. (n=6). (Error bars: S.E.M, Statistical analysis: Student's t-test. ** P value <0.01, *** P value < 0.001.)

In further experiments carried out by Dr. Marco Saparano analysing a published dataset using BRD4 degradation (Muhar, Ebert et al. 2018), we showed that 1 hour after BRD4 degradation, genome-wide net occupancy of RNA Pol II increased by 53.8% (Bowry, Piberger et al. 2018) (Figure 8.1/Appendix A2). This increase was shown to be particularly high for transcribed genes that produce non-polyadenylated RNAs such as histone and non-coding RNA genes. qRT-PCR was undertacken to see whether this increased RNA Pol II occupancy was also increasing gene expression. Expression of candidate genes we selected were also up-regulated by JQ1 in U2OS cells after 1 and 8 hour treatments (Bowry, Piberger et al. 2018) (Figure 8.2/Appendix A2). Taken together this data shows that JQ1 treatment leads to an observed increase in total RNA synthesis as short as 1 hour after treatment.

3.4: JQ1 phenotypes are mirrored in different cell lines, and by different BET inhibitors

The previous experiments were all undertaken in U2OS cells. We wanted to test whether the same phenotypes could be shown in other cell lines. Previously, fork slowing had been shown in (ALL) cell line Nalm6 (Da Costa, Agathanggelou et al. 2013). We then tested three other cell lines: fibroblasts BJ-hTert, Chronic lymphocytic leukemia (CLL) cell lines Mec1 and C2. Firstly, JQ1 was added for 1 hour before fibre labelling and staining in these cells (Figure 3.6 A). JQ1 had no effect on fork speed in C2 cells (Figure 3.6 B). However, JQ1 induced significant

fork slowing in MEC1 (1.2 kb/min in control to 0.65 kb/min) and BJ-hTert (1.03 kb/min to 0.73 kb/min) cell lines (Figure 3.6C-D).

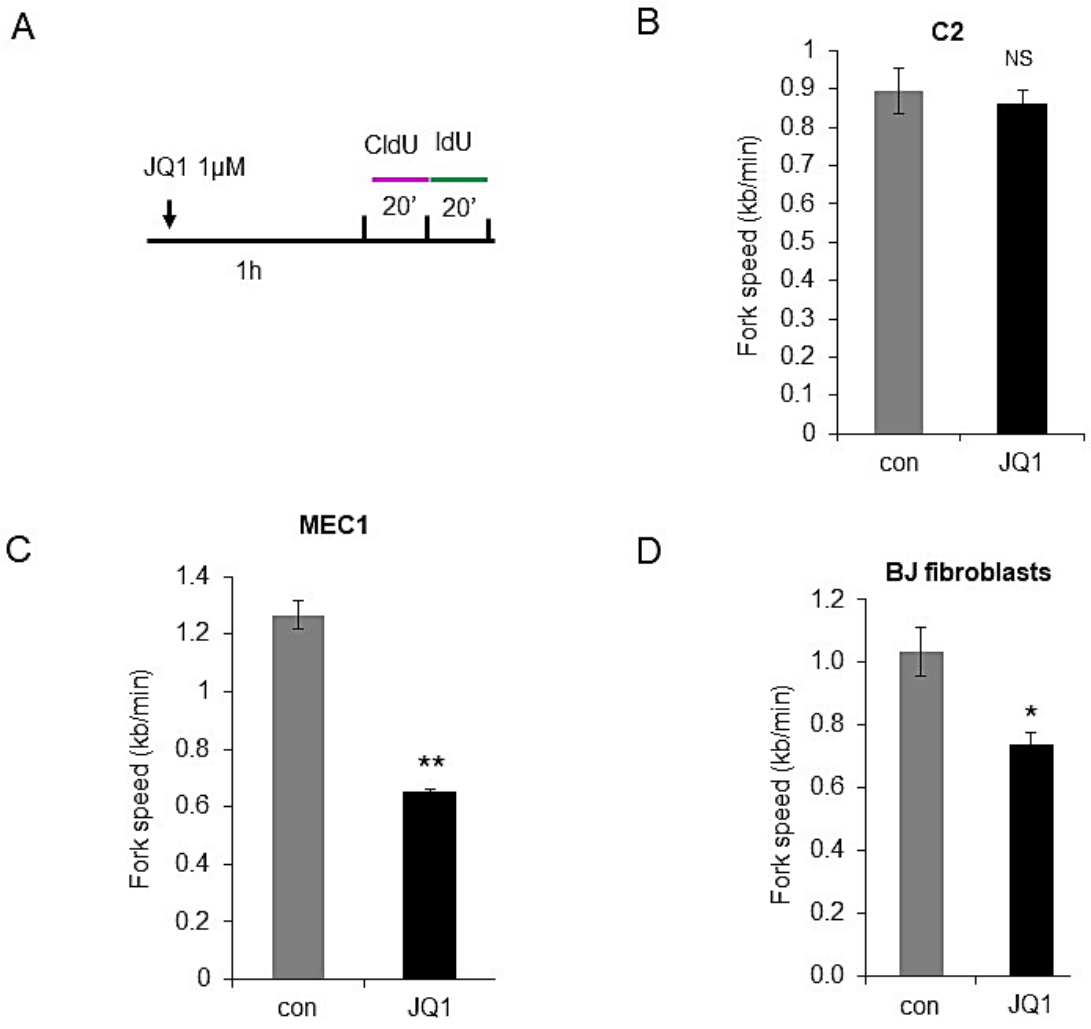


Figure 3.6: BJ-h-Tert, and MEC1 cells show JQ1 induced replication stress, C2 cells show no visible change in fork speeds.

A) DNA fibre labelling performed in C2, MEC1 and BJ-hTert cells with 1 μm JQ1 treatment for 1 hour. B) Average median replication fork speeds in C2 cells after 1 hour 1 μM JQ1 treatment. C) Average median replication fork speeds in MEC1 cells after 1 hour 1 μM JQ1 treatment. D) Average median replication fork speeds in BJ-hTert cell lines after 1 hour 1 μM JQ1 treatment. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01, NS=Not significant.)

The EU assay was performed the same way as stated previously in these cells to see the effect on total RNA synthesis (Figure 3.7 A). Preliminary data done previously by the lab showed JQ1 treatment after 1 hour in Nalm6 cells lead to increased EU incorperation. JQ1 had no effect on EU incorperation in C2 cells (Figure 3.7 B). However, JQ1 induced significant increases in EU incorperation in MEC1 (18%) and BJ-hTert (21%) cell lines (Figure 3.7C-D). These results show that three cell lines tested, Nalm6, BJ-hTert, MEC1, all showed the exact same phenotype as observed in U2OS cell lines, however one cell line C2 had no phenotypic response to JQ1 treatment.

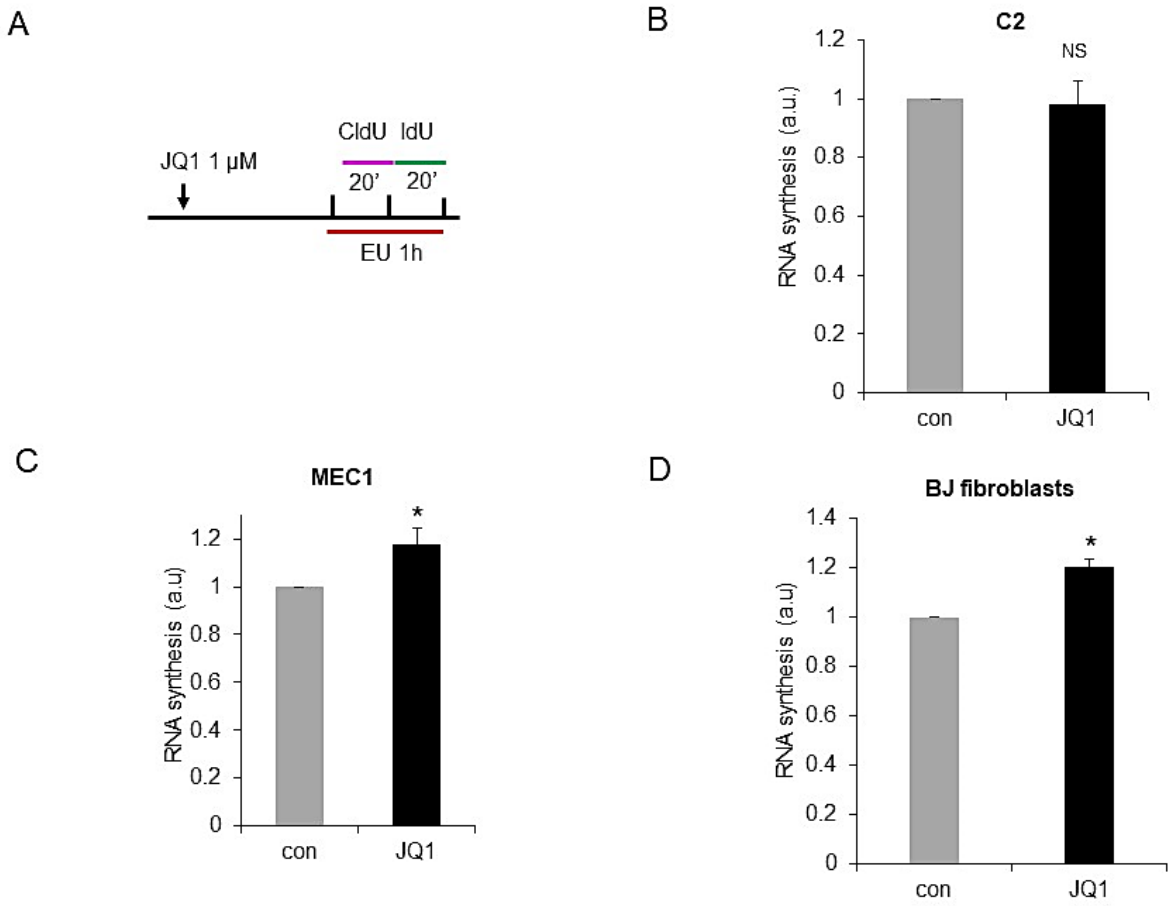


Figure 3.7: BJ-h-Tert, and MEC1 cells show JQ1 induced increase in total RNA synthesis, C2 cells show no visible change in total RNA synthesis.

A) MEC1, C2 and BJ-hTert cells were treated with JQ1 for 1, 4, 8 and 24 hours and transcription elongation were quantified by incorporation of EU into the cells. B) Nuclear EU intensities after 1-hour JQ1 treatment in C2 cells. C) Nuclear EU intensities after 1-hour JQ1 treatment in MEC1 cells. D) Nuclear EU intensities after 1-hour JQ1 treatment in BJ-h-Tert cells. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, NS=Not significant)

The experiments so far involve the use of one BET inhibitor JQ1. To confirm that the previous shown effects caused by JQ1 treatment were due to BET inhibition in general and not due to some specific effect of JQ1, we tested another BET protein inhibitor, I-BET151. I-BET151 works similarly to JQ1 by preventing binding of acetylated histones by BET proteins (Dawson, Prinjha et al. 2011, Dawson, Kouzarides et al. 2012, Hewings, Fedorov et al. 2013). I-BET151 was added for 1 hour before cells were labelled and stained (Figure 3.8 A). A range of concentrations of 500 nM, 750 nM and 1 µM were used. I-BET151 led to significantly decreased fork speeds even at the lowest concentration of 500 nM (Figure 3.8 B-C). An EU assay was then performed using 1 µM I-BET151. I-BET151 caused an increase in EU incorporation (Figure 3.8 D). These results mirror what was previously seen with JQ1. This suggests that the effects seen in replication speeds and transcription levels are due to BET inhibtion rather than JQ1 specifically.

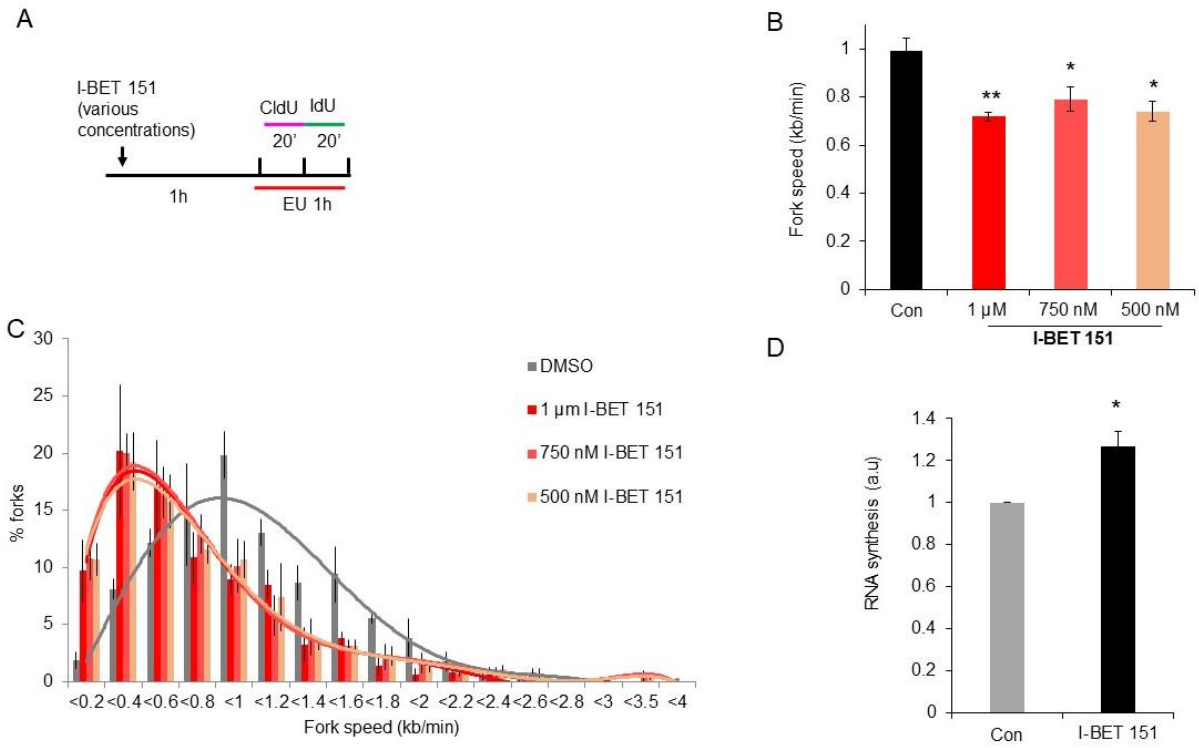


Figure 3.8: I-BET151 mirrors the phenotypes in fork slowing and RNA synthesis change as observed with JQ1 treatment.

A) DNA fibre labelling and quantification of incorporation of EU were performed in U2OS cells after 1-hour treatment with 1 µM I-BET151. B) Average median replication fork speeds treated with 1 µM, 750 nM, and 500 nM I-BET151 for 1 hour. C) Distribution of replication fork speeds in cells treated, 1 µM, 750 nM, and 50 0nM I-BET151 for 1 hour. D) Nuclear EU intensities after 1 hour I-BET151 treatment in U2OS cells. (N=4, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01, NS=Not significant.)

3.5: BET inhibitor-induced replication stress depends on transcription-replication conflicts

Replication-transcription conflicts as mentioned earlier are a major cause of replication fork slowing in cells (French 1992, Poveda, Le Clech et al. 2010, García-Muse and Aguilera 2016). JQ1 had shown increases in RNA synthesis as well as causing forks to be slowed down, therefore transcription-replication conflicts seemed to be a likely candidate for causing JQ1-induced fork slowing. To test this hypothesis we used three different transcription inhibitors, DRB, α-amanitin and triptolide to inhibit transcription and see if this rescued the replication fork slowing induced by JQ1 treatment. DRB is known to prevent activating phosphorylation of the RNA Pol II C-terminal domain, α-amanitin which is a potent inhibitor of RNA Pol II, and triptolide which inhibits transcription initiation by inhibiting XPB/TFIIH (Bensaude 2011, Wang, Johnson et al. 2014, Chen, Gao et al. 2015). Firstly EU assays were performed with transcription inhibitors DRB, triptolide, and α-amanitin to check they work in presence of JQ1. α- amanitin was added 3 hours before JQ1 was added for 1 hour, as α-amanitin takes approximatley 4 hours to suppress transcription (Bensaude 2011, Kotsantis, Silva et al. 2016). DRB and triptolide were added at the same time as JQ1 for 1 hour (Figure 3.9 A). All three transcription inhibitors inhibited EU incorperation into the cells in the presence of JQ1 treatment (Figure 3.9 B). Using the fibre technique where α-amanitin was added 3 hours before JQ1 was added, while DRB and triptolide were added at same time as JQ1, before labelling and staining the fibres (Figure 3.9 A). The fork speeds where transcription inhibitors were added in presence of JQ1 showed a partial rescue

towards control levels (Figure 3.9 C-D). JQ1 caused an average median fork speed of around 0.52 kb/min compared to 1 kb/min in control treated samples, while treatment with transcription inihbitors caused fork speeds to increase to between 0.76-0.83 kb/min (Figure 3.9 C). Transcription inhibitor treatment without the treatment of JQ1 had no effect on fork speeds compared to control, showing transcription inhibitors have no effect on replication by themselves (Figure 3.9 C). We observed a similar effect in BJ-hTert cells, observing a partial rescue of fork speeds in cells treated with both JQ1 and transcriptional inhibitor DRB towards control levels (Figure 3.9 E). All together, these results indicate that by inhibiting transcription we can rescue JQ1-induced fork slowing in cells, indicating transcription-replication interference as a major source of JQ1 induced replication stress.

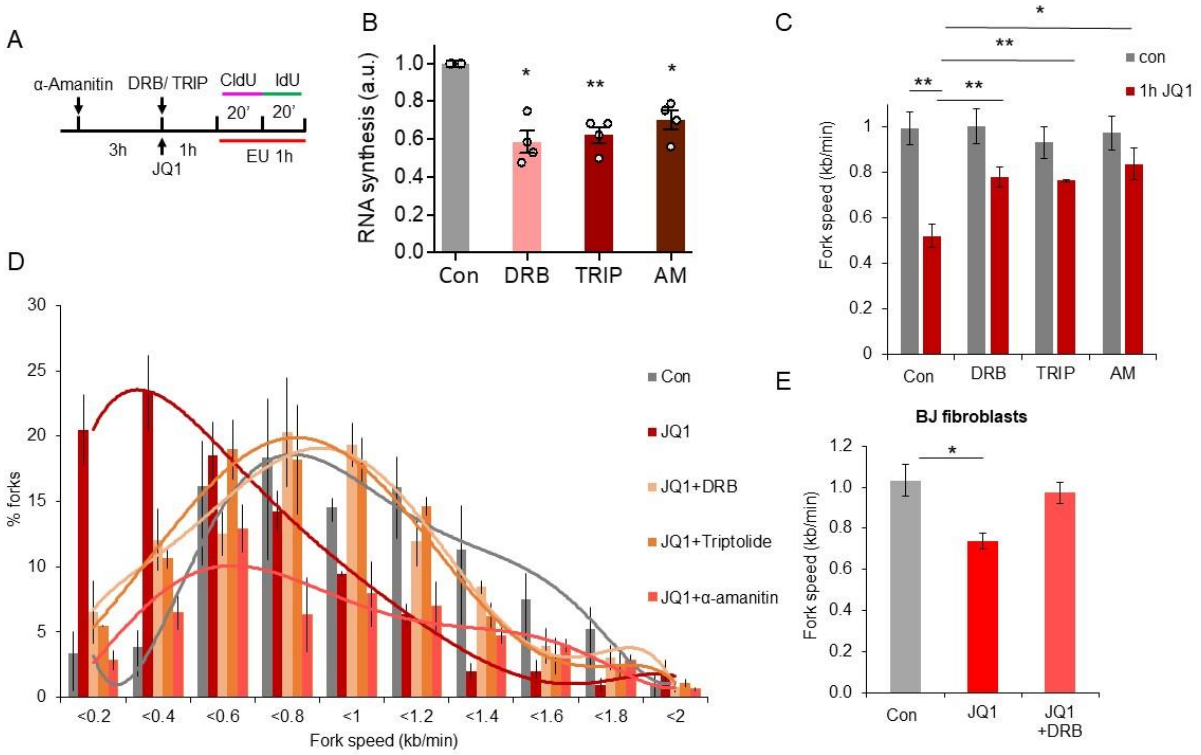


Figure 3.9: Transcriptional inhibitors rescue BET inhibitor induced fork slowing.

A) DNA fibre labelling and quantification of incorporation of EU were performed in U2OS cells with transcriptional inhibitors α-amanitin (AM, 10 µg/ml) for 3 hours or DRB (100 µM) or Triptolide (TRIP, 1 µM) for 1 hour, +/- 1 µM JQ1 treatment for 1 hour B) Nuclear EU intensities in cells treated with transcription inhibitors α-amanitin/DRB/Triptolide with 1 µM JQ1 treatment for 1 hour. C) Average median of replication fork speeds treated with transcriptional inhibitors α-amanitin/DRB/Triptolide +/- 1 µM JQ1 treatment for 1 hour. D) Distribution of replication fork speeds in cells treated transcriptional inhibitors α-amanitin or DRB or Triptolide +/- 1 µM JQ1 treatment for 1 hour. E) Average median of replication fork speeds in BJ-hTert cells treated with transcriptional inhibitors DRB +/- 1 µM JQ1 treatment for 1 hour. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01.)

Approximately 80% of total RNA synthesis in cells is performed by RNA Pol I and III (Paule and White 2000). This large amount of total RNA synthesis could also contribute to replication-transcription interference in cells. To test whether RNA Pol I

and Pol III has a contributing role in BET inhibtion-induced replication slowing we used the DNA fibre technique with 100 µM RNA Pol I inhibitor (CX-5461) and 25 µM or 100 µM of RNA Pol III inhibitor (ML-60218). Cells were treated with these inhbitors in combination with JQ1 for 1 hour before cells were labelled, fibres spread and stained (Figure 3.10 A). RNA Pol I and Pol III inhibitors in combination with JQ1 led to a rescue in fork speeds. In particular RNA Pol I inhibitor showed a complete resuce of JQ1-induced fork slowing (Figure 3.10 B). The RNA Pol I inhbitor treatment showed a small increase in replication fork speeds (0.92 kb/min in control compared to 1 kb/min with RNA Pol I inhibitor treatment), indicating that it has no large effect on replication by itself (Figure 3.10 B). RNA Pol III inhibitor caused slight slowing of replication fork speeds (Figure 3.10 B), but this effect should have no bearing on our conclusion that these inhibitors rescue JQ1-induced fork slowing.

An EU assay was also conducted using these RNA polymerase inhibitors. Interestingly, we see no effect of RNA Pol I or Pol III inhibitor alone on EU incorporation, but see a decrease when combined with JQ1 treatment compared to JQ1 treatment alone (Figure 3.10 C). DRB, a potent transcription inhibitor was used as a positive control, caused EU intensity decrease as expected. RNA Pol I and Pol III inhibitors have been shown to be potent inhibitors of these RNA polymerases (Wu, Pan et al. 2003, Drygin, Lin et al. 2011). We conclude from this that RNA synthesis is decreased when these inhibitors are combined with JQ1, and hence the effects we see in the fibre assay could be due to the suppression of RNA synthesis. These results show that RNA Pol I and Pol III may have a role in BET inhibitor induced transcription-replication interference leading to impeded fork progression.

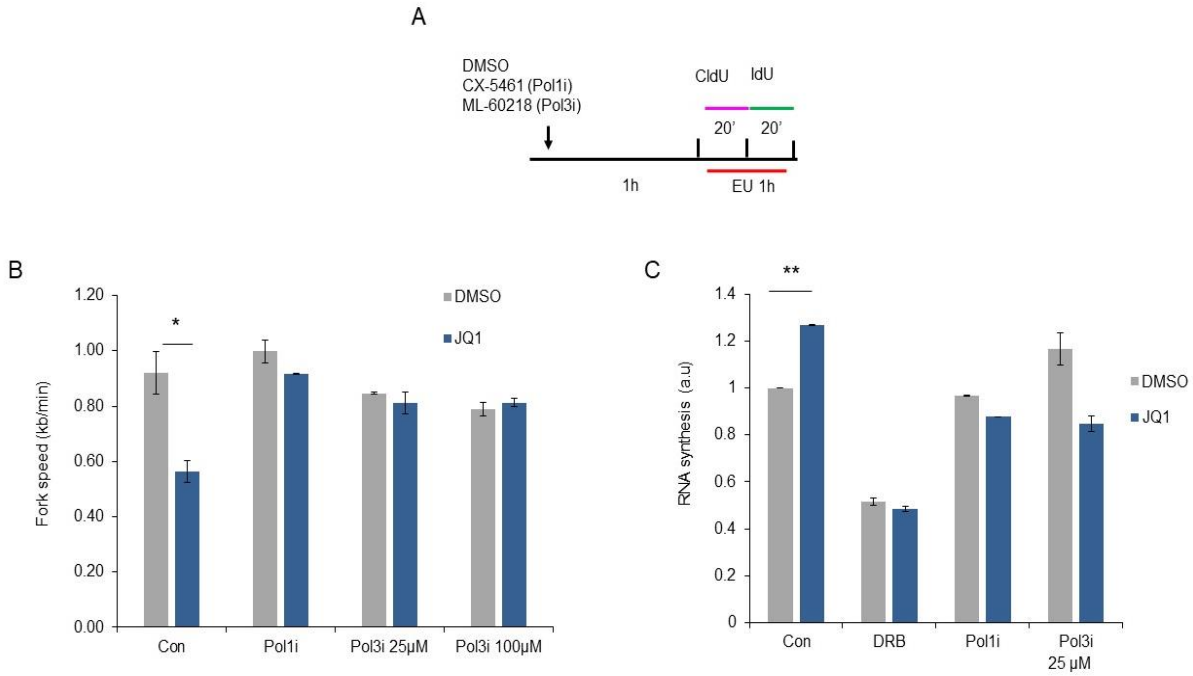


Figure 3.10: Inhibiting RNA polymerase 1 or 3 rescues BET inhibitor induced fork slowing.

A) DNA fibre labelling and quantification of incorporation of EU were performed in U2OS cells with RNA polymerase I (RNA POL 1) inhibitor CX-5461(250 nM) and RNA polymerase 3 (RNA POL 3) inhibitor ML-60218 (100 µM or 25 µM) for 1 hour +/-1 µM JQ1 treatment for 1 hour. B) Average median fork speeds in cells treated with RNA POL 1 inhibitor, 25 µM RNA POL 3 inhibitor and 100 µM RNA POL 3 inhibitor +/- JQ1 treatment for 1 hour. C) Nuclear EU intensities in cells treated with RNA POL 1 inhibitor and RNA POL 3 inhibitors for 1 hour +/- 1 µM JQ1 treatment for 1 hour (n=2). (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01, NS=Not significant.)

3.6: BET inhibition leads to increased origin firing

Replication stress can be caused by a myriad of different sources on the DNA causing replication fork blocks leading to fork slowing and replication stress (Zeman and Cimprich 2014). As shown previously, BET inhibition leads to the quick slowing of fork speeds which indicates an important role for BET proteins in the maintenance of fork progression. Understanding how this occurs is important for a more detailed understanding of how these proteins work and how BET inhibition can be used as a therapeutic. Previously, our work showed transcription-replication interference as a major source of BET inhibition induced fork slowing. It has been shown before, using Cyclin E overexpressing cells, that replication stress can be caused by increased transcription as well as increased number of replication origins being fired (Jones, Mortusewicz et al. 2013).

Eukaryotic cells have a multitude of licenced replication origins in the genome which can be fired to allow establishment of bi-directional replication forks. Licencing and firing of replication origins is tightly regulated. If the tight regulation of the number of active origins is lost this can lead to aberrant fork progression and can cause later DNA damage and genomic instability. Tight control of origin firing is crucial for allowing normal fork progression (Woodward, Gohler et al. 2006, Masai, Matsumoto et al. 2010, McIntosh and Blow 2012).

We then decided to test whether BET inhibition has any effects on the numbers of new origins that are fired and whether any changes observed in replication origin firing may be causing replication fork progression to be slowed down.

To test whether BET inhibition may increase origin firing, we used the DNA fibre technique. 1 µM of JQ1 was added 1 hour before cells were labelled and stained in exactly the same way as mentioned previously. Instead of measuring fork speeds we quantified replication fork structures. As seen in Figure 3.11 A, we quantified five different fibre structures, of which green only labelled (IdU) and green labels on either side of a red label (CldU) show origins that have been newly fired. Green only labelled are 2nd labelled origins and green labels on either side of a red label are 1st labelled origins. By counting the number of first and second labelled origins in each treatment as a proportion of all labelled fibre structures, we found that BET inhibition led to increased origin firing (Figure 3.11 B). For first label origins we observed almost four times as many origins being fired (4-15%) and also observed a significant increase in second label origins (15-25%) after BET inhibition (Figure 3.11 B). This result shows that 1 hour of BET inhibition leads to increased new origin firing.

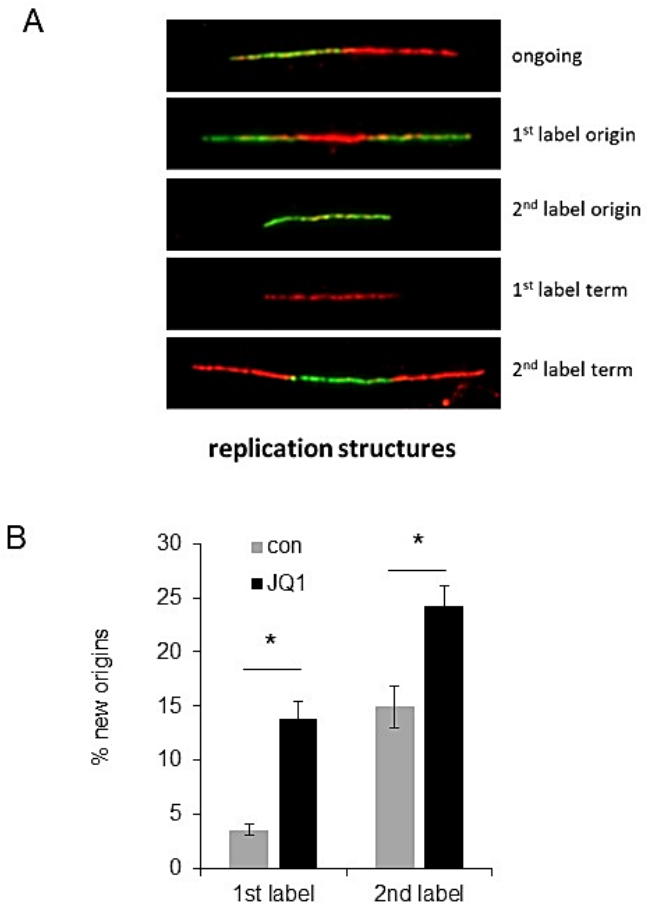


Figure 3.11: JQ1 leads to increased origin firing.

A) Diagram showing the replication structures formed during the fibre assay. B) % of 1ST label and 2nd label new origin firing in U2OS cells after treatment with 1 μM JQ1 for 1 hour (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05)

3.7: Inhibition of CDKs rescues BET inhibitor-induced fork slowing

We wanted to elucidate whether this increase in origin firing was a causative source of replication stress or just a downstream effect to compensate for replication fork slowing seen with BET inhibitor treatment. To do this the DNA fibre technique was used again by using three CDK inhibitors RO3306, PHA-767491 and Roscovotine to inhibit origin firing. RO3306 is a specific Cyclin dependent Kinase 1 (CDK1) inhibitor (Vassilev 2006). PHA-767491 is a potent dual Cdc7/CDK9 inhibitor (Montagnoli, Valsasina et al. 2008). Roscovotine inhibits cell division cycle 2(Cdc2), Cyclin dependent kinase 2 (CDK2) and Cyclin dependent kinase 5 (CDK5) (Meijer, Borgne et al. 1997). RO3306 and PHA-767491 were used for 1-hour treatment and Roscovotine for 30 minutes before fibre labelling in combination with JQ1 treatment (Figure 3.12 A). Firstly, we quantified the amount of 1st and 2nd labelled origins in the same method as before to check that these inhibitors did cause suppression of origin firing in the presence of JQ1. For each CDK inhibitor tested we saw a major decrease in both 1st and 2nd label origin firing in response to JQ1 treatment (Figure 3.12 B). The levels of 1st and 2nd label origins also decreased below control levels, indicating that these inhibitors where acting to suppress origin firing in cells. Thus, we quantified fork speeds in the same samples and observed a full rescue of fork speeds to that of control cells with combination treatment of JQ1 and the CDK inhibitors (average median fork speed around 1.3 kb/min) (Figure 3.12 C-D). There were slight decreases in average fork speeds of fibres treated with only CDK inhibitors compared to control fork speeds (Figure 3.12 C-D). This did not change

the conclusion that the CDK inhibitors are rescuing BET inhibitor induced fork slowing.

These results taken together strongly indicate that BET inhibition increases origin firing which may be a mechanism by which BET inhibition can causes fork speeds to slow down.

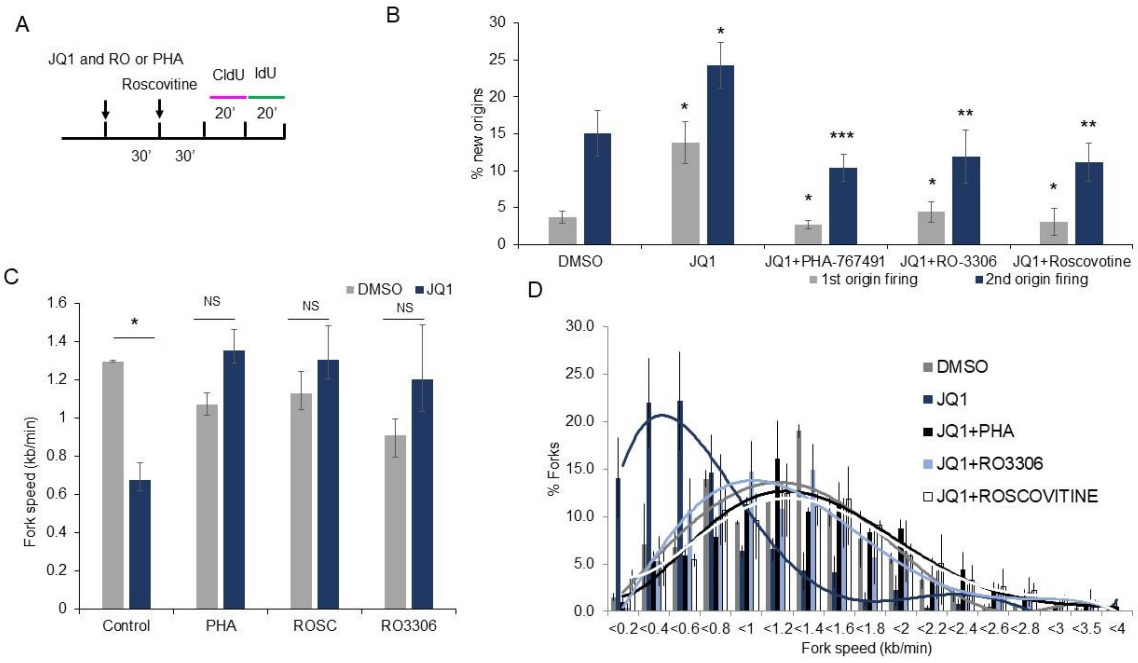


Figure 3.12: CDK inhibitors rescue BET inhibitor-induced fork slowing.

A) DNA fibre labelling in U2OS cells treated with JQ1 and either PHA (10 µM) or RO3306 (10 µM) for 1 hour or roscovitine (25 µM) for 30 mins. B) % of 1ST label and 2nd label new origin firing in cells treated with JQ1, JQ1+roscovitine, JQ1+ RO3306 and JQ1+PHA shown as percentages of labelled tracks. Statistics for P-values correspond to values compared to JQ1 only. JQ1 only is compared to DMSO. C) Average median replication fork speed in cells treated with CDK inhibitors RO3306 or roscovitine or PHA +/- 1 µM JQ1 treatment for 1 hour. D) Distribution of replication forks in cells treated with CDK inhibitors PHA, RO3306 and Roscovitine and 1µM JQ1 treatment for 1 hour. (N=3 error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01, *** P value < 0.001 NS= Not significant.)

3.8: The interplay between origin firing and transcription-replication interference during BET inhibition

Our experiments now indicated that there were two potential mechanisms, increased transcription-replication interference and increased firing of origins that may act to cause BET inhibition induced fork slowing. Whether these two mechanisms may act separately or together to cause this fork slowing is undefined. To better gain an insight into how these two potential mechanisms may work together we undertook a further experiment. Global RNA synthesis is measured after adding CDK inhibitors to control and JQ1 treated cells (Figure 3.13 A). We observed quite prominent decreases in EU intensity after treatment with CDK inhibitors in both combination with JQ1 or by themselves (Figure 3.13 B). This indicated that CDK inhibitors were suppressing total RNA synthesis in cells, the mechanism of which is still unclear. This means that we cannot distinguish whether CDK inhibitors rescue JQ1-induced fork slowing by inhibiting origin firing or by inhibiting RNA synthesis.

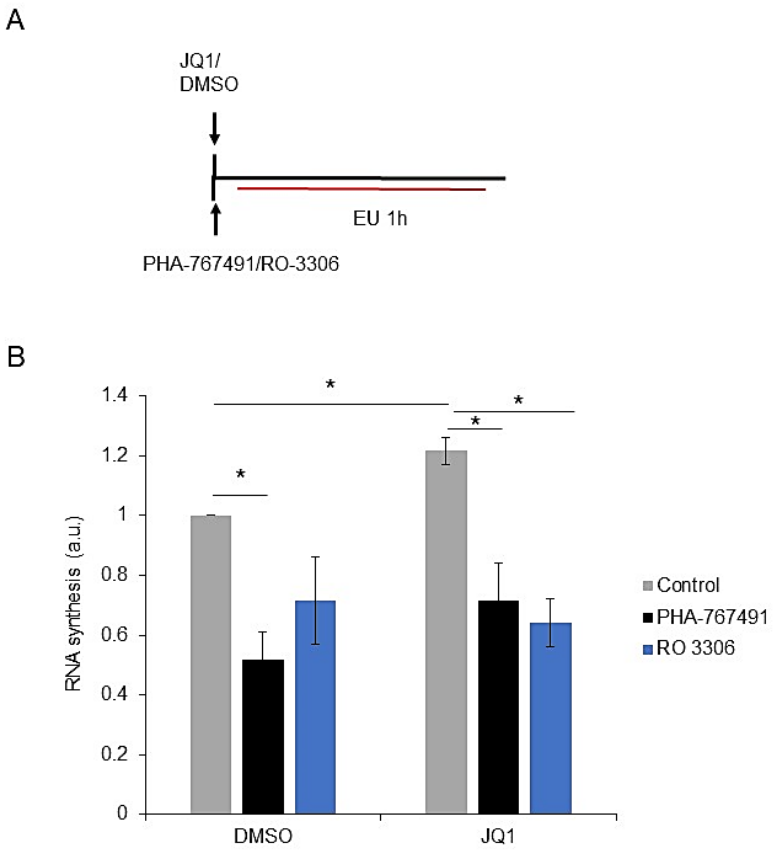


Figure 3.13: CDK inhibitors suppress nascent RNA synthesis.

A) U2OS cells were treated with CDK inhibitors RO-3306 (10 µM) and PHA (10 µM) for 1 hour and 1 µM JQ1 for 1 hour and transcription elongation was quantified by incorporation of EU into the cells. B) Nuclear EU intensities in cells treated with CDKi and 1 µM JQ1 for 1 hour. (N=3 error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05)

3.9: BET inhibition does not activate a replication stress response

Replication fork slowing is often a hallmark of replication stress. Persistent replication stress in cells leads to activation of a replication stress reponse pathway involving ATR kinase (Ciccia and Elledge 2010). The ATR response firstly results in the accumulation of RPA followed by increased levels of γH2AX at the site of stress (Flynn and Zou 2011). Persistent replication stress can lead to fork collapse into DSBs and recruitment of 53BP1 into nuclear foci (Zeman and Cimprich 2014). As BET inhibtion has been shown to slow down fork progression, it was important to test whether this leads to acitvation of the replication stress repsonse. To do this we used immunofluorescence to look at damage foci. Staining for γH2AX and RPA was used as replication stress markers and 53BP1 was used to visualise formation of DSBs after JQ1 treatment for 1, 4 and 8 hours. Interestingly, we saw no increases in either γH2AX, 53BP1 or RPA foci at these time points compared to control treatment (Figure 3.14 A-B). 8 hour treatment with 2 mM HU acted as a positive control. HU depletes the cells of dNTPs, which initially results in stalled replication forks that, after prolonged treatment, collapse into DSB's (Koç, Wheeler et al. 2004). HU treatement induced significant increases in all three DNA damage response foci (Figure 3.14 A-B). This suggests JQ1-induced fork slowing does not seem to activate a DNA damage response despite inducing replication fork slowing.

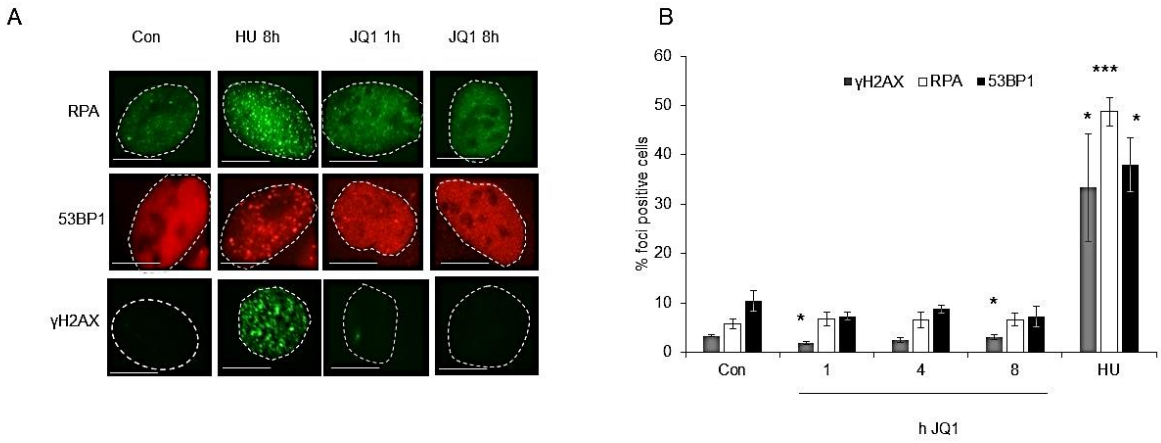


Figure 3.14: JQ1 treatment does not activate a replication stress response.

A) Nuclear foci of 53BP1, γH2AX, RPA in U2OS cells treated with DMSO, HU (2 mM), and JQ1 treatment for 1, 4 and 8 hours. Scale bar 10 µm**.** B) Quantification of % foci positive cells (more than 8 foci). Statistics for P-values correspond to values compared to respective control. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, *** P value < 0.001.)

There are two possible explanations for the observation seen with BET inhibition and DNA damage response markers: either JQ1-induced fork slowing does not result in DNA damage, or JQ1 inhibits the ATM- and ATR-dependent DNA damage signalling pathways that recruit 53BP1 and promote phosphorylation of H2AX and other targets. To investigate whether BET inhibition may lead to suppression of DNA damage signalling, further DNA damaging agents were used: HU, etoposide and Camptothecin (CPT). CPT inhibits the nuclear enzyme DNA topoisomerase I, causing SSBs and DSBs when replication encounters the initial SSBs (Liu, Desai et al. 2000). Etoposide induces DNA damage by inducing DSBs in cells by inhibiting DNA

topoisomerase II (Hande 1998). We tested whether JQ1 suppressed the response to DNA damaging agents HU, CPT and etoposide, using phosphorylation of CHK1 (serine 317) as readout for ATR activity and RPA32 (serine 4/8) as readout for ATR, ATM and DNA-PK activity (Ciccia and Elledge 2010, Liaw, Lee et al. 2011). JQ1 treatment alone did not seem to lead to an observed increase in either CHK1 or RPA32 phosphorylation either by itself or when combined with HU or CPT treatment (Figure 3.15 A). γH2AX levels were also looked at in response to these damage-inducing drugs. JQ1 treatment leads to a decrease in γH2AX levels compared to control, when HU and CPT were added together with JQ1, similar effects were seen with γH2AX levels decreasing compared to control treated samples (Figure 3.15 B-C). However, with etoposide the opposite effect on γH2AX and RPA32 phosphorylation was observed (Figure 3.15 A-B). In agreement with a previous report showing that JQ1 amplifies the ATM-mediated response to directly induced DSBs, combination of JQ1 and etoposide led to increased RPA phosphorylation compared to etoposide alone (Floyd, Pacold et al. 2013).

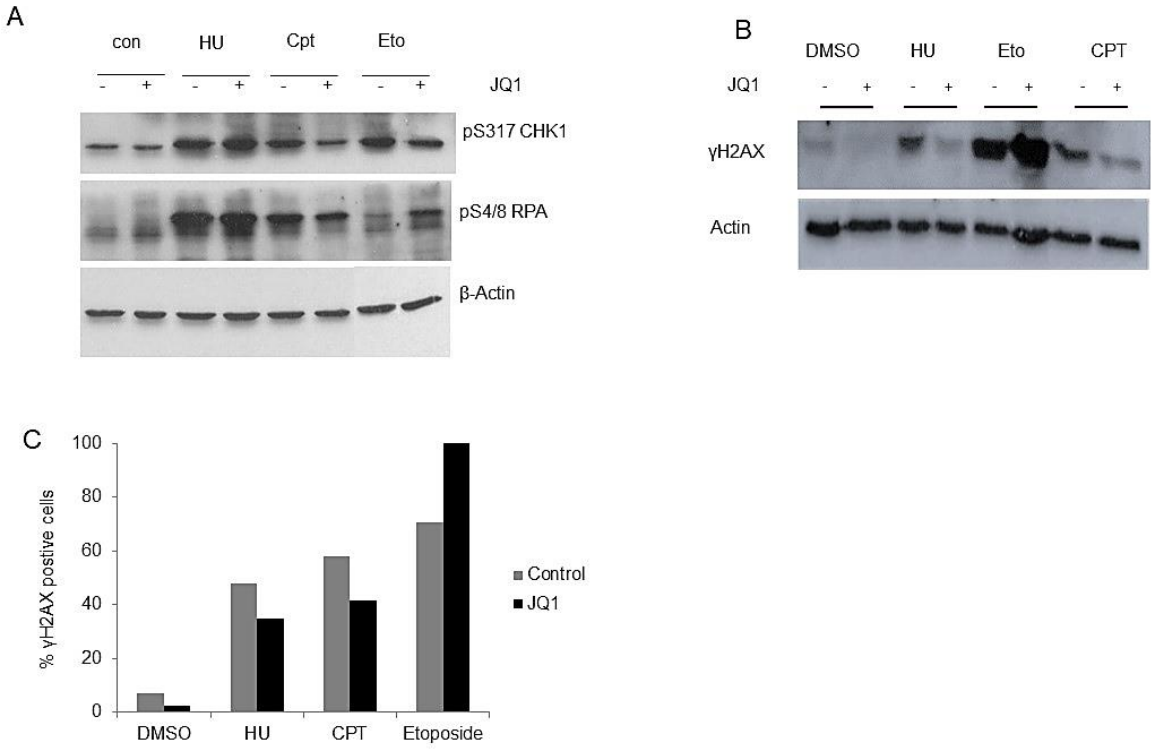


Figure 3.15: JQ1 may lead to a suppression in the replication response pathway.

A) Protein levels of phospho-S4/8 RPA32, phospho-S317 CHK1 and Actin after treatment with HU (2mM 8hrs), Camptothecin (CPT, 1 µM 1 hour) and Etoposide (Eto, 25 µM 2 hours) +/- JQ1 treatment for 8 hours (N=1). B) Protein levels of γH2AX and actin (loading control) after treatment with JQ1, HU, CPT and Etoposide (N=1). C) Quantification of cells containing 8 or more γH2AX or 53BP1 foci after treatment with JQ1, HU, CPT and Etoposide (N=1).

Secondly, we looked into whether inhibition of the replication stress response proteins ATR, CHK1 or BLM lead to an increase in DNA damage signalling in JQ1-treated cells, because these proteins can protect stressed replication forks by binding to them and help to stabilise stalled forks and prevent the formation of DNA damage or fork collapse via various cellular processes as discussed in more detail in Chapter 1 (Cortez, Guntuku et al. 2001, Ammazzalorso, Pirzio et al. 2010, Ciccia and Elledge 2010, Zeman and Cimprich 2014). BLM inhibitor (ML216) and JQ1 lead to no increase in DNA damage foci (Figure 3.16 A). In response to ATR inhibitor (AZ20) and CHK1 inhibitor (PD-407824) (1-hour treatment) combination with JQ1, an increase in γH2AX positive cells was seen indicating more DNA damage signalling (Figure 3.16 B-C). These data suggest that some DNA lesions are formed at JQ1-slowed replication forks, and that the functions of replication stress response pathways ATR and CHK1 may stabilise these stalled forks and help prevent them from being converted into downstream DNA damage/DSB's that would activate the replication response signal more strongly.

This data firstly suggests a potential mechanism where JQ1 could prevent the formation of DNA damage, as shown when there were reduced signal of DNA damage markers with DNA damage inducing agents when treated in combination with JQ1. There is also a suggestion that replication stress factors such as ATR and CHK1 which are involved in the replication stress response pathway could help prevent DNA damage as inhibition of both factors led to increased signalling of DNA damage markers. The precise mechanism of how JQ1 could prevent DNA damage still requires further research.

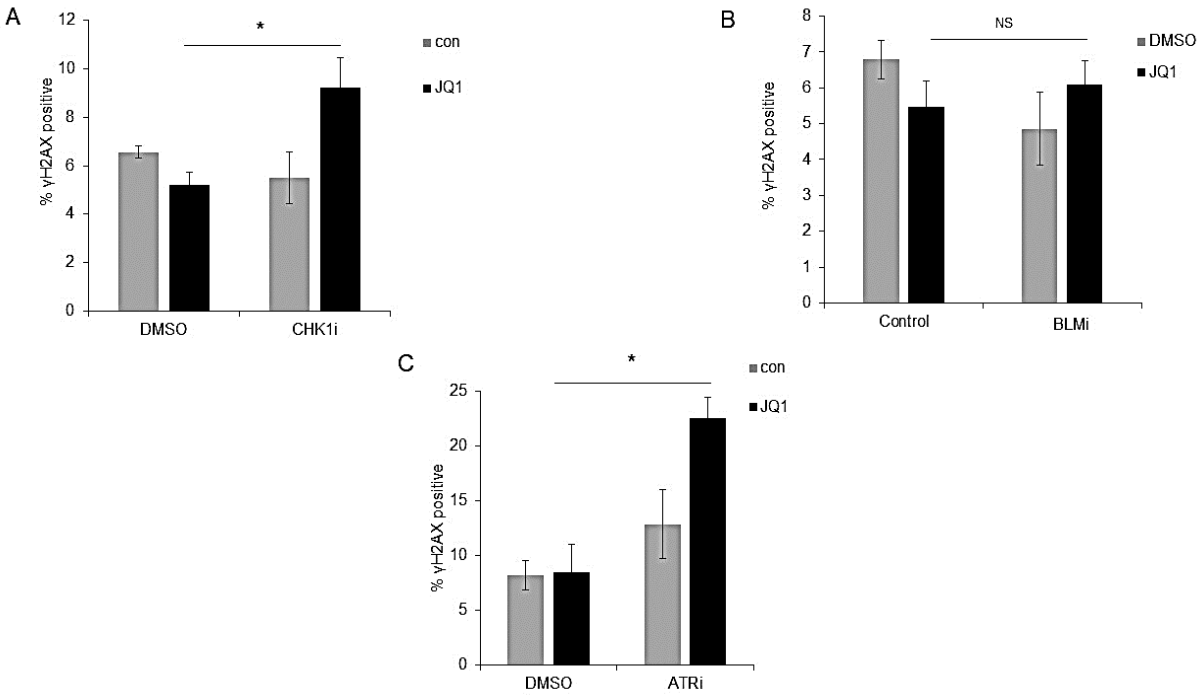


Figure 3.16: CHKi and ATRi co-treatment with JQ1 gives rise to a replication stress response.

A) Percentages of cells containing more than 8 γH2AX foci after treatment with ML216 (1.4 µM) with and without 1 µM JQ1 8-hour treatment. B) Percentages of cells containing more than 8 γH2AX foci after treatment with AZ20 (2.4 µM) +/- 1 µM JQ1 8-hour treatment. C) Percentages of cells containing more than 8 γH2AX foci after treatment with PD-407824 (300nM) +/- 1 µM JQ1 8-hour treatment. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05)

3.10: Summary

Preliminary data from the lab suggested a role for BET proteins in the maintenance of replication progression. JQ1, a BET inhibitor, had been previously shown to slow down replication fork speeds. Work presented in this chapter firstly shows that JQ1 can cause replication progression impedement as quickly as 1 hour of treatment (Figure 3.1 C-D). This occurred over a range of different cell lines including U2OS, NALM6, BJ-hTert and MEC1 cell lines (Figure 3.6 C-D). This observation shows that the effect of BET inhibtion is not limited to just cancer cell lines, but may also have similar effects on non cancer cell lines as well. The effect was not seen in C2 CLL cells which appeared resistance to JQ1 treatments (Figure 3.6 B). The reason for the apparent resistant is unknown and requires further investigation. JQ1 induced significant fork slowing over numerous concentrations starting from 250 nM to 1 µM. This finding was important as a lot of research currently published on JQ1 as a potential therapeutic drug has been done at these concentrations. As all concentrations showed the same effect, 1 µM JQ1 was further used in the rest of the experiments as it provided the most robust and striking phenotypic change. JQ1 is a synthesised chemical with a specific chemical structure used to inhibit BET proteins, but is one of a number of of different BET inhibitors. Our works suggest that it is not only JQ1 that provides this phenotypic effect. I-BET151 is another BET inhibitor and works to inhibit BET proteins as well. By mirroring the effect shown by JQ1 it provides evidence that inhibiting BET proteins is the mechanism of action rather than some other effect of JQ1. This is important as we can assume that inhibiting BET proteins is essential for replication fork impedement and therefore

BET proteins do have a functional role in assisting normal replication fork progression.

Work in this chapter showed that BET inhibitor treatment increases total RNA synthesis (Figure 3.5 C-F). This is rather a surprising result as the prevailing view is that BET proteins act as transcription factors and BET inhibitors therefore would act as transcription inhibitors. Our results however report that treatment with BET inhibitors actually has the reverse effect leading to increased RNA synthesis, especially at highly transcribed histone and other non poly-adenylated non-coding RNA genes.

A major question that needed to be adressed in this work was what were the causes for this replication fork slowing. There are a many of different obstacles on DNA that have potential to cause replication fork slowing (Zeman and Cimprich 2014). One particular obstacle is transcription-replication interference that has been shown to cause replication stress in cells (French 1992, Poveda, Le Clech et al. 2010, García-Muse and Aguilera 2016).

After observing fork slowing and RNA synthesis increase after BET inhibitor treatment, we hypothesised that transcription-replication interference may be a major source of BET inhibitor induced replication stress. Our work with three different transcription inhibitors showed that by inhibiting transcription, replication progression is rescued in BET inhibitor treated cells (Figure 3.9 B-C). Each inhibitor works differrently as explained previously, and may have some other cellular side effects. By using three different inhibitors, especially α-amanitin which is a known potent RNA Pol II inhibitor, we can be more certain that this rescue in phenotype is

due to transcription inhibition. RNA Pol I and Pol III inhibitors were also tested in coordination with JQ1 and showed similar results (Figure 3.10 B-C). Although the EU assay proved unable to show that these inhibitors worked effectively, they have been widely studied and shown to be potent inhibitors (Wu, Pan et al. 2003, Drygin, Lin et al. 2011). There could be many reasons for this such as RNA Pol I and III transcribe in small regions of the genome, for example RNA Pol I only transcribes ribosomal RNA which is concentrated in the nucleoli and therefore less likely to affect the mean EU intensity across the nucleus (White 2005). Another method such as qRT-PCR could be used in further assays to ensure that the inhibitors are effectively working. We decided not to persue this further as we concluded that these inhibitors were effectively working, as we saw a change in phenotype in replication and could visualise reductions in EU intensity around the nuclei coupled with the literature of them being potent inhibitors and previous data in our lab showing that these inhibitors worked in BJ-hTert cells. However, our results so far are in agreement with the idea that BET inhibition may lead to increased transcription of highly transcribed non-coding RNA genes. These results provide a clear mechanism, in which BET inhbitors lead to increase in RNA synthesis which causes increased transcription-replication interference that leads to replication fork slowing. How BET inhibition can lead to this increase in RNA synthesis is unclear and required further work which will be discussed later.

Timecourse analyses showed that RNA synthesis was increased for up to 72 hours after JQ1 addition, while replication fork slowing was slowed down from 1 to 8 hours, before being rescued back to normal fork speeds by 24 hours after JQ1 addition (Figure 3.3 B-C, Fig 3.5 C-D). This shows that the initial increase in RNA

synthesis coincides with fork slowing. It is however unclear how replication fork speeds can be recovered later on, whilst RNA synthesis is still increased. This will be futhur investigated in chapters 5 and 6.

Previous work in the lab had shown that replication fork slowing could occur via two different mechanisms. The first of which, transcription-replication interference, had been demonstrated to be a major cause for $HRAS^{V12}$ overexpression induced fork slowing (Kotsantis, Silva et al. 2016). The second was that of increases in newly firing replication origins, which has been shown in the overexpression of oncogenes such as C-MYC or Cyclin E which then induces replication fork slowing (Jones, Mortusewicz et al. 2013, Srinivasan, Dominguez-Sola et al. 2013).

Another aim of this chapter was to see whether this could be seen with BET inhibitor induced fork slowing too. Firstly, BET inhibitor JQ1 treated cells have a dramatic increase in origin firing compared to that of control cells, suggesting that an increase in origin firing may be a cause of fork slowing (Figure 3.11 B). However, an increase in origin firing could also be due to BET inhibitors inducing replication fork slowing and hence more origins are fired to rescue fork speeds. To further investigate whether increased origin firing caused fork slowing three different CDK inhibitors were used. The CDK inhibitors, which reduce origin firing below control levels shown in (Figure 3.12 B), rescued BET inhibitor-induced fork slowing back to control levels when co-treated with BET inhibitor JQ1 (Figure 3.12 C). This potentially suggests a mechanism in which increase origin firing in JQ1 treated cells can cause the fork slowing associated with JQ1. There are various ways increased origins can cause replicative stress such as depletion of nucleotides or other replication factors due to more active replication forks. This was interesting as it

suggested another potential mechanism of BET inhibitor induced fork slowing, but more research was needed to fully characterise this as a potential mechanism for JQ1-induced replication stress in cancer cells.

As there were now two potential mechanism for fork slowing to occur, we decided to investigate whether they acted separately to cause fork slowing, or together where increased origin firing leads to more transcription replication interference. Before we could address this question, we first wanted to test if CDK inhibitors had any effect on total RNA synthesis. CDK inhibitors decreased total RNA synthesis compared to JQ1 and control (Figure 3.13 B). This meant that CDK inhibitors also acted as transcriptional inhibitors as they inhibited RNA synthesis below the control level just as the transcriptional inhibitors did in figure 3.6. This meant that it was very hard for us to interpret whether CDK inhibitors were rescuing fork speeds due to the fact that they can decrease origin firing or because these inhibitors are able to reduce transcription levels in the cell. It may well be that increased origins are having a role in causing BET inhibitor induced fork slowing or that they are a by-product of the cell already being stressed due to slow forks.

At this point it is hard to resolve this issue, as it quite hard for us to try and further investigate origin firing as a mechanism of BET inhibitor induced fork slowing. Our data doesn’t allow us to dissect and distinguish these as two separate mechanisms and it is unclear whether increased firing of origins play any role in causing BET inhibitor induced fork slowing. As our data provided clear evidence of transcription-replication interference causing BET inhibitor induced fork slowing, we decided to concentrate on understanding the mechanism of transcription-replication interference rather than pursue further research on the origin firing mechanism.

Work from this chapter has reported that inhibition of BET proteins in U2OS cells caused no increases in nuclear foci of γH2AX, 53BP1 and RPA (Figure 3.14 A-B). H2AX is normally phosphorylated by activated ATR in a response to replication fork stalling. RPA foci are normally seen in response to excessive amounts of ssDNA which it can bind to before recruiting ATR-ATRIP (Flynn and Zou 2011).This would suggest that JQ1 induces fork slowing but does not cause excessive ssDNA. Also despite persistent fork slowing there does not seem to be formation of DSBs. These methods are measuring DNA damage signalling in response to BET inhibitor induced fork slowing, but may not prove that there is no damage in cells. The signalling pathway may have been surppressed and research has shown that both HR factors and ATR factors such as TOBP1 and WEE1 are downregulated in response to BET inhibitor treatment (Karakashev, Zhu et al. 2017, Sun, Yin et al. 2018). When JQ1 was combined with DNA damaging agents, γH2AX, p-CHK1 and p–RPA32 induction was lower in cells treated with JQ1 compared to the control (Figure 3.15 A-C). This indicates some suppression of the DNA damage response pathways when BET proteins are inhibited. With DNA damaging agents HU and CPT, JQ1 caused a decrease in γH2AX, p-CHK1 and p–RPA32. However, when etoposide was added the opposite effect was seen. Etoposide is known to directly induce DSBs in DNA, while HU and CPT interfere with replication to cause DNA damage and DSBs. Previous research showed increased γH2AX in BRD4-depleted cells after ionising irradiation, which induces DSBs directly similar to etoposide. This was due to enhanced signalling from DSBs in BRD4 depleted cells, rather than increased amount of damage. It was suggested that BRD4-depleted cells have more open chromatin that facilitates γH2AX foci formation (Floyd, Pacold et al.

2013). Our results so far would indicate that there is reduced ATR activation in BET inhibited cells which is also supported by recent publications (Pawar, Gollavilli et al. 2018, Sun, Yin et al. 2018, Zhang, Dulak et al. 2018). There is also evidence supporting that BET inhibition may be prevented from causing DNA damage by replication stress response factors such as ATR or CHK1, which are known to help stabilise the replication fork and prevent DNA damage (Figure 3.16 A-B).

To summarise this chapter, BET inhibitors cause replication fork slowing by increasing transcription leading to transcription-replication interference. This does not activate the normal replication stress response pathways, and the replication stress response could even be supressed.

The main questions that arise from the work in this chapter are firstly how RNA synthesis is increased after BET inhibitor treatment, and secondly, what happens downstream of the replication fork slowing. There may be damage occuring in the cells which is not inducing a signal or there may be another mechanism which happens after fork slowing which may cause no DNA damage signalling to occur. These questions are investigated in further depth in chapters 6 and 7.

4. Loss of BRD4 causes replication-transcription conflicts

4.1: Introduction

As mentioned previously, BET proteins consist of four different BET family members BRD2, BRD3, BRD4 and BRDT. All members act as transcription factors and epigenetic regulators (Zeng and Zhou 2002). Each BET protein family member has a conserved architecture consisting of two N-terminal tandem bromodomains and an ET domain (Filippakopoulos, Qi et al. 2010). BET proteins have important functions in transcription and are recruited to chromatin by acetylated histones on lysine tails, before recruiting various transcription factors to chromatin (Sanchez and Zhou 2009).

All the BET proteins have been implicated in cancer and so are important proteins to study. Each BET protein has specific functions in regulating transcription as well as possessing specific diverse cellular roles. BET inhibitors work by binding to the acetylated lysine binding pockets of BET proteins and therefore stopping their recruitment to chromatin. BET inhibitors equally affect each BET protein, stopping all BET protein functions in the cell (Filippakopoulos, Qi et al. 2010).

To further understand the role of BET inhibitors in DNA replication, it is important to know which BET protein is crucial for this function. To do this we would want to investigate which BET protein specifically is causing this phenotype shown when

treating with BET inhibitors. Thus, the specific aim of this chapter is to elucidate the BET protein involved in DNA replication function. By understanding which BET protein is required for normal fork progression, we will have new insights into biological functions of BET proteins which may be important in developing better therapeutics.

4.2: Loss of BRD4 leads to replication stress

To begin to investigate which BET proteins are the targets responsible for BET inhibitors induced fork slowing, we used siRNA depletion to test which BET protein prevents replication stress following the Dharmafect protocol. First, we depleted BRD4 as it was the protein that is known to interact with P-TEFB as well as replication factor proteins such as Replication factor C (RFC), TICRR and CDC6 (Maruyama, Farina et al. 2002, Quaresma, Bugai et al. 2016, Sansam, Pietrzak et al. 2018, Zhang, Dulak et al. 2018). Firstly, we treated cells with siRNA specifically for BRD4 for 48 hours (Figure 4.1 A). By Western blot we showed that BRD4 protein levels are reduced indicating that the BRD4 siRNA works (Figure 4.1 B). 48 hours after treatment with the siRNA, DNA fibre labelling was undertaken. BRD4 depleted cells showed replication fork speeds decreased significantly compared to cells treated with control siRNA, mirroring the effects of treatment with JQ1 (Figure 4.1 C-D). No additional fork slowing was seen when JQ1 was added to BRD4 depleted cells (Figure 4.1 C-D).

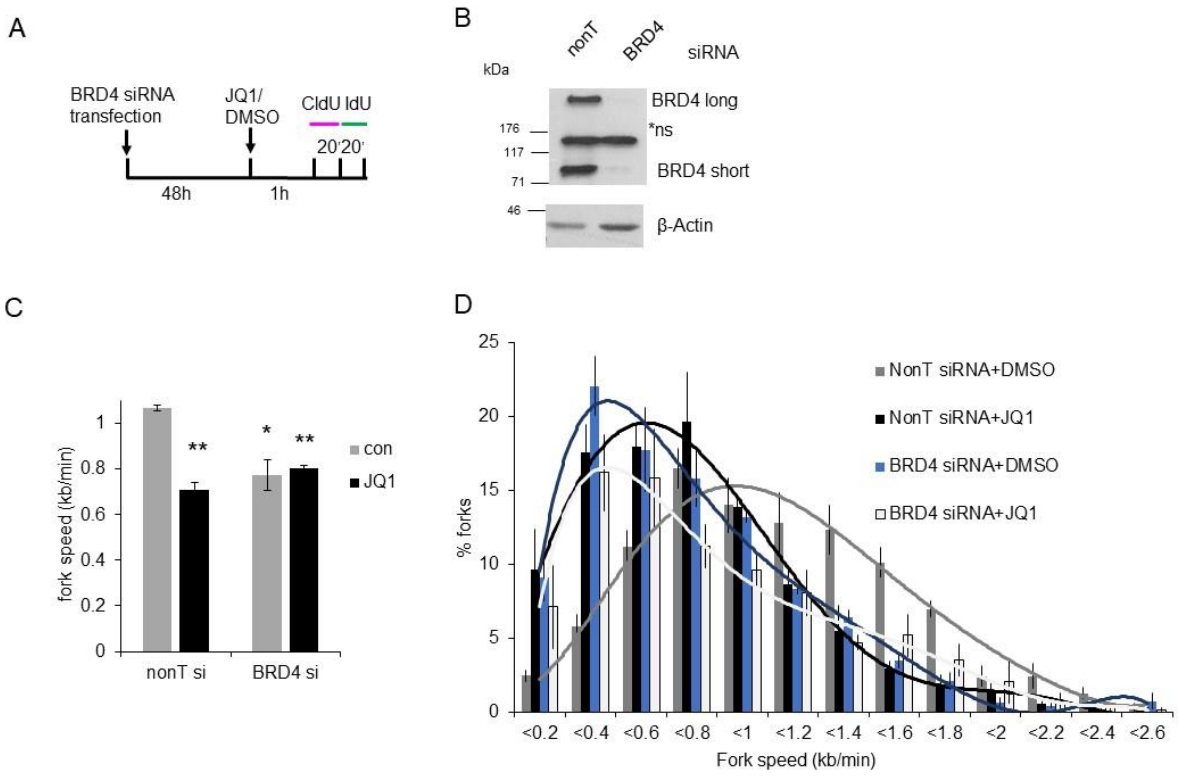


Figure 4.1: BRD4 depletion induces fork slowing.

A) U2OS cells were transfected with BRD4 siRNA or control siRNA (nonTsi). Cells were processed for DNA fibre analysis 48 hours later. B) Protein levels of BRD4 and Actin after siRNA transfection. C) Average median replication fork speeds of cells treated with NonTsi or BRD4 siRNA +/- 1 µM JQ1 treatment for 1 hour. D) Distribution of replication forks in cells treated with NonTsi or BRD4 siRNA +/- 1 µM JQ1 treatment for 1 hour. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01.)

Rescue experiments were also performed to test whether the BRD4 siRNA-induced replication fork slowing can be rescued when re-expressing wild type BRD4. To do this we combined siRNA and plasmid transfections for 48 hours using the Transit-X2 transfection reagent. Either cells were treated with control siRNA or BRD4

siRNA with either empty vector or BRD4 long isoform-expressing plasmid (Figure 4.2 A). To show that the plasmid transfections had worked, we first tried a Western blot. However, due to technical difficulties in trying to visualise the antibody against the long isoform plasmid we decided to use an alternate method. We showed that the plasmid transfections worked by observing emGFP-BRD4 expression in cells. Cells that were treated with the BRD4 plasmid showed Green fluorescent protein (GFP) expression indicating successful transfections (Figure 4.2 B). DNA fibres were performed after these transfections. We observed that expressing BRD4 long isoform in cells treated with BRD4 siRNA restored fork speeds towards control fork speeds (Figure 4.2 C-D). When BRD4 plasmid transfection was combined with control siRNA, this had a slight effect on fork speeds, causing mild replication fork slowing (Figure 4.2 C-D). This data together shows fork speeds in BRD4-depleted cells can be rescued by re-expression of BRD4. This indicated that the BRD4 siRNA specifically knocked down BRD4 in U20S cells including BRD4 long isoform. This also indicates that the loss of BRD4 alone impacts on normal ongoing replication in the cell.

Thus, all this data taken together supports that BRD4 plays an important role in maintaining DNA replication in cells.

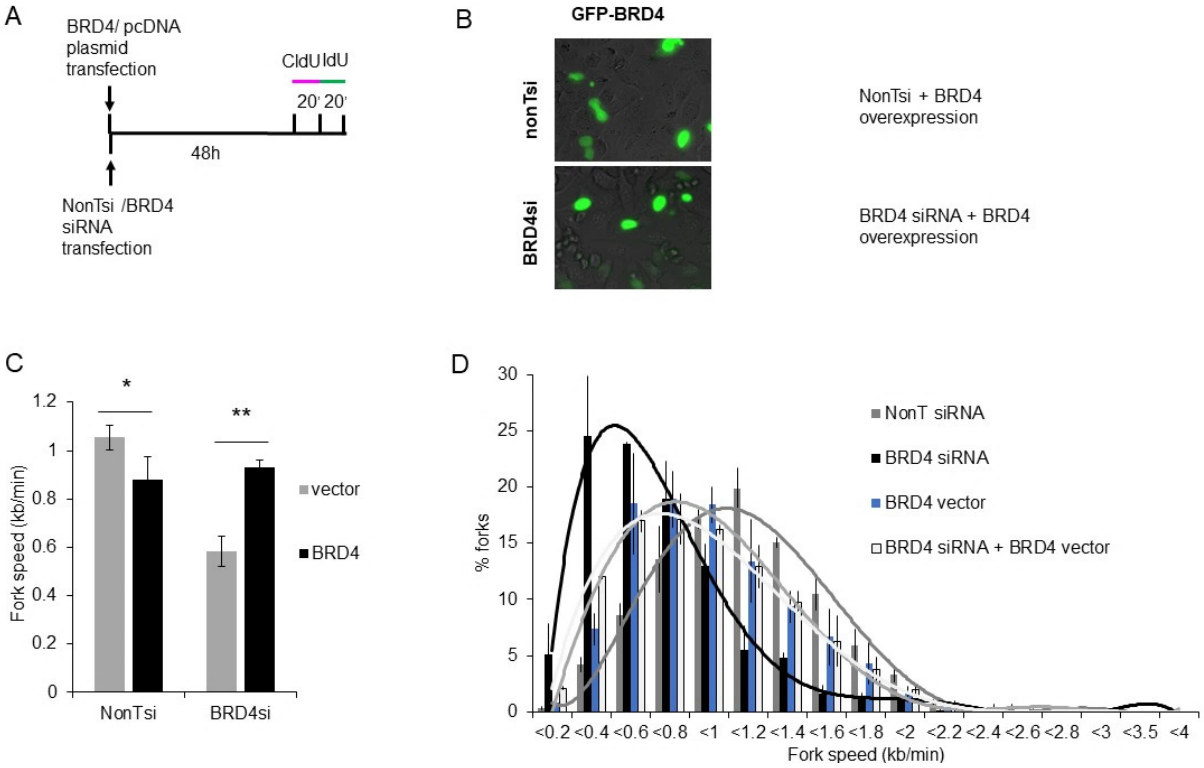


Figure 4.2: BRD4 long isoform-expressing plasmid rescues BRD4 depleted fork slowing.

A) DNA fibre labelling after cells treated with either pcDNA or BRD4 long isoform expression for 48 hours with either nonTsi or BRD4 siRNA treatment. B) Representative pictures of EmGFP-BRD4 expression in U2OS cells after plasmid transfection with and without BRD4 siRNA treatment. C) Average median replication fork speeds with cells treated pcDNA or BRD4 long isoform expression plasmids +/- BRD4 siRNA treatment. D) Distribution of replication forks with cells treated pcDNA or BRD4 long isoform expression plasmids +/- BRD4 siRNA treatment. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01.)

4.3: BRD4 depletion leads to increased RNA synthesis

BRD4 depletion led to fork slowing, similar to that seen with BET inhibitor treatment. This then led to the idea that BRD4 depletion may also promote increased RNA synthesis mirroring BET inhibitor treatment. An EU assay was performed after both 24 and 48 hours BRD4 siRNA treatment (Figure 4.3 A). We observed that as early as 24 hours of BRD4 depletion, there is an increase in total RNA synthesis (30% increase) which is similarly maintained in cells after 48 hours of BRD4 depletion (Figure 4.3 B-C). This is similar to what is observed with BET inhibitor treatment. This data shows that BRD4 depletion leads to increased RNA synthesis again mirroring the phenotypes shown with BET inhibitor treatment.

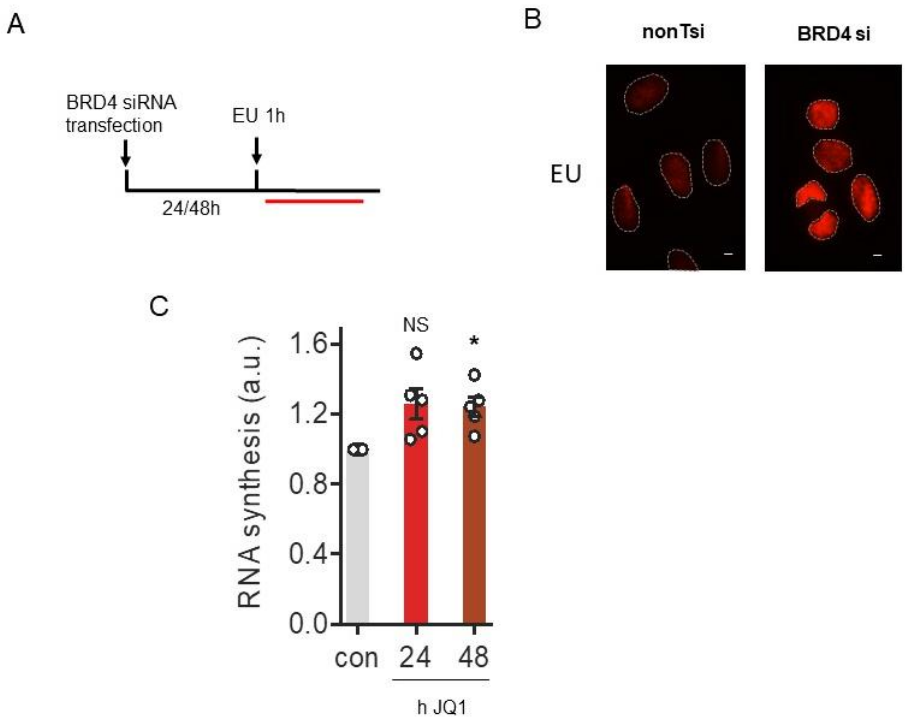


Figure 4.3: BRD4 depletion causes increase in total RNA synthesis.

A) U2OS cells were treated with nonTsi or BRD4 siRNA treatments and transcription elongation was quantified by incorporation of EU into the cells after 24- or 48-hours siRNA treatment. B) Representative images of click-stained EU cells with either nonTsi or BRD4 siRNA treatments for 48 hours. Scale bar 10 µm C) Nuclear EU intensities after BRD4 siRNA treatment after 24 and 48 hours. (N=5, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, NS= Not significant.)

4.4: BRD4 loss-induced replication fork slowing depends on transcription-replication conflicts

As the phenotypes of increasing RNA synthesis and replication fork slowing were seen in cells depleted of BRD4 protein, we wanted to investigate whether transcription-replication interference was a major cause of this fork slowing. To do this we used a similar DNA fibre protocol as previously with JQ1 (Figure 3.9 A). Three transcriptional inhibitors DRB (100 µM for 1 hour), triptolide (1 µM for 1 hour) and α-amanitin (10 µg/µl for 4 hours) were added after BRD4 siRNA treatment for 48 hours followed by DNA fibre labelling (Figure 4.4 A). The same effect was seen as was observed earlier with JQ1 treatment, wherein all three transcriptional inhibitors lead to a rescue of BRD4 depletion-induced fork slowing towards the control levels (0.66 kb/min in BRD4 siRNA treated cells to 0.84-1.03 kb/min in BRD4 siRNA treated cells combined with transcription inhibitors (Figure 4.4 B-C). The transcriptional inhibitors had slight effects of fork slowing when combined with control siRNA (Figure 4.4B). However, the levels of fork slowing observed where quite minimal, with forks still progressing at least 0.83 kb/min. This also does not impact the conclusion of this experiment that by inhibiting transcription in BRD4 depleted cells we observe a rescue in fork speeds.

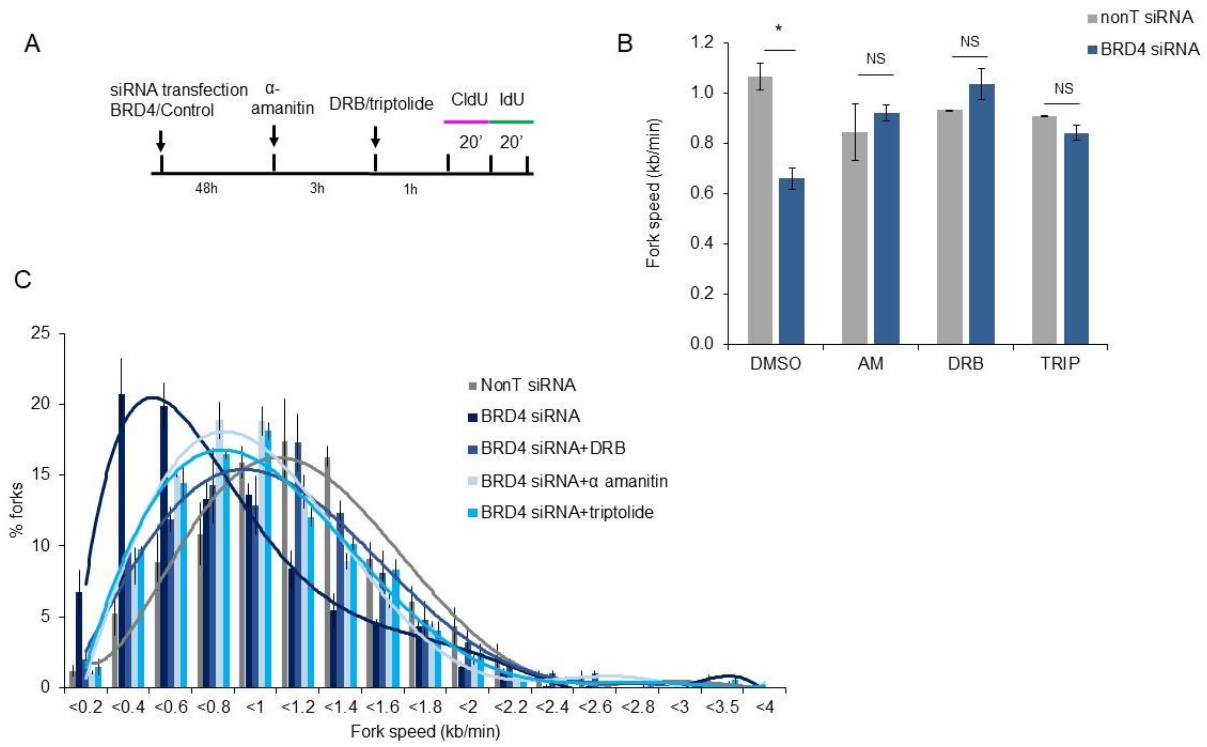


Figure 4.4: Transcriptional inhibitors rescue BRD4 depletion induced fork slowing.

A) DNA fibre labelling in U2OS cells with transcriptional inhibitors α-amanitin (AM,10 µg/ml) for 3 hours or DRB (100 µM) or Triptolide (TRIP,1 µM) for 1 hour with BRD4 siRNA or nonTsi treatment for 48 hours. B) Average median of replication fork speeds treated with transcriptional inhibitors α-amanitin, DRB or Triptolide with BRD4siRNA or nonTsi treatment for 48 hours. C) Distribution of replication fork speeds in cells treated transcriptional inhibitors α-amanitin, DRB or Triptolide with BRD4siRNA or NonTsi treatment for 48 hours. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, NS= Not significant.)

4.5: BRD2 and BRD3 loss has no major effect on replication and RNA synthesis

In the previous experiments we had observed that BRD4 depletion had mirrored the phenotype of BET inhibitor treatment, hence indicating it as a target of BET inhibitor induced fork slowing. However, BRD2 and BRD3 had not been tested to see if they may also have the same effect. BRDT was excluded from this as it is only expressed in testes so would not be expressed in U2OS cells (Pivot-Pajot, Caron et al. 2003) (Appendix A1).

Firstly, we investigated the effects of BRD2 depletion. We treated the cells with BRD2 siRNA for 48 hours (Figure 4.5 A). Using Western blot, we observed that BRD2 siRNA treatment for 48 hours led to the depletion of BRD2 protein in the cell (Figure 4.5 B). To test whether the same phenotypic effects observed with BET inhibitor treatment were seen in BRD2 depleted cells we used the DNA fibre assay and EU assay. Using the same approach detailed previously for BRD4, we observed that BRD2 depletion caused no major changes in fork speed (0.91 kb/min – 0.84 kb/min) (Figure 4.5 C). JQ1 treatment still caused fork slowing in combination with BRD2 depletion (Figure 4.5 C). BRD2 depletion also caused a slight decrease in EU intensity (by 0.18%) indicating a slight decrease of total RNA synthesis in BRD2 depleted cells (Figure 4.5 C). These results show that BRD2 depleted cells do not mirror the phenotype induced by BET inhibitors or BRD4 depletion.

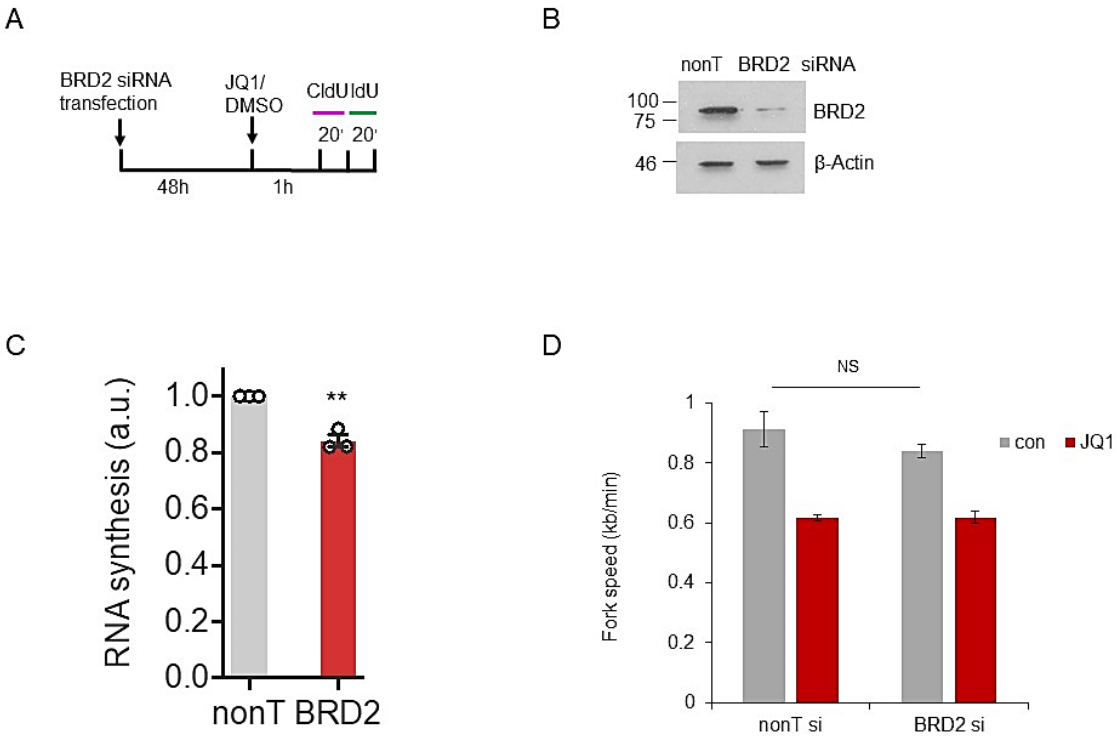


Figure 4.5: BRD2 depletion shows no significant changes in fork slowing or total RNA synthesis.

A) DNA fibre labelling in U2OS cells with BRD2 siRNA or NonTsi treatment for 48 hours. B) Protein levels of BRD2 after siRNA depletion. C) Nuclear EU intensities after BRD2 siRNA treatment after 48 hours. D) Average median replication fork speeds of cells treated with NonTsi or BRD2 siRNA +/- 1 µM JQ1 treatment for 1 hour. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. ** P value <0.01, NS= Not significant.)

Lastly, we looked into the effects of BRD3 depletion. We treated the cells with BRD3 siRNA for 48 hours (Figure 4.6 A). Using Western blot, we observed that this led to the depletion of BRD3 protein in the cell (Figure 4.6 B). To test whether the same phenotypic effects caused by BET inhibitor treatment were seen in BRD3 depleted cells we used the DNA fibre assay and EU assay. Using the same approach detailed previously for BRD4, we observed that BRD3 depletion caused no major changes in fork speed (1.10 kb/min – 0.95 kb/min) (Figure 4.6 D). JQ1 treatment induced fork slowing in combination with BRD3 depletion. BRD3 depletion also caused no change in EU intensity indicating there are no changes of total RNA synthesis in BRD3 depleted cells (Figure 4.6 D). These results show that BRD3 depleted cells do not mirror the phenotypes induced by BET inhibitors or BRD4 depletion.

As this excluded roles for BRD2 and BRD3 in replication-transcription conflicts induced by BET inhibitor treatment, we decided not to pursue any additional experiments with these proteins.

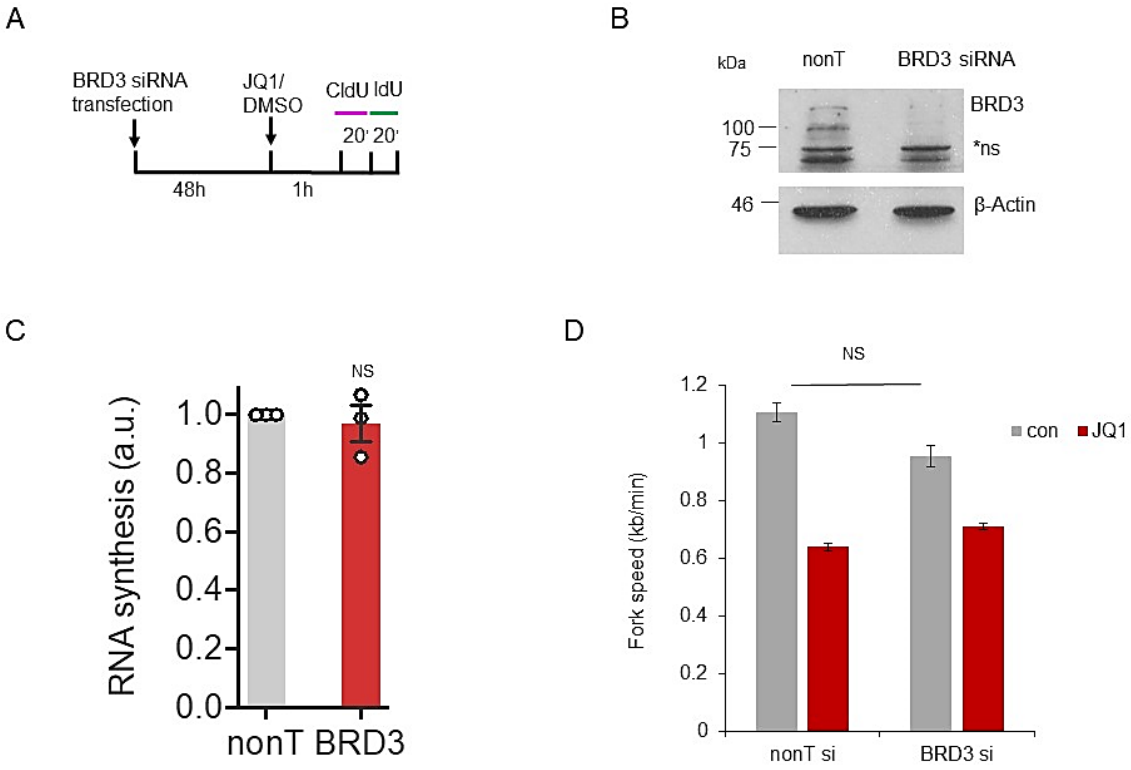


Figure 4.6: BRD3 depletion shows no significant changes in fork slowing or total RNA synthesis.

A) DNA fibre labelling in U2OS cells with BRD3 siRNA or nonTsi treatment for 48 hours. B) Protein levels of BRD3 after siRNA depletion. C) Nuclear EU intensities after BRD3 siRNA treatment after 48 hours. D) Average median replication fork speeds of cells treated with NonTsi or BRD3 siRNA +/- 1 µM JQ1 treatment for 1 hour. (N=3, error bars: S.E.M.)

4.6: BRD4 knockdown does not activate a replication stress response

As previously shown in Chapter 3, JQ1 does not induce a DNA damage response despite replication fork slowing. BRD4 depleted cells had shown a similar phenotype to JQ1 with induced fork slowing and increased RNA synthesis. We now wanted to test whether BRD4 depletion could activate a DNA damage response. To look at this we used the same method as before with JQ1, performing immunofluorescence to analyse γH2AX and 53BP1 foci in presence of BRD4 depletion.

Like JQ1 treatment, BRD4 depletion caused no increase in the amount of foci compared to control (Figure 4.7). This data indicates that when cells are BRD4 depleted an ATR response is not activated and there is no DNA damage response despite replication fork slowing.

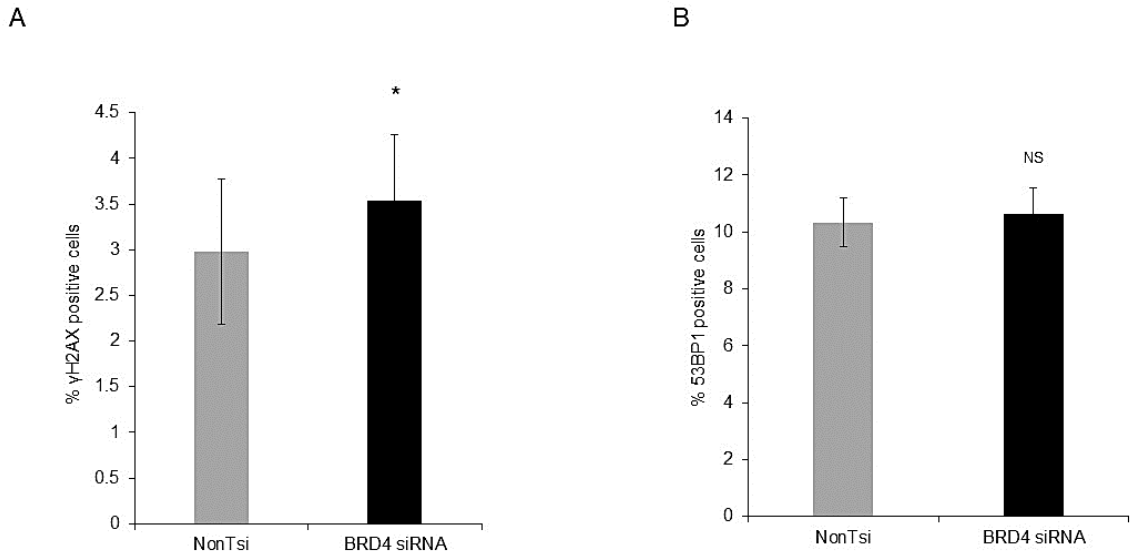


Figure 4.7: BRD4 depletion does not activate the replication stress response pathway.

A) Percentages of cells containing more than 8 γH2AX after BRD4 depletion. B) Percentages of cells containing more than 8 53BP1 after BRD4 depletion. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, NS= Not significant.)

4.7: Summary

The main aim for this chapter was to establish which BET protein was a specific target for BET inhibitors in inducing transcription-replication interference. There are four members of BET proteins of which three were thought to be a possible target for BET inhibitor-induced transcription-replication interference in U2OS cells.

This chapter first investigated the effects of loss of BRD4 activity. BRD4 is the most studied member of the BET proteins. BRD4 is a transcriptional and epigenetic regulator but also has a number of different functions ranging from regulating development to regulating cell cycle progression (Yang, He et al. 2008, Di Micco, Fontanals-Cirera et al. 2014). When cells were depleted of BRD4, the effects mirrored those seen when cells were treated with BET inhibitors JQ1 or IBET151. Forks were slowed down, and total RNA synthesis had increased too (Figures 4.1 and 4.3). By inhibiting RNA synthesis in the same way as done with JQ1 this fork slowing could be rescued (Figure 4.4). This indicated to us that BRD4 was important in preventing transcription-replication interference and was crucial in maintaining normal fork speeds in the cell. We can also conclude that BET inhibitors work by inhibiting BRD4 and hence we see a similar phenotype. This can be shown when JQ1 addition to BRD4 depleted cells caused no additional fork slowing (Figure 4.1). It is important to note that fork speeds were rescued after 24 hours with JQ1 but not after BRD4 depletion. The exact reason for this is still unclear but may be due to cells being able to adapt to drug treatment, but unable to adapt when BRD4 is depleted.

While this was a positive result, there were still BRD2 and BRD3 which had not been tested before. Both BRD2 and BRD3 are involved in transcriptional regulation as well. We depleted both BRD2 and BRD3 in cells and observed that depletion of either protein caused no fork slowing or significant changes in RNA synthesis. This indicated that neither BRD2 nor BRD3 were involved in maintaining replication fork progression (Figures 4.5 and 4.6). As neither BRD2 nor BRD3 mirrored what was seen during BET inhibitor JQ1 treatment we concluded that they must not be the target of BET inhibitor induced replication-transcription interference. From these results we can be pretty certain that BRD4 is the most important BET protein target in replication fork progression that prevents fork slowing via transcription-replication interference. By establishing BRD4 as the only BET protein family member to phenotypically mirror BET inhibitors in our system, we have established a potential new role of BRD4 in fork maintenance. This could be important in a better understanding of BRD4 role in the cell as well as potential importance in future therapeutics. Recently studies have used a specific molecule (dBET6) to degrade BRD4 (Winter, Mayer et al. 2017). As BET inhibitors are non-specifically targeting all BET proteins this new molecule could provide useful in future therapeutics, especially since only BRD4 is implicated in maintaining normal replication fork progression.

BRD4 depletion led to replication fork slowing which normally leads to the activation of replication stress response pathway driven by ATR. Previously, we observed that there was no activation of this response with JQ1 (Figure 3.14). We also observed the same lack of response with BRD4 depletion (Figure 4.7). There was no clear

increase in γH2AX or 53BP1 foci. This again mirrors what is seen with BET inhibition. This showed that the lack of replication stress response activation in BET inhibitor treated cells is not due to a side effect of JQ1 treatment on the cell, but potentially a mechanism that involves BRD4 being inhibited in cells.

Now we know that BRD4 is the target of BET inhibition and it leads to increased transcription-replication interference, the main issues arising from this chapter are firstly what happens after BRD4 depletion induced fork slowing. Is there some mechanism preventing damage in these cells? Secondly, what causes this increase in RNA synthesis and whether it is a similar mechanism to BET inhibitors?

5. BET inhibitor-induced replication-transcription conflicts require HEXIM1

5.1: Introduction

Our previous results showed that BET inhibition, and BRD4 depletion, led to an increased amount of total RNA synthesis. Increased RNA synthesis led to more transcription-replication interference causing a slowing of replication forks. Thus far the mechanism of how RNA synthesis increases after BET inhibition was unknown and required further investigation.

As mentioned previously, BET proteins are recruited to acetylated lysine residues on histone tails which then act to further recruit transcription factors to chromatin. One such transcription factor is P-TEFb which is required for RNA Pol II to undergo productive elongation. P-TEFb is a heterodimeric complex consisting of the catalytic CDK9 kinase and a regulatory CycT1 subunit (Price 2000, Zhou, Li et al. 2012). P-TEFb can form active complexes with either BRD4 or the Super Elongation complex (SEC) and once activated can lead to the phosphorylation of CTD on RNA Pol II activating transcription initiation and elongation. The understanding of P-TEFb function was obtained using transcriptional inhibitor DRB which inhibits the CDK9 kinase activity, hence stops CTD phosphorylation and inhibits transcription elongation (Marshall, Peng et al. 1996). P-TEFb can also be held in an inactive complex with 7SK-snRP which forms with HEXIM1 (Quaresma, Bugai et al. 2016).

Recent studies have shown a mechanism in which loss of P-TEFb activation by BRD4, induced by BET inhibitors JQ1 or I BET-151, leads to compensatory disruption of the 7SK-P-TEFb complex leading to increased free (active) P-TEFb in the cells (Bartholomeeusen, Xiang et al. 2012, Chaidos, Caputo et al. 2014). Interestingly JQ1 treatment has been shown to increase HEXIM1 levels across cell lines (Bartholomeeusen, Xiang et al. 2012, Devaraj, Fiskus et al. 2016, Huang, Garcia et al. 2016, Zhu, Enomoto et al. 2017).

The main aim of this chapter is to try and elucidate how this increase in RNA synthesis occurs after BET inhibition or BRD4 loss. Secondly, we will try and investigate the role of HEXIM1 and 7SK-P-TEFb in this process and test whether they could be involved in a potential mechanism of increased total RNA synthesis.

5.2: BET inhibition leads to RNA Pol II phosphorylation

Firstly, we wanted to investigate the potential evidence for BET inhibition leading to the disruption of the 7SK complex. As shown in figure 5.1 A, P-TEFb can be sequestered by HEXIM1 of the 7SK complex. JQ1 can lead to the disruption of this complex, which leads to more free P-TEFb which can then be recruited to the RNA Pol II either via BRD4 or the SEC. This can lead to increased phosphorylation of serines on the CTD on RNA Pol II by P-TEFb. These phosphorylation events lead to RNA Pol II being able to escape from a paused state to be able to perform productive elongation. This leads to increased RNA synthesis. A feedback loop occurs where RNA Pol II activation leads to more mRNA transcripts of HEXIM1 of

the 7SK complex. This allows 7SK complex to form more complexes with P-TEFb again, hence leading to re-inhibition of P-TEFb as observed by Western blot probing for free P-TEFb (Bartholomeeusen, Xiang et al. 2012).

To look at whether this mechanism could be why we see increased RNA synthesis, we first looked at whether we observed an increase in serine phosphorylation of RNA Pol II. To investigate this we looked at CTD serine 2 and serine 5 phosphorylation. Both of these residues are on the CTD and involved in helping RNA Pol II produce stable elongation (Buratowski 2009, Zaborowska, Egloff et al. 2016). We first tried to use Western blots to look at serine 2 phosphorylation, however we had some technical problems with this approach. We then used immunofluorescence after BET inhibitor treatment and quantified serine 2 phosphorylation intensity. We observed an increase in this phosphorylation after BET inhibition treatment for 1 to 8 hours which returns to normal levels after 24 hours (Figure 5.1 B-C). We looked at the effects on serine 5 phosphorylation as well and observed an increase in phosphoylation after 1 hour using Western blots (Figure 5.1 D-E). However, the results for later timepoints were not as reproducible and gave rise to inconsistent results (Figure 5.1 E). Potentially, this could be due to technical issues with the Western blot and using another technique may have helped to produce more re-producible results.

Taken together these results indicate that BET inhibitor treatment in the first few hours leads to increased phosphorylation of the CTD on RNA Pol II.

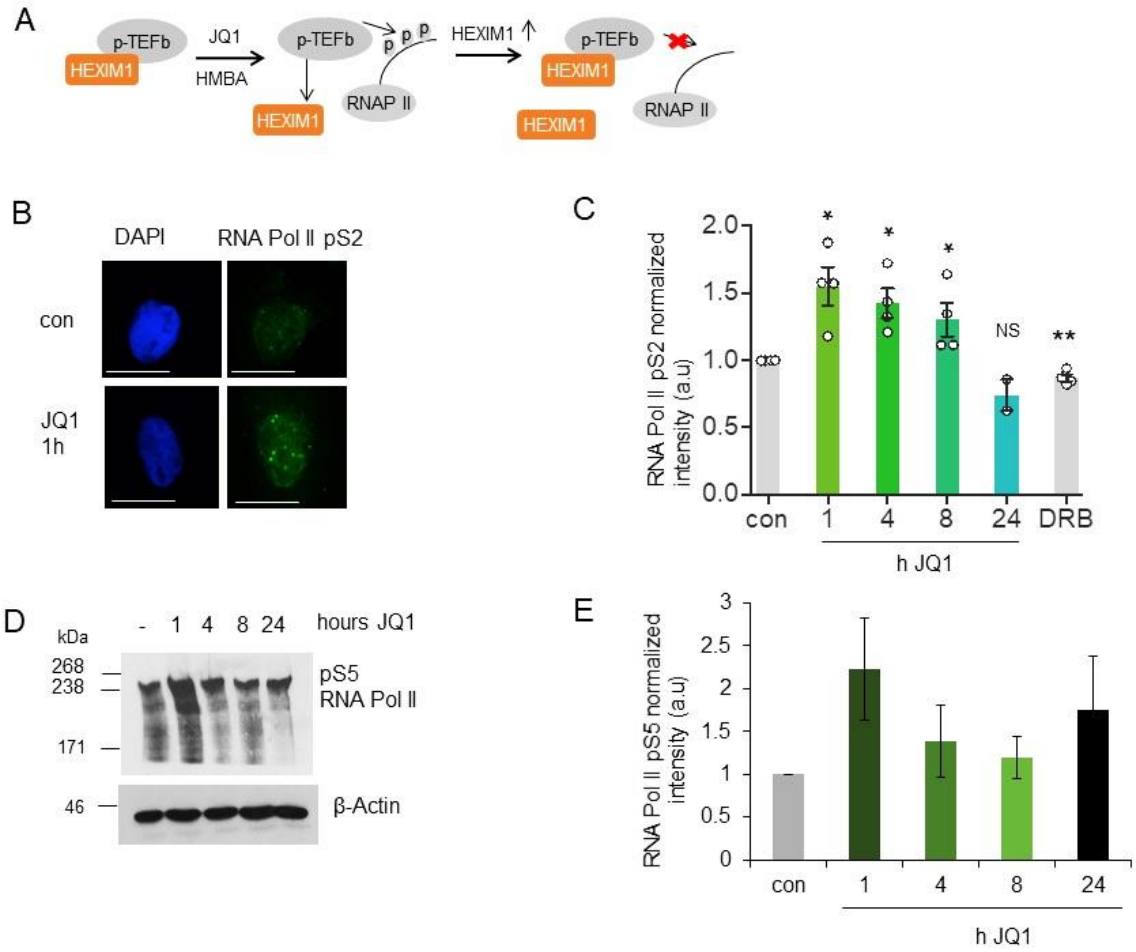


Figure 5.1: JQ1 leads to increases in RNA polymerase II S2 phosphorylation.

A) Current model of the role of HEXIM1 in JQ1-induced transcription increase. B) Representative images of nuclear foci for phospho-S2 RNA polymerase II and DAPI +/- JQ1 1 µM JQ1 treatment. Scale bar 10 µm C) Nuclear phospho-S2 RNA polymerase II intensity after 1µM treatment for 1, 4, 8- and 24-hour treatments or 10 µM DRB. D) Representative protein levels of phospho-S5 RNA polymerase II after 1 µM JQ1 treatment for 1, 4, 8 and 24 hours. E) Quantification of protein levels after 1 µM JQ1 treatment for 1, 4, 8 and 24 hours (N=4, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value <0.01, NS= Not significant).

5.3: HEXIM1-7SK disruption leads to same phenotype as BET inhibition

The previous result supported the hypothesis shown in Figure 5.1 A. We further wanted to confirm that disrupting the complex between 7SK-HEXIM1 and P-TEFb can lead to the same phenotypes observed with BET inhibitor treatment. This would provide further evidence that the disruption if the 7SK-HEXIM1 complex is the underlying cause of BET inhibitor-induced fork slowing.

To test this, we needed a drug that is known to disrupt the 7SK-HEXIM1 complex from P-TEFb. HMBA is a drug known to be able to dissociate 7SK-snRP from P-TEFb. HMBA is a hybrid bipolar compound which can induce cell differentiation in transformed cells. HMBA leads to the activation of the PI3K/Akt signalling pathway, which in turn leads to HEXIM1 being phosphorylated which disrupts the 7SK-HEXIM1 complex leading to increasd free P-TEFb (Contreras, Barboric et al. 2007, Fujinaga, Luo et al. 2015).

To test whether HMBA mirrors the phenotype of BET inhibitor treatment we first looked at fibre experiments to see the effect on fork speeds. HMBA has no known effect on replication so any effects seen would be due to the 7SK complex disruption. We used two concentrations, 5 mM and 10 mM, as these concentrations had been effective in previous literature (Napolitano, Varrone et al. 2007, Bartholomeeusen, Xiang et al. 2012). We tested the effect of HMBA on RNA synthesis using the EU assay. We observed that the nuclear EU intensity increased with HMBA treatment with 5 mM (+21%) and 10 mM (+14%) indicating an increased amount of total RNA synthesis (Figure 6.2 B). We then performed DNA fibre assays

where HMBA was added to the cells 1 hour before cells were labelled, spread and stained (Figure 6.2 A). HMBA treatment led to signinifcant fork slowing compared to control with either 5 mM (1.05 kb/min in control treated samples to 0.66 kb/min) or 10 mM concentrations (1.05 kb/min in control treated samples to 0.65 kb/min) (Figure 5.2 C-D). There was no additional fork slowing with JQ1 co-treatment with HMBA, indicating that JQ1 had an identical method of fork slowing (Figure 5.2 C-D).

These results taken togther show HMBA mirrors the phenotype seen with BET inhibitors, indicating that BET inhibitor-induced fork slowing is due to the 7SK complex being disrupted. A study has suggested that HMBA can function as a BET inhibitor and disrupts BET proteins from chromatin (Nilsson, Green et al. 2016). As we know that HMBA disupts the 7SK complex, this would again support that BET inhibitors cause fork slowing by disturbing the inhibitory complex of P-TEFb.

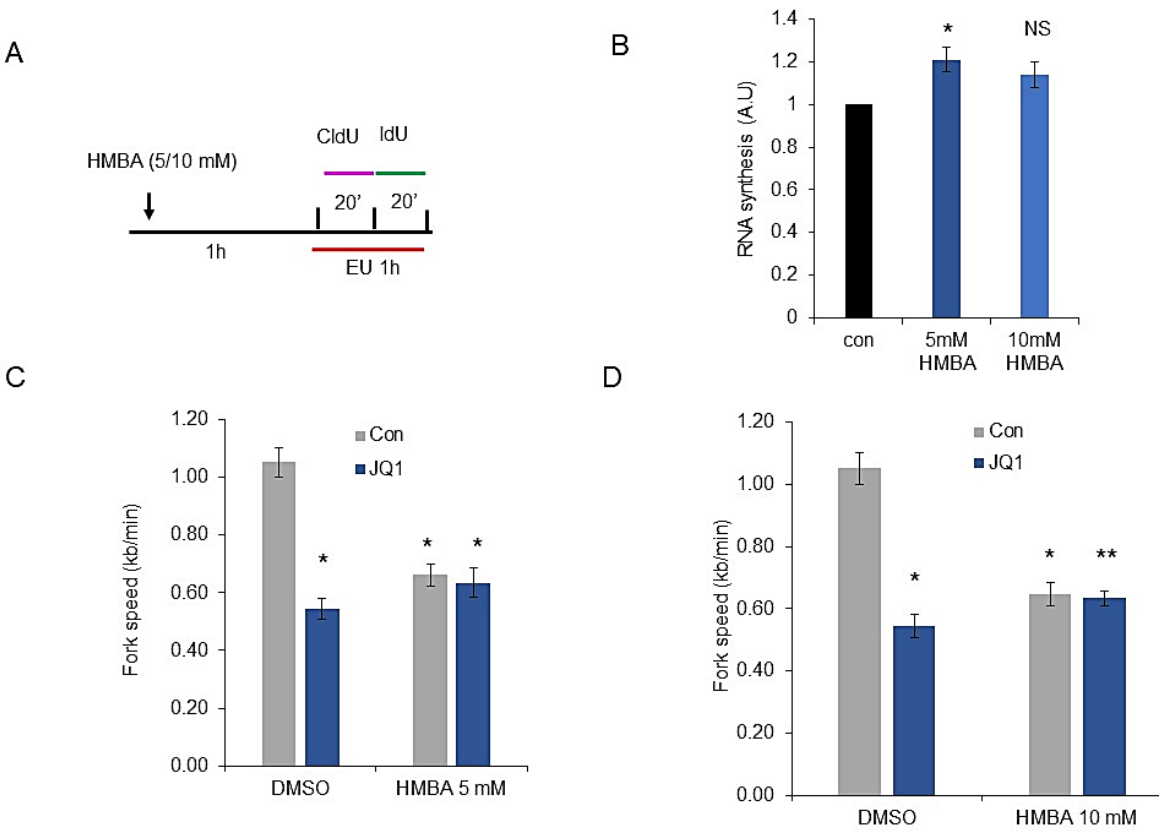


Figure 5.2: HMBA treatment leads increased RNA synthesis and increased fork slowing.

A) DNA fibre labelling and quantification of incorporation of EU were performed in U2OS cells after treatment with HMBA (10 or 5 mM) for 1 hour. B) Average median replication fork speeds with cells treated with HMBA 5 mM +/- 1 µM JQ1 treatment for 1 hour. C) Average median replication fork speeds with cells treated with HMBA 10 mM +/- 1 µM JQ1 treatment for 1 hour. D) Nuclear EU intensities after treatment with HMBA 5 mM or 10 mM for 1-hour treatment. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value <0.01, NS= Not significant.)

5.4: BRD4 requires interaction with P-TEFb to maintain normal fork progression

As we hypothesised that BET inhibitor induced fork slowing was caused by the disruption of the inhibitory complex of P-TEFb, we wanted to invesitgate if loss of the BRD4-P-TEFb interaction is involved in fork slowing when BRD4 is depleted from cells.

BRD4 has three different isoforms, isoform A, isoform B and isoform C. Isoform A, which is commonly referred to as the long isoform, includes a CTD which can interact with P-TEFb. Isoform B lacks the CTD but has a unique 75 amino acid extension instead. Isoform C, commonly referred to as the short isoform, lacks this CTD and therfore cannot interact with P-TEFb (Figure 5.3 A) (Sakamaki, Wilkinson et al. 2017). BRD4 siRNA had been shown to knock down both the long and short isoforms (Figure 4.1 B). We had also shown that we could rescue the BRD4 depletion-induced fork slowing by re-expressing BRD4 long isoform plasmid (Figure 4.2 D). We wanted to see what the effect would be when re-transfecting BRD4 depleted cells with the short isoform which cannot interact with P-TEFb but still interacts with replication proteins RFC,TICRR and CDC6 (Schroder, Cho et al. 2012, Sakamaki, Wilkinson et al. 2017).

To do this we used combined siRNA and plasmid transfections for 48 hours using the Transit-X2 reagent. Either cells were treated with control siRNA or BRD4 siRNA

with either pEGFP-C2 control plasmid or the BRD4 short form isoform-expressing plasmid (Figure 5.3 B). We first used Western blots to confirm that we can re-express the BRD4 short isoform even in combination with BRD4 siRNA treatment (Figure 5.3 C). We next performed DNA fibre assays after these transfections (Figure 5.3 B). We observed that when combined with BRD4 depletion, short isoform re-expression does not rescue the fork speeds, which are still slowed to the same level as after BRD4 depletion alone (from 1.01 kb/min to 0.63 kb/min) (Figure 5.3 D).

These results showed that, unlike the long isoform of BRD4, the short isoform of BRD4 cannot rescue the BRD4 loss-induced fork slowing. This indicates a role for the interaction of BRD4 with P-TEFb, but not the known interactions with replication proteins, in maintaining replication fork progression.

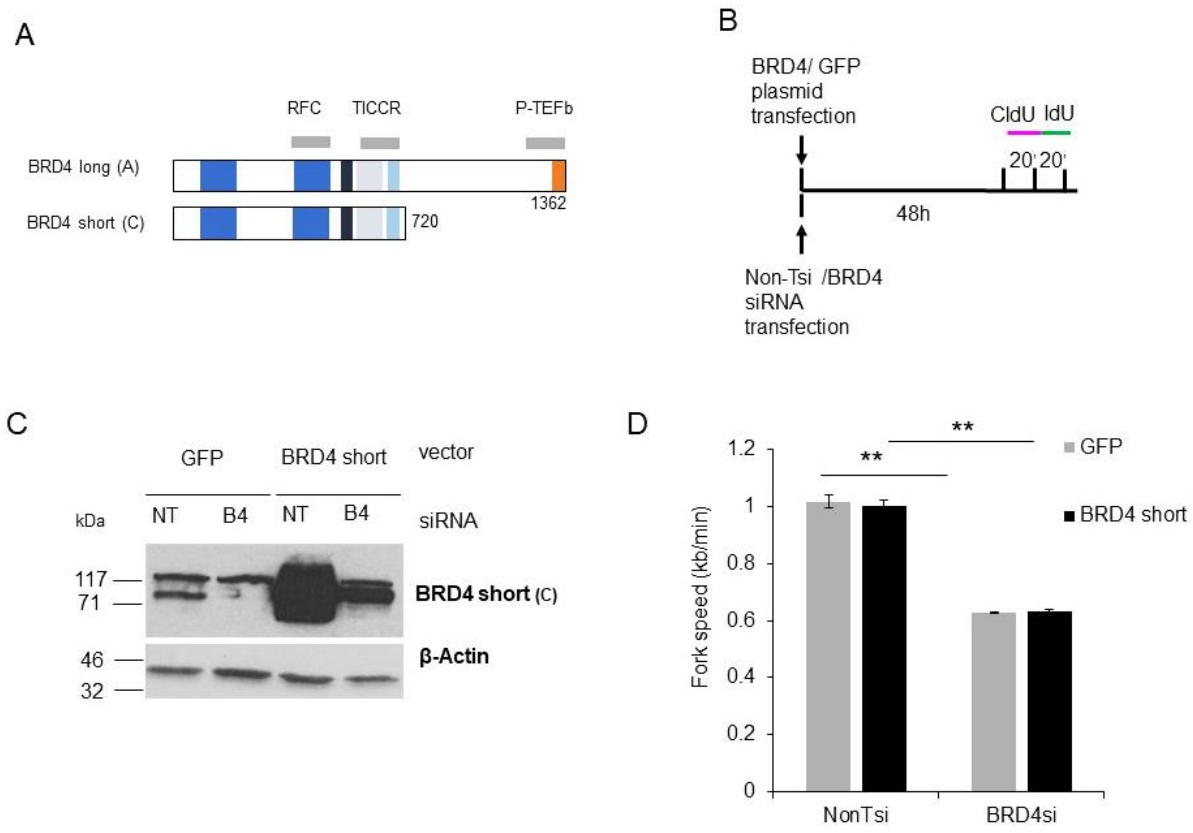


Figure 5.3: BRD4 short isoform expression does not rescue fork slowing after BRD4 depletion.

A) Schematic of BRD4 long and short isoforms. B) DNA fibre labelling after cells treated with either GFP or BRD4 short isoform expression for 48 hours with either nonTsi or BRD4 siRNA treatment. C) Protein levels of BRD4 short isoform +/- BRD4 siRNA treatment and +/- BRD4 short isoform plasmid transfection. D) Average median of replication fork speeds with cells treated GFP or BRD4 short isoform expression plasmids +/- BRD4 siRNA treatment. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test: ** P value <0.01)

5.5: Replication-transcription conflicts induced by BET inhibition require HEXIM1

We had shown that P-TEFb had a role in BET inhibitor induced fork slowing and increased total RNA synthesis. Our hypothesis stated that after P-TEFb was realeased from its 7SK-HEXIM1 inhibitory complex, it led to more RNA Pol II phosphorylation, which would lead to an increase in HEXIM1 levels leading back to P-TEFb being inhibited by the 7SK complex again. We therefore decided it was important to further investigate the role that HEXIM1 played in BET inhibitor induced fork slowing.

Firstly, we wanted to test what happened to the levels of HEXIM1 after 1 to 72 hours of BET inhbitor treatment (Figure 5.4 A). To do this we performed a Western blot with cells treated with either control or HEXIM1 siRNA for 48 hours before being JQ1 treated for another 1 to 24 hours. We firstly observed that the HEXIM1 levels were depleted after treatment with HEXIM1 siRNA for all timepoints with JQ1 treatment. We also observed no significant change in HEXIM1 levels across these treatment times (Figure 5.4 B). To investigate this further we looked at HEXIM1 levels for 48-72 hours after JQ1 treatment and observed a slight increase in HEXIM1 levels compared to control levels (Figure 5.4 C).

These results indicate that we see an increase in HEXIM1 levels after BET inhibitor treatment supporting the model outlined in Figure 5.1 A, however this increase is quite slow and it takes up to 48 hours of JQ1 treatment for HEXIM1 protein levels to increase.

We also tested the HEXIM1 baseline protein expression among all cell lines we had previously tested (Figures 1.6 and 1.7), to try and elucidate whether the baseline HEXIM1 levels correlates with BET inhibitor-induced replication stress.

We tested HEXIM1 protein levels in these cell lines and found that the cell lines in our panel displayed comparable HEXIM1 protein levels (Figure 5.4 D). The non-cancer BJ cells had lower HEXIM1 levels than the cancer lines, the reason for this is unknown. This suggests that JQ1 should be able to induce RNA synthesis and replication stress in all cell lines tested. The reason for the lack of replication stress in C2 cells is still unknown and will require further investigation.

This cell line panel currently does not allow us to draw conclusions about the correlation between HEXIM1 levels and JQ1-induced replication stress, and this would have to be addressed in a larger follow-up study.

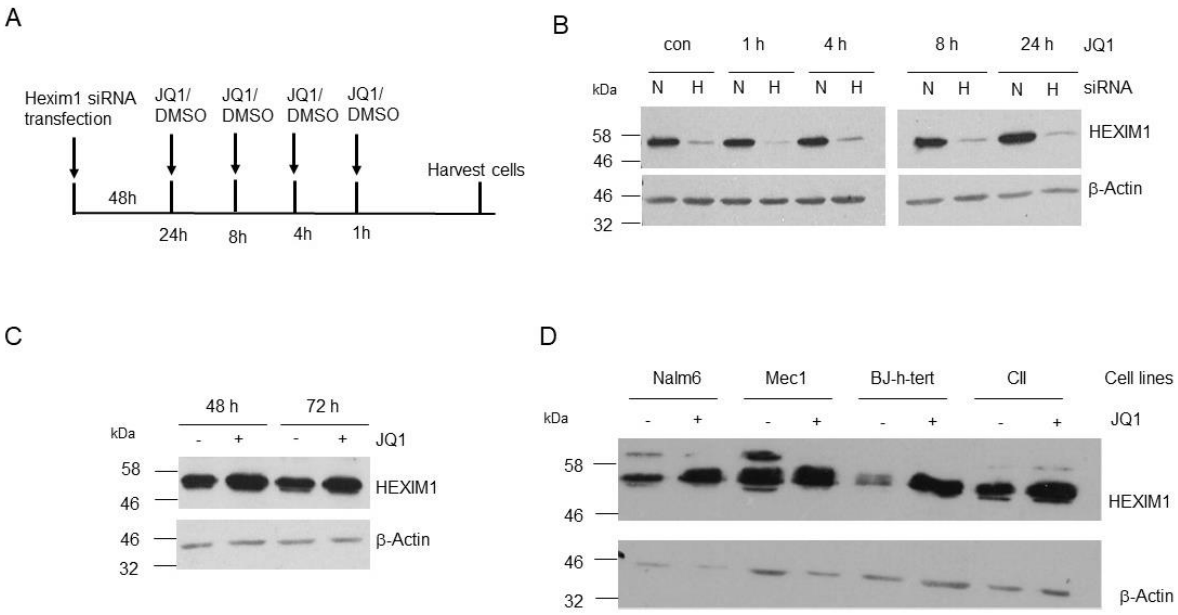


Figure 5.4: HEXIM1 levels after HEXIM1 siRNA depletion with JQ1 treatment.

A) Cells were treated with HEXIM1 siRNA for 48 hours before JQ1 treatment for indicated times before cells were harvested. B) Protein levels of HEXIM1 after 48 hours siRNA transfection followed by 1 µM JQ1 treatment for 1, 4, 8 and 24 hours (n=3). C) Protein levels of HEXIM1 after 1 µM JQ1 treatment for 48 and 72 hours (n=3). D) Protein levels of HEXIM1 with and without 1 µM JQ1 treatment for 1 hour in Nalm6, Mec1, BJ-hTert and C2 cells (n=1).

We decided to further study the role HEXIM1 plays in BET inhibitor induced fork slowing and increased RNA synthesis. To do this we used both the DNA fibre and EU assays. After HEXIM1 siRNA transfection for 48 hours followed by JQ1 treatment for different times, fibres were labelled and spread, or EU was added for 1 hour (Figure 5.5 A). HEXIM1 depletion seemed to delay the BET inhibitor-induced increase in EU intensity (Figure 5.5 B). After 1 hour of BET inhibitor treatment, HEXIM1 depletion actually led to a decrease in EU intensity. The increase in RNA synthesis was comparable to values in NonTsi treated cells after 24 hours after BET inhibitor treatment, where we saw the same levels of EU intensity as seen with JQ1 treatment alone (Figure 5.5 B). We also observed this delayed effect when HEXIM1 is depleted in the DNA fibre assay (Figure 5.5 C). Here when cells are depleted of HEXIM1, cells show no visible fork slowing after BET inhibitor treatment from 1-8 hours. However, cells depleted of HEXIM1 showed fork slowing when combined with JQ1 treatment for 24 hours (Figure 5.5 C).

Together, this data shows that HEXIM1 depletion delays the BET inhibitor induced increase in RNA synthesis as well as delaying fork slowing until after 24 hours of BET inhibitor treatment. This data suggests that HEXIM1 is required for the rapid induction of RNA synthesis and rapid induction of fork slowing by JQ1 treatment.

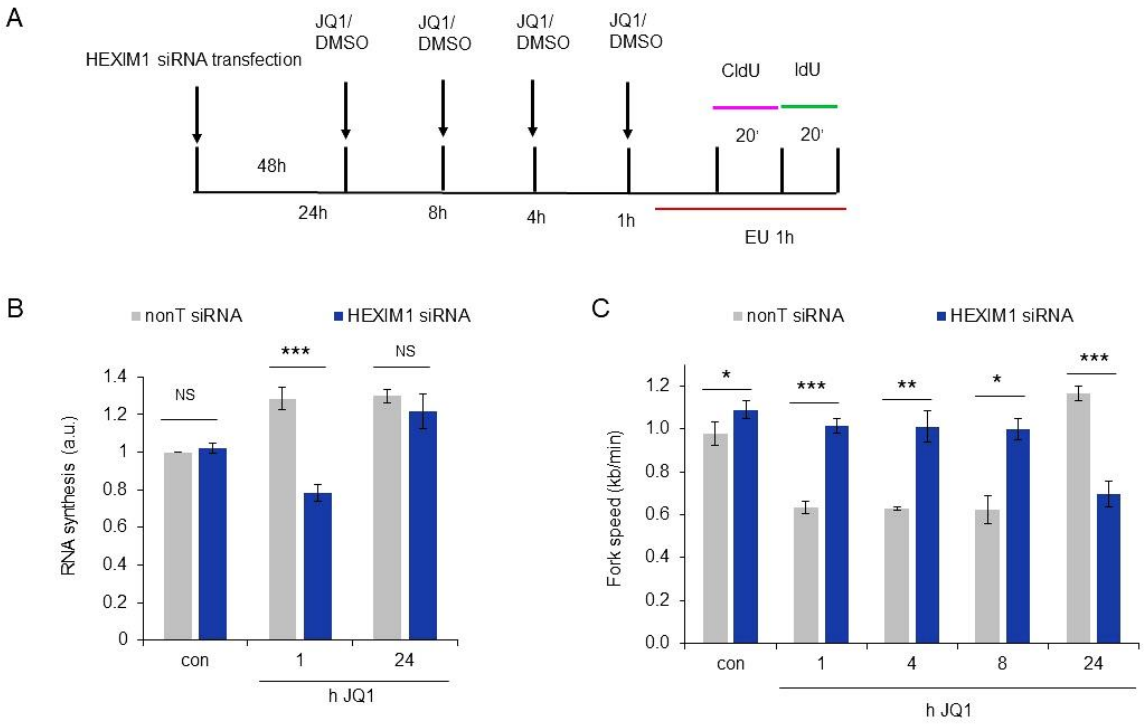


Figure 5.5: HEXIM1 depletion reverses effect on fork speeds and RNA synthesis of JQ1.

A) DNA fibre labelling in U2OS cells were treated with HEXIM1 siRNA for 48 hours before 1 µM JQ1 treatment for indicated times. B) Nuclear EU intensities with HEXIM1 siRNA or NonTsi siRNA treatment with +/- 1 µM JQ1 treatment for 1 and 24 hours. C) Average median replication fork speeds after HEXIM1/ nonTsi siRNA and +/- 1 µM JQ1 treatment for 1, 4, 8 and 24 hours. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value <0.01, *** P value <0.001, NS= Not significant.)

5.6: HEXIM1 depletion leads to increased DNA damage during BET inhibition

We had previously observed that JQ1 treatment or BRD4 loss slowed fork speeds but showed no induction of a downstream replication stress response (Figure 3.14, Figure 4.7). We decided to investigate the relationship between BET inhibition, HEXIM1 and DNA damage. HEXIM1 depletion causes replication forks to slow later during JQ1 treatment, when gene expression changes might make them more vulnerable to damage.

To test this, we used the same methods as before, using immunofluorescence to look at γH2AX and 53BP1 foci after HEXIM1 depletion coupled with JQ1 treatment (Figure 5.6 A). HEXIM1-depleted cells showed more active DNA damage signalling after 24 h JQ1, in contrast to control cells as there were increases in percentage of 53BP1 foci positive cells (from 9% to 15%) (Figure 5.6 B-C) and γH2AX positive cells (from 2% to 5%). Unexpectedly, HEXIM1-depleted cells also accumulated DNA damage early during JQ1 treatment (Figure 5.6 B-C). This suggests that HEXIM1 prevents JQ1-induced DNA damage. DNA damage can contribute to cell death, so Dr Eva Petermann performed colony assays to see how sensitive cells depleted of HEXIM1 were to JQ1. HEXIM1-depleted cells were more sensitive to 24-hour JQ1 treatment than control-depleted cells (Figure 5.6 D). We also compared the effect of HEXIM1 depletion to the effect of ATR inhibition, a treatment that induces high amounts of DNA damage. We observed that both HEXIM1

depletion alone and ATR inhibitor alone lead to similar sensitivity and observed that ATR inhibition in combination with HEXIM1 depletion leads to increased sensitivity of cells to JQ1. Cells depleted of HEXIM1 were just as sensitive as cells treated with ATR inhibitor which correlated with the damage seen in cells where HEXIM1 is depleted at this time point.

These results taken together suggest that HEXIM1 is important in helping prevent DNA damage in response to JQ1 treatment and also contributes to helping cells survive cytotoxic effects of BET inhibitor treatment.

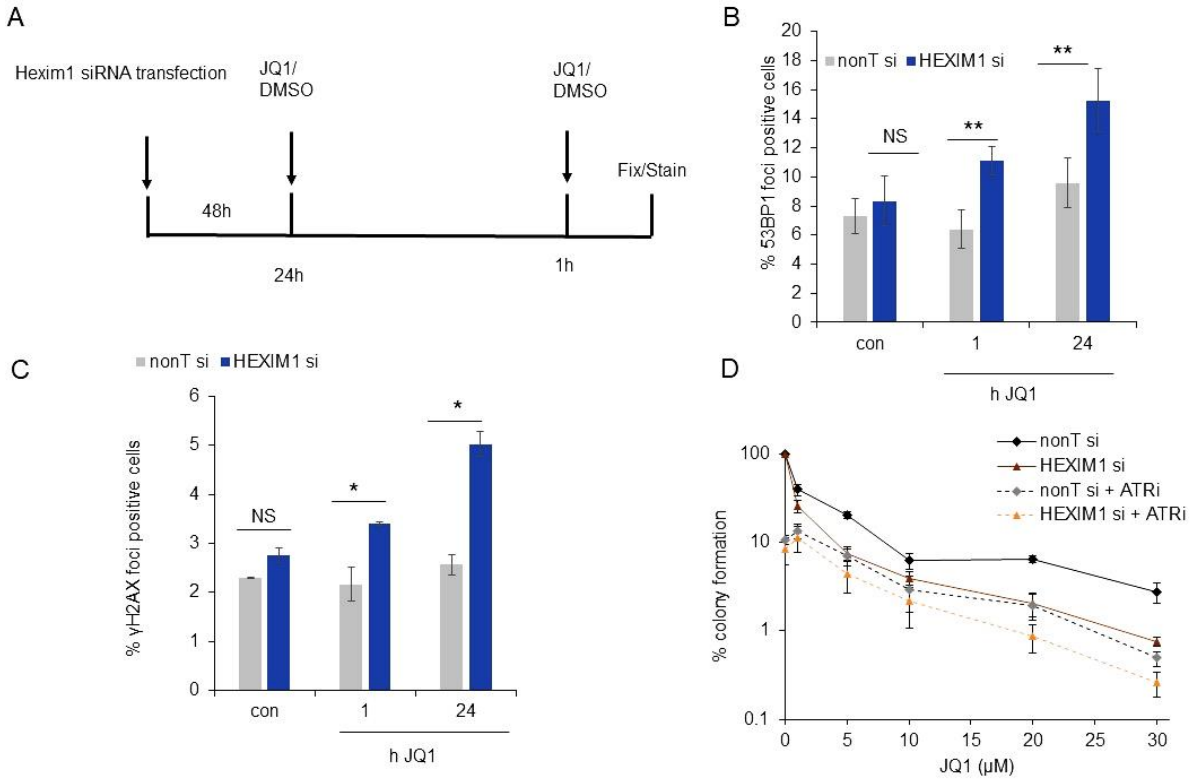


Figure 5.6: HEXIM1 depletion activates a BET inhibitor replication stress response.

A) Cells were treated with HEXIM1 siRNA for 48 hours before 1 μM JQ1 treatment for 1- or 24-hours treatment before cells were fixed and stained. B) Percentages of cells containing more than 8 53BP1 after HEXIM1 depletion +/- 1 μM JQ1 for 1- and 24-hour treatments. C) Percentages of cells containing more than 8 γH2AXafter HEXIM1 depletion +/- 1 μM JQ1 for 1- and 24-hour treatments. D) Colony survival of U2OS cells treated with indicated concentrations of JQ1 for 24 hours, +/- HEXIM1 siRNA and 2.4 μM ATRi AZ20 for 24 hours. The statistical test used was 2-way ANOVA with Tukey's, **** = p<0.0001. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value <0.01, NS= Not significant.)

5.7: BET inhibition leads to increased genomic instability

Persistent replication stress can lead to genomic instability in cells (Carr and Lambert 2013, Zeman and Cimprich 2014, Gaillard, Garcia-Muse et al. 2015). Genomic instability is a major hallmark of tumorigenesis in cells (Hanahan and Weinberg 2000). Understanding what can drive genomic instability is crucial, especially in the case of therapeutics or drugs where genomic instability could promote secondary cancers. BET inhibitors had not shown to induce DNA damage signalling, however they induced transcription-replication conflicts, RAD51 recruitment and potentially still induce DNA damage. It therefore was important to check if BET inhibitors may potentially promote genomic instability.

To investigate this further we looked to see if JQ1 treatment could increase micronuclei formation. Micronuclei are biomarkers of genomic instability (Fujisawa, Nakajima et al. 2015, Higgs, Reynolds et al. 2015, Reynolds, Bicknell et al. 2017). They are extra-nuclear bodies that contain displaced or damaged chromosomes that are not incorporated into the daughter nucleus during mitosis (Luzhna, Kathiria et al. 2013, Gekara 2017). They can be visualised around the cells under the microscope with DAPI stained cells (Figure 5.7 A). We measured micronuclei in both control treated cells and JQ1 treated cells and calculated the percentage of cells with micronuclei over number of cells counted. We observed that JQ1 treatment after 24 hours leads to increased amount of micronuclei (Figure 5.7 B). To see if this effect would be amplified after depletion of proteins that prevent DNA damage signalling, we measured micronuclei after HEXIM1 depletion with JQ1 co-treatment. We observed that although HEXIM1 depleted cells did increase

micronuclei, this increase was similar with and without JQ1 treatment (Figure 5.7 C).

Overall this data suggests that BET inhibitors and HEXIM1 depletion lead to increased genomic instability in the cell, possibly in an independent manner.

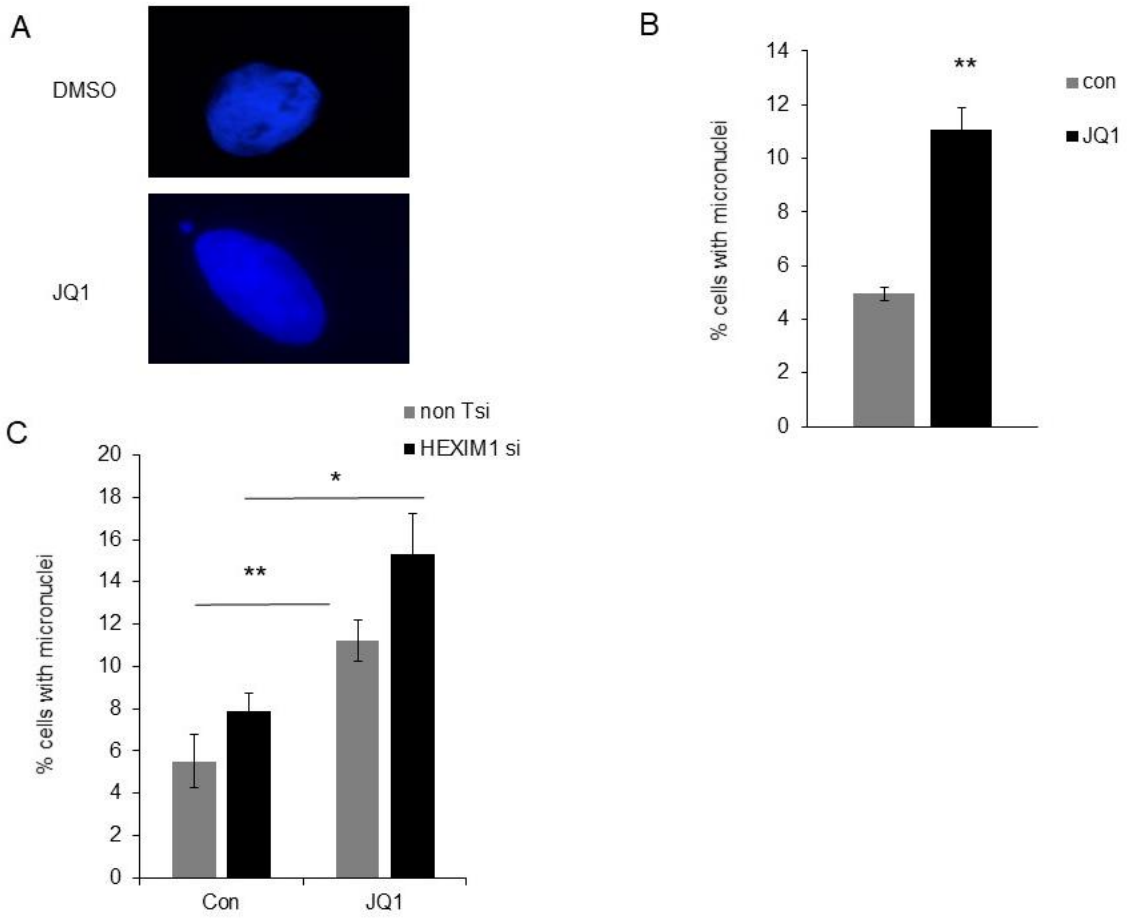


Figure 5.7: BET inhibitor treatment increases genomic instability.

A) Representative images showing micronuclei formation of DAPI stained cells after 1 µM JQ1 treatment for 24 hours. Scale bar 10 µm B) Percentage of total cells containing micronuclei after 24 hours JQ1 treatment. C) Percentage of total cells containing micronuclei after HEXIM1 siRNA treatment +/- 1 µM JQ1 treatment for 24 hours. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01)

5.8: Summary

The main aim of the chapter was to try and elucidate the mechanism of increased RNA synthesis. *HEXIM1* is the only gene whose expression is regularly up-regulated after JQ1 treatment across cell lines (Bartholomeeusen, Xiang et al. 2012, Devaraj, Fiskus et al. 2016, Huang, Garcia et al. 2016, Zhu, Enomoto et al. 2017). Previous literature indicated a role for JQ1 to disrupt the 7SK-HEXIM1 inhibitory complex of P-TEFb (Bartholomeeusen, Xiang et al. 2012, Chaidos, Caputo et al. 2014). Hence, we hypothesised that BET inhibitors increase RNA synthesis by disruption of the P–TEFb and 7SK-HEXIM1 inhibitory complex.

Our data suggests a mechanism of how BET inhibitors can lead to increased RNA synthesis. Increased CTD phosphorylation after JQ1 treatment (Figure 5.1 B-E) agrees with increased activity of P-TEFb, which phosphorylates and activates RNA Pol II. We showed that by using a drug HMBA that can disrupt the inhibitory complex of P-TEFb and 7SK-HEXIM1 but doesn't impair any factors involved in allowing faithful DNA replication, we can induce fork slowing (Figure 5.2). This agrees with the model that disrupting the P-TEFb-7SK-HEXIM1 complex can lead to fork slowing. This fork slowing was not exacerbated by JQ1 addition, which may suggest that the disruption of the 7SK complex may act as a primary cause of fork slowing. Hence, from our data we suggest a potential mechanism where BET inhibitors can disrupt the 7SK-HEXIM1 complex which may liberate P-TEFb from its inhbitory complex, which can cause more RNA Pol II phosphorylation which suggests more RNA synthesis which would lead to increased transcription-replication interference.

We showed that BRD4 regulates DNA replication via its P-TEFb interaction domain (Figure 6.3). The sites for the interactions with replication proteins TICRR and RFC are contained in the BRD4 short isoform but the CDC6 interaction has not been mapped as of yet (Maruyama, Farina et al. 2002, Sansam, Pietrzak et al. 2018, Zhang, Dulak et al. 2018). We showed that re-expressing the BRD4 short isoform, containing the interaction domains for TICRR and RFC but not P-TEFb, was unable to rescue replication fork slowing, while the long isoform with a P-TEFb interaction domain could rescue fork speeds (Figure 3.2 and 5.3). Hence, our data suggest that TICRR and RFC replication proteins are not involved in the phenotypes seen with BRD4 loss. Instead, our data supports a novel mechanism of how BRD4 regulates DNA replication progression via its P-TEFb interactions. There is a caveat in that BRD4 long isoform has recently been shown to interact with NELF, which is also an important factor in transcription elongation (Lambert, Picaud et al. 2019). Further work is required to see if the interaction between NELF and BRD4 is playing a role in the regulation of replication fork speeds.

As HEXIM1 protein is consistently up-regulated by JQ1 treatment, we decided to investigate if HEXIM1 displayed the same behaviour in the U2OS cell line being tested (Bartholomeeusen, Xiang et al. 2012, Devaraj, Fiskus et al. 2016, Huang, Garcia et al. 2016, Zhu, Enomoto et al. 2017). HEXIM1 protein levels slowly increased during 48–72 hr JQ1 treatment (Figure 5.4 C), although this was a very slow induction of HEXIM1 levels compared to what had been shown in other studies (Bartholomeeusen, Xiang et al. 2012, Devaraj, Fiskus et al. 2016, Huang, Garcia et al. 2016, Zhu, Enomoto et al. 2017). The increase in HEXIM1 (Figure 5.4 C)

correlated with no decrease in nascent RNA synthesis at these time points (Figure 5.4 D). These results suggest that the hypothesised mechanism of a feedback loop highlighted in Figure 5.1 A is not occurring with the U2OS cells. This suggests that the process of replication adaptation after 24 hours of JQ1 (Figure 3.2 B-C) is more complex than the hypothesised HEXIM1-mediated feedback loop suppressing transcription. How cells adapt to BET inhibitor treatment to rescue fork speeds requires further investigation.

In cells depleted of HEXIM1, JQ1 treatment caused delayed replication fork slowing and delayed increases in RNA synthesis (Figure 5.5). Our findings suggest that HEXIM1 is required for BET inhibitor-induced replication fork slowing, which is delayed by at least 8 hours in the absence of HEXIM1. This data suggests that both these processes are altered when HEXIM1 is depleted, but we have no model for how this works yet. The most likely explanation would be that in absence of HEXIM1, the initial disrupting of the P-TEFb-7SK-HEXIM1 complex does not occur, but cells eventually adapt to this as well. There could be numerous explanations such as JQ1 inducing gene expression changes after 24 hours (Delmore, Issa et al. 2011, Muralidharan, Bhadury et al. 2016). Another explanation may be compensation by HEXIM2, which can bind to P-TEFb when HEXIM1 is depleted compensating for HEXIM1 loss. Studies have shown that these two proteins are regulated differently in response to external stimuli so may act differenty to BET inhibitor treatment (Byers, Price et al. 2005). However, this again requires further investigation.

Interestingly, the lack of replication fork slowing in HEXIM-depleted cells is associated with increased DNA damage (Figure 5.6 B-C). We also showed that

HEXIM1 depletion leads to JQ1 sensitivity in response to short treatments of 1 hour (Figure 5.6 D). Research had shown that HEXIM1 depletion could lead to long-term BET inhibitor resitance, which did not conflict with our findings as it was only seen with over 24 hours treatment. The authors proposed that loss of HEXIM1 decreases overall JQ1 effectiveness by allowing higher P-TEFb activity (Devaraj, Fiskus et al. 2016). In addition to modulating transcription-dependent fork slowing, HEXIM1 might have more undiscovered roles in controlling the replication stress response pathway and DNA damage signalling.

We also observed an increase in micronuclei, a genomic instability marker in cells after BET inhibitor treatment (Figure 5.7). Genomic instability is crucial in driving tumorigenesis and could be an important side-effect if BET inhibitor drugs are used as cancer therapeutics (Hanahan and Weinberg 2000, Gaillard, Garcia-Muse et al. 2015).

How BET inhibitor-treated cells acquire more micronuclei is unclear. It may be due to reversed forks collapsing before they can be restarted or being subjected to nucleophilic attack. This can lead to cells with under-replicated DNA after S-phase, which when undergoing mitosis can generate ultra-fine anaphase bridges and ultimately lead to micronuclei or chromosome breakage (Naim and Rosselli 2009). However, it must be noted that micronuclei generation is not solely caused by replication stress. There is potential that BET inhibitors could be interfering with the mitotic machinery which could give rise to mis-segregation of chromosomes and form micronuclei. Another mechanism could be BET inhibitor causing increased DNA damage which is not activating a DNA damage signal. PFGE or a comet assay

may help determine if there is more total DNA damage in the cell after BET inhibitor treatment.

So far, we have provided data supporting that disruption of the 7SK-HEXIM1 inhibitory complex with P-TEFb leads to increased RNA synthesis after BET inhibitor treatment, hence leading to replication fork slowing. BRD4 regulates replication fork speeds via its interaction with P-TEFb, and loss of this interaction causes replication fork slowing. We have also shown that HEXIM1, which is upstream of transcription-replication conflicts, is required for BET inhibitor-induced fork slowing and increased RNA synthesis.

Our data also suggests that HEXIM1 prevents DNA damage signalling after BET inhibitor fork slowing, but the underlying mechanism is also unclear at this point.

6. RAD51 modulates BET inhibitor-induced DNA damage

6.1: Introduction

Despite replication fork slowing in response to either BET inhibition or BRD4 loss, there was no activation of the replication stress response or any DNA damage signalling. Fork speeds were also rescued after 24 hours of BET inhibition, suggesting that there is some adaptation mechanism in response to BET inhibition.

RAD51 is a homologous recombination protein involved in helping cells deal with DSB's (West 2003, Ciccia and Elledge 2010, Bhat and Cortez 2018). RAD51 also has a role in fork re-modelling and reversal in a DSB independent manner (Hanada, Budzowska et al. 2007, Petermann, Orta et al. 2010, Carr and Lambert 2013, Zellweger, Dalcher et al. 2015, Bhat and Cortez 2018). RAD51 has been proposed to help replication restart after replication forks slow or stall (Petermann, Orta et al. 2010, Zellweger, Dalcher et al. 2015, Bhat and Cortez 2018). This is thought to involve the formation of a reversed fork which can help stabilise and promote fork restart. Fork reversal is a tightly regulated process whereby the replication fork regresses into a Holliday junction (chicken foot structure) (Neelsen, Chaudhuri et al. 2014, Zellweger, Dalcher et al. 2015).

Recent studies show that HR and RAD51 are able to play roles at replication fork processes such as replication stress. Studies have indicated that RAD51 can be recruited to stalled forks after treatment with stress inducing compounds such as HU (Petermann, Orta et al. 2010). RAD51 can bind at these forks and prevent

replication fork collapse and potentially lead to fork restart as well (Arnaudeau, Lundin et al. 2001, Lundin, Schultz et al. 2003, Carr and Lambert 2013, Willis, Chandramouly et al. 2014). Further research has indicated a role for fork reversal by RAD51 has been shown to be able to modulate fork progression in response to genotoxic stress (Zellweger, Dalcher et al. 2015). RAD51 loss led to the rescue of stressed replication fork speeds indicating a role of RAD51 slowing forks down in response to replication stress. They also observed no ATR activation upon treatment with several genotoxic compounds such as Etoposide, Mitomycin C, Aphidicolin, Doxorubicin and Cisplatin all of which caused fork slowing under the same conditions. These results have similarity to our data with BET inhibitors (Zellweger, Dalcher et al. 2015). RAD51 levels have also been shown to be gradually depleted after JQ1 treatment across a variety of cell lines (Yang, Zhang et al. 2017, Pawar, Gollavilli et al. 2018). These observations led us to hypothesise a potential role for RAD51 in modulating fork progression and reversing and stabilising stressed replication forks after BET inhibitor treatment.

In this chapter we aim to investigate the cell's response to BET inhibitor-induced replication stress. In particular we want to investigate if there is a potential role RAD51 plays at BET inhibitor-induced slowed replication forks in either modulating fork progression or stabilising these replication forks after replication stress has occurred. We also try to elucidate whether RAD51 levels are important in rescuing fork speeds after 24 hours.

6.2: BET inhibition increases RAD51 foci formation

To firstly test whether RAD51 is recruited to chromatin during BET inhibitor induced fork slowing, we used immunofluorescence. Cells were treated with 1 µM JQ1 for 8 hours, with or without the presence of ATR inhibitor and then stained with RAD51 antibody. We observed an increase in positive RAD51 foci cells with JQ1 treatment from 7.4% to 14%. This increase in RAD51 foci was lost when JQ1 was co-treated with 2.4 µM ATRi (Figure 6.1 A-B). This suggested that RAD51 recruitment after JQ1 treatment requires a basal level of ATR activation in response to BET inhibitor treatment. In addition, a time-course of JQ1 treatment from 1 to 24 hours was performed by Dr Ann Liza Piberger in our laboratory. Dr Piberger observed a significant increase in RAD51 foci after 4 hours of treatment (16%-26%), while after 24 hours RAD51 foci were reduced back to control levels (Figure 6.1 C). As this was observed with BET inhibitor treatment, we next tested whether RAD51 foci formation could also be seen after BRD4 depletion. After treating cells with control siRNA or BRD4 siRNA, we again observed similar results where BRD4 depleted cells showed increased percentages of RAD51 foci positive cells, from 11% to 18% (Figure 6.1 D).

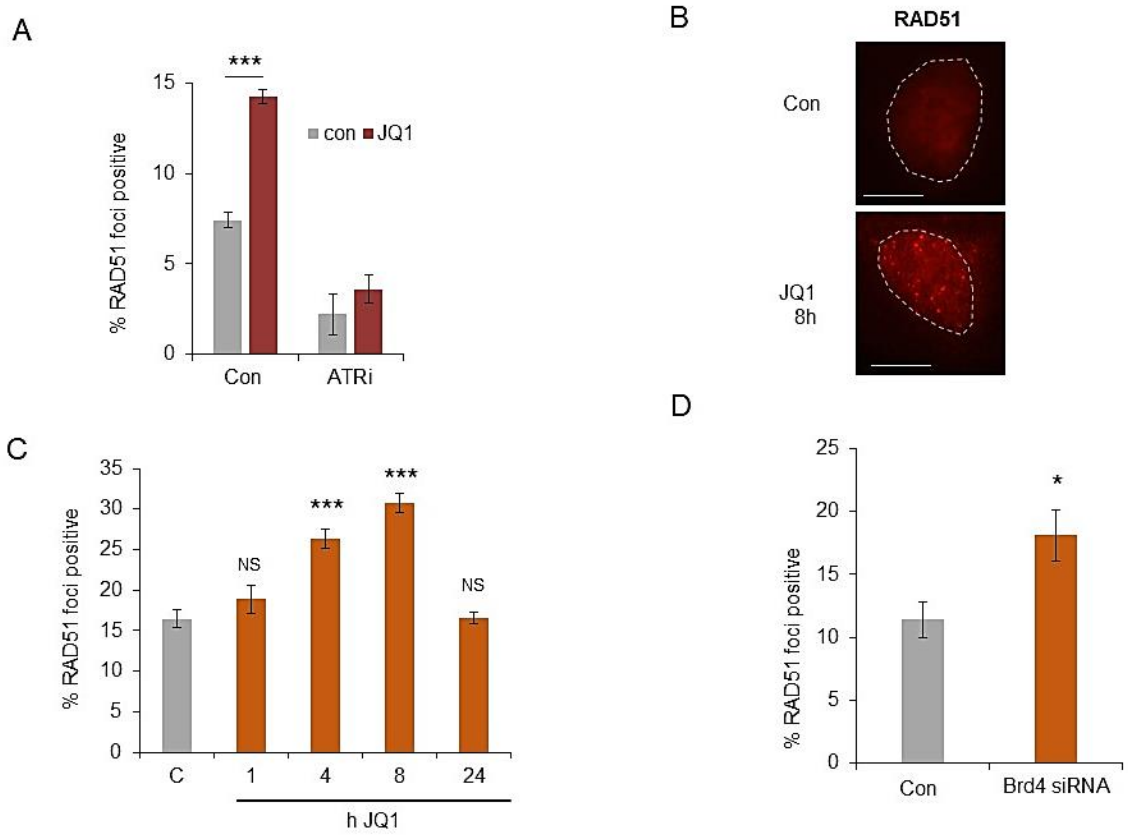


Figure 6.1: JQ1 and BRD4 depletion leads to increased RAD51 foci.

A) Percentages of cells containing more than 5 RAD51 foci after 1 hour 1 µM JQ1 treatment +/- ATR inhibitor 2.4 µM AZ20. B) Representative images of RAD51 foci after 8 hour 1 µM JQ1 treatment. Scale bar 10 µm. C) Percentage of cells containing more than 5 RAD51 after 1 µM JQ1 treatment for 1, 4, 8- and 24-hour treatment. D) Percentages of cells containing more than 5 RAD51 foci after BRD4 depletion for 48 hours. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, *** P value <0.001, NS= Not significant.)

RAD51 foci were induced after JQ1 treatment, suggesting that RAD51 is recruited to sites of transcription-replication conflicts. To see if RAD51 foci formation was dependent on ongoing transcription, we looked at RAD51 foci formation with or without treatment with potent transcriptional inhibitor α-amanitin as used previously in chapter 3. This experiment was carried out by Dr.Piberger. Cells were co-treated with JQ1 and α-amanitin for 4 hours. Again we observed an increase in the percentage of RAD51 foci positive cells after JQ1 treatment, which after co-treatment with α-amanitin was reduced from 26% to 19% (Figure 6.4 A). α-amanitin by itself had no effect on the recruitment of RAD51 foci compared to control. This suggests that RAD51 is recruited in response to the transcription-replication conflicts, as transcription inhibition prevents the increase in RAD51 foci.

Overall these results indicate that BET inhibitor treatment and BRD4 loss lead to the induction of RAD51 foci formation in correspondence with transcription-associated replication fork slowing, dependent on a basal level of ATR activity.

6.3: RAD51 promotes BET inhibitor-induced fork slowing

A previous report had showed a role for RAD51 in slowing down fork progression in response to genotoxic stress (Zellweger, Dalcher et al. 2015). As we observed an acummlation of RAD51 foci after BET inhibitor treatment, we decided to investigate if RAD51 could potentially be playing a similar role in fork slowing in response to BET inhibitor treatments. To do this we used RAD51 siRNA. We transfected cells

with either control or RAD51 siRNA for 48 hours before JQ1 treatment for 1 or 24 hours, followed by harvesting the cells (Figure 6.2 A). We used Western blots to confirm the effect of siRNA treatment on RAD51 protein levels. RAD51 siRNA treatment depleted RAD51 protein levels in presence or absence of JQ1 (Figure 6.2 B). We also observed that the levels of RAD51 were slowly decreasing after JQ1 treatment in control siRNA treated cells (Figure 6.2 B). This mirrored what had previously be seen in the other reports (Yang, Zhang et al. 2017, Pawar, Gollavilli et al. 2018).

To test the effects of RAD51 on BET inhibitor fork progression we used DNA fibre assays, using the same method as above but labelling and spreading fibres after the JQ1 treatment (Figure 6.2 A). We observed that RAD51 depletion rescued the fork slowing induced by 1 hour BET inhibitor treatment (Figure 6.2 C). There was no change in fork speeds after 24 hours with or wihout RAD51 depletion, as fork speeds were not slowed in either case. RAD51 siRNA by itself had no effect on fork speeds compared to control indicating RAD51 was having no effects on replication progression in the absence of BET inhibitor treatment. This would indicate that RAD51 has an important role in controlling fork speeds in response to BET inhibitors, similar to what was observed in response to genotoxic stress.

We observed that in response to JQ1 treatment, RAD51 levels were slowly being decreased (Figure 6.2 B). This effect was most prominently seen after 24 hours where a major reduction in RAD51 levels was occuring. This lead us to hypothesise that a decrease in RAD51 levels might help rescue fork speeds at 24 hours.

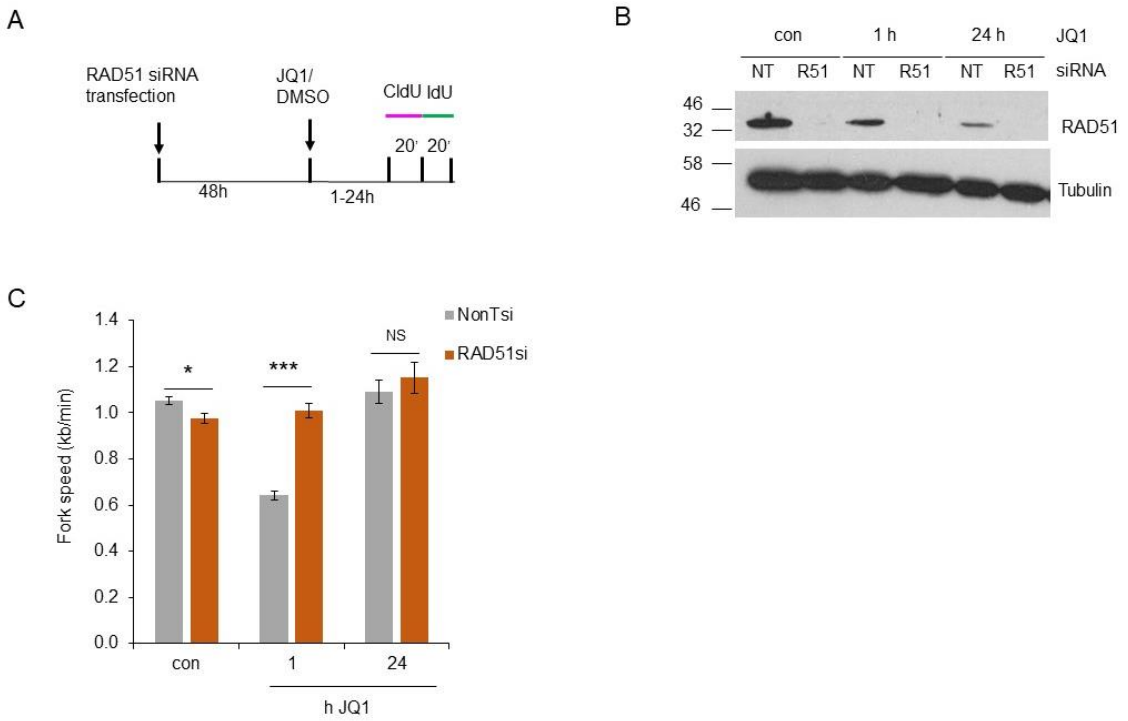


Figure 6.2: RAD51 depletion rescues BET inhibitor induced fork slowing.

A) DNA fibre labelling in U2OS cells treated with RAD51 siRNA for 48 hours 1 µM JQ1 treatment for 24 or 1 hour. B) Protein levels of RAD51 and actin after nonTsi or RAD51 siRNA treatment +/- 1 µM JQ1 treatment for 1 or 24 hours. C) DNA fibre labelling in U2OS cells were treated with RAD51 siRNA for 48 hours before 1 µM JQ1 treatment for 1 or 24 hours. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, *** P value <0.001).

To investigate this further, we transfected cells with a RAD51 expression plasmid using to overexpress RAD51 in cells. We performed DNA fibre assays, after cells were treated with either control or RAD51 plasmid for 24 hours, followed by treatment with or without JQ1 for 24 hours (Figure 6.3 A). We first tested that the transient plasmid transfection worked using a Western blot. Here we observed that RAD51 plasmid transient overexpression with or without JQ1 increased levels of RAD51 compared to control (Figure 6.3 B). This indicated that the plasmid transient transfection was working.

We observed that RAD51 transient overexpression after 24 hour JQ1 treatment led to fork speeds being reduced compared to the control as usually obserevd after 1 hour BET inhibitor treatment (Figure 6.3 C). RAD51 overexpression in control treated cells had no effect on fork speeds, indicating that RAD51 overexpression has no adverse effects on fork speeds by itself. Overall this data provides another indicator that RAD51 is slowing forks on response to BET inhibition. This data also provide a potential mechanism of how fork speeds are rescued after 24 hours of BET inhibitor treatment due to depleted RAD51 levels.

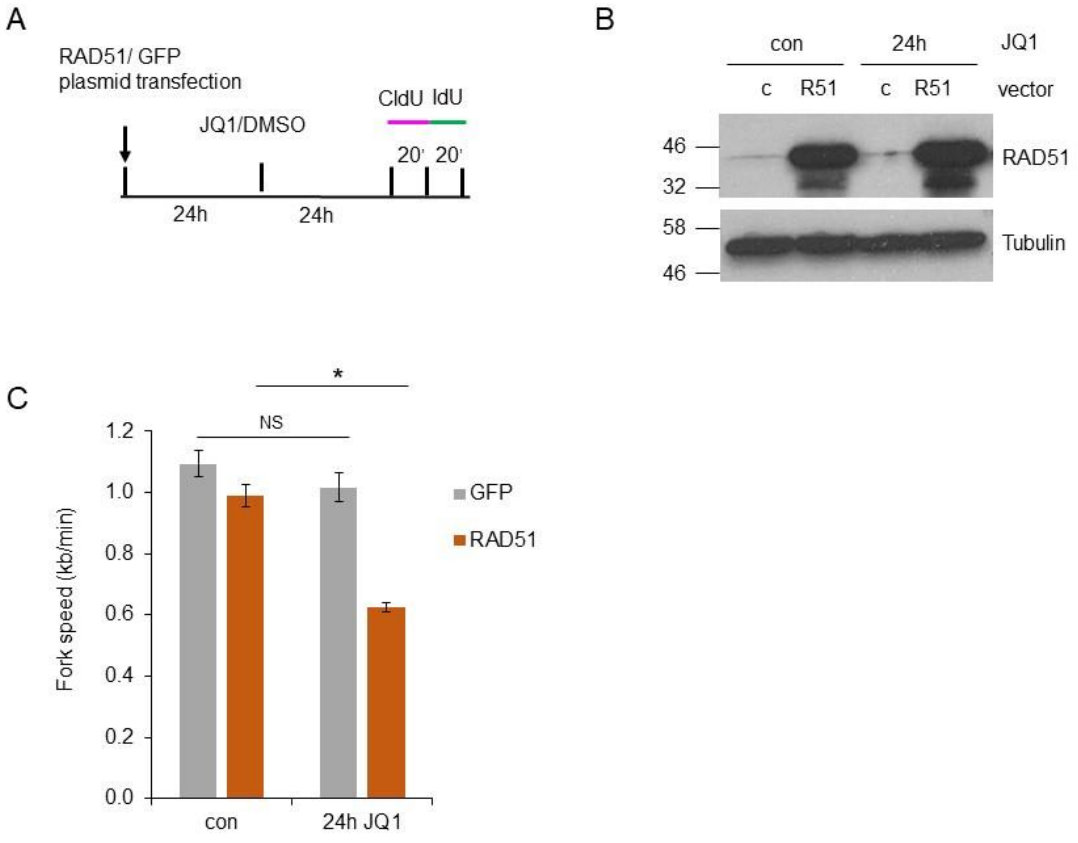


Figure 6.3: RAD51 overexpression stops BET inhibitor adaptation to replication stress after 24 hours.

A) DNA fibre labelling after cells treated with either GFP or RAD51 expression plasmid for 48 hours with or without 1 µM JQ1 treatment for 1 or 24 hours. B) Protein levels of RAD51 +/- RAD51 plasmid transfection and +/- 1 µM JQ1 treatment. C) Average median of replication fork speeds after transient overexpression of RAD51 or eGFP (control) +/- 1 µM JQ1 treatment for 24 hours. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, NS= Not significant.)

We wanted to see whether this rescue in fork speeds after RAD51 depletion was due to reduced RNA synthesis as we observed with HEXIM1 depletion (Figure 5.5). To do this we used the EU assay, depleting RAD51 for 48 hours before adding EU for an additional hour (Figure 6.4B). RAD51 knockdown cells showed high levels of RNA synthesis and produced variable results with or without JQ1 treatment (Figure 6.4 C). This makes interpreting the data difficult, but it can be concluded that there is no decrease in total RNA synthesis after RAD51 depletion.

Overall this data would indicate that RAD51-induced fork slowing is in response to transcription-replication conflicts, and that the rescue seen when RAD51 is depleted is not due to a suppression of RNA synthesis.

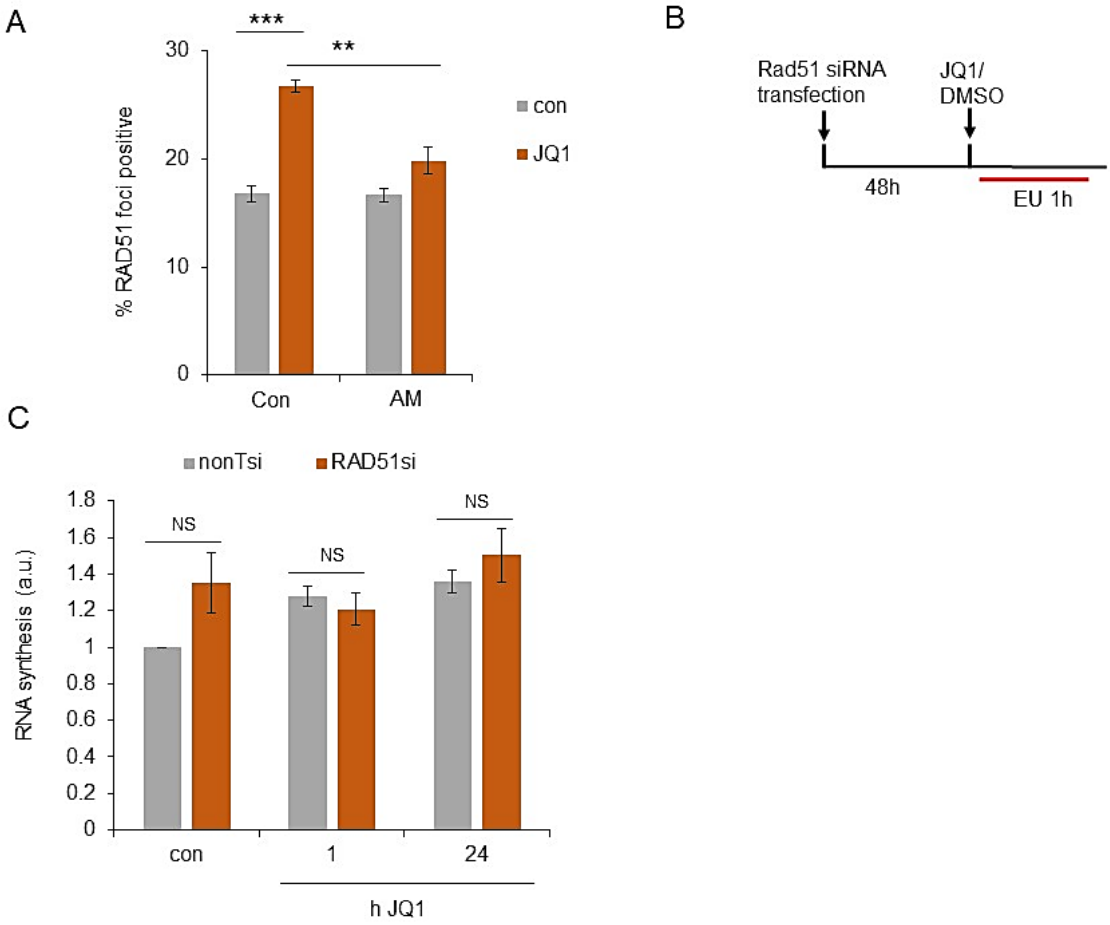


Figure 6.4: RAD51 foci formation after BET inhibitor treatment depends on RNA synthesis.

A) Percentages of cells with 8 or more RAD51 foci after 4-hour treatment of 1 μM JQ1 and transcription inhibitor α-amanitin (AM, 10μg/ml) for 4 hours. B) Quantification of incorporation of EU performed in U2OS cells after 48 hours RAD51 siRNA or NonTsi treatment followed by +/- 1 μM JQ1 treatment for 1 hour. C) Nuclear EU intensity after RAD51 siRNA treatment and +/- 1 μM JQ1 treatment for either 1 or 24 hours (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. ** P value <0.01, *** P value < 0.001, NS= Not significant.)

6.4: Fork reversal may play a role in response to BET inhibition

RAD51 had previously been shown to slow forks down in response to genotoxic compounds. The authors had suggested that RAD51 slows forks by mediating fork reversal (Zellweger, Dalcher et al. 2015). Fork reversal helps stabilise the replication fork until the lesions or other sources of replication slowing have been removed (Branzei and Foiani 2010, Neelsen and Lopes 2015). This suggested a role for fork reversal in response to BET inhibition as a means to stabilise and slow down fork speeds in response to transcription-replication conflicts.

To test this hypothesis, we used DNA fibre assays, firstly using 5 μm PARP inhibitor Olaparib (PARPi) for 2 hours before labelling the cells (Figure 6.5 A). PARP1 is a crucial mediator of fork reversal and is needed for fork stabilisation (Ding, Ray Chaudhuri et al. 2016). PARP1 acts to inhibit RECQ1, a helicase that counteracts fork reversal (Berti, Ray Chaudhuri et al. 2013). Hence using PARPi can prevent the accumulation and stabilisation of reversed forks. We observed that when PARPi was combined with JQ1 this helped rescue fork speeds towards the control levels. PARPi alone had no effects on fork speeds, indicating the effect was specific to the combination with JQ1 treatment (Figure 6.5 B-C).

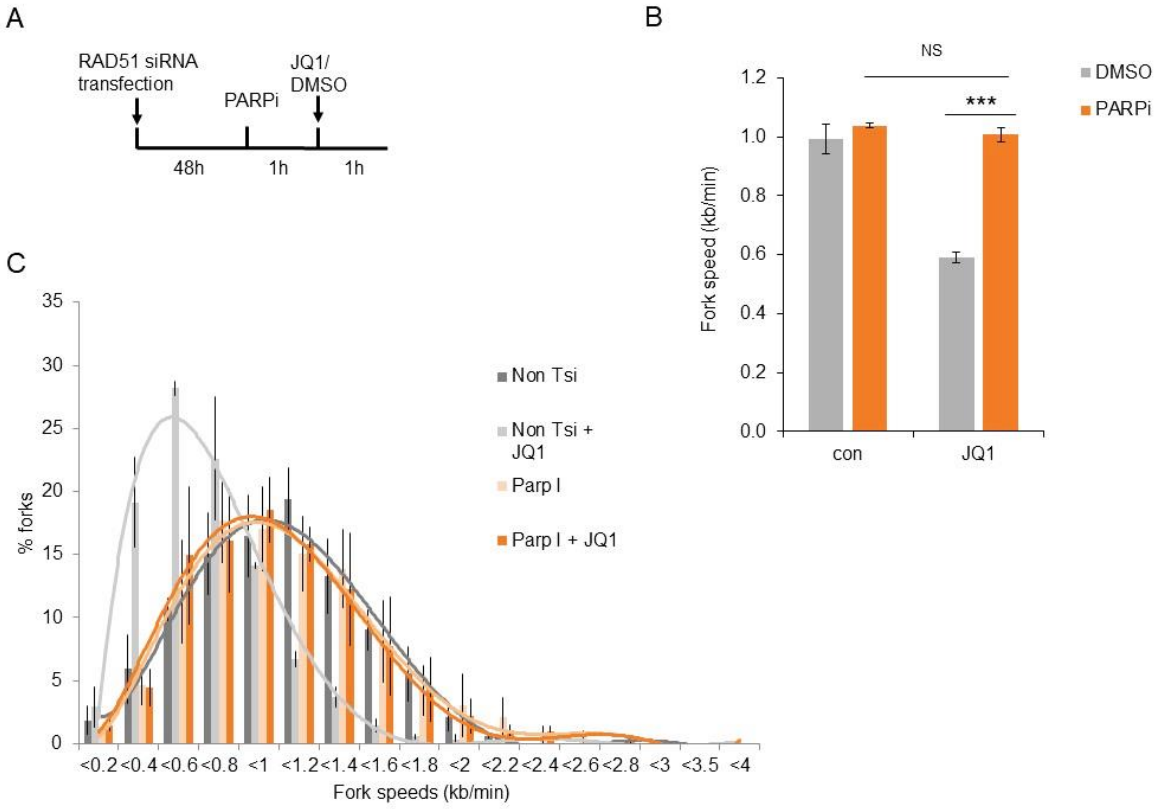


Figure 6.5: PARP inhibition rescued fork slowing induced by BET inhibition.

A) DNA fibre labelling performed in U2OS cells treated with PARP-I olaparib for 2 hours followed by JQ1 for 1 hour. B) Average median replication fork speeds after treatment +/- PARP-I olaparib (5 µM) followed by +/- 1 µM JQ1 treatment. C) Distribution of replication fork speeds in cells treated with +/- PARPi olaparib followed by +/- 1 µM JQ1 treatment. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. *** P value < 0.001, NS= Not significant.)

We also looked at other fork reversal factors to test if the same effect was seen. We used siRNA to deplete DNA translocases SMARCAL1 and ZRANB3. Both of these proteins have been shown to use their translocase activity to reverse replication forks, and inhibiting or depleting these proteins would block fork reversal (Betous, Mason et al. 2012, Couch, Bansbach et al. 2013, Vujanovic, Krietsch et al. 2017).

To investigate the effects that these proteins have on BET inhibitor induced fork speeds, DNA fibre assays were performed. Either protein was depleted with specific siRNA targeted against each protein for 48 hours before DNA fibre labelling. To check that both proteins were depleted we used Western blots. We observed that SMARCAL1 protein and ZRANB3 protein levels were strongly reduced after siRNA treatments, indicating that both proteins were being successfully knocked down (Figure 6.6 A, C).

We observed that when either protein was depleted, fork speeds were significantly increased towards the control levels (0.93 kb/min for SMARCAL1 depletion in presence of JQ1 and 0.90 kb/min for ZRANB3 depletion in presence of JQ1 from 0.65 kb/min after JQ1 treatment alone) in the presence of JQ1 (Figure 6.6 B, D). Depletion of both proteins in absence of JQ1 treatment showed no impact on replication fork speeds, again indicating the effect was specific to JQ1 treatment.

Overall, these results suggest that fork reversal proteins PARP1, ZRNAB3 and SMARCAL1 are crucial for forks to be slowed down in response to BET inhbitor treatment. This suggests a role for forks being reversed to help fork slowing during BET inhibitor induced transcription-replication conflicts.

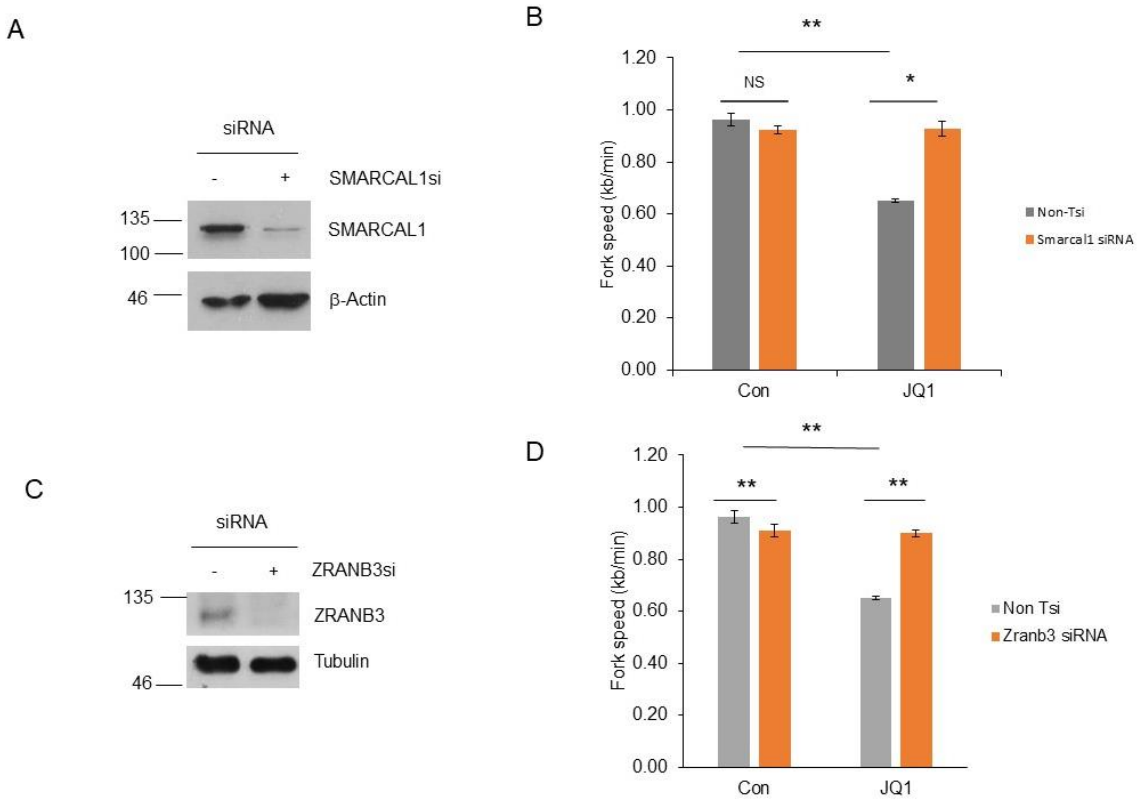


Figure 6.6: SMARCAL1 and ZRANB3 depletion both rescue BET inhibitor induced fork slowing.

A) Protein levels of SMARCAL1 after 48 hours treatment with NonTsi or SMARCAL1 siRNA transfection. B) Average median replication fork speeds with cells treated with NonTsi or SMARCAL1 siRNA +/- 1 µM JQ1 treatment for 4 hours. C) Protein levels of ZRANB3 after 48-hour treatment with nonTsi or ZRANB3 siRNA transfection. D) Average median replication fork speeds with cells treated with nonTsi or ZRANB3 siRNA +/- 1 µM JQ1 treatment for 4 hours. (N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01, NS= Not significant.)

6.5: RAD51 depletion leads to increased DNA damage during BET inhibition

RAD51 slows fork speeds down in response to BET inhibitor treatment, suggesting that it acts downstream of the transcription-replication conflicts that occur. Previously, we had yet to detect any DNA damage signalling in response to BET inhibitor treatment, but saw increased DNA damage signalling after depletion of HEXIM1 which acted upstream of these collisions. We hypothesised that RAD51 depletion may also serve to proctect cells from DNA damage by slowing and stabilising replication forks.

We used immunofluorescence microscopy of γH2AX and 53BP1 foci after RAD51 depletion. Cells were treated with RAD51 siRNA for 48 hours followed by control or JQ1 treatment for 8 hours before fixing and staining. We observed that RAD51 depletion in presence of JQ1 led to the increase in both γH2AX and 53BP1 foci formation compared to control (6% in control to 15% in presence of JQ1 for Yh2ax and 14% in control to 21% in presence of JQ1 for 53BP1) (Figure 6.7 A-C). RAD51 depletion in the absence of JQ1 showed only a small increase in DNA damage foci, indicating that the effect was specific to JQ1.

These results indicate that RAD51 is involved in preventing DNA damage after BET inhibitor treatment, and depletion of RAD51 serves to promote BET inhibitor-induced DNA damage.

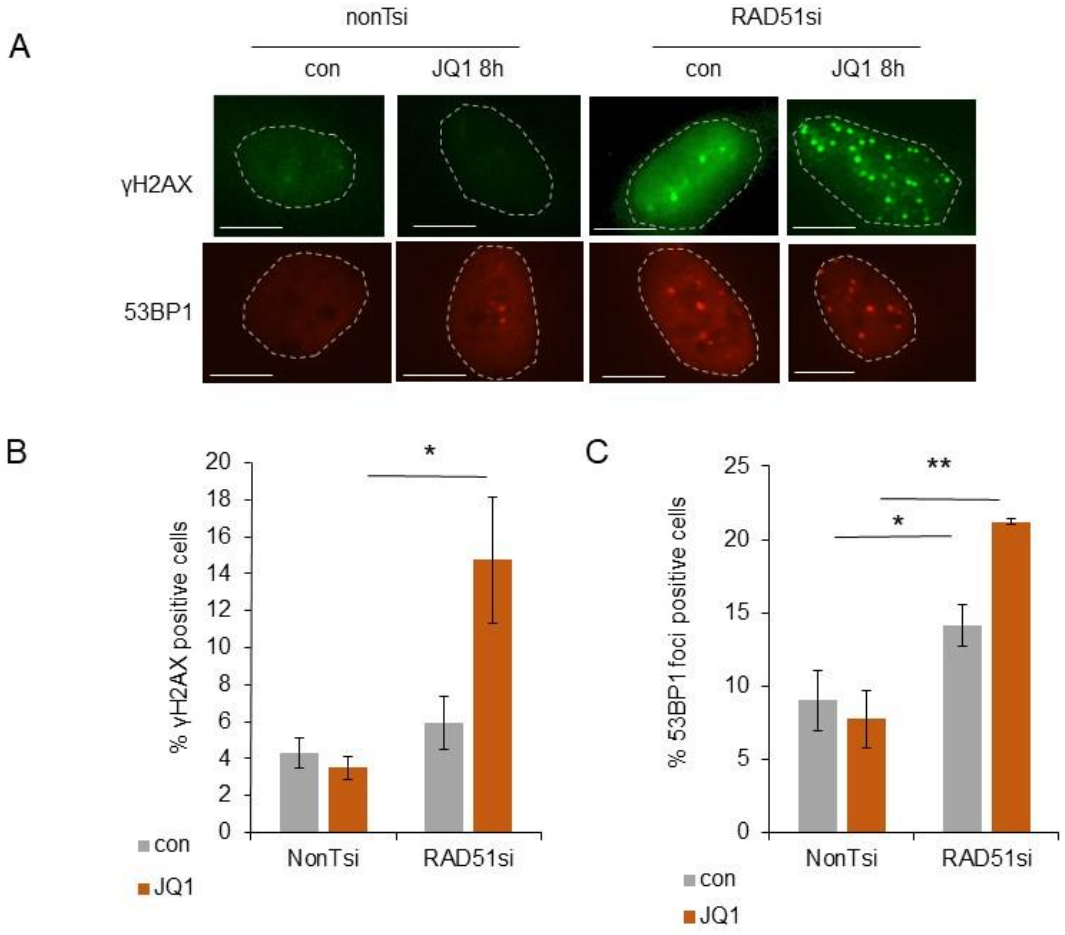


Figure 6.7: RAD51 depletion activates a BET inhibitor replication stress response.

A) Representative images of γH2AX and 53BP1 foci after 1 μM JQ1 treatment with RAD51 or NonTsi siRNA transfection. Scale bar 10 μM. B) Percentages of cells with 8 or more γH2AX foci after 1 μM JQ1 treatment for 8 hours with either RAD51 or NonTsi siRNA transfection. C) Percentages of cells with 8 or more 53BP1 foci after 1 μM JQ1 treatment for 8 hours with either RAD51 or NonTsi siRNA transfection. N=3, error bars: S.E.M, Statistical analysis: Student's t-test. * P value <0.05, ** P value < 0.01, NS= Not significant.)

6.6: Summary

The main aim of this chapter was to try and further characterise the downstream DNA damage response to BET inhibitor-induced fork slowing. We had previously shown no activation of DNA damage signalling after fork slowing. In this chapter we showed that BET inhibitor treatment or BRD4 depletion activate RAD51 (Figure 6.1), a homologous recombination factor which has been shown to play a crucial role during replication stress in cells (Petermann, Orta et al. 2010, Zellweger, Dalcher et al. 2015, Bhat and Cortez 2018). The RAD51 response is transient occurring at the same time as fork slowing, but this may also be due to RAD51 protein levels being reduced after 24 hours as well (Figure 6.2 B) or due to cell cycle arrest. This illustrates a picture where RAD51 is activated but there is not a full damage response to BET inhibitor treatment.

Previous literature indicated a role for RAD51 in directly slowing replication fork speeds in response to specific types of DNA damage (Zellweger, Dalcher et al. 2015). We observed that RAD51 also plays a role in slowing fork speeds during transcription-replication conflicts (Figure 6.4). Depletion of RAD51 lead to fork speeds being rescued towards control speeds during BET inhibitor treatment (Figure 6.2). RAD51-mediated fork slowing might involve fork reversal. Inhibition of PARP1, and depletion of ZRNAB3 and SMCARCAL1, all of which are crucial for formation and stabilisation of reversed forks, also showed fork speeds being returned to control levels (Figure 6.5-6.6). This potentially suggests a mechanism where RAD51 is reversing forks in response to transcription-replication conflicts

which leads to fork stabilization and prevents further DNA damage signalling. We are yet to determine whether fork reversal happens directly at sites of these conflicts or is due to indirect effects of transcription-replication conflicts.

One potential way to answer this question is to see if the transcription machinery is in close proximity to proteins that are involved in fork reversal such as SMARCAL1. This may suggest that fork reversal is happening at sites of transcription-replication conflicts. One way to try and address this question would be to carry out a proximity ligation assay. This technique would allow us to see whether fork reversal proteins are in close proximity to either transcription factors or RNA Pol 2, which has been phosphorylated at either serine 2 or serine 5. We could also look to see whether fork reversal proteins or RAD51 co-localize with RNA Pol 2 or other transcription proteins after JQ1 treatment. This may help address the question as to whether fork reversal happens at sites of transcription-replication conflicts.

RAD51 protein levels are significantly reduced after 24 hours of BET inhibitor treatment (Figure 6.2 B). This provides a potential mechanism where RAD51 loss allows fork speeds to be rescued after 24-hour BET inhibitor treatment. We observed that by over-expressing RAD51, we can promote BET inhibitor-induced fork slowing even after 24 hours JQ1 (Figure 3.2). This again shows RAD51 playing a role in fork progression and a potential mechanism of how BET inhibitor treated cells rescue fork speeds after this time point. Whether this is the main mechanism of rescue requires more investigation. Fork reversal can lead to forks being restarted which may also explain this rescue of fork speeds.

RAD51 not only controlled fork speeds in response to BET inhibitor treatment, but also prevented DNA damage signalling (Figure 6.7). Here when RAD51 is depleted, there are increases in DNA damage foci formation. RAD51 might prevent BET inhibitor-induced DNA damage signalling by helping to slow down and stabilise replication forks hence preventing accumulation of ssDNA for an ATR response, or by preventing forks from collapsing into DNA breaks after persistent replication stress.

In this chapter, I have described a new downstream DNA damage response mechanism that occurs during BET inhibitor induced transcription-replication conflicts. RAD51 is activated in response to transcription-replication conflicts and helps slow down fork speeds and prevents DNA damage signalling. Furthermore, decreased levels of RAD51 after 24 hours of BET inhibitor treatment may explain the rescue of fork speeds at this time point. BET inhibitor treatment also leads to signs of increased genomic instability after 24 hours. RAD51 could play a crucial role in the response to BET inhibitors and BET inhibitor-induced fork slowing.

7. Discussion

BET bromodomain proteins are epigenetic transcriptional regulators that bind to acetylated lysine's in proteins, including histone tails on the chromatin. They have been shown to promote oncogenic functions such as MYC-dependent transcription driving cancer formation (Delmore, Issa et al. 2011). BET proteins have established roles in transcription functions. Research over the past few years has also led to the discovery of BET protein involvement in a number of different cancer types (Dawson, Prinjha et al. 2011, Delmore, Issa et al. 2011, Cheng, Gong et al. 2013, Da Costa, Agathanggelou et al. 2013). Interestingly BET proteins have recognition sites that allow for the development of specific inhibitors, which can target the function of BET proteins (Filippakopoulos, Qi et al. 2010, Dawson, Prinjha et al. 2011). These inhibitors have emerged as a new approach for targeted cancer therapy.

The lab had previously shown that treatment with BET inhibitor JQ1 had slowed replication fork progression in Nalm 6 cells (Da Costa, Agathanggelou et al. 2013). Slowed replication fork progression, if persistent, can lead to promotion of DNA damage and genomic instability in the cell (Zeman and Cimprich 2014, Kotsantis, Jones et al. 2015, Kotsantis, Silva et al. 2016). Research performed in this thesis aimed to uncover the causes of BET inhibitor-induced fork slowing and the effect that this would have on the cell. A more detailed understanding of the impact of BET proteins on processes such as DNA replication is necessary for development of future therapeutic applications.

7.1: BET inhibition causes P-TEFb induced replication-transcription conflicts

In this thesis, we first sought out how BET inhibitors were causing replication stress. Here we identified that BET inhibition leads to an increase in total nascent RNA synthesis as measured by EU assay and total RNA quantification. In contrast to our findings, recent papers had shown that BET inhibitor treatment led to suppression of nascent transcription of protein encoding genes such as *MYC* (Winter, Mayer et al. 2017, Muhar, Ebert et al. 2018). By re-analysing the RNA Pol II ChIP-seq data-sets published in Muhar et al, we showed that following BRD4 depletion after 1 hour led to an increase in genome-wide occupancy of RNA Pol II (Bowry, Piberger et al. 2018) (Figure 7.2). This was seen especially over transcription of non-poly-adenylated histone and non-coding RNA genes. More analysis using qRT-PCR showed increased gene expression of candidate genes and also corresponded with previous reports where we observed decreases in MYC transcript levels (Bowry, Piberger et al. 2018) (Figure 7.1). These experiments were carried out by the Saporano group. This suggested that BET inhibition leads to increased transcription of non-poly-adenylated histone and non-coding RNA genes suggesting that this was the reason for observed increased in nascent RNA synthesis.

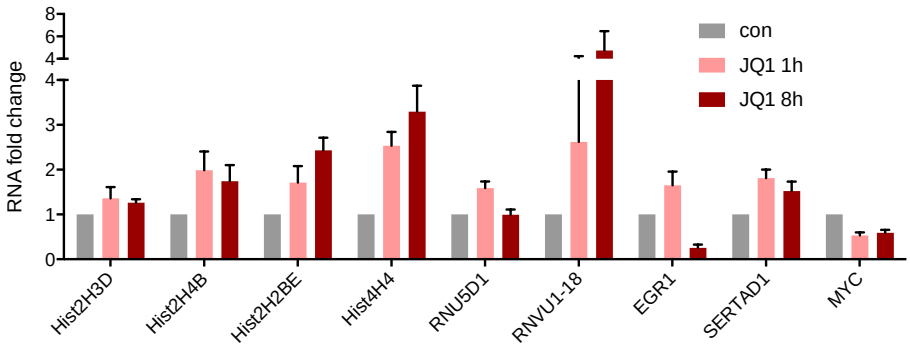


Figure 7.1: Fold change in the normalized expression levels of indicated transcripts ± JQ1 as indicated (n = 4). Retrieved from (Bowry, Piberger et al. 2018)

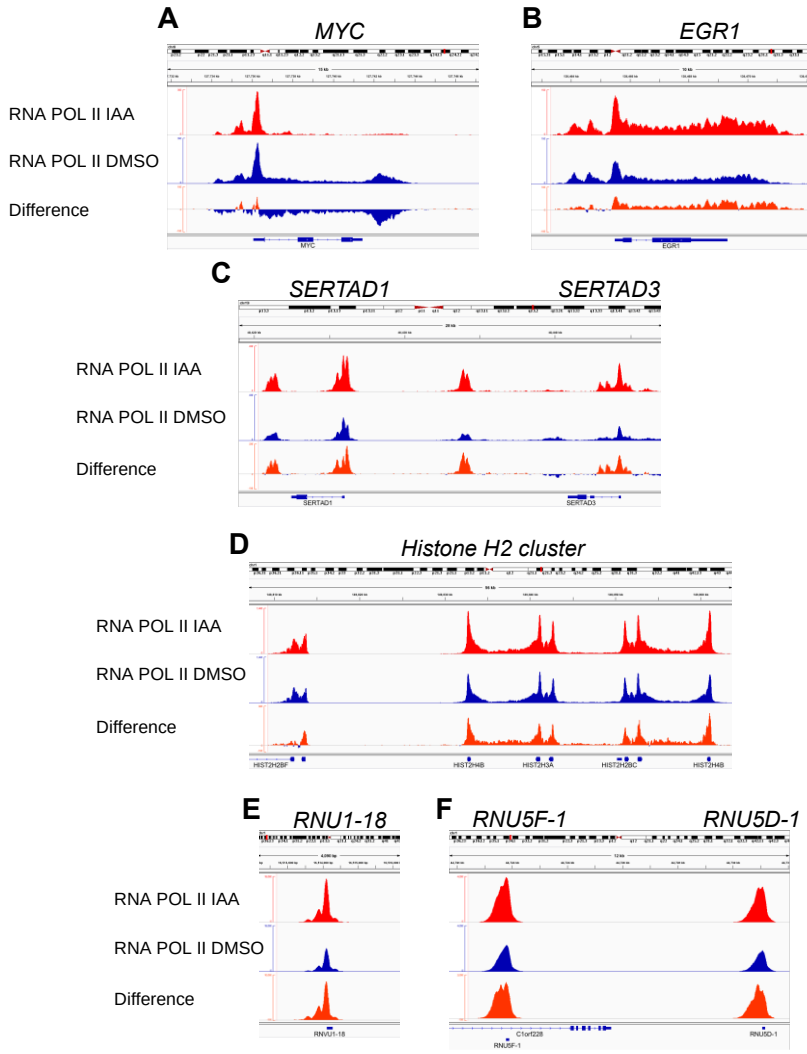


Figure 7.2: BRD4 degradation increases RNA Pol II occupancy in histone and non-coding RNA genes. Retrieved from (Bowry, Piberger et al. 2018).

Increased transcription has recently been shown to cause replication fork slowing in cells (Kotsantis, Silva et al. 2016, Stork, Bocek et al. 2016, Gorthi, Romero et al. 2018, Lavado, Park et al. 2018, Nojima, Tellier et al. 2018). We identified that this increase in transcription indeed was the cause of fork slowing after BET inhibitor treatment by using transcriptional inhibitors.

The mechanism of how BET inhibitors cause this increase in RNA synthesis was next investigated. Research had suggested that BET inhibitors could lead to disruption of a 7SK inhibitory complex of P-TEFb (Bartholomeeusen, Xiang et al. 2012). This would lead to increase in free and active P-TEFb in the cell. P-TEFb is crucial in allowing productive elongation of RNA Pol II via CTD phosphorylation (Peterlin and Price 2006, Jonkers, Kwak et al. 2014, Jonkers and Lis 2015). We showed that BET inhibitor treatment lead to increased RNA Pol II CTD phosphorylation indicating that BET inhibitor treatment lead to more free P-TEFb. To confirm that disruption of the 7SK complex can lead to increased replication-transcription interference we used a chemical called HMBA. HMBA is known to disrupt 7SK inhibitory complex, but has no known effects on DNA replication (Contreras, Barboric et al. 2007, Fujinaga, Luo et al. 2015). We showed that HMBA treatment also caused increased transcription-replication interference via a mechanism that involved the disruption of the 7SK complex. A caveat is that a recent paper showed that HMBA may act as a BET inhibitor by disrupting BET proteins binding to chromatin (Nilsson, Green et al. 2016). This could mean that our results seen with HMBA are due to the fact that it is a BET inhibitor and that it functions similarly to JQ1. However, it also shows further evidence that BET inhibitors function to slow down fork speeds via disrupting the 7SK-PTEF-b

complex. As stated HMBA is known not to have any effects on replication, so as a BET inhibitor it causes fork slowing via the disruption of 7SK-PTEF-b. The role of HMBA as a potential BET inhibitor is dose dependent. HMBA has a weaker IC50 to that of BET inhibitors such as JQ1 (Nilsson, Green et al. 2016). We could therefore potentially use concentrations of HMBA where its ability to function as a BET inhibitor is poor to confirm whether the fork slowing phenotype is due to non-BET-targeted effects.

All together, the HMBA results provide information that disrupting the 7SK-P-TEFb complex provides a mechanism in which forks can be slowed down due to the increased transcription-replication interference that it causes.

BET inhibition illustrates a model in how small-molecule inhibitors can also lead to the formation of replication stress via increased RNA synthesis after treatment. We hypothesise that the increase in RNA synthesis we observe after BET inhibitor treatment represents a global increase in transcription activity. RAS activation was shown to lead to a global increase in RNA synthesis (Kotsantis, Silva et al. 2016). We observed similar phenotypic changes in RNA synthesis after BET inhibition treatment that were seen in cells after RAS activation (Kotsantis, Silva et al. 2016). We observed that BET inhibition led to increased EU incorporation. The EU assay is designed to show global changes in RNA synthesis. Furthermore, the observed increase in RNA synthesis after BET inhibitor treatment was lost when BET inhibitor treated cells were also co-treated with transcriptional inhibitors known to inhibit global transcription (Bensaude 2011, Wang, Johnson et al. 2014, Chen, Gao et al. 2015). This loss of RNA synthesis by transcription inhibitors also coincided with fork speeds being rescued. This again suggests that loss of global transcription is

stopping BET inhibitor induced transcription-replication conflicts. To further address this we could perform a global RNA-seq to look at what genes are up-regulated after BET inhibition and allows us to look at the differences in global transcriptomes after BET inhibition.

Other cancer treatments such as HDAC inhibitors that led to the disruption of the P-TEFb-7SK-snRNP inhibitory complex could also lead to increased transcription-replication interference (Bartholomeeusen, Xiang et al. 2012, Bartholomeeusen, Fujinaga et al. 2013).

Hence our data strongly suggests that BET inhibitor-induced fork slowing is caused by increased transcription-replication interference via disruption of the 7SK-snRNP complex. This can allow for increase in free P-TEFb leading to more CTD phosphorylation, signalling from RNA Pol II to escape from promotor pausing.

7.2: BET inhibition leads to increased origin firing:

We observed that BET inhibitor treatment led to an increase in origins being fired. Origins are known to be fired locally in response to increased fork slowing as a mechanism to help rescue stalled forks (Woodward, Gohler et al. 2006, Ge, Jackson et al. 2007, Blow, Ge et al. 2011). There is also evidence to suggest that the regulation of origin firing is key to maintaining normal fork speeds, and that an increase in the number of origins being fired can cause increased replication stress (Petermann and Helleday 2010, Beck, Nahse-Kumpf et al. 2012, Jones, Mortusewicz et al. 2013). Our data suggested that the increased amount of origins being fired in response to BET inhibitor treatment may be inducing the fork slowing

phenotype observed. However, the CDK inhibitors used to suppress origin firing also seemed to interfere with transcription making it difficult to interpret our results. Further work is needed to understand why we see an increase in the number of origins fired after BET inhibitor treatment. To investigate this further we could deplete cells of either CDC6 or CDC7 and observe whether this would lead to a rescue in replication fork speeds. This would enable us to know whether increased origin firing is causing slower fork speeds. Increased firing of origins may also be involved in transcription induced replication fork slowing by altering the spatial coordination of replication initiation and transcription after BET inhibitor treatment (Helmrich, Ballarino et al. 2011, Jones, Mortusewicz et al. 2013, Sansam, Pietrzak et al. 2018).

We have shown that after 24 hours of BET inhibitor treatment fork speeds are rescued to speeds observed in control treated samples. As mentioned earlier, an increase in the number of origins fired can rescue fork speeds and allow for the continuation of normal replication afterwards. This may indicate that the increase in the number of origins being fired after BET inhibitor treatment is helping rescue reversed forks. This would lead to replication fork speeds returning back to normal control speeds. This could be tested by analysing fork speeds after 24 hours of BET inhibitor treatment in cells depleted of either CDC6 or CDC7.

If this increase in origin firing is lost, it could lead to increased genomic instability or increased damage, as JQ1 induced reversed forks are unable to be rescued. We could test this by looking at whether BET inhibitor treatment leads to any increases in DNA damage markers such as γH2AX or 53BP1 in cells depleted of either CDC6 or CDC7. Increased origin firing may also help prevent under-replication which

could also lead to increased genomic instability (Shima, Alcaraz et al. 2007, Debatisse, Le Tallec et al. 2012). Again this could be investigated by analysing BET inhibitor treated cells depleted with CDC6 or CDC7 and seeing whether we observe increases of 53BP1 bodies or anaphase bridges.

7.3: BRD4, the BET protein required for normal fork progression

Our data provide insight into the roles of BRD4 in DNA replication. BRD4 depletion caused replication stress in cells that was not seen when BRD2 and BRD3 were depleted. BRDT is expressed only in the testis and is not expressed well in a whole range of cancer cell lines including U2OS especially compared to other BET proteins. This was further illustrated using the CCLE database where mRNA expression levels for BRDT across a range of cancer cells was miniscule (Appendix A1). BRD4 has been shown to have numerous roles in the cell, however our data suggests that it has a novel role in DNA replication. Cells depleted of BRD4 were unable to show normal fork speeds, suggesting that BRD4 is important in maintaining normal replication fork progression in the cell. Our data implied that loss of BRD4 led to increased RNA synthesis which caused more transcription-replication interference hence leading to slower fork speeds. This result mirrored the phenotype observed with BET inhibition. However, BET inhibition caused transient fork slowing while BRD4 consistently slowed forks, suggesting that there is some mechanism of fork speed adaptation with BET inhibitor treatment not seen with BRD4 depletion. Our data suggest that BRD4 regulated DNA replication via the

interaction with P-TEFb and not via the interaction with the replication factors RFC and TICRR. This was shown via the inability of BRD4 short isoform containing RFC and TICRR interactions to rescue replication stress (Maruyama, Farina et al. 2002, Sansam, Pietrzak et al. 2018). The CDC6 interaction site in BRD4 is still unmapped, so we could not test it (Zhang, Dulak et al. 2018). Overall our data supports that BRD4 loss rapidly leads to increased P-TEFb activity.

Together this data suggests a newly discovered role of BRD4 in the process DNA replication which may be potentially important in future cancer therapy applications.

7.4: BET inhibitor-induced transcription-replication conflicts depend on HEXIM1

Our results have suggested that HEXIM1 plays an important role in BET inhibitor induced replication fork slowing. Cells depleted of HEXIM1 do not show BET inhibitor-induced fork slowing for up to at least 8 hours. HEXIM1 depletion also leads to suppression of BET inhibitor induced RNA synthesis at early time points as well. The mechanism of how HEXIM1 depletion causes delay in BET inhibitor-induced changes to fork progression and RNA synthesis is still unclear at this moment. What we do know is that HEXIM1 is required for the BET inhibitor induced fork slowing.

After 24-hour treatment with BET inhibitor in HEXIM1 depleted cells, there is significant fork slowing. Why we observed this switch in phenotype around this time point is still unclear. We suggest that this phenotypic switch could be caused by

extensive gene expression changes or possibly HEXIM2 which may compensate for HEXIM1 loss (Byers, Price et al. 2005). HEXIM2 has been shown to compensate for HEXIM1 loss in cells and has been shown to be regulated differently in response to external stimulus. Hence, at this time point HEXIM2 could carry out the function of HEXIM1 and hence may explain the switch in phenotype (Byers, Price et al. 2005). Extensive gene changes through 24 hours of BET inhibitor treatment may also affect how the cell deals with HEXIM1 depletion (Delmore, Issa et al. 2011, Muralidharan, Bhadury et al. 2016).

We also show that HEXIM1 loss can promote JQ1 effects, such as DNA damage. In addition to modulating transcription-dependent fork slowing, HEXIM1 might play undiscovered roles in replication stress and DNA damage response. It may be relevant that HEXIM1 also regulates p53 (Lew, Chia et al. 2012). HEXIM1 has been found to interact with p53 and enhances its stability and function. Hence by down-regulating HEXIM1, the observed effects we see may be due to the loss of p53 stability and loss of p53 induction. However, we have observed JQ1-induced replication fork slowing in a p53 mutant cell line MEC1 (Agathanggelou, Smith et al. 2017).

7.5: The importance of RAD51 in BET inhibitor response to transcription-replication conflicts

If the cause of replication stress is persistent, it normally results either in DNA damage or genomic instability (Kotsantis, Jones et al. 2015). An ATR driven response is normally seen in situations of prolonged fork slowing (Ciccia and Elledge 2010, Zeman and Cimprich 2014). In this thesis we had shown that there was little to no replication stress response after BET inhibitor treatment or after BRD4 depletion. We found that there was little activation of ATR response markers such as RPA and γH2AX or p-CHK1 S317. This observation has also been reported in recent studies using CRPC cells where the authors also observed BET inhibitor-induced decreases in γH2AX and 53BP1 foci (Pawar, Gollavilli et al. 2018). A report last year also had similar results shown in both U2OS and OVCAR3 cell lines where p-CHK1 S317 was reduced after BET inhibitor treatment. p-CHK1 activation was also reduced when BET inhibitor treatment was combined with HU compared to HU treatment alone (Zhang, Dulak et al. 2018). This confirms our results that BET inhibitor induced fork slowing does not activate an ATR response. This was followed up by investigating whether slowed replication forks may activate a different pathway in response to fork slowing induced by BET inhibition or BRD4 depletion. The homologous recombination factor RAD51 has been established to be important in binding to stalled forks and stabilizing forks that have encountered stress (Arnaudeau, Lundin et al. 2001, Lundin, Schultz et al. 2003, Petermann, Orta et al. 2010, Carr and Lambert 2013, Willis, Chandramouly et al. 2014, Kolinjivadi, Sannino et al. 2017). RAD51 loading onto replication forks is known to slow forks in response to a variety of genotoxic agents

(Zellweger, Dalcher et al. 2015). Our data shows that BET inhibition leads to increased recruitment of RAD51 shown by the increase in the percentage of RAD51 foci positive cells. We still need to know whether this occurs transcriptionally or post-translationally. Firstly, we could look at levels of RAD51 mRNA using qRT-PCR, and RAD51 protein levels by Western blot, after BET inhibitor treatment. Western blotting has already shown that RAD51 levels were not increased after JQ1 treatment (Figure 6.2 B). To see whether BET inhibitor treatment is modifying RAD51 post-translationally, we can use techniques such as tandem mass spectrometry or IP to look at PTMs. Tandem mass spectrometry can be used to identify and quantify PTMs on proteins. Here we could isolate and purify RAD51 before digesting the protein with trypsin, both with control and BET inhibitor treatments. This would be followed by peptide enrichment before analysis can take place. Shifts in mass indicate a modified amino acid. This technique allows for broad spectrum analysis of novel PTMs on proteins (Larsen, Trelle et al. 2006, Silva, Vitorino et al. 2013, Ratovitski, O'Meally et al. 2017). Another experiment that could be used is immunoprecipitation. Here a protein, both from untreated and treated samples, can be isolated and enriched before Western blotting using antibodies against specific PTMs. By carrying out these experiments we would hope to further understand how of RAD51 is regulated after BET inhibitor treatment and have a clearer understanding of the mechanism of RAD51 recruitment. Our data strongly suggests that this depends on RNA synthesis and that RAD51 slows down forks after BET inhibition. RAD51 has been shown to be able to lead to replication fork reversal to help stabilise stalled replication forks (Zellweger, Dalcher et al. 2015). Our data also support that fork reversal could play a role at BET inhibitor affected forks. Cells depleted of BRD4 also showed an increased

amount of RAD51 foci indicative of a similar mechanism, however more research needs to be done to see if this mirrors the BET inhibitor phenotype completely. This data suggests that RAD51 binds to forks that have been slowed due to either direct or indirect effects of increased transcription-replication interference, which can lead to fork reversal.

We had also shown that RAD51 helps suppress the DNA damage response in presence of BET inhibitors. Cells depleted of RAD51 had increased DNA damage signalling after BET inhibitor treatment. This would indicate that RAD51 helps not only to slow down fork speeds but helps prevent the formation of DNA damage. We also observed that BET inhibitors reduced RAD51 expression levels after treatment. Other research had also showed similar results, that RAD51 expression is downregulated in response to BET inhibition and in models of acquired BET inhibitor resistance (Yang, Zhang et al. 2017, Pawar, Gollavilli et al. 2018). Interestingly, acquired BET inhibitor resistance models also showed more DNA damage signalling (Pawar, Gollavilli et al. 2018). This agrees with our data that RAD51 downregulation increases DNA damage signalling.

Our data also showed that JQ1 treatment could lead to genomic instability in cells. Genomic instability is a key hallmark for tumour progression (Hanahan and Weinberg 2000). Potential causes of micronuclei formation could be the inability to resolve replication stress intermediates so that the cell can continue normal replication. Here, the cell is unable to remove the replication stress source which results in replication fork to collapse leading to DNA breaks and potential micronuclei formation. This could be due to the collapse or degradation of the suggested reversed fork after BET inhibitor treatment. Other leading causes of

micronuclei normally occur from faulty DNA repair mechanisms which lead to mis-repaired or unrepaired DNA breaks. Micronuclei formation may also be due to mitosis and cell cycle effects. This includes faulty spindle formation, de-regulation of cell cycle checkpoints, defects in kinetochore proteins and assembly or improper chromosome segregation during mitosis (Thomas, Fenech et al. 2011, Luzhna, Kathiria et al. 2013). Whether JQ1 causes micronuclei formation by improper resolution of replication stress or via another mechanism in the cell is unclear. However, this finding is still important as the formation of genomic instability could be a much unwanted side-effect of JQ1 treatment if pursued further as a cancer therapy treatment, as genome instability in the cell has the potential to be a major driving force in tumorigenesis and allow cancer cells to evolve and adapt to maximise cell survival.

In this thesis we have presented novel roles of BET proteins and their inhibitors in the process of DNA replication. Put together we outline a model where firstly BET inhibitors can lead to increased RNA synthesis via disruption of 7SK-snRNP complex. The increase in transcription leads to increased transcription-replication interference causing slow fork speeds. Loss of BRD4 protein also leads to replication fork slowing due to increased RNA synthesis. This fork slowing mechanism requires the loss of BRD4 interaction with P-TEFb. HEXIM1 protein is involved upstream of these replication-transcription conflicts, although the mechanism is still unclear. These replication-transcription conflicts activate RAD51, which binds and slows down fork speeds. Our data also suggests that these forks are reversed too. Both RAD51 acting downstream of these conflicts and HEXIM1 upstream of these conflicts help prevent the formation of DNA damage. This model

is outlined below (Figure 7.3). How BET inhibitor treatment leads to increased RNA Pol 1 and RNA Pol 3 transcription and how this contributes mechanistically to BET inhibitor induced fork slowing is unclear. How the observed increase in fired origins after BET inhibitor treatment contributes mechanistically to BET inhibitor induced fork slowing is also unclear at this moment. This is depicted in Figure 7.3.
Studies have indicated that BET inhibitor resistance shows increased DNA damage and that cells depleted with either RAD51 or HEXIM1 showed increased BET inhibitor resistance. This would highlight that these factors are involved in BET inhibitor resistance by potentially preventing increased DNA damage in response to BET inhibitor treatment.

BET inhibitors have been fast-tracked into clinical trials and it is important for cancer therapy that we know as much about the effects that these drugs have on different cellular processes. In this thesis we provide insights into the role of BET proteins, DNA damage and DNA replication which could be useful in future cancer therapy research.

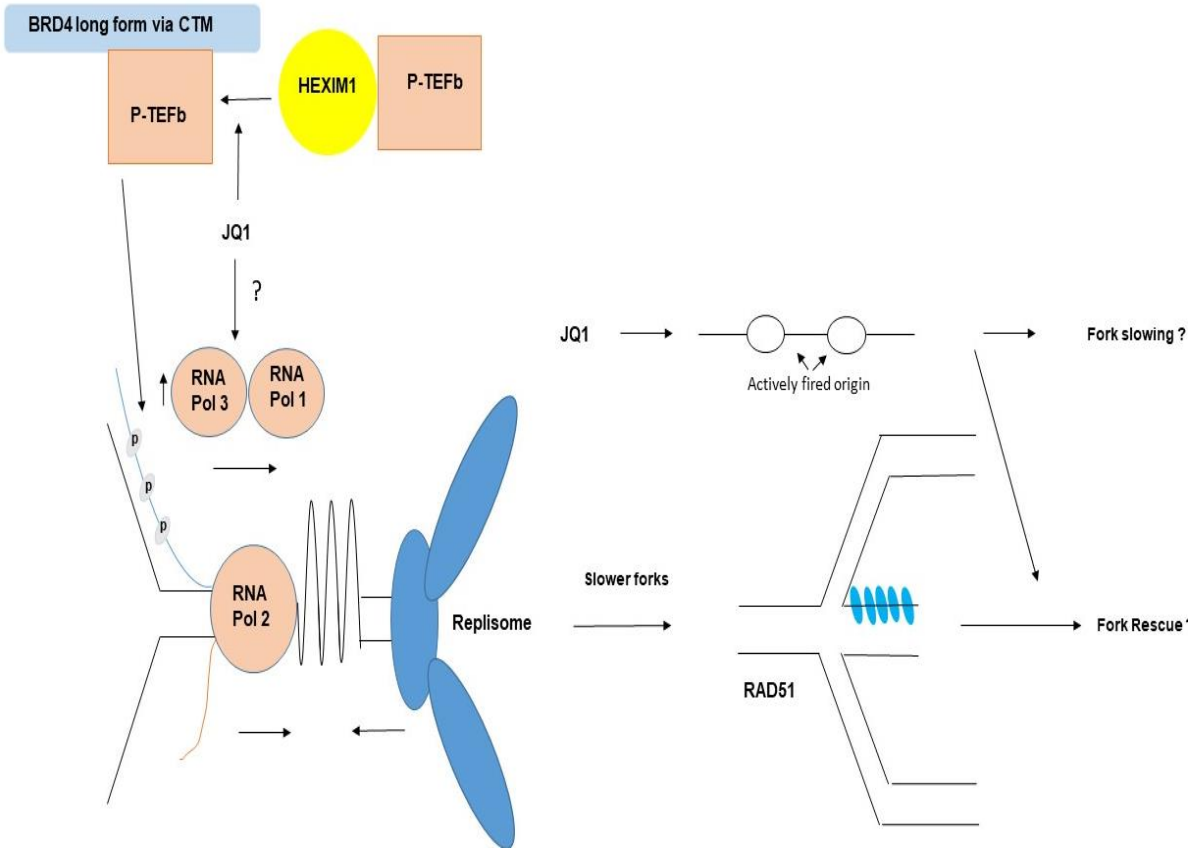


Figure 7.3: Diagram representing the proposed model for this thesis.

7.6: Future questions and directions

In this thesis we have provided a function for BET proteins in DNA replication and have shown a mechanism where inhibition or loss of BET proteins can cause impaired DNA replication fork progression and lead to downstream activation of homologous recombination. However, there are still some key questions to be resolved in the future.

Throughout the thesis we have performed our experiments with inhibitor concentrations that have been used in previous literature. However, the IC50 for many drug inhibitors we used are much lower. For example, JQ1 has an IC50 of 77 nM (Filippakopoulos, Qi et al. 2010). Using a higher concentration of drugs could increase the chances of causing off target effects. There is a potential danger that some of the results we have observed may be due to some off target effects induced by using higher drug concentrations. It is therefore crucial in future experiments to use drug inhibitor concentrations that are closer to their IC50s. This will serve to increase the confidence that my results are due to the specific inhibiting of the proteins or signalling pathways that we aim to target. It is also important to carry out experiments to observe whether there are dose dependent effects seen with using these drugs on our experiments. This again allows us to be more confident in concluding our results are due to the specificity of the drugs being able to inhibit our desired targets rather than some off target effects.

Both BRD4 depletion and BET inhibition led to increased RNA synthesis and replication fork slowing. However, after 24 hours of BET inhibition, we see a rescue in replication fork speeds that we do not see with BRD4 depletion. The mechanism

for this is still not clear suggesting that more research using BRD4 depletion is required. We see that after BET inhibitor treatment for 24 hours when RAD51 levels have been depleted, RAD51 overexpression leads to fork speeds being decreased. This suggests that when RAD51, which slows forks speeds down in response to transcription-replication conflicts, is downregulated due after 24 hours BET inhibitor treatment, this could help rescue replication fork speeds. We observed that BRD4 depletion led to more RAD51 foci similar to BET inhibitor treatment, however we did not measure the protein levels of RAD51 after 48 hours of BRD4 depletion. The RAD51 downregulation after BET inhibitor treatment may be one explanation for this difference and experiments looking into levels of RAD51 protein after BRD4 depletion will need to be done in the future. There may be other factors involved in this adaptation to BET inhibitor treatment, as there is potential for BET inhibitors to change the expression or activation of a number of target genes resulting in DNA replication adaptation. Again, this would require more research to look into the difference between the impact of BET inhibitor treatment and BRD4 depletion on changes in the cell.

Due to time constraints, work on the mechanism downstream of BRD4 depletion is not complete. More experiments on whether RAD51 slows down replication forks in BRD4 depleted cells and whether BRD4 depletion can cause fork reversal and genomic instability needs to be looked at in the future. It would also help to see whether RAD51 depletion suppresses DNA damage in BRD4 depleted cells too. This will give insights into how BRD4 depletion and BET inhibitor treatments work and would identify whether their mechanisms downstream of these replication-transcription conflicts are the same. Recently, more work has been done using

specific drugs that lead to BRD4 disruption and degrade BRD4 (Lu, Qian et al. 2015, Winter, Buckley et al. 2015, Winter, Mayer et al. 2017) to counteract potential side-effects from using pan-BET inhibitors which inhibit numerous transcriptional pathways. Understanding the full roles of BRD4 loss in DNA replication and DNA damage may provide to be useful if these specific drugs were to be used in future cancer therapy.

We showed how crucial HEXIM1 is to replication fork slowing and DNA damage in BET inhibitor treated cells. The full mechanism of how HEXIM1 depletion can delay BET inhibitor-induced fork slowing is still unclear. We speculated that perhaps there is a role for HEXIM2 that helps to compensate for HEXIM1 loss. Further experiments in depleting both HEXIM2 and HEXIM1 would likely shine more light onto this mechanism.

Interestingly, reduced HEXIM1 protein levels have been observed in metastatic breast cancer, melanoma, acute leukaemia and colon rectal cancer (Ketchart, Smith et al. 2013, Huang, Garcia et al. 2016, Tan, Fogley et al. 2016). In our data reduced HEXIM1 levels prevent transcription-replication interference but lead to amplified DNA damage and genomic instability in the cells. Other research has shown that KRAS mutations can correlate with BET inhibitor resistance and mitogen-activated protein kinase (MAPK) inhibition sensitises colorectal cancer (CRC) lines to BET inhibition (Ma, Wang et al. 2017, Sun, Yin et al. 2018). The lab has also observed similar preliminary results to BET inhibitors with HRAS overexpressed cells, where HEXIM1 depletion causes amplified DNA damage signalling. This suggests that HEXIM1 could be an important new player in oncogene- and drug-induced

replication-transcription conflicts. As HEXIM1 loss has been reported to promote resistance to long-term BET inhibition, its impact on replication stress, survival and genomic instability in cancer require urgent investigation (Devaraj, Fiskus et al. 2016).

Key questions arising from this research are: What is the role of HEXIM1 in modulating replication-transcription conflicts? How does HEXIM1 interact with existing oncogene-induced replication stress? Can this be exploited as a biomarker and target to improve cancer therapy? Tackling these questions may help to better understand the function of HEXIM1 in BET inhibitor induced transcription-replication conflicts as well as uncover novel roles of HEXIM1 in cancer and potentially establish a new biomarker for future clinical work in cancer therapy research.

To address these questions, we could firstly look to induce transcription-replication conflicts by treating cells with drugs, specifically BET inhibitors, to induce HEXIM1 complex disruption. We could also use biochemistry assays to assess changes in the interaction between P-TEFb (consisting of CDK9 and Cyclin T1) and HEXIM1, in response to BET inhibition, by immunoblotting for RNA Pol II CTD phosphorylation for P-TEFb activity. Another experiment that could be used to assess changes in P-TEFb and HEXIM1 interaction, is performing site directed mutagenesis targeting the phosphorylation sites T270 and S278 in HEXIM1. These sites are targeted by oncogenic signalling through PI3K-AKT and play an important role in regulating the dissociation of HEXIM1 and P-TEFb (Contreras, Barboric et al. 2007). We could also look at the effects of oncogenes on RNA synthesis after HEXIM1 depletion or overexpression to see how HEXIM1 affects oncogenic changes in RNA synthesis.

HEXIM1 has not been detected at unperturbed or stressed replication forks, but we could test to see if HEXIM1 localises to replication forks or DNA damage sites under wither oncogene- or BET inhibitor induced replication stress using Isolation of Proteins on Nascent DNA (Ipond). This will help us address both the role of HEXIM in replication stress and its role in oncogenic stress as well.

To see if HEXIM1 may be a potential biomarker we can look at CRC cells or organoid tissues that have low levels of 7SK or HEXIM1 and investigate the potential effects on DNA replication and DNA damage. We can also test whether cells or organoids with low levels of HEXIM1 are more resistant to drugs or cancer treatments that either induce replication stress or induce HEXIM1 via P-TEFb disruption by performing cell survival assays.

In addition to the work being done on HEXIM1 more research is needed to further elucidate the exact downstream effects of BET inhibitors. It is clear that RAD51 and HEXIM1 prevent DNA damage signalling. It is unclear still if BET inhibitor induces any physical DNA damage in the cell and further experiments to measure DNA breaks using comet assays and pulse field gel electrophoresis should allow us to see if DNA damage is occurring. Previous reports have only looked into the comet assay comparing BET inhibitor treatment in normal cells compared to BET inhibitor resistant cells (Pawar, Gollavilli et al. 2018). It will be more informative to compare total damage with BET inhibitor treatment compared to control treatment in U2OS cells especially at the time points where we have observed fork slowing. This would allow us to know whether we see suppression of DNA damage signalling pathways as suggested by our results and other studies or whether no damage is occurring in the cell after BET inhibitor induced fork slowing. It is surprising that we do not see

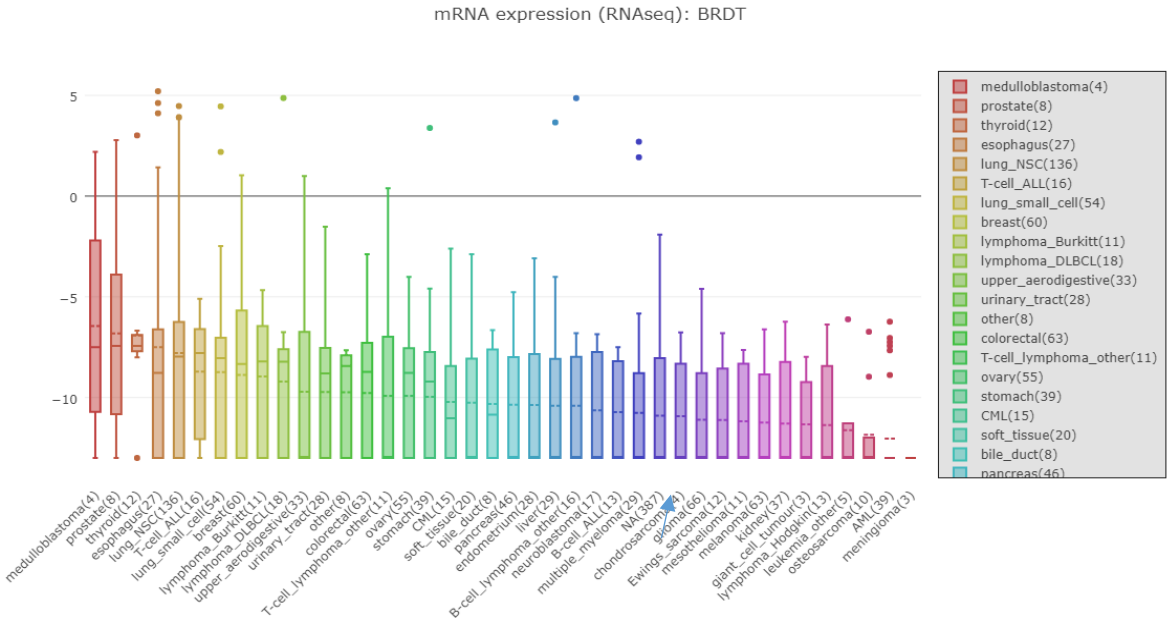
the formation of RPA foci after BET inhibitor treatment but do see RAD51 foci formation. Further research into how RAD51 is loaded without prior RPA loading will be necessary. The first step would be to see if BET inhibitor treatment leads to ssDNA formation, potentially using electron micrographs. This would allow us to see if there is either no ssDNA formed in which case maybe RAD51 is binding to other structures such as regressed forks. If there is ssDNA formation then maybe RPA binding is being suppressed, or potentially RAD51 may quickly replace RPA. RAD51 foci could be formed either to help reverse forks or to stabilise regressed forks, however this would require more research as well. It would seem that RAD51 could bind to slowed forks helping them reverse but this is just a hypothesis and would require further evidence. RAD51 seems to play a role in BET inhibitor induced fork reversal at earlier time points after BET inhibitor addition, however we are unsure on what happens next. Carrying out experiments such as looking at the percentage of stalled forks through a JQ1 treatment time-course to see whether we have re-start of stalled forks after 24 hours or beyond may help us answer this question. This would allow us to explain the mechanism more clearly. If forks do not restart, then perhaps collapse of these forks later on could potentially be a cause of genomic instability that is observed with BET inhibitor treatment. If reversed forks are not being re-started it would be interesting to see whether we see more DNA damage signalling at later time points after BET inhibitor treatment (24 hours and beyond) without RAD51 depletion. This might then support reversed forks collapsing. It is not yet clear whether the genomic instability caused by BET inhibitor treatment is directly due to the effect of increased transcription-replication interference. BET inhibitors may have a number of effects on the cell, as BET

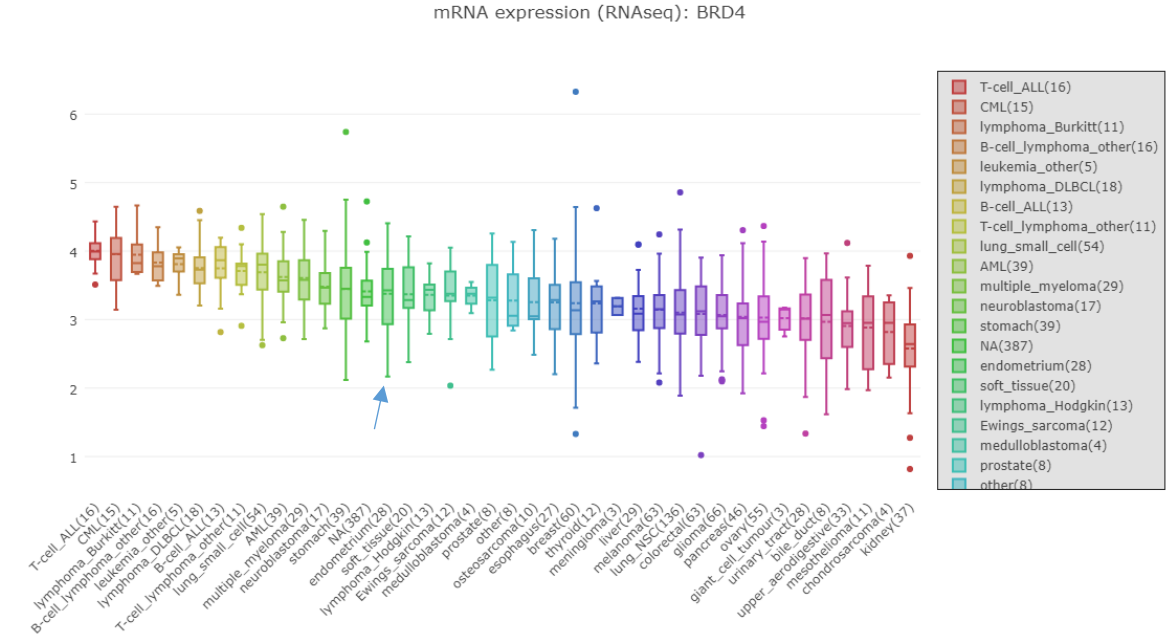
proteins are implicated in many cellular processes such as cell cycle regulation and regulation of many transcriptional pathways. BET inhibitors may impede the role of BRD4 to regulate normal mitosis in the cell which could give rise to micronuclei formation (Yang, He et al. 2008). Potential changes in gene expression, especially in the expression of DNA repair genes could also have an effect on the amount of micronuclei formation in the cell. More research is required to understand the exact mechanism of BET inhibitor-induced genomic instability.

Further research is required to fully understand the biological functions of BET proteins and their role in DNA replication and DNA damage, and in understanding the impact of BET inhibitors on these processes too. However, our results provide some novel insights onto this area which will be useful in the future. It is important to understand the complexity and wide-ranging consequences and implications of using BET inhibitors as cancer therapeutics and it is vital that more research is done to understand the roles of BET proteins in the cell more fully.

Appendix

A1: Expression levels of BET proteins across human cell lines





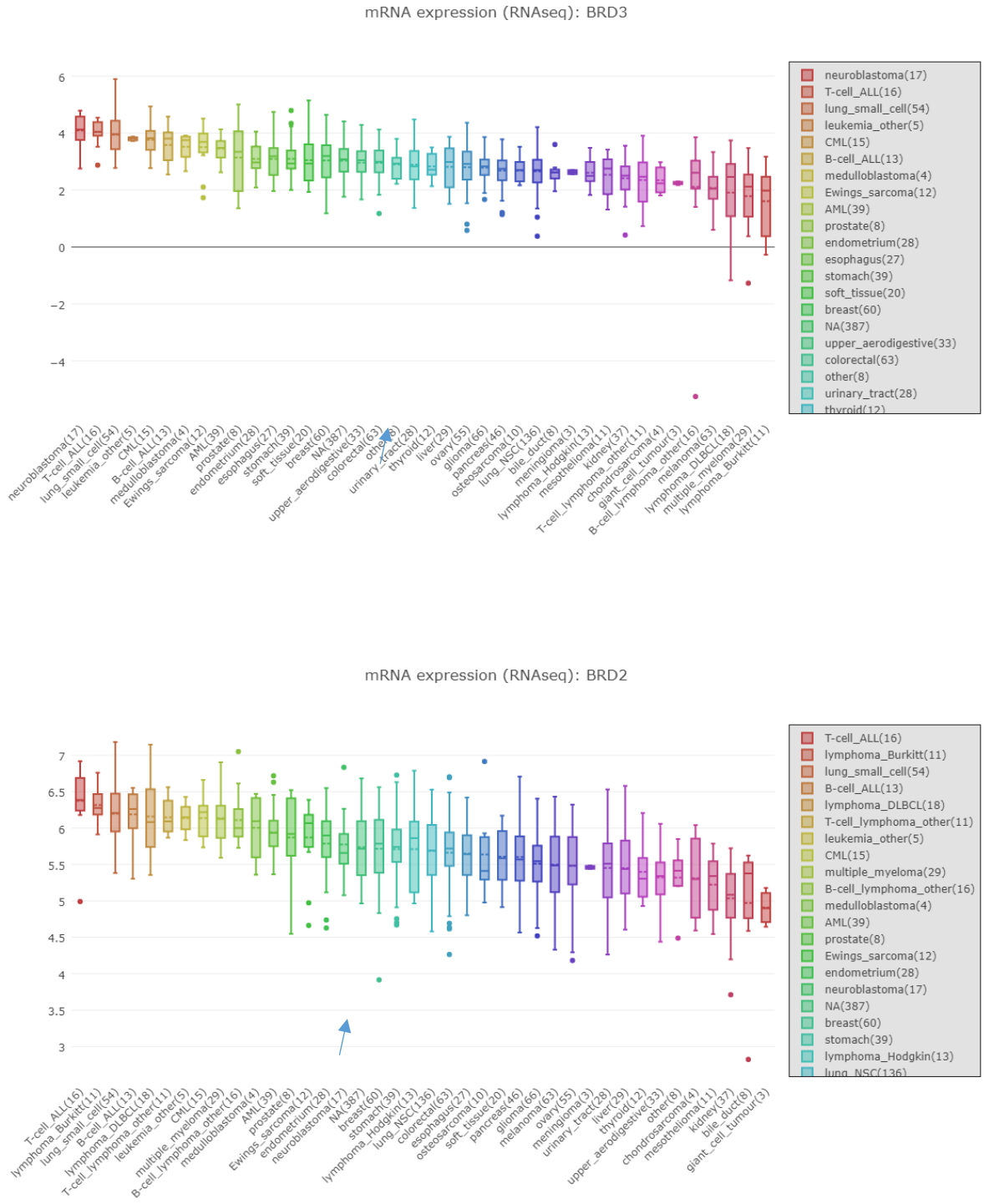
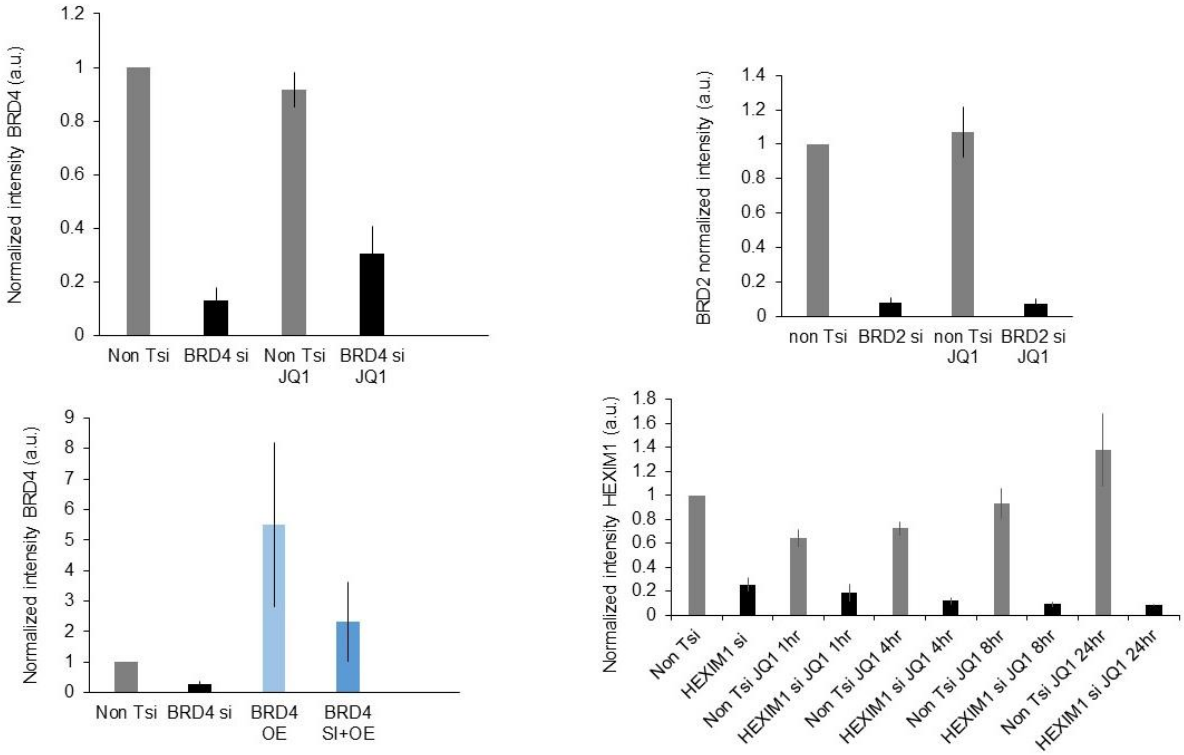
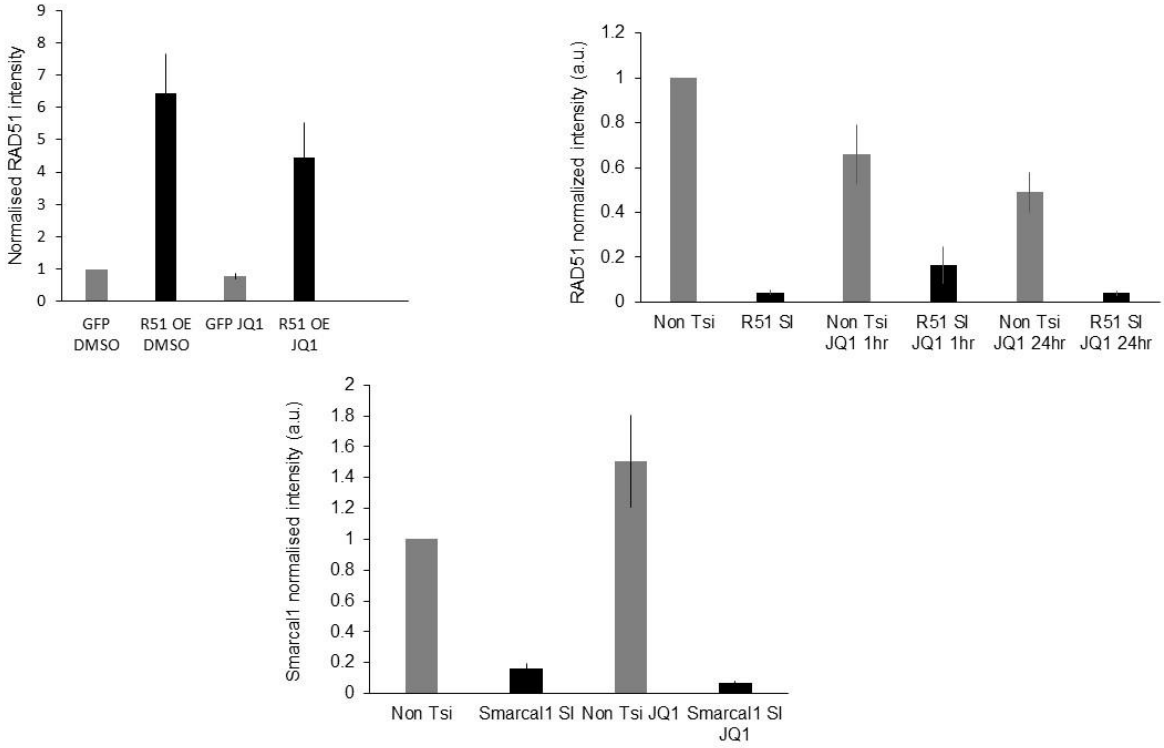


Figure A1: Data showing mRNA levels of BET proteins BRDT, BRD4, BRD3 and BRD2 in selected cancer cells. Arrow shows osteosarcoma cells (U2OS cells). Data retrieved from **https://portals.broadinstitute.org/ccle**

A2: Western blot Quantification



Quantification of protein levels for BRD4 siRNA fibre experiments, BRD2 siRNA fibre experiments, BRD4 short isoform overexpression experiments and HEXIM1 siRNA fibre experiments.



Quantification of protein levels for RAD51 siRNA fibre experiments, RAD51 overexpression experiments and Smarcal1 siRNA fibre experiments.

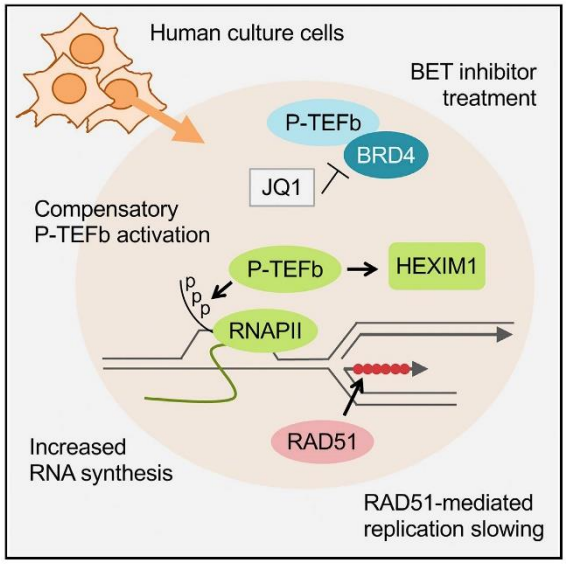
A3: Published work

Cell Reports

Report

BET Inhibition Induces HEXIM1- and RAD51-Dependent Conflicts between Transcription and Replication

Graphical Abstract



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In Brief

Bowry et al. show that BET inhibitors, emerging cancer therapeutics that target transcription programs, cause conflicts between transcription and replication by deregulating P-TEFb. These conflicts recruit the homologous recombination factor RAD51, which slows down replication and suppresses DNA damage. This highlights the importance of replication stress for BET inhibitor treatment.

Highlights

- BET inhibitors and BRD4 depletion increase overall RNA synthesis
- Loss of BRD4 activity causes conflicts between transcription and DNA replication
- These transcription-replication conflicts depend on P-TEFb inhibitor HEXIM1
- BET inhibition activates RAD51 to slow replication forks and suppress DNA damage

Bowry et al., 2018, Cell Reports *25*, 2061–2069
November 20, 2018
https://doi.org/10.1016/j.celrep.2018.10.079

CellPress

Cell Reports
Report

BET Inhibition Induces HEXIM1- and RAD51-Dependent Conflicts between Transcription and Replication

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https://doi.org/10.1016/j.celrep.2018.10.079

SUMMARY

BET bromodomain proteins are required for oncogenic transcription activities, and BET inhibitors have been rapidly advanced into clinical trials. Understanding the effects of BET inhibition on processes such as DNA replication will be important for future clinical applications. Here, we show that BET inhibition, and specifically inhibition of BRD4, causes replication stress through a rapid overall increase in RNA synthesis. We provide evidence that BET inhibition acts by releasing P-TEFb from its inhibitor HEXIM1, promoting interference between transcription and replication. Unusually, these transcription-replication conflicts do not activate the ATM/ATR-dependent DNA damage response but recruit the homologous recombination factor RAD51. Both HEXIM1 and RAD51 promote BET inhibitor-induced fork slowing but also prevent a DNA damage response. Our data suggest that BET inhibitors slow replication through concerted action of transcription and recombination machineries and shed light on the importance of replication stress in the action of this class of experimental cancer drugs.

INTRODUCTION

Members of the BET bromodomain-containing protein family bind to lysine-acetylated histone tails and regulate transcription by recruiting and activating positive transcription elongation factor b (P-TEFb). P-TEFb can occur in two active complexes with BET protein BRD4 or the super elongation complex (SEC) and an inactive complex with 7SK-snRP (7SK RNA, HEXIM1, LARP7, and MEPCE). BRD4 activates P-TEFb by releasing it from 7SK-snRP and recruits active P-TEFb to gene promoters (Quaresma et al., 2016). Active P-TEFb facilitates RNA polymerase II (Pol II) pause release by phosphorylating the RNA Pol II C-terminal domain (CTD) and other targets.

BET proteins promote oncogenic transcription programs, and specific small-molecule inhibitors of BET bromodomains promise a targeted cancer treatment (Delmore et al., 2011). BET inhibition downregulates MYC protein levels and kills tumor cells independently of p53 (Da Costa et al., 2013). In solid tumor cells, BET inhibitor responses can be *MYC* independent (Lockwood et al., 2012). Although the molecular mechanisms surrounding BET inhibitor action are still poorly understood, BET inhibitors are already undergoing clinical trials in a wide range of cancers (Andrieu et al., 2016; Fujisawa and Filippakopoulos, 2017).

More recently, BRD2 and BRD4 have been implicated in DNA replication and DNA damage responses (Da Costa et al., 2013; Floyd et al., 2013; Sansam et al., 2018). BRD4 in particular interacts with DNA replication factors RFC, TICRR, and CDC6 (Maruyama et al., 2002; Sansam et al., 2018; Zhang et al., 2018). Inhibiting the interaction between BRD2/4 and TICRR slowed euchromatin replication, suggesting that BET proteins control DNA replication initiation to prevent interference between replication and transcription (Sansam et al., 2018). BET inhibitors cause little or no DNA damage but promote downregulation of DNA replication stress-response and stress-repair genes (Pawar et al., 2018; Zhang et al., 2018). It is not known whether the latter are specific responses to BET inhibition affecting replication and repair. Investigating more direct effects of BET proteins and BET inhibition on DNA replication might help understand BET inhibitor action independently of cell-type-specific transcription programs and provide insights into potential side effects and resistance mechanisms.

We previously reported that JQ1 treatment slows replication fork progression in NALM-6 leukemia cells, indicative of replication stress (Da Costa et al., 2013). Replication stress occurs when the transcription machinery or other obstacles hinder replication fork progression, which promotes formation of mutagenic or cytotoxic DNA damage, especially double-strand breaks (DSBs). This is highly relevant to cancer therapy, as many conventional chemotherapies act by causing severe replication stress and collapse of replication forks into DSBs. However, non-toxic levels of replication stress can promote genomic instability, an unwanted side effect of cancer therapy (Kotsantis et al., 2015).

Here we describe a mechanism by which BET inhibition causes replication stress. We show that BET inhibition and loss of BRD4 cause rapid upregulation of RNA synthesis and transcription-dependent replication fork slowing in a pathway that depends on HEXIM1 and RAD51. Unexpectedly, combination of BET inhibitor with HEXIM1 or RAD51 depletion prevents fork slowing but activates a DNA damage response, suggesting that replication fork slowing might help suppress BET inhibitor-induced DNA damage.

RESULTS

U2OS osteosarcoma cells were used as a well-characterized model for replication stress and DNA damage. Osteosarcoma is one of many cancers proposed to benefit from BET inhibitor treatment (Lamoureux et al., 2014). We confirmed that JQ1 treatment slowed replication within 1 hr (Figures 1A and 1B). Replication was also slowed by lower concentrations of JQ1 and another BET inhibitor, I-BET151 (Figures S1A and S1B).

As reported previously (Da Costa et al., 2013), replication forks speeds were recovered to control levels after 24 hr incubation with JQ1 and remained at control levels for up to 72 hr (Figure S1C). This was not due to loss of JQ1 activity, because adding fresh JQ1 after 23 hr did not slow fork speeds (Figures 1A and 1B). This suggests that replication forks are rapidly slowed by JQ1 treatment, but they eventually adapt. Cell cycle distribution remained unaffected between 1 and 8 hr JQ1 treatment, but cells accumulated in G1 after 24 hr JQ1 treatment (Figure S1D). The lack of S-phase arrest could be explained by compensatory new origin firing (Figure S1E).

To investigate whether ongoing transcription contributes to JQ1-induced replication slowing, we quantified nascent RNA synthesis using nuclear incorporation of 5-ethynyluridine (EU) (Figure 1C). EU incorporation increased by about 35% after 1 hr JQ1 treatment and remained increased up to 72 hr of JQ1 treatment (Figures 1D, 1E, and S1F). Increased RNA synthesis was also observed in U2OS cells treated with I-BET151 and in NALM-6 cells (Figures S2A and S2B). For an alternative approach, we isolated total RNA and normalized yields to cell numbers, showing that JQ1-treated cells contained more RNA overall (Figure 1F).

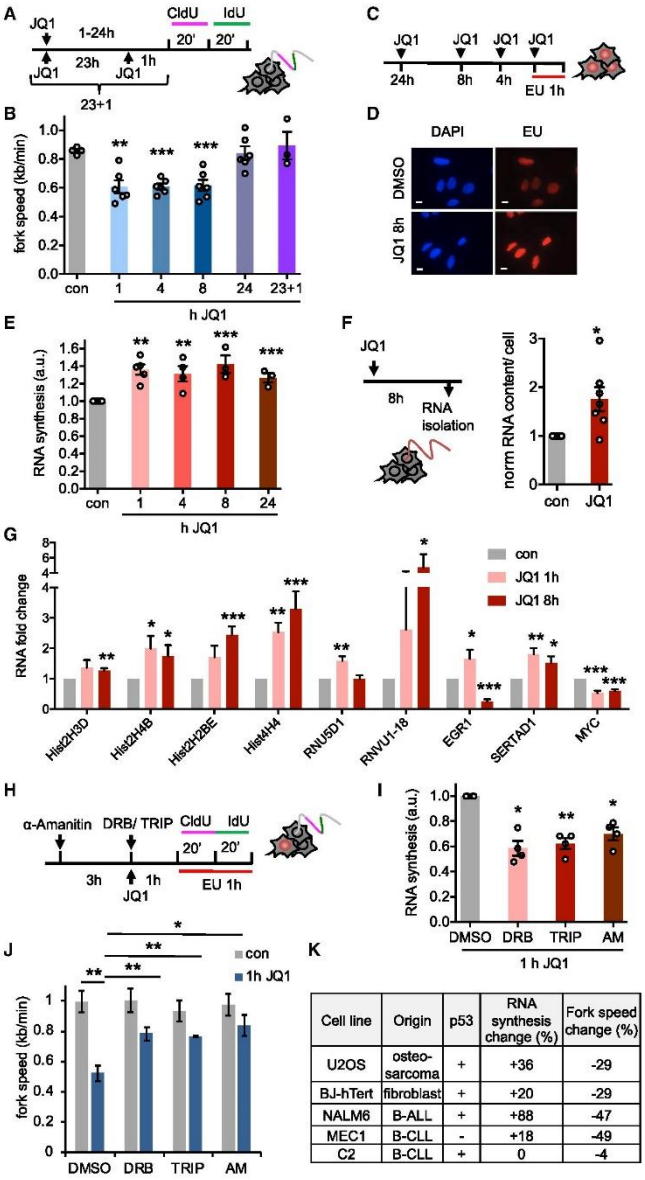
In contrast to our findings, it was previously reported that JQ1 treatment or BRD4 degradation quickly suppress nascent transcription of most protein-coding genes such as *MYC*, with only a few genes such as *EGR1* and *SERTAD1* upregulated (Muhar et al., 2018; Winter et al., 2017). As previous work focused on poly-adenylated mRNA sequencing, we decided to investigate the effect of BET inhibition on non-poly-adenylated RNA species. First, we re-analyzed the published RNA Pol II chromatin immunoprecipitation sequencing (ChIP-seq) datasets (Muhar et al., 2018). These showed that 1 hr after BRD4 degradation, genome-wide net occupancy of RNA Pol II actually increased by 53.8%, particularly over a set of highly transcribed genes that produce non-poly-adenylated RNAs such as histone and non-coding RNA genes (Figure S3). We used qRT-PCR to test whether this increased RNA Pol II occupancy was also increasing gene expression. Indeed, expression of all selected candidate genes was also upregulated by JQ1 in U2OS cells (Figure 1G). Our data support that although BET inhibition suppresses transcription of poly-adenylated protein-coding genes, highly transcribed histone and other non-poly-adenylated non-coding RNA genes are upregulated, and this may explain the observed increase in total nascent RNA synthesis.

To test whether replication fork slowing was transcription dependent, we used short treatments with the transcription inhibitors triptolide, DRB, and α-amanitin (Figure 1H). These inhibited ongoing RNA synthesis and increased replication fork speeds specifically in the presence of JQ1 (Figures 1I and 1J). Similar results were observed in human non-cancer BJ-hTert fibroblasts and in two chronic leukemia cell lines, C2 and MEC1 (Figures S2C–S2H). These data suggest that JQ1-induced replication stress depends on active RNA synthesis and that this is not restricted to cancer cells. One hour JQ1 treatment increased RNA synthesis in four of five cell lines tested, which was always accompanied by fork slowing (Figure 1K). Fork slowing was more dramatic in leukemia lines compared with U2OS or fibroblasts. Only C2 cells appeared resistant to JQ1 effects, displaying neither increased RNA synthesis nor fork slowing.

We used small interfering RNA (siRNA) to investigate which BET protein was the target of JQ1-induced replication-transcription conflicts. We first depleted BRD4, which can interact with DNA replication proteins (Maruyama et al., 2002; Sansam et al., 2018; Zhang et al., 2018). BRD4 depletion increased RNA synthesis (Figures 2A–2D) and reduced fork speeds (Figure 2E). Adding JQ1 did not further affect fork speeds in BRD4-depleted cells (Figure 2E). BRD4 siRNA-induced fork slowing was rescued by ectopic expression of the long isoform of BRD4 (Figures 2F and 2G) and short transcription inhibitor treatments (Figure 2H). In contrast, depletion of BRD2 or BRD3 did not increase RNA synthesis and caused negligible fork slowing (Figures 2I–2N). These data suggest that BRD4 is the BET protein that prevents replication-transcription conflicts and is required for BET inhibitor-induced replication stress.

We next investigated the mechanism of increased RNA synthesis. It has been shown that both JQ1 and I-BET151 can disrupt the 7SK-snRP-P-TEFb complex, increasing the proportion of active P-TEFb in complex with the SEC (Bartholomeeusen et al., 2012; Chaidos et al., 2014). This promotes transcription of genes including the 7SK-snRP component *HEXIM1* (Bartholomeeusen et al., 2012). Increased HEXIM1 protein levels eventually re-establish the 7SK-snRP-pTEFB complex and therefore P-TEFb inhibition (Figure 3A). We hypothesized that JQ1-induced replication stress might result from 7SK-snRP dissociation and increased RNA Pol II activity. In line with this, RNA Pol II CTD serine2 phosphorylation was transiently increased during the first hours of JQ1 treatment (Figures 3B and 3C). Several drugs including hexamethylene bis-acetamide (HMBA) can dissociate 7SK-snRP from P-TEFb (Fujinaga et al., 2015). If 7SK-snRP dissociation underlies BET inhibitor-induced fork slowing, then HMBA treatment should also slow replication, even though HMBA has no known connection to replication forks. HMBA treatment slightly increased RNA synthesis and slowed replication forks, which was not further exacerbated by co-treatment with JQ1 (Figures 3D and 3E). Finally, the short isoform of BRD4, which cannot effectively interact with P-TEFb (Schröder et al., 2012), failed to rescue the effect of BRD4 knockdown on replication fork slowing (Figures 3F–3H).

We decided to further investigate the roles of HEXIM1 in JQ1-induced RNA synthesis and replication fork slowing. HEXIM1 depletion prevented the early JQ1-induced increase in RNA synthesis and replication fork slowing. After 24 hr JQ1 treatment, however, RNA synthesis increased, accompanied by fork slowing (Figures 3I–3L). These data suggest that HEXIM1 depletion delayed the effects of JQ1. Although HEXIM1 protein levels slowly increased during 48–72 hr JQ1 treatment, there was no decrease in nascent RNA synthesis (Figures 3J and S1F),



Cell line	Origin	p53	RNA synthesis change (%)	Fork speed change (%)
U2OS	osteo-sarcoma	+	+36	-29
BJ-hTert	fibroblast	+	+20	-29
NALM6	B-ALL	+	+88	-47
MEC1	B-CLL	-	+18	-49
C2	B-CLL	+	0	-4

Figure 1. BET Inhibition Induces Replication-Transcription Conflicts

(A) DNA fiber labeling in U2OS cells treated with JQ1.

(B) Replication fork speeds after JQ1 treatment (n = 3–6).

(C) EU labeling after JQ1 treatment.

(D) Representative images of click-stained EU labeled cells ± 8 hr JQ1.

(E) Nuclear EU intensities after JQ1 treatment (n = 3–5).

(F) RNA was extracted after 8 hr JQ1 treatment and yield normalized to cell number and DMSO (n = 7).

(G) Fold change in the normalized expression levels of indicated transcripts ± JQ1 as indicated (n = 4).

(H) Cells were treated with transcription inhibitors before and during EU or DNA fiber labeling. AM, α-amanitin; TRIP, triptolide.

(I) Nuclear EU intensities in cells treated with transcription inhibitors and JQ1 (n = 4).

(J) Replication fork speeds after 1 hr JQ1 ± transcription inhibitors (n = 3 or 4).

(K) JQ1 effect on nascent RNA synthesis and replication fork speeds in a panel of human cell lines.

Data are represented as mean ± SEM. Scale bars, 10 μm. See also Figures S1–S3 and Table S1.

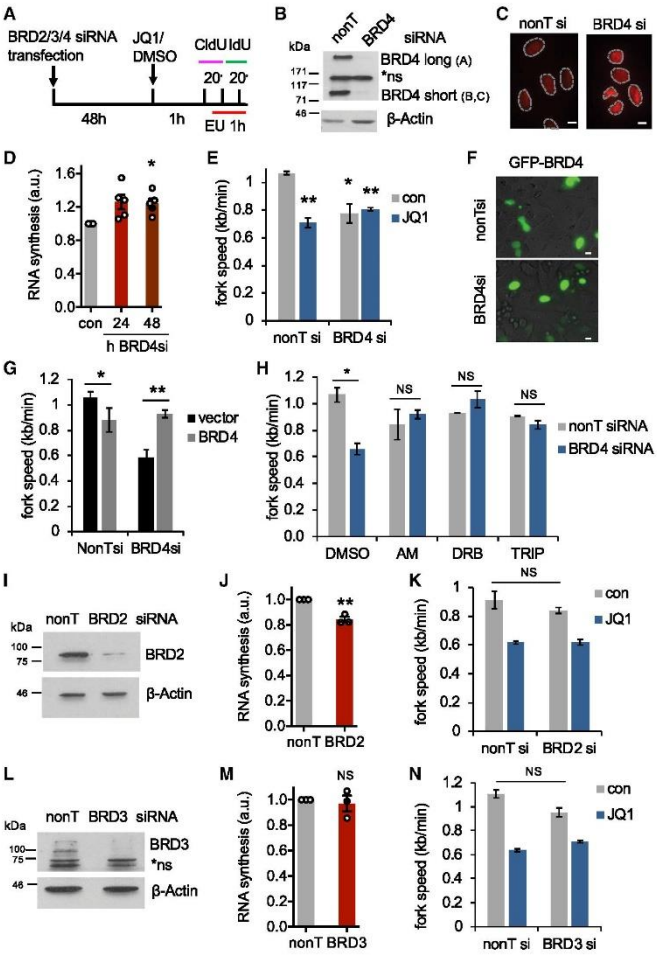


Figure 2. Loss of BRD4 Causes Replication-Transcription Conflicts

(A) DNA fiber labeling after BRD2/3/4 depletion.
(B) Protein levels of BRD4 isoforms after siRNA depletion. NS, non-specific bands.
(C) Representative EU images ± BRD4 siRNA.
(D) Nuclear EU intensities ± BRD4 siRNA (n = 5).
(E) Replication fork speeds after BRD4 depletion ± 1 hr JQ1 (n = 3).
(F) Equal EmGFP-BRD4 expression 48 hr after plasmid transfection ± BRD4 siRNA.
(G) Replication fork speeds after BRD4 siRNA ± BRD4 long isoform expression plasmid (n = 3).
(H) Median replication fork speeds after BRD4 siRNA ± transcription inhibitors (n = 3).
(I) Protein levels of BRD2 after siRNA depletion.
(J) Nuclear EU intensities ± BRD2 siRNA (n = 3).
(K) Replication fork speeds after BRD2 siRNA ± 1 hr JQ1 (n = 3).
(L) Protein levels of BRD3 after siRNA depletion. NS, non-specific.
(M) Nuclear EU intensities ± BRD3 siRNA (n = 3).
(N) Replication fork speeds after BRD3 siRNA ± 1 hr JQ1 (n = 3).
Data are represented as mean ± SEM. Scale bars, 10 μm.

suggesting that the process of replication adaptation is more complex than a HEXIM1-mediated feedback loop suppressing transcription.

We then investigated the relationship between JQ1-induced fork slowing and DNA damage. Replication fork slowing can expose single-stranded DNA (ssDNA) and, if forks collapse, cause DSBs. These activate the ATR and ATM checkpoint kinases and p53. However, JQ1 does not induce DNA damage (Pawar et al., 2018) or activate p53 (Da Costa et al., 2013). In line with this, we observed no JQ1-induced increase in ssDNA or DSBs as measured by nuclear foci formation of RPA and 53BP1 or phosphorylation of the ATM and ATR targets histone H2AX (γH2AX; Figure 4A), CHK1, and RPA (Figure S4A). BRD4 depletion also failed to induce DNA damage foci (Figure S4B).

Unexpectedly however, JQ1 induced foci formation of the homologous recombination factor RAD51, which depended on ongoing transcription and ATR activity (Figures 4B–4D and S4C). This suggested that RAD51 is recruited in response to JQ1-induced transcription-replication conflicts, aided by basal ATR activity. We used siRNA to investigate the impact of RAD51 on replication fork progression in JQ1-treated cells (Figures 4E and 4F). Interestingly, RAD51 depletion prevented JQ1-induced fork slowing (Figure 4G). We investigated whether RAD51 suppresses RNA synthesis, like HEXIM1. RAD51-depleted cells displayed high levels of RNA synthesis in both the presence and absence of JQ1, making these data difficult to interpret (Figure S4D). Nevertheless, we concluded that RAD51-dependent rescue of fork speeds was not due to decreased transcription. Transient overexpression of RAD51 promoted fork slowing after 24 hr JQ1 treatment, additionally supporting that RAD51 directly slows forks in response to JQ1 (Figures S4E and S4F).

RAD51-mediated fork slowing was previously reported for DNA-damaging treatments and is suggestive of replication fork reversal (Zellweger et al., 2015). In support of a fork reversal model, PARP inhibition and depletion of SMARCAL1 or ZRANB3 (Bhat and Cortez, 2018) also prevented JQ1-induced fork slowing (Figure 4H-J).

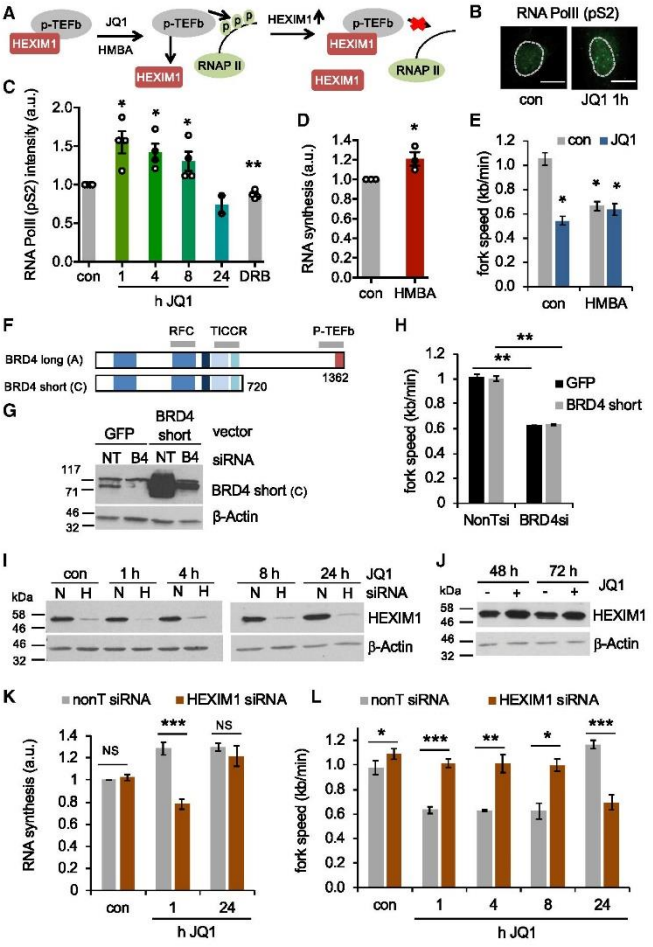


Figure 3. BET Inhibitor-Induced Replication-Transcription Conflicts Require HEXIM1

(A) Current model HEXIM1 role in JQ1-induced transcription increase.

(B) Nuclear immunostaining for phospho-S2 RNA Pol II ± JQ1.

(C) Nuclear phospho-S2 RNA Pol II intensities as in (B) (n = 4; n = 2 [24 hr]).

(D) Nuclear EU intensities ± 5 mM HMBA (1 hr) (n = 3).

(E) Replication fork speeds after HMBA treatment ± JQ1 (1 hr) (n = 3).

(F) Schematic of BRD4 isoforms used.

(G) Protein levels of BRD4 short isoform after full-length BRD4 siRNA ± BRD4 short isoform expression plasmid.

(H) Replication fork speeds after full-length BRD4 siRNA ± BRD4 short isoform expression plasmid (n = 3).

(I) Protein levels of HEXIM1 after 48 hr siRNA transfection followed by JQ1 for times indicated.

(J) Protein levels of HEXIM1 after 48–72 hr JQ1.

(K) Nuclear EU intensities after HEXIM1 siRNA and JQ1 treatment (n = 3–9).

(L) Replication fork speeds after HEXIM1 siRNA and JQ1 treatment (n = 3 or 4, n = 2 [nonT 8 hr]).

Data are represented as mean ± SEM. Scale bars, 10 μm.

We then tested the effect of RAD51 depletion on JQ1-induced DNA damage. Interestingly, RAD51 depletion actually promoted DNA damage, as indicated by γH2AX and 53BP1 foci formation (Figures 4K–4M). This suggests that RAD51 prevents DNA damage at JQ1-slowed replication forks.

As HEXIM1 depletion also rescued fork progression, we tested the effect of HEXIM1 on DNA damage. HEXIM1-depleted cells accumulated DNA damage early during JQ1 treatment that persisted for 24 hr (Figures 4N and 4O). In line with the increased DNA damage, HEXIM1-depleted cells were more sensitive to 24 hr JQ1 treatment than control cells, as were ATR inhibitor-treated cells (Figure S4G). This suggests that both HEXIM1, which is upstream of transcription-replication conflicts, and RAD51, which acts downstream of these conflicts, contribute to replication fork slowing and prevent JQ1-induced DNA damage (Figure 4P).

DISCUSSION

We report that BET inhibition increases transcription especially of highly transcribed histone and other non-poly-adenylated non-coding RNA genes and causes transcription-dependent replication fork slowing. This depends on HEXIM1, a central factor in the BET inhibitor response, and the homologous recombination factor RAD51, which is central in the replication stress response. Unusually, BET inhibitor-induced replication stress is transient and, despite engaging RAD51, does not activate a full DNA damage response. We speculate that these unusual transcription-replication conflicts will help illuminate some aspects of BET inhibitor treatment responses.

Our data provide insight into the roles of BRD4 in DNA replication. Reports show that BRD4 interacts with DNA replication factors RFC, TICRR, and CDC6 (Maruyama et al., 2002; Sansam et al., 2018; Zhang et al., 2018). We show here that BRD4 also regulates DNA replication via its P-TEFb interaction. The BRD4 short isoform contains the interaction domains for TICRR and RFC (Maruyama et al., 2002; Sansam et al., 2018) but failed to rescue replication stress. The interaction with CDC6 is not yet

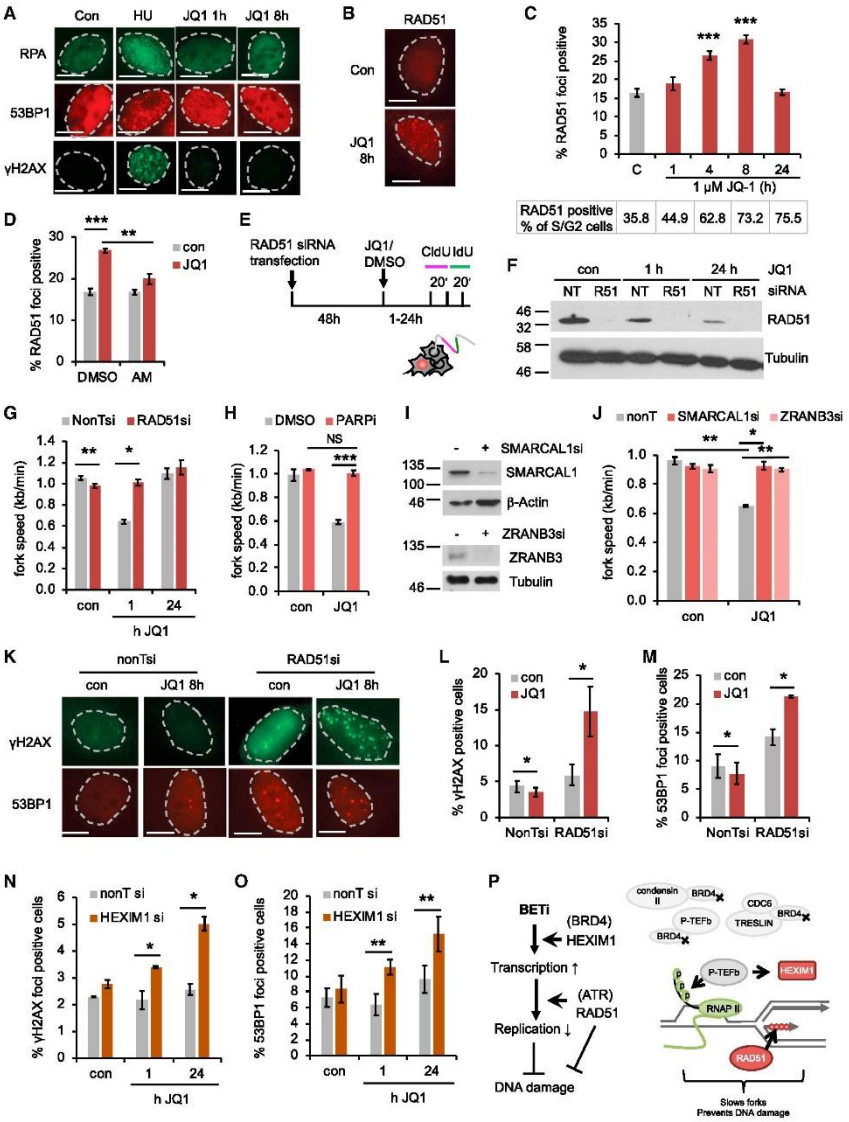


Figure 4. RAD51 and HEXIM1 Modulate BET Inhibitor-Induced DNA Damage

(A) Representative images of DNA damage foci after JQ1 treatment. HU was 2 mM 8 hr.

(B) Representative images of RAD51 foci after JQ1 treatment.

(C) Total percentages (top) and percentage normalized to S/G2 content (bottom) of cells with RAD51 foci after JQ1 treatment (n = 3–6).

(D) Percentages of cells with RAD51 foci ± 4 hr JQ1 and α-amanitin (AM) (n = 3).

(E) EU and DNA fiber labeling after RAD51 depletion.

(legend continued on next page)

mapped (Zhang et al., 2018). Our data suggest that TICRR and RFC are not involved in the phenotypes described here. Instead, they support previous reports that BET inhibition rapidly increases P-TEFb activity (Bartholomeeusen et al., 2012; Chaidos et al., 2014). We show that this also involves increased RNA synthesis. Our data support that although BET inhibition suppresses transcription of poly-adenylated protein-coding genes (Muhar et al., 2018; Winter et al., 2017), non-poly-adenylated RNA Pol II transcripts such as histones and non-coding RNA genes are upregulated. We previously reported that overexpression of H-RAS^{V12} or transcription factor TBP increases nascent RNA synthesis and causes replication stress (Kotsantis et al., 2016). BET inhibition shows how small-molecule inhibitor treatments can also cause replication stress by increasing RNA synthesis. Any treatments that disrupt the complex of P-TEFb with 7SK-snRP, including other cancer drugs, such as HDAC inhibitors and azacytidine (Fujinaga et al., 2015), could potentially cause transcription-replication conflicts in this way.

Our findings suggest that HEXIM1 is required for BET inhibitor-induced replication fork slowing, which is delayed by at least 8 hr in the absence of HEXIM1. After 24 hr JQ1, extensive gene expression changes and possibly HEXIM2 (Byers et al., 2005) may compensate for HEXIM1 loss. HEXIM1 depletion has been associated with long-term BET inhibitor resistance. Our findings do not conflict with this, as BET inhibitor resistance in HEXIM1-depleted cells was observed only after prolonged (>24 hr) treatment (Devaraj et al., 2016). It was proposed that HEXIM1 loss counteracts JQ1 by increasing P-TEFb activity (Devaraj et al., 2016). Importantly, we show that HEXIM1 loss can also promote JQ1 effects, such as DNA damage. In addition to modulating transcription-dependent fork slowing, HEXIM1 might play undiscovered roles in replication stress and DNA damage response. It may be relevant that HEXIM1 also regulates p53 (Lew et al., 2012). Nevertheless, we observed JQ1-induced replication fork slowing in a p53 mutant cell line. Reduced HEXIM1 protein levels have been observed in metastatic breast cancer (Ketchart et al., 2013), melanoma (Tan et al., 2016), and acute leukemia (Devaraj et al., 2016; Huang et al., 2016). It will be important for cancer researchers to further investigate the relationship between HEXIM1, replication stress, and the response to cancer treatment.

Homologous recombination capacity of cancer cells is a well-established predictive biomarker for the response to replication stress-inducing treatments. RAD51 loading is known to actively slow forks in response to a variety of genotoxic agents (Zellweger et al., 2015). We show that BET inhibition also activates RAD51, likely because of increased transcription, and that RAD51 promotes fork slowing in response to BET inhibitor, which may involve fork reversal. Forks may reverse at sites of direct collisions between replication and transcription machineries or in response to indirect effects of transcription on replication. RAD51 expression is downregulated in response to BET inhibition (Yang et al., 2017) and in models of acquired BET inhibitor resistance (Pawar et al., 2018). Intriguingly, acquired BET inhibitor resistance models also displayed increased DNA damage signaling (Pawar et al., 2018). This agrees with our data that RAD51 downregulation increases DNA damage signaling.

Our data support a speculative model whereby loss of HEXIM1 or RAD51 allows normal replication fork progression in the presence of BET inhibitor at the expense of DNA damage. This may even contribute to long-term BET inhibitor resistance. Although it is extensively documented that transcription-replication conflicts promote DNA damage, BET inhibition seems to induce DNA damage when such conflicts are prevented. This latter damage might, for example, result from reduced chromatin recruitment of chromatin remodelers (Floyd et al., 2013) or DNA replication proteins (Sansam et al., 2018; Zhang et al., 2018) (Figure 4P). The underlying mechanisms, and how they relate to BET inhibitor response, will require future investigation. In summary, we provide insights into the relationship among BET proteins, DNA replication, and DNA damage response that will be relevant to cancer therapy research.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Drug treatments
  - DNA fiber analysis
  - EU incorporation assay
  - siRNA and DNA transfection
  - Immunofluorescence
  - Western blotting
  - RNA isolation

(F) Protein levels of RAD51 after siRNA depletion.
(G) Replication fork speeds after 1 hr JQ1 ± RAD51 siRNA (n = 3 and 4).
(H) Replication fork speeds after 1 hr JQ1 ± PARP inhibitor (n = 3).
(I) Protein levels of SMARCAL1 or ZRANB3 48 hr after siRNA transfection.
(J) Replication fork speeds after 4 hr JQ1 ± SMARCAL1 or ZRANB3 siRNA (n = 3).
(K) Representative images of γH2AX and 53BP1 foci after JQ1 ± RAD51 siRNA.
(L) Percentages of cells with γH2AX foci after 8 hr JQ1 ± RAD51 siRNA (n = 4).
(M) Percentages of cells with 53BP1 foci after 8 hr JQ1 ± RAD51 siRNA (n = 4).
(N) Percentages of cells with γH2AX foci after JQ1 ± HEXIM1 siRNA (n = 3).
(O) Percentages of cells with 53BP1 foci after JQ1 ± HEXIM1 siRNA (n = 3).
(P) Model: BET inhibition disrupts recruitment of chromatin and replication factors; increased RNA synthesis and RAD51 activity (e.g., fork reversal) slow replication forks without DNA damage.
Data are represented as mean ± SEM. Scale bars, 10 μm. See also Figure S4.

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Supplemental Information

BET Inhibition Induces HEXIM1- and RAD51-Dependent Conflicts between Transcription and Replication

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Supplemental Figures

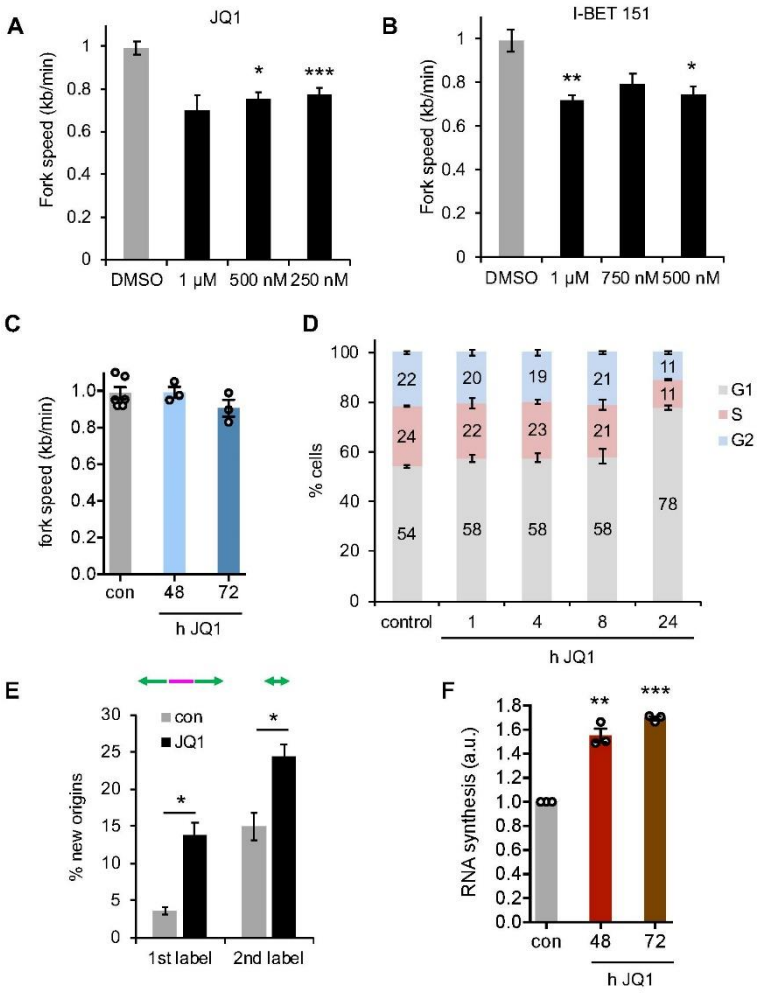

Figure S1. Related to Figure 1. BET inhibition induces replication fork slowing. A) Replication fork speeds in U2OS cells after treatment with 0.25-1 μM JQ1 for 1 h. n=3. B) Replication fork speeds in U2OS cells after treatment with 0.5-1 μM I-BET151 for 1 h. n=4. C) Replication fork speeds in U2OS cells treated with 1 μM JQ1 for 48 and 72 h. n=3. D) Cell cycle distribution measured by flow cytometry of U2OS cells after 1-24 h treatment with 1 μM JQ1. n=3. E) New origin firing in U2OS cells after treatment with 1 μM JQ1 for 1 h. n=3. F) Nuclear EU intensity in U2OS cells treated with 1 μM JQ1 for 48 and 72 h. n=3. Data are represented as mean ± SEM.

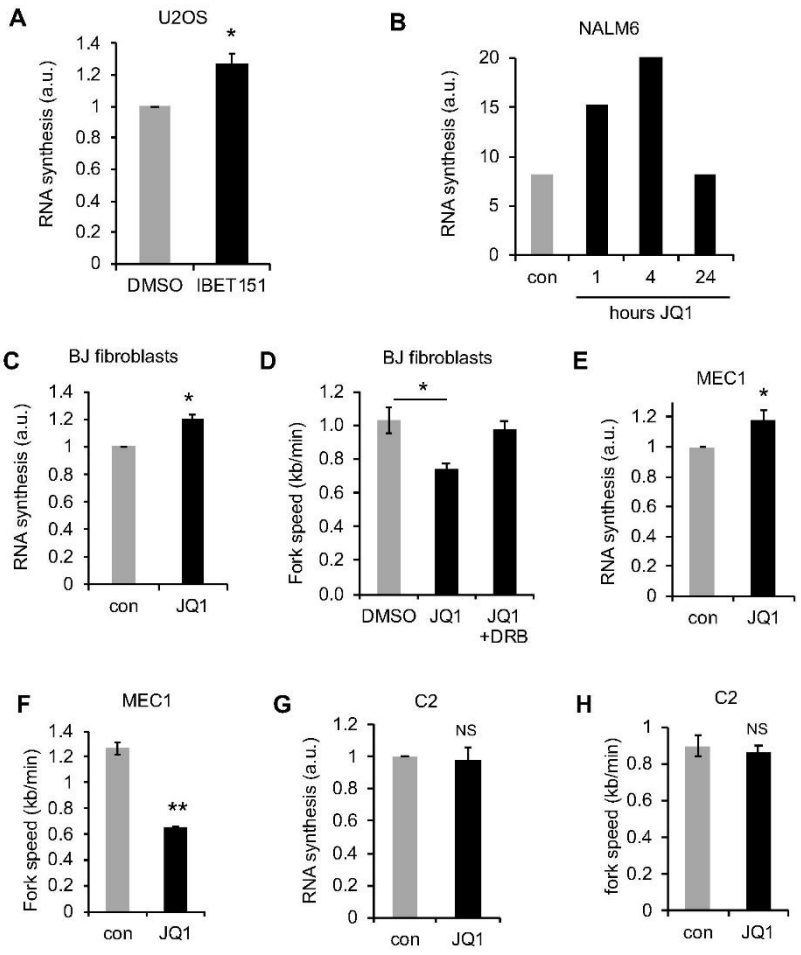

Figure S2. Related to Figure 1. Replication-transcription conflicts induced by I-BET151 and in a number of cell lines. A) Quantification of nuclear EU intensity in U2OS cells after 1 μM I-BET151 (1h). n=4. B) Nuclear EU intensity in NALM6 cells after 1-24h 1 μM JQ1. n=1. C) Nuclear EU intensity in BJ-hTert fibroblasts +/- 1 μM JQ1 (1h). n=3. D) Median replication fork speeds in BJ-hTert fibroblasts +/- 1 μM JQ1 (1h), and treated with JQ1 and DRB for 1h. n=3. E) Nuclear EU intensity in MEC1 cells +/-1 μM JQ1 (1h). n=3. F) Median replication fork speeds in MEC1 cells +/- 1 μM JQ1 (1h). n=5. G) Nuclear EU intensity in C2 cells +/-1 μM JQ1 (1h). n=3. H) Median replication fork speeds in C2 cells +/- 1 μM JQ1 (1h). n=3. Data are represented as mean ± SEM.

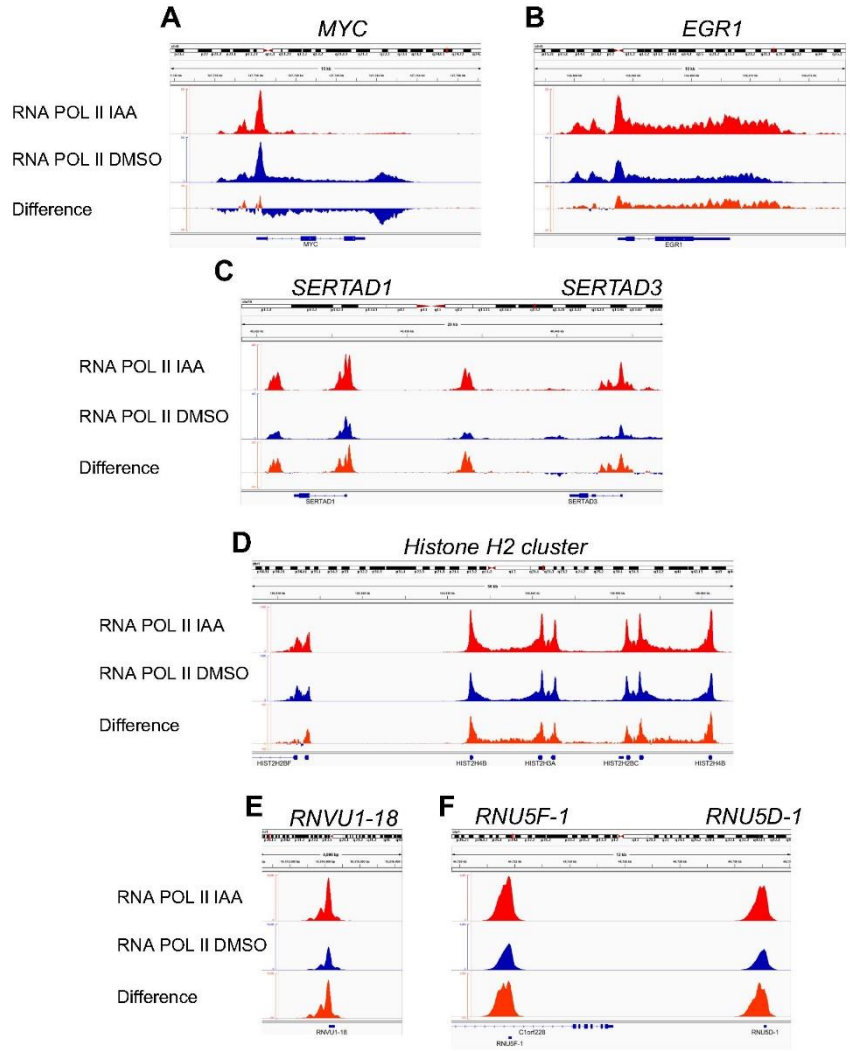

Figure S3. Related to Figure 1. BRD4 degradation increases RNA Pol II occupancy in histone and non-coding RNA genes. Analysis of published RNA Pol II ChIP-seq data sets (Muhar et al., 2018; GEO Series GSE111463). RNA Pol II ChIP was performed 1h after BRD4 degradation using auxin inducible degron (AID) approach in K562 leukaemia cells. The mRNA expression of *MYC*, *EGR1* and *SERTAD1* was analysed in parallel (Muhar et al., 2018). A) *MYC* (mRNA decreased 1h after BRD4 degradation), B) *EGR1* (mRNA increased 1h after BRD4 degradation), C) *SERTAD1* (mRNA increased 1h after BRD4 degradation), D) histone gene cluster (non-polyadenylated transcripts), also with large increase in RNA Pol II levels outside genes boundaries, E) non-coding RNA *RNVU1-18* (non-polyadenylated transcript), F) non-coding RNAs *RNU5F-1* and *RNU5D-1* (non-polyadenylated transcripts). Data are represented as mean ± SEM.

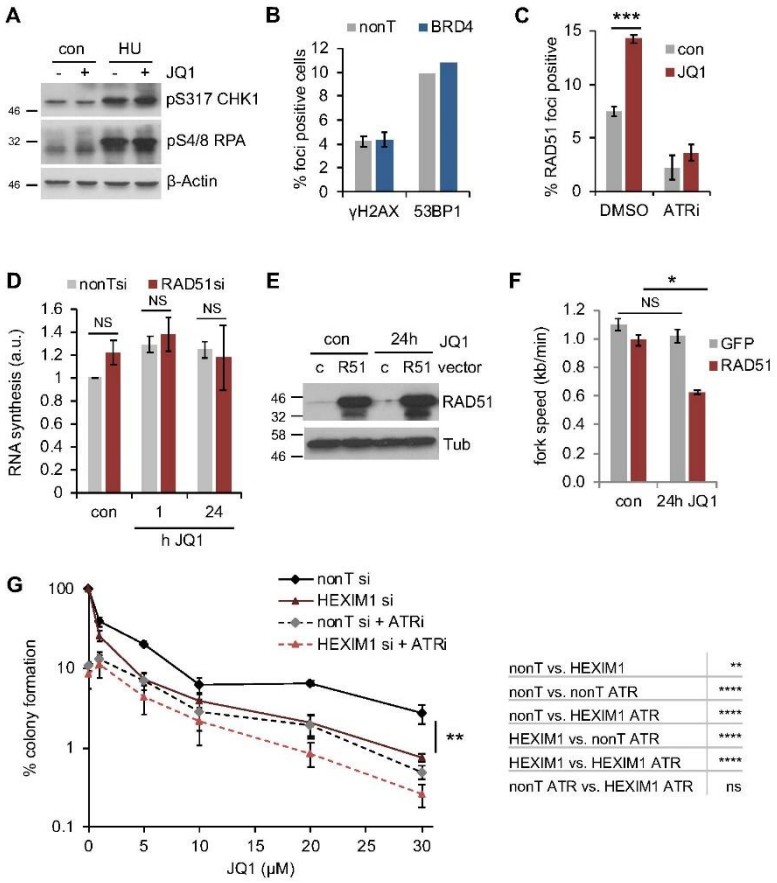

Figure S4. Related to Figure 4. DNA damage response to BET inhibition. A) Levels of phospho-S4/8 RPA32, phospho-S317 CHK1 and β-Actin after treatment with JQ1 and HU as indicated. B) Percentages of cells containing more than 8 γH2AX or 53BP1 foci after BRD4 depletion. n = 2 (γH2AX), n = 1 (53BP1). C) Percentages of cells containing more than 8 RAD51 foci after JQ1 treatment +/- ATR inhibitor AZ20. n=3 (DMSO), n=2 (ATRi). D) Quantification of nuclear EU intensity +/- RAD51 siRNA and JQ1 treatment. n=3. E) Levels of RAD51 and loading control after transient overexpression of RAD51 or eGFP (control) for 24 h +/- JQ1. F) Replication fork speeds after transient overexpression of RAD51 or eGFP (control) for 24 h +/- JQ1. n=3. G) Colony survival of U2OS cells treated with indicated concentrations of JQ1 for 24h, +/- HEXIM1 siRNA and AZ20. n=3. The statistical test used was 2-way ANOVA with Tukey's. Data are represented as mean ± SEM.

Supplementary Table S1. Related to STAR Methods. Sequences of PCR primers

Name	Sequence (5' → 3')
HIST2H3D For	CGAGATCGCGCAGGACTTTA
HIST2H3D Rev	TGTCCTTGGGCATGATGGTC
HIST2H4B For	GAAGCTGTCTATCGGGCTCC
HIST2H4B Rev	ACTGCGTCCCGAATCACATT
HIST2H2BE For	GCCACCCACCTAATCACTAGAAA
HIST2H2BE Rev	CAATGACGCACTGGGGACC
HIST4H4 For	GGGTCGCACCCTTTATGGTT
HIST4H4 Rev	GTATCCGGAAGGCGACATCA
EGR1 For	TGCAGATCTCTGACCCGTTC
EGR1 Rev	CAGGAAAAGACTCTGCGGTCA
SERTAD1 For	GAGGACAGCCAACAAGCGAT
SERTAD1 Rev	TGCTCAGCATCTTGCTCACTA
MYC For	CAGCGACTCTGAGGAGGAAC
MYC Rev	GCTGCGTAGTTGTGCTGATG
RNU5D1 For	GCTCTGGTTTCTCTTCAAAT
RNU5D1 Rev	AACCCCACCAACATAGGG
RNVU1-18 For	GGTTTTCCCAGGGCGAGG
RNVU1-18 Rev	CCACTACCACAAATTATGCAGTC
RPLP0 F2 (control)	CAGATTGGCTACCCAACTGTT
RPLP0 R2 (control)	GGAAGGTGTAATCCGTCTCCAC

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