Replication Fork Stability in Mammalian Cells

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Nothing in life is to be feared, it is only to be understood.

- Marie Curie
Abstract

Maintaining replication fork integrity is vital to preserve genomic stability and avoid cancer. Physical DNA damage and altered nucleotide or protein pools represent replication obstacles, generating replicative stress. Numerous cellular responses have evolved to ensure faithful DNA replication despite such challenges. Understanding those responses is essential to understand and prevent or treat replication-associated diseases, such as cancer.

Re-priming is a mechanism to allow resumption of DNA synthesis past a fork-stalling lesion. This was recently suggested in yeast and explains the formation of gaps during DNA replication on damaged DNA. Using a combination of assays, we indicate the existence of re-priming also in human cells following UV irradiation.

The gap left behind a re-primed fork must be stabilised to avoid replication-associated collapse. Our results show that the checkpoint signalling protein CHK1 is dispensable for stabilisation of replication forks after UV irradiation, despite its role in replication fork progression on UV-damaged DNA. It is not known what proteins are necessary for collapse of an unsealed gap or a stalled fork. We exclude one, previously suggested, endonuclease from this mechanism in UV-irradiated human fibroblasts. We also show that focus formation of repair protein RAD51 is not necessarily associated with cellular sensitivity to agents inducing replicative stress, in rad51d CHO mutant cells.

Multiple factors are required for replication fork stability, also under unperturbed conditions. We identify the histone methyltransferase SET8 as an important player in the maintenance of replication fork stability. SET8 is required for replication fork progression, and depletion of SET8 led to the formation of replication-associated DNA damage.

In summary, our results increase the knowledge about mechanisms and signalling at replication forks in unperturbed cells and after induction of replicative stress.
List of original publications

This thesis is based on the following publications, which will be referred to by their Roman numerals:

I UV stalled replication forks restart by re-priming in human fibroblasts
Elvers I, Johansson F, Groth P, Erixon K, Helleday T.
Submitted

II CHK1 activity is required for fork elongation but not fork stabilisation after UV irradiation
Elvers I, Johansson F, Djureinovic T, Lagerqvist A, Stoimenov I, Klaus E, Helleday T.
Submitted

III UV-induced replication fork collapse in DNA polymerase η deficient cells is independent of the MUS81 endonuclease
Manuscript

IV The histone methyltransferase SET8 is required for S-phase progression
J Cell Biol. 2007 Dec 31;179(7):1337-45

V Uncoupling of RAD51 focus formation and cell survival after replication fork stalling in RAD51D null CHO cells
Urbin SS, Elvers I, Hinz JM, Helleday T, Thompson LH.
Submitted
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<th>Description</th>
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<tbody>
<tr>
<td>(6-4)PP</td>
<td>pyrimidine pyrimidone (6-4) photo product</td>
</tr>
<tr>
<td>AraC</td>
<td>cytarabine, cytosine arabinoside</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3 related</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CHK1</td>
<td>checkpoint kinase 1</td>
</tr>
<tr>
<td>CHK2</td>
<td>checkpoint kinase 2</td>
</tr>
<tr>
<td>CPD</td>
<td>cyclobutane pyrimidine dimer</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>GG-NER, GGR</td>
<td>global genome repair</td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
</tr>
<tr>
<td>ORC</td>
<td>origin of replication complex</td>
</tr>
<tr>
<td>ORI</td>
<td>origin of replication</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>pre-RC</td>
<td>pre-replication complex</td>
</tr>
<tr>
<td>pre-IC</td>
<td>pre-initiation complex</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modification</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPIIo</td>
<td>RNA polymerase II, elongating</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TC-NER, TCR</td>
<td>transcription-coupled repair</td>
</tr>
<tr>
<td>TLS</td>
<td>translesion synthesis</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet radiation</td>
</tr>
<tr>
<td>XP</td>
<td>xeroderma pigmentosum</td>
</tr>
<tr>
<td>XP-V</td>
<td>XP variant cells or phenotype</td>
</tr>
<tr>
<td>γH2AX</td>
<td>phosphorylated histone H2AX</td>
</tr>
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</table>
Introduction

Replication is the process by which DNA is copied to generate identical daughter chromosomes, thereby passing on the genetic material to the daughter cells. The DNA synthesis phase is part of the cell division cycle, which is necessary for tissue growth and renewal. Some tissues stop proliferating while others, such as the skin, continue to grow during our entire lives. Replication, being vital to all aspects of life, is tightly regulated by the cell. As DNA is constantly exposed to exogenous and endogenous damaging factors, DNA lesions are frequently occurring. If not repaired before replication, these perturbed structures may become an obstacle during the progression of replication. DNA lesions may cause stalling and collapse of a replication fork structure, and can also give rise to mutations eventually leading to cancer.

Several DNA repair pathways have evolved to remove DNA damage and restore the genetic material. Maintaining the integrity of the genome is essential to avoid cancer, but to some extent, DNA damage may be tolerated by the cell. However, replication of damaged DNA may induce changes in the genetic sequence inherited by the daughter cells, the original sequence no longer identifiable.

DNA damaging agents are frequently used in cancer therapy, which may seem contradictory as they can also cause cancer. The aim of this study has been to use well-known DNA damaging agents to further understand the mechanisms and signalling involved in replication fork stability in mammalian cells. Additional understanding in this field may be useful in the struggle to prevent cancer, and to cure such disease, but also in the general understanding of the function of cells, the diverse components forming our bodies.

Genomic integrity maintenance

Every single day, tens of thousands of DNA lesions are formed in a mammalian cell (Beckman and Ames, 1997; Lindahl, 1993). Most lesions are small, such as the spontaneous oxidation of a nucleotide, while other lesions may induce loss of genetic material if not repaired correctly. Indeed, if left unrepaired, DNA damage may cause misregulation of, or be a physical
obstacle to, the transcription of genes. Furthermore, if encountered by the replication fork, DNA lesions may induce mutations or cause loss of genetic information, and hence they pose a severe threat to the cell and the organism. Several specialised DNA repair pathways have evolved to take care of the numerous lesions before replication occurs. In addition, DNA damage may activate other cellular responses including cell cycle arrest and apoptosis. To some extent, damage may also be tolerated during replication.

Numerous proteins are involved in maintaining genomic integrity. The physical connection between sister chromatids, termed cohesion, is achieved by ring-like cohesion protein complexes which hold the sister chromatids together. Cleavage of this connection is vital for proper segregation of sister chromatids during anaphase (Uhlmann et al., 1999). By facilitating DNA repair, cohesin is important for genomic integrity (Strom et al., 2004; Unal et al., 2004).

Failure to maintain the genomic material may lead to accumulation of DNA damage. Loss of genome integrity may also lead to changes in gene expression, as a result of deletions or duplications. Genomic instability is associated with diseases such as cancer, Fragile X, and Huntington’s disease.

**DNA damage response**

Being constantly exposed to many different types of stress and DNA damaging agents, the cell has evolved several pathways to repair DNA lesions. A damaged protein can be replaced by a new one, but since the cell doesn’t have any backup copies of its nuclear genome, DNA repair is vital. Several different repair pathways, specialising in different types of damage, are present in eukaryotic cells in combination with fine-tuned sensing mechanisms. Many types of DNA damage can become more harmful for the cell if a replication fork runs into the site of damage before repair has taken place. Ultraviolet radiation emitted by the sun is an important environmental source of DNA damage. The damaged bases may be detected by the DNA repair system, and repaired. However, if not repaired before replication, this will cause an obstacle to the replication machinery. Another agent influencing replication forks is hydroxyurea, which depletes the nucleotide
pool by inhibiting ribonucleotide reductase, thereby stalling replication forks.

When damage is detected, the cell cycle may need to be halted to allow repair of the damage. The signalling for repair of DNA and restoration of chromatin is an intricate system, tightly regulated by the cell. The above mentioned DNA damage inducing agents as well as damage signalling and DNA repair pathways are described in more detail below.

**Induction of DNA damage**

Paracelsus (1493-1541) stated that all compounds can be poisonous, the toxicity being only a matter of dose. This is a central dogma in the field of toxicology, as the concentration of a compound affects the response in the cell. Below, two commonly used agents for induction of DNA damage are discussed; ultraviolet radiation and hydroxyurea. Ultraviolet radiation-induced DNA damage and hydroxyurea both block the progression of replication forks, but by different mechanisms. These two agents can be used to study different and overlapping aspects of replication fork stability and repair at replication forks.

**Ultraviolet radiation**

One of the constant exogenous threats to DNA is ultraviolet (UV) radiation, an electromagnetic radiation emitted by the sun. The energy carried by the ultraviolet radiation is taken up by the matter with which the wave collides when reaching the earth's surface. Ultraviolet radiation is subdivided into UVA (400-320nm), UVB (320-295nm), and UVC (295-100nm). UVC and most of the UVB radiation is absorbed by the ozone layer, and the majority of UV radiation reaching the surface of earth consists of UVA.

While UVA and UVB primarily cause protein damage, the energy peak of UVC radiation is preferentially absorbed by DNA. Hence UVC irradiation induces mainly the same types of lesions as the other ultraviolet radiation types together, but no protein damage. This makes UVC irradiation a useful tool for studying DNA repair after sun exposure. The most frequent DNA
lesion observed after UV irradiation is cyclobutane pyrimidine dimers (CPDs), where two adjacent pyrimidines in the same strand become covalently linked to each other (Kuluncsics et al., 1999), destabilising the helical structure. This covalent bond can theoretically occur in several different conformations, but in DNA sterical hindrance allows mainly the cis-syn CPD to form. In addition, two pyrimidines can be linked by a covalent bond forming between the C6 of the 5' pyrimidine and the C4 of the 3' pyrimidine, a structure identified as a pyrimidine-pyrimidone (6-4) photoproduct ((6-4)PP) (Varghese and Wang, 1967). UVC irradiation induces CPDs and (6-4)PPs in the ratio 3:1 (Mitchell et al., 1990). Both possible lesions, originating from two adjacent thymidines are shown in figure 1. (6-4)PPs cause a higher distortion to the DNA backbone compared to a CPD. A (6-4)PP induces a 44º bending of the backbone (Kim and Choi, 1995), while a cis-syn CPD causes less backbone bending, still allowing some Watson-Crick base-pairing (Park et al., 2002). This difference in backbone distortion makes the (6-4)PPs more easily recognised by the cellular DNA repair machinery, and hence this lesion is more quickly repaired following UV exposure even though (6-4)PPs comprise only a fraction of the total amount of DNA lesions induced (Mitchell and Nairn, 1989; Tijsterman et al., 1999). Additionally, 8-oxo-Guanine (8-oxoG) is produced following UVA exposure (Kino and Sugiyama, 2005).

**Hydroxyurea**

Hydroxyurea (HU) is occasionally used as an antitumor agent and in the treatment of HIV infections, reviewed in (Szekeres et al., 1997). Its effect comes from the inhibition of ribonucleotide reductase (RNR), a rate-limiting
enzyme of DNA synthesis. RNR reduces ribonucleotides to deoxyribonucleotides, the source for deoxynucleotides in the cell. HU is a radical scavenger and prevents the electron transport from a tyrosine in the R2 subunit of RNR, which is required for reduction of the 2’-hydroxy group, as described below. This effect can be used to slow down and eventually stall replication forks in living cells. As HU is a general radical scavenger, it may also disturb other free radical chemistry.

Currently, the known RN Rs are divided into three classes. Class I is the only class described to carry out ribonucleotide reduction in mammals. It has been proposed that the existence of the three classes of ribonucleotide reductases and their differences in regulation is reflecting the changes in oxygenic environment during evolution (Poole et al., 2002).

The class I RN Rs are tetramers consisting of two nonidentical homodimers, the larger subunit R1, and the smaller subunit R2. Both are required for enzymatic activity, although the active site is restricted to the R1 subunit. Ribonucleotide reductase capacity is controlled through cell-cycle regulated transcription and proteolysis of the two subunits (Bjorklund et al., 1990; Chabes et al., 2003; Engstrom et al., 1985). For the enzyme to function, a stable tyrosyl radical is formed in the R2 subunit. This radical is to be transferred via several other amino acid residues to a cysteine residue in the active site of the R1 subunit, generating a thiyl radical. The thiyl radical may transfer the extra electron onto the C3 of a ribose, from where it is transported via the ribose C2 onto another cysteine residue of the active site of the R1 subunit, resulting in reduction of the C2. This involves the formation of several intermediates, which are stabilised by interactions with other amino acid side chains of the active site.

The same RNR enzyme can reduce ADP, CDP, GDP, and UDP. dUMP is later methylated and phosphorylated to generate dTTP. The activity of class I RN Rs is controlled by the binding of deoxyribonucleotides to an allosteric site of the R1 subunit, regulating which ribonucleotides are reduced (Eliasson et al., 1996).

Hydroxyurea scavenges the free radical of RNR, by a mechanism not yet completely understood. The result is a decrease in the concentration of the dNTP pool and stalled replication forks, which eventually collapse producing double-strand breaks (Lundin et al., 2002). Interestingly,
following genotoxic stress, the yeast small RNR subunit has been shown to redistribute to the cytoplasm, while both human subunits are relocalised from the cytoplasm to the nucleus in response to UV irradiation (Xue et al., 2003; Yao et al., 2003).

**DNA damage signalling**

The DNA damage response (DDR) is a complex network of proteins activated by induction of DNA damage or replicative stress. In a generalised view, DNA damage can be recognised by sensor proteins that recruit and activate transducer proteins that phosphorylate effector or mediator proteins, inducing cell cycle arrest (Petrini and Stracker, 2003). In addition to cell cycle arrest, transcription is downregulated in damaged areas by regulation of DNA methyltransferase DNMT, which methylates CpG islands in promoters (O'Hagan et al., 2008).

Besides signalling for DNA repair, this signalling cascade may induce cell cycle arrest to allow time for repair of DNA, or cell death via apoptosis. In addition, the DNA damage response may induce cellular senescence (von Zglinicki et al., 2005), a permanent cell cycle arrest that is associated with ageing (reviewed in (Herbig et al., 2006)). Recently, ageing has been discussed as an antagonist of cancer, as ageing induced by cell death can be seen as an alternative pathway to the accumulation of mutations and subsequent transformation into a malignant cell that characterises cancer development (recently reviewed in (Hoeijmakers, 2009)). Furthermore, there are a number of genetic disorders caused by deficiencies in genomic maintenance that are characterised by increased ageing (Lombard et al., 2005; Schumacher et al., 2008), e.g. ATM deficiency which causes premature ageing (Wong et al., 2003). Interestingly, mice expressing a mutant version of the DNA damage signalling protein BRCA1 show premature ageing accompanied by an increased risk for cancer (Cao et al., 2003). In contrast, mice with elevated levels of active p53, also showing premature ageing, are protected from cancer (Tyner et al., 2002). Expression of p16INK4a, associated with senescence, elevates with age (Collins and Sedivy, 2003). Some of the proteins and interactions of the DNA damage response are summarised in figure 2.
Figure 2. Schematic and simplified view of the DDR. Dotted lines indicate crosstalk between ATM and ATR after double-strand break induction.

ATM and ATR

The ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases play a major role in the DNA damage response and are vital to maintain genomic stability (Shiloh, 2003). They belong to the phosphatidyl-inositol 3-kinase related kinase (PIKK) family, as do DNA-dependent protein kinase catalytic subunit (DNA-PKcs), suppressor of morphogenesis in genitalia-1 (SMG-1), mammalian target of rapamycin (mTOR), and transformation/transcription domain-associated protein (TRRAP). DNA-PK is primarily involved in non-homologous end joining (NHEJ) mediated repair of DNA double-strand breaks (DSBs) (reviewed in (Meek et al., 2008)).

In response to a DSB, the MRE11-RAD50-NBS1 (MRN) sensory complex activates and recruits ATM (Lee and Paull, 2005; van den Bosch et al., 2003). Under normal conditions, ATM exists as inactive dimers in the cell (Bakkenist and Kastan, 2003). Upon recruitment to damaged DNA, ATM is acetylated by TIP60, stimulating ATM autophosphorylation which results in its disassociation into active monomers (Bakkenist and Kastan, 2003; Sun et al., 2005). The active ATM monomers phosphorylate multiple effector proteins, including the MRN complex (Gatei et al., 2000; Kim et al., 1999). The roles for ATM include damage signalling for repair after ionising...
radiation (IR) and other DSB-inducing agents, and cell cycle control (recently reviewed in (Derheimer and Kastan, 2010)).

When the replicative polymerases are stalled, either from slowing down by hydroxyurea treatment or by a direct stalling on one strand by a physical DNA lesion or adduct, the MCM helicase continues to unwind DNA in front of the fork. This exposes long stretches of single-stranded DNA (ssDNA), which are stabilised by rapid binding of replication protein A (RPA). RPA then acts as a sensor protein and recruits ATR through interaction with ATRIP, which directly binds RPA-coated ssDNA (Zou and Elledge, 2003). Like ATM, ATR belongs to the PIKK protein family and these two related kinases play fundamental roles in the DDR. Although ATM is generally activated by DSBs and ATR signals in response to replicative stress, the two proteins have overlapping roles, especially if one is missing or downregulated (Bartek and Lukas, 2003; Lindsay et al., 1998; Zachos et al., 2003).

Both ATM and ATR phosphorylate BRCA1 as well as p53 (Cortez et al., 1999; Siliciano et al., 1997; Tibbetts et al., 1999), thereby promoting nucleotide excision repair (NER) (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997). ATR also promotes NER by phosphorylation of and physical interaction with XPA after UV exposure (Shell et al., 2009; Wu et al., 2007; Wu et al., 2006) and by promoting repair in S phase (Auclair et al., 2008). Conversely, NER intermediates activate ATR leading to phosphorylation of H2AX (Hanasoge and Ljungman, 2007). In addition, although ATR is the main signalling kinase after UV exposure, ATM-deficient cells show a deficiency in repair of UV-induced lesions (Hannan et al., 2002).

The checkpoint kinases CHK1 and CHK2 play major roles in the signalling after DNA damage and replicative stress. Although ATM primarily signals through CHK2 and ATR through CHK1, there is some crosstalk between the two pathways (Gatei et al., 2003). In line with the roles of ATM and ATR, CHK2 is constitutively expressed throughout the cell cycle and is activated in response to DNA damage (Lukas et al., 2001). In contrast, the structurally different but functional analogue CHK1 (Bartek and Lukas, 2003) is preferentially expressed during S and G2 (Lukas et al.,
having a constitutive activity that is amplified in response to DNA damage induction (Kaneko et al., 1999; Zhao et al., 2002).

**Replication protein A in DNA damage signalling**

RPA is a conserved heterotrimeric protein consisting of three subunits of different sizes (70, 32, and 14 kDa, respectively), found in all eukaryotes (Wold, 1997). Binding specifically to single-stranded and not double-stranded DNA (Nasheuer et al., 1992; Wobbe et al., 1987; Wold and Kelly, 1988), RPA plays a crucial role in DNA replication, DNA repair, and DNA recombination (Wold, 1997). Stabilisation of ssDNA by RPA is required for the loading of replication factors onto replication origins (Walter and Newport, 2000), and RPA is involved in the switch from polymerase α to polymerase δ (Yuzhakov et al., 1999). In addition, in NER the polarity of RPA helps in the positioning of XPF and XPG at the lesion (de Laat et al., 1998b). Human RPA has been reported to show a preference for ssDNA but not for DNA ends in *in vitro* experiments using oligonucleotides (Ristic et al., 2003). However, when plasmid DNA was used, no preference was observed toward either ss or dsDNA (Van Dyck et al., 1998).

In response to replication damage, ATRIP is recruited to RPA-coated ssDNA, stimulating ATR activation (Cortez et al., 2001; Costanzo et al., 2003; Zou and Elledge, 2003). The 32 (-34) kDa-subunit of RPA is phosphorylated by CDKs in the S and M phases of the cell cycle, and dephosphorylated in late M (Din et al., 1990; Dutta and Stillman, 1992; Oakley et al., 2003). This phosphorylation affects the binding of RPA to DNA and replication factors (Oakley et al., 2003). In addition to this, RPA is also phosphorylated in response to DNA damage (Carty et al., 1994; Liu and Weaver, 1993; Shao et al., 1999; Zernik-Kobak et al., 1997; Zou and Elledge, 2003). This modification induces a conformational change, lowering the interaction of RPA with replication proteins, while interactions with DNA repair proteins are unaffected (reviewed in (Binz et al., 2004)). UV induces RPA phosphorylation only during replication (Rodrigo et al., 2000) in an ATR-dependent manner (Olson et al., 2006) but also involving DNA-PK (Cruet-Hennequart et al., 2006). In contrast, ionising radiation induces phosphorylation of RPA during all cell cycle phases, and is
dependent on ATM and DNA-PK (Stephan et al., 2009). The UV-induced phosphorylation of RPA increases in the absence of polymerase η (Cruet-Hennequart et al., 2006).

**Damage signalling is a complex network**

The signalling events after DNA damage requires a complex network of interactions. For example, the Rad9-Rad1-Hus1 (9-1-1) clamp activates ATR by recruiting TopBP1 to the site of DNA damage, thereby facilitating the phosphorylation of CHK1 (Delacroix et al., 2007; Kumagai et al., 2006; Lee et al., 2007). However, efficient accumulation of 9-1-1 at damaged DNA requires ATR-mediated phosphorylation of Rad17 (Bao et al., 2001; Medhurst et al., 2008), which together with four small RFC subunits acts as a clamp loader of 9-1-1 at sites of DNA damage (Bermudez et al., 2003; Ellison and Stillman, 2003; Zou et al., 2002). Like ATR, Rad17 is recruited by RPA (Zou et al., 2003). Although ATR and Rad17/9-1-1 are recruited to damaged DNA independently of each other (Kondo et al., 2001; Zou et al., 2002), they interact with each other in the regulation of CHK1 phosphorylation. Interestingly, *Xenopus* TopBP1 is required for 9-1-1 clamp recruitment to stalled replication forks (Yan and Michael, 2009).

**ATR activation after double-strand break induction**

ATR recognises ssDNA regions present after uncoupling of the replication fork due to replicative stress (Byun et al., 2005) or formed by resection of DNA ends (Adams et al., 2006; Jazayeri et al., 2006; Zou and Elledge, 2003).

In response to DSBs, ATM activates ATR through phosphorylation of TopBP1, strengthening the interaction between ATR and TopBP1 (Yoo et al., 2007). This phosphorylation is mediated through MRE11 (Yoo et al., 2009). Furthermore, after IR exposure, ATM and MRE11 generate ssDNA regions that, upon RPA binding, recruit ATR to the site of a DSB, leading to activation of CHK1 (Cuadrado et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006).
In yeast, the 9-1-1 complex is also recruited to DSBs, activating ATR (Kondo et al., 2001; Melo et al., 2001).

**CHK1 in DNA damage signalling**

ATR phosphorylates Claspin, creating a docking site for CHK1 recruitment allowing phosphorylation by ATR (Kumagai and Dunphy, 2003). CHK1 suppresses origin firing after induction of DNA damage, and is essential for cell cycle arrest in S and G2 phases (Zachos et al., 2003). CHK1 also prevents hydroxyurea-stalled replication forks from collapse (Syljuasen et al., 2005). In response to DNA damage, ATR-dependent CHK1 activation releases CHK1 from chromatin (Smits et al., 2006), allowing it to phosphorylate downstream targets such as CDC25A inducing a cell-cycle arrest (Zhao et al., 2002). In response to UV exposure, ATR signals through CHK1 giving an S phase arrest and preventing replication initiation (Heffernan et al., 2002) as well as slowing down replication forks (Guo et al., 2000; Heffernan et al., 2002; Liu et al., 2000). The slowing of replication forks also requires RAD51 (Henry-Mowatt et al., 2003), and in addition to this, CHK1 interacts with RAD51 to promote homologous recombination at stalled replication forks (Sleeth et al., 2007; Sorensen et al., 2005). CHK1 is also required for proper ubiquitylation of PCNA for translesion synthesis (Yang and Zou, 2009).

**Cell cycle arrest**

The cell cycle is driven by non-catalytic, regulatory cyclin proteins in complex with cyclin-dependent kinases (CDKs). The CDKs are active only when bound to a cyclin, and can then phosphorylate target proteins. The activity of Cyclin/CDK complexes is regulated by cyclin levels, CDK inhibitors, and regulatory phosphorylations (Morgan, 1995; Sherr and Roberts, 1999; Waga et al., 1994). In response to DNA damage, the cell cycle is arrested to allow repair. Three major checkpoints where the cell cycle can be stalled have been found – the G1/S, the intra-S, and the G2/M checkpoints (figure 3).
Figure 3. (a) Overview of the cell cycle and the major cyclin and CDK complexes. (b) DNA damage checkpoints summary. The impact of the three CDC25 proteins in different checkpoints are discussed in (Bucher and Britten, 2008)

G1/S checkpoint

During G1, CyclinD/CDK4/6 phosphorylates the retinoblastoma (Rb) protein, releasing E2F and allowing progression through G1 (Sherr, 1996; Sherr and Roberts, 1999; Weinberg, 1995). At the end of the G1 phase, Cyclin E levels increase and the CyclinE/CDK2 complex form (Figure 3 a), being activated through dephosphorylation by CDC25A (Xu and Burke, 1996). Both Cyclin E and CDC25A are transcriptional targets of E2F (Bartek and Lukas, 2001; Dyson, 1998; Vigo et al., 1999). The active CyclinE/CDK2 complex marks the transition into S phase.

In response to DNA damage, the cell cycle is temporarily halted before S phase begins by phosphorylation of CDC25A (Hoffmann et al., 1994). A slower but more permanent arrest is achieved by p16^{INK4a} induction and p21 activation by p53, leading to inhibition of the Cyclin/CDK complex, thereby preventing replication of the damaged DNA (Dulic et al., 1994; Serrano et al., 1993; Xiong et al., 1993). This is known as the G1/S checkpoint (Figure 3 b) (reviewed in (Massague, 2004)).

S phase checkpoint

Once replication has begun, the intra-S checkpoint is activated both by DNA damage and replication stress (Figure 3 b) (reviewed in (Bartek et al., 2004)).
2004)), leading to inactivation of CDC25 phosphatases and downregulation of origin firing (Larner et al., 1999). Although the ATR-CHK1 pathway is considered to be the main pathway induced after replication-associated DNA damage, the ATM-CHK2 pathway may also induce CDC25A phosphorylation, targeting it for destruction (Falck et al., 2001; Sorensen et al., 2003). ATM also phosphorylates the cohesin subunit SMC1 (Kim et al., 2002; Kitagawa et al., 2004; Yazdi et al., 2002), constituting a CHK2-independent pathway signalling for S phase arrest (Yazdi et al., 2002). This pathway also requires NBS1 and BRCA1 (Kitagawa et al., 2004; Yazdi et al., 2002). In addition to the actions of CHK1 and CHK2, the MAPK-activated protein kinase-2 (MK2) is also believed to play a role in the destruction of CDC25 phosphatases (Falck et al., 2001; Reinhardt and Yaffe, 2009; Sanchez et al., 1997) as part of the intra-S checkpoint. p38-activated MK2 directly phosphorylates CDC25B and C, inducing cell cycle arrest after UV exposure (Manke et al., 2005). MK2 is also activated after other DNA damaging agents (Reinhardt et al., 2007).

G2/M checkpoint

Both the G1/S and the intra-S checkpoints ensure that replication is stalled until DNA damage has been repaired. DNA damage that has remained undetected by these checkpoints or has occurred during the G2 phase, may activate the G2/M checkpoint (Figure 3 b). This checkpoint stalls the cell cycle and prevents entry into mitosis in response to DNA damage (reviewed in (Bucher and Britten, 2008)). During late G2 phase, CyclinB/CDK1 complexes start to assemble but are kept inactive by the Wee1 and Myt1 kinases (Booher et al., 1997; Parker and Piwnica-Worms, 1992). After faithful finishing of DNA replication, CDC25 phosphatases remove this inhibitory phosphorylation, allowing entry into mitosis (Dunphy, 1994). In response to DNA damage CHK1, and to some extent CHK2, phosphorylates the CDC25 phosphatases preventing CyclinB/CDK1 complexes from assembling (Falck et al., 2001; Jin et al., 2008; Schmitt et al., 2006; Xiao et al., 2003; Zhao et al., 2002). In addition to the cell cycle arrest caused by DNA damage, anaphase will not begin until all
chromosomes are aligned on the metaphase plane (recently reviewed in (Ito and Matsumoto, 2010).

Both ATM and ATR are crucial for the G2/M checkpoint-induced cell cycle arrest. CHK1 inactivation abolishes the G2/M checkpoint, resulting in cell death (Chen et al., 2003). Interestingly, the unspecific kinase inhibitor caffeine has been shown to inhibit ATM-dependent G2/M arrest after nitrogen mustard treatment without affecting the other checkpoints (Das, 1987).

**DNA repair**

Several specialised repair pathways have evolved in the cell. Smaller base damages such as alkylations and oxidations, as well as apurinic (AP) sites and single-strand breaks, are repaired by base excision repair. In this repair pathway, the damaged base is cut out by a glycosylase, after which the abasic site formed is cleaved by an AP endonuclease and up to a few new bases are synthesised. In the case of larger lesions that are also restricted to one strand, such as UV-induced lesions inducing a distortion to the DNA backbone, the nucleotide excision repair (NER) pathway opens up a bubble in the DNA and removes about 24-32 bases around the damage, followed by repair synthesis and ligation. DNA double-strand breaks are repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). In NHEJ the two double-strand ends are trimmed and quickly joined together, often resulting in a small deletion. HR uses a homologous sequence, preferably the sister chromatid, as a template for correct repair of the DNA double-strand break, but since this pathway requires a template it is mainly used following DNA replication. HR also plays a role in resolving replication forks stalled at a lesion. The fifth main mammalian repair pathway is mismatch repair, repairing mismatches induced during replication of the genome. Some damage can also be removed from DNA by direct reversal mechanisms such as photoreactivation.

Below, NER and HR are discussed in detail.
**Nucleotide excision repair**

Nucleotide excision repair (NER) is a repair pathway that removes UV-induced CPDs and (6-4)PPs, bulky adducts etc. Deficiencies in NER are seen in disorders such as xeroderma pigmentosum (XP), cockayne syndrome (CS), and trichothiodystrophy (TTD), which are all heritable disorders associated with sunlight sensitivity. The XP patients can be subdivided into eight complementation groups, out of which seven are defective in different proteins involved in NER. These proteins are named, as their complementation groups, XPA to XPG. Similarly, the CS patients lack either the CSA or the CSB protein, both involved in NER. TTD patients are a heterogenous group, characterised by brittle hair and growth retardation, due to defective synthesis of certain genes. Most of the patients show mutations on their XPD alleles, leading to impaired NER. Schematically, NER can be divided into five steps; (a) recognition of the lesion, (b) DNA unwinding creating an open bubble structure, (c) incisions resulting in DNA single-strand breaks and removal of the damaged region followed by (d) new DNA synthesis to cover the gap and (e) ligation. In total, about 24-32 bases are removed (de Laat et al., 1999). The recognition step may differ and NER has hence been subdivided into two separate pathways, global genome nucleotide excision repair (GG-NER or GGR) and transcription-coupled nucleotide excision repair (TC-NER or TCR).

**Damage recognition during NER**

Removal of UV-induced (6-4)PPs and CPDs exemplifies the difference in damage recognition by TC-NER, which recognises lesions blocking transcription, and GG-NER, detecting and repairing lesions that induce a distortion on the DNA helix. As mentioned previously, UV irradiation induced (6-4)PPs cause a much stronger distortion on the DNA backbone compared to the more common lesion, CPDs. These two lesion are removed with equal efficiency from the transcribed strand while (6-4)PPs are removed considerably faster than CPDs by GG-NER (Tijsterman et al., 1999).

XPC-hHR23B has a binding preference for UV-damaged over undamaged DNA, and can bind both single-stranded and double-stranded
DNA (Masutani et al., 1994; Reardon et al., 1996; Shivji et al., 1994), changing the conformation of DNA around the lesion (Sugasawa et al., 1998). The binding of XPC-hHR23B to a DNA lesion leads to assembly of repair proteins and removal of the damaged part in GG-NER. Lesions inducing only a small backbone distortion, such as CPDs, can be identified by the E3 ubiquitin ligase complex UV-DDB, consisting of DDB1 and DDB2 (Takao et al., 1993). The smaller subunit DDB2, also known as XPE (Nichols et al., 1996), is absent in rodents (Tan and Chu, 2002; Tang et al., 2000). DNA damage binding by the UV-DDB complex is followed by recruitment of the SWI/SNF chromatin-remodelling protein complex XPC-hHR23B (Fitch et al., 2003). The direct recognition of (6-4)PPs explains why these lesions are removed more quickly from the genome.

However, transcriptionally active DNA has been shown to be repaired faster than the overall repair rate. This is explained by the specific recognition of damage being an obstacle to transcription, resulting in repair by TC-NER. This subpathway of NER results in rapid removal of damage on the template strand of transcriptionally active DNA, and the repair is triggered by the stalling of RNA pol II by a lesion (Tornaletti and Hanawalt, 1999). Repair signalling following the stalling of elongating RNA pol II (RNAPIIio) requires several specific factors, including the CSA and CSB proteins. CSA is part of a ubiquitin ligase complex also involving Cullin 4A, which is neddylated by the COP signalosome (CSN) in response to UV irradiation (Fousteri and Mullenders, 2008). CSB is related to the SWI/SNF family of ATP-dependent chromatin remodelers, displaying nucleosome remodelling activity in vitro, and is also active in the initiation of transcription of certain genes as a response to UV irradiation (Citterio et al., 2000).

**Damage excision and new DNA synthesis during NER**

Following recognition of the damage, the same repair factors are assembled on DNA in both TC-NER and GG-NER. Among the first repair proteins to be loaded at the site of damage is XPA, believed to verify the damage, the single-stranded DNA coating protein replication protein A (RPA), and the nine-subunit protein complex TFIH (Yokoi et al., 2000), which plays a role in both GG-NER, TC-NER, and transcription. Similarly
to XPC, XPA preferentially binds damaged DNA and recognises various DNA damages, preferring (6-4)PPs over CPDs (Asahina et al., 1994; Jones and Wood, 1993; Robins et al., 1991). The TFIIH complex includes the helicase subunits XPB (ERCC3) and XPD (ERCC2), and is required in both transcription initiation and nucleotide excision repair to open up the DNA helix (Evans et al., 1997; Gerard et al., 1991). Both XPB and XPD have helicase activity: XPB unwinds DNA in the 3' → 5' direction and XPD in the opposite direction (Schaeffer et al., 1994; Schaeffer et al., 1993). This opens up a bubble around the lesion, an action requiring TFIIH (Evans et al., 1997) and in GG-NER also XPC-hHR23B (Evans et al., 1997; Mu et al., 1997). XPC-hHR23B is not required for TC-NER, possibly because the DNA helix is already partly opened due to ongoing transcription.

The opening of DNA is followed by cleavage of single-stranded DNA by the two structure-specific endonucleases XPG (ERCC5) and ERCC1-XPF (ERCC4), on the 3' and 5' sides of the bubble, respectively.

XPG is a member of the FEN-1 family of endonucleases, a protein family known to incise DNA in loops and at other junctions between single-stranded and double-stranded DNA (Harrington and Lieber, 1994), and has been shown to make a 3' incision in nucleotide excision repair (O'Donovan et al., 1994). XPG is also required for formation of the open complex (Evans et al., 1997) and an active-site mutant of XPG has been found to stabilise a pre-incision complex with XPC-hHR23B, TFIIH, XPA, and RPA (Mu et al., 1997) although removal of XPG does not prevent TFIIH and XPA from binding RNAPII (Laine and Egly, 2006).

Stabilisation of the open complex by XPG is independent of its catalytic activity (Mu et al., 1997), and although XPG has to be present for efficient cleavage by ERCC1-XPF the catalytic activity of XPG is not required (Wakasugi et al., 1997). Notably, even though some cleavage by XPG in the absence of ERCC1-XPF has been reported (Matsunaga et al., 1995; Mu et al., 1996), there is substantial evidence indicating that the cleavage by ERCC1-XPF occurs before the incision by XPG (Staresincic et al., 2009). The ERCC1-XPF heterodimer binds to single-stranded DNA protruding in either a 3' or 5' direction and can incise several different DNA substrates, including bubbles and stem loops, although a minimal loop size of 4-8 nucleotides is required (de Laat et al., 1998a). The incisions made by
ERCC1-XPF are always on the 5' side of the border between duplex and single-stranded DNA (Sijbers et al., 1996).

Following incision of the damage, new DNA is synthesised by a DNA polymerase. *In vivo* and *in vitro* studies using different chemical inhibitors suggest that Polδ and Polε, but not Polα, function during NER synthesis (Coverley et al., 1992; Dresler and Frattini, 1986; Hunting et al., 1991; Popanda and Thielmann, 1992), and this is supported by the need for PCNA in repair synthesis to convert UV irradiated nicked repair intermediates to fully repaired templates (Shivji et al., 1992). The replication factor C (RFC) protein complex is also required for nucleotide excision repair (Shivji et al., 1995). It is possible that Polβ seals small gaps left by DNA Polδ or Polε (Popanda and Thielmann, 1992). After finished DNA synthesis, the resulting nick at the 5' end of the newly synthesised patch is sealed by DNA ligase I (Aboussekhra et al., 1995; Araujo et al., 2000).

**Homology-directed repair**

Homologous recombination (HR) is a general name for several DNA repair pathways used by the cell to accurately repair DNA double-strand breaks (DSBs). HR is also used during replication to repair one-ended DSBs induced by replication forks running into single-strand breaks, and to overcome lesions stalling the replication fork.

DSBs can be induced by exogenous sources such as γ-irradiation, or enzymatically for instance by endonucleases or by DNA topoisomerase II. DSBs induced by a restriction enzyme are rather 'clean', while radiation-induced DSBs are surrounded by other damages such as single-strand breaks and oxidative damage, or may even be due to a cluster of single-strand breaks. The nature of the break will affect the repair, however much of the knowledge about DSB repair comes from studies using the HO endonuclease in yeast, and from experiments using high doses of γ-irradiation. A schematic illustration of DSB repair pathways is shown in figure 4. After induction of the DSB (figure 4 a), the two ends may be ligated in a the quick but error-prone non-homologous end-joining repair pathway (figure 4 b). Alternatively, homologous recombination may repair the break without any loss of information, using the complementary sequence of a sister chromatid.
as a template for new DNA synthesis. Occasionally, a homologous chromosome can be used as a template in homologous recombination, potentially resulting in loss of heterozygosity. HR is initiated by resection of the 5' ends of the break, creating 3' ssDNA overhangs (figure 4 c), enabling strand invasion (figure 4 e) and repair by classical HR (figure 4 h - j) or synthesis-dependent strand annealing (figure 4 f - g). If the DSB is induced in a repetitive area, the ssDNA overhangs may be directly ligated with each other in a process termed single-strand annealing (figure 4 d), a homology directed repair that requires resection but not recombination.

Figure 4. The different possible outcomes of double-strand break repair in eukaryotic cells.

**DSB damage recognition during HR**

The first recognition signal of DNA double-strand break repair in yeast is the phosphorylation of the SQ motif on histone H2A ($\gamma$H2A) around the break. In mammals, the phosphorylation motif is present on the histone H2A variant H2AX, comprising a minor fraction of the H2A present in the cell (Kinner et al., 2008; Rogakou et al., 1998). Consequently, the H2A phosphorylation signal is seen at 50 kbp on either side of a double-strand break in yeast (Unal et al., 2004), while $\gamma$H2AX is seen at a 2 Mbp area around a DNA double-strand break in mammals (Rogakou et al., 1998). In yeast, new cohesin molecules are loaded onto DNA following a double-strand break, and the enrichment of cohesion correlates with the $\gamma$H2A domain (Strom et al., 2004; Unal et al., 2004). H2AX can be phosphorylated
by ATM, ATR, and DNA-PKcs, as part of the DNA damage response. They further phosphorylate and activate proteins involved in the cell cycle checkpoint control, to stop the cell cycle progression until the damage is repaired. The phosphorylation signal on histone H2AX works as a platform for the recruitment of repair factors to the site of the break. In homologous recombination, some of the first proteins to be recruited include MRE11, RAD50, and NBS1, which together form the MRN complex known to promote 5' → 3' resection in yeast (Ivanov et al., 1994), although the exonucleolytic activity of MRE11 proceeds in the 3' → 5' direction (Paull and Gellert, 1998). The resection creates 3' single-stranded overhangs of several hundred bases (White and Haber, 1990) (figure 4 c), and is immediately followed by RPA binding to and protecting the single-stranded DNA (Wang and Haber, 2004). As more DNA is resected, RPA binding spreads from the double-strand break (Wang and Haber, 2004).

**Strand invasion and branch migration during HR**

The 3' overhang generated by DNA resection may invade a homologous sequence (fig 4 e). This requires several proteins, including RAD51, a homologue of the E. coli protein RecA, and cofactors including RPA (Baumann and West, 1997). RAD51 binds ssDNA and dsDNA with similar affinity and forms helical filaments with ssDNA and dsDNA in the presence of ATP (Benson et al., 1994; Sung, 1994), and has been shown to catalyse strand exchange between single-stranded and double-stranded DNA *in vitro* (Baumann et al., 1996). However, binding of RPA impedes yeast Rad51p binding, and *in vitro* studies have shown that if RPA is allowed to bind DNA before the addition of Rad51p, other factors are needed for branch migration (Shinohara and Ogawa, 1998). One such factor shown to improve the actions of Rad51 is Rad52, a protein conserved among eukaryotes (Bezzubova et al., 1993; Muris et al., 1994). Yeast Rad52 has been shown to bind ssDNA, dsDNA, and RPA (Shinohara et al., 1998). Human RAD52 proteins interact with each other, forming heptameric rings with a central channel, where DNA is bound along the positively charged peripheral groove (Stasiak et al., 2000). About 4 nucleotides of single-stranded DNA are suggested to bind each Rad52 subunit (Singleton et al., 2002), yielding a beads on a string-like pattern (Van Dyck et al., 1998). RAD51 binds RAD52, (Kurumizaka et al., 28
1999; Milne and Weaver, 1993), and yeast Rad52p has been suggested to counteract the inhibitory effect of RPA on ssDNA binding by Rad51p (Sung, 1997). RAD51 binding displaces RAD52 from DNA (Van Dyck et al., 1998). Another factor proposed to facilitate the invasion mediated by RAD51 is the SNF2-related protein RAD54 (Eisen et al., 1995), inducing supercoiling of the intact template which facilitates separation of the two strands to allow invasion (Petukhova et al., 1999; Sigurdsson et al., 2002).

BRCA2 is a large nuclear protein binding RAD51 through its BRC repeats (Wong et al., 1997), and is involved in mediating RAD51-dependent strand invasion by transporting RAD51 into the nucleus and regulating its DNA binding activity (Davies et al., 2001; Moynahan et al., 2001). Consequently, BRCA2 mutations predispose to breast and ovarian cancers (Venkitaraman, 2002), supposedly because BRCA2 deficient cells use non-homologous end joining or single-strand annealing to repair DNA double-strand breaks, both being error-prone repair pathways (Tutt et al., 2001; Xia et al., 2001). However, RAD51 foci do form at stalled replication forks even in the absence of BRCA2 (Tarsounas et al., 2003). Due to its requirement in homologous recombination, RAD51 can be used as an indicator of homologous recombination.

**Repair synthesis during HR**

Once the ssDNA overhang has invaded the dsDNA template, repair synthesis by replicative polymerases occurs (figure 4 e). In the classical homologous recombination scheme, a double Holliday junction forms (figure 4 h) that is resolved resulting in a non-crossover (figure 4 i) or crossover (figure 4 j) event. Alternatively, the formed heteroduplex may be opened up, allowing the invading, prolonged strand to reanneal with its original complementary strand in a process designated synthesis-dependent strand annealing (SDSA) (figure 4 f, g). Both pathways are reviewed in (Helleday et al., 2007).

Another double-strand break repair mechanism reported in yeast and mammals, is the RAD51 independent single-strand annealing (SSA), occurring in regions of repeated sequences (Lin et al., 1984). Strand resection following a double-strand break in such a region results in overhangs that are partially homologous, and may be annealed (figure 4 d).
Removal of the 3' non-homologous overhangs and DNA synthesis to fill in shorter single-stranded DNA sections results in a repaired DNA molecule containing a deletion.

**Replication fork stabilisation**

The double-helical nature of DNA provides the basis of replication, where the separated strands are used as templates. Accurate and complete copying of the genome is essential to maintain genomic stability in the cell, and is hence subjected to tight regulations. The initiation of replication happens at one point on the bacterial chromosome and on several spots simultaneously in eukaryotic genomes. The number of origins fired and at what stage during replication, is tightly regulated in the eukaryotic cell.

**Replication on undamaged DNA**

Once the cell has passed the G1/S checkpoint, the double helix is unwound and DNA replication is initiated from origins of replication. This is illustrated in figure 5. Two replication forks, or replisomes, will emerge in opposite directions from the origin, creating a bubble on the DNA (figure 5 b). The sister replisomes are uncoupled from each other (Yardimci et al., 2010).

An origin from which replication has been initiated may not be fired again until the next round of replication. This is partly regulated through the levels of cyclin-dependent kinases controlling cell cycle progression. Replication initiation and elongation are well studied, although the mechanisms are not yet fully understood, while the termination of replication forks still leaves a lot to explore.

**Replication initiation and origin firing**

In bacteria, replication is initiated at one single point in the circular chromosome. This point is called the origin of replication (ORI). Mammalian cells, having a much greater amount of DNA, start their replication at several points on each individual chromosome.
Replication is controlled by the selection of such potential replication origins and the firing of a subset of them. Some origins are fired early during replication, and others late. The origin of replication in budding yeast *Saccharomyces cerevisiae*, is often intergenic (Raghuraman et al., 2001; Wyrick et al., 2001) and is about 100 bp in length including a consensus sequence (Palzkill and Newlon, 1988). In fission yeast, *Schizosaccharomyces pombe*, replication origins often appear in clusters where normally only one origin is used in each replication round (Dubey et al., 1994; Patel et al., 2006). The typical *S. pombe* replication origin is about 1000 bp in length and AT-rich (Segurado et al., 2003) but lacking a clear consensus sequence (Clyne and Kelly, 1995; Dubey et al., 1996; Kim et al., 2001). The increase in A and T is probably energetically favorable for the cell since A-T pairs are held together by fewer hydrogen bonds than G-C basepairs, and are thereby easier to pull apart in the DNA melting process necessary for replication initiation. Even though no consensus sequence has been identified in metazoans, certain intergenic regions have been found to be enriched in the origin-binding proteins, as well as some CpG islands (Keller et al., 2002; Ladenburger et al., 2002), in striking contrast to the finding that human replication origins in general are AT-rich (Falaschi et al., 2007).

### Assembly of pre-replication complexes

Pre-replication complexes (pre-RCs) can only assemble at the potential replication origins when levels of cyclin-dependent kinases (CDKs) are low and APC/C protein levels are high; that is, from late mitosis and until early G1 phase (Dahmann et al., 1995; Piatti et al., 1996). Origin firing on the other hand can only occur following APC/C inactivation and increased CDK levels (Diffley, 2004). This prevents re-accumulation of pre-RC complexes at fired origins before the end of S phase. Formation of the pre-RC at potential replication origins involves the assembly of several proteins, that are not highly conserved (Falaschi et al., 2007). In total, the pre-RC consists
of more than ten different proteins (Gillespie et al., 2001), some of which are discussed below.

First, the ATPase origin of replication complex (ORC) binds DNA. \textit{S. cerevisiae} and \textit{S. pombe} ORC both interact preferentially with A-rich DNA sequences, which are typical for the \textit{S. cerevisiae} autonomously replicating sequence (ARS) consensus sequences (ACS) (Van Houten and Newlon, 1990) whereas in \textit{S. pombe} the ORC uses an AT-hook to bind the origin sequence (Chuang and Kelly, 1999). In contrast, no consensus sequence have been found for human origins of replication, and the binding of human ORC to origins could depend on other protein factors or on local chromatin structure (Bell, 2002). Consistent with the lack of a conserved sequence in replication origins, \textit{in vitro} studies have shown that the ORCs from higher eukaryotes have very little DNA sequence specificity (Vashee et al., 2003).

After binding DNA, the ORC uses the energy of ATP hydrolysis to recruit several other proteins (Bowers et al., 2004), working as a platform for the assembly of these additional replication factors. Early proteins to be assembled at the origins include Cdc6p / Cdc18p / p62 (Cdc6); Tah11p (Sid2p) / Cdt1p / CDT1, and the Mcm2-7 / Mcm2-7 / MCM2-7 proteins in \textit{S. cerevisiae} / \textit{S. pombe} / human, respectively. Cdc6, CDT1, and MCM2-7 together form the pre-replication complex (pre-RC). MCM2-7 is a six-subunit ATPase with helicase activity while the main actions of Cdc6 and CDT1 seem to be regulatory. The expression levels of these proteins are controlled to prevent re-replication. Cdc6 is an ATP-binding protein (Neuwald et al., 1999) that is required for association of Mcm2-7 with chromatin (Coleman et al., 1996). When the cell enters S phase, ScCdc6p is ubiquitinated and degraded by the proteasome (Drury et al., 1997). Similarly, the levels of SpCdt1p have been shown to peak in G1 and decrease when the cell passes through S phase (Bell and Dutta, 2002). In metazoans, an additional regulatory protein named geminin (McGarry and Kirschner, 1998) has been described. Human geminin interacts with CDT1, preventing the loading of the MCM proteins (Wohlschlegel et al., 2000), and thereby inhibiting the formation of pre-replicative complexes. Consistent with this, geminin depletion induces re-replication in human cells (Melixetian et al., 2004). Together, Cdc6 and CDT1, and in metazoans also geminin, are involved in preventing re-replication while the main function of
the MCM complex appears to be its helicase activity. 20 to 40 MCM complexes have been found to bind at individual origins in *Xenopus* sperm DNA (Edwards et al., 2002). The six MCM proteins in the hexamer have high sequence similarity but they all contain unique and conserved sequences that distinguish them from each other, and interestingly all studied eukaryotes appear to have all six MCM protein analogs, one of each class (Mcm2-7p) (Bell and Dutta, 2002). Additional MCM proteins have also been found in higher eukaryotes, reviewed in (Maiorano et al., 2006).

**The existence of dormant origins**

Not all origins bound by the ORC are fired (Raghuraman et al., 2001; Wyrick et al., 2001), and the exact regulation of this is not known, although some argue that firing of an origin is a stochastic event (Ge et al., 2007; Goldar et al., 2008). Loading of the MCM helicase onto an origin is referred to as origin licensing, since only these origins can be activated (DePamphilis et al., 2006). Chromatin loops are physically attached to the nuclear matrix, and the effect of this on DNA replication is reviewed in (Anachkova et al., 2005). Recently it has been shown that replication fork speed can change the binding of loops and thereby the firing of origins (Courbet et al., 2008). Furthermore, human ORC has been shown to interact with the histone acetyl transferase HBO1, which by acetylating histones can increase the negative charge on chromatin, opening up the local chromatin structure and possibly making firing more likely by increasing the accessibility of DNA (Iizuka and Stillman, 1999). HBO1 also interacts with mammalian MCM proteins (Burke et al., 2001). Similarly, loss of the budding yeast histone deacetylase RPD3 or targeting of the histone acetyl transferase Gcn5p results in accelerated origin firing (Vogelauer et al., 2002). Consistent with this, targeting of *Drosophila* Rpd3 close to a replication origin resulted in a decrease in origin firing (Aggarwal and Calvi, 2004), and an increased histone acetyl transferase activity is seen as DNA replication is initiated in mammalian cells (Weiss and Puschendorf, 1988).

Following genotoxic stress, origin firing is decreased through activation of the checkpoint protein CHK1 (Petermann et al., 2010b; Zachos et al., 2003). Interestingly, it was recently shown dormant origins close to the
induced damage are still fired despite the general decrease in origin activation (Ge and Blow, 2010).

Firing of a replication origin

Following pre-RC assembly, the origin may be fired, generating two sister replisomes moving in opposite directions (figure 5 a - b). Vital for the firing of an origin is the loading of the cell division cycle protein Cdc45p onto DNA (Kukimoto et al., 1999; Mimura and Takisawa, 1998). The loading of Cdc45p onto DNA correlates with replication initiation (Aparicio et al., 1999; Zou and Stillman, 2000), and requires CDK activity (Zou and Stillman, 1998). Cdc45p loading precedes the loading of DNA polymerases and is required for the assembly of PCNA and replication protein A (RPA) at replication forks (Mimura et al., 2000). These proteins are required for replication elongation, and are discussed below. Following assembly, the resulting structure at the replication origin is called a pre-initiation complex (pre-IC) as the origin can now be fired and replication initiated. Pre-ICs are seen on early fired origins before they are fully assembled on late origins (Mendez and Stillman, 2003). As the cell progresses through S phase, human CDC45 is released from chromatin, which might prevent re-replication (Saha et al., 1998).

Two kinases are involved in the control of replication initiation; CDK1 mentioned above and Cdc7-Dbf4 kinase (DDK). DDK phosphorylates MCM proteins and other replication factors at replication origins (Foiani et al., 1995; Nougarede et al., 2000; Sclafani, 2000), and CDK1 phosphorylates MCM proteins, RPA, and DNA polymerase alpha (Polα) (Dutta and Stillman, 1992; Foiani et al., 1995; Hendrickson et al., 1996). Phosphorylation of pre-RC components by CDK1 also prevents reassembly and re-replication (Coverley et al., 1998; Hendrickson et al., 1996).

In bacteria, replication starts by the denaturation of DNA at the origin of replication by DnaA, which is called an initiator factor. This allows the binding of the helicase DnaB in complex with the helicase loading factor DnaC. DnaA belongs to the AAA+ initiator factor protein family of ATPases, and in metazoans, melting of the double-stranded DNA helix at the origin is also believed to be achieved by AAA+ ATPases. Both the ORC complex and the MCM helicase complex belong to this family (Iyer et al., 2005).
The ability of AAA+ ATPases to melt DNA at the origin might be conserved in all three domains of life (Costa and Onesti, 2008). It is still unclear whether it is the MCM helicase or the ORC complex that is responsible for melting the DNA at the replication origin.

Figure 5. (a) Pre-RC assembly allows origin firing. (b) Sister replisomes travel in opposite directions on DNA. (c) In each replication fork, the leading and lagging strand are coupled together by interactions between PCNA, replication factor C, and the MCM helicase complex. Figure from (Groth, 2011).

Replication elongation

The unwinding of DNA requires the MCM helicase complex, whose helicase activity is dependent on the presence of single-stranded DNA (Lee and Hurwitz, 2001). Also, the helicase activity of the MCM complex have been reported to be stimulated by thymine-rich sequences (You et al., 2003), a feature commonly seen at replication origins, see above. The MCM complex is regulated by phosphorylations (Ishimi et al., 2000; Lei et al.,
and by Cdc45p, which might be the critical factor converting the MCM helicase complex to an active DNA helicase, allowing DNA replication (Masuda et al., 2003). As Cdc45p is loaded at an origin, unwinding starts resulting in an opened bubble in DNA (Walter and Newport, 2000) from which replication proceeds in both directions (figure 5). While MCM complexes are present in large excess at replication origins, only a fraction of the chromatin-bound MCM helicase complexes recruit Cdc45p, explaining why Cdc45p is limiting for DNA replication (Edwards et al., 2002). Cdc45p will stay associated with the MCM complex at each replication fork (Gambus et al., 2006), being required not only for replication initiation but also for replication elongation (Hopwood and Dalton, 1996). The unwinding activity by the MCM helicase complex at each replication fork requires the prior removal of histones from the nucleosomes. This is thought to be achieved by the chromatin remodelling complex "facilitates chromatin transactions" (FACT), also referred to as SPN (yeast) or DUF (Xenopus), reviewed by (Tabancay and Forsburg, 2006), known to promote human RNA polymerase II elongation in vitro (Orphanides et al., 1998).

SV40 replication elongation

Much of our original understandings about eukaryotic DNA replication come from biochemical studies of replication of plasmid DNA containing the Simian Virus 40 (SV40) replication origin. These studies, reviewed in (Waga and Stillman, 1998), revealed the combined action of two essential DNA polymerases, Polα and Pol delta (Polδ). Polα, also designated the primase, makes short RNA primers and extends them on DNA to about 30 nucleotides. This double-stranded DNA template is recognised and bound by replication factor C (RFC), which then expels Polα and facilitates the loading of proliferating cell nuclear antigen (PCNA) (Stillman, 2008). PCNA is a three subunit ring-shaped clamp that after loading can slide along DNA, and by binding Polδ, ensuring the progression of DNA replication. On the SV40 leading strand, priming is only needed to initiate replication at the origin of replication, while at the lagging strand, short Okazaki fragments are formed and Polα is needed for initiation of replication of each new fragment. In eukaryotic cells, Okazaki fragments are about 100-200 base pairs, while
in prokaryotes they are about ten times that size.

**Eukaryotic replication elongation**

Eukaryotes have another essential DNA polymerase, Pol epsilon (Pol\(\varepsilon\)), initially designated Pol\(\varepsilon\) II (Burgers et al., 1990). Originally, contradicting data was presented regarding the need for the catalytic activity of this polymerase during replication in *S. cerevisiae* and *S. pombe* (D'Urso and Nurse, 1997; Feng and D'Urso, 2001; Kesti et al., 1999; Navas et al., 1995). However, *in vivo* inhibition of human Pol\(\varepsilon\) in cell nuclei blocks genomic DNA synthesis (Pospiech et al., 1999). The presence of a proofreading exonuclease activity in Pol\(\varepsilon\), as in Pol\(\delta\), indicates that the DNA synthesis activity by Pol\(\varepsilon\) is actually required for proper replication of genomic DNA. Recent experiments from the Kunkel and Johansson labs have shed some light on the separable roles of Pol\(\delta\) and \(\varepsilon\). By placing a reporter gene in either direction and at either side of an origin of replication, a given strand in the reporter gene will be replicated as either leading or lagging strand, depending on the orientation of the gene. In yeast mutated in Pol\(\delta\), mutations were primarily found on the lagging strand, while the opposite was seen in yeast mutated in Pol\(\varepsilon\) (Nick McElhinny et al., 2008; Pursell et al., 2007). This idea is supported by the finding that Pol\(\delta\) and Pol\(\varepsilon\) edit replication errors on opposite DNA strands, indicating that they would replicate these opposite strands (Shcherbakova and Pavlov, 1996), and the reporting of strand-specific replication of plasmid DNA (Karthikeyan et al., 2000). Consistent with these findings, Pol\(\delta\) but not Pol\(\varepsilon\) proofreads errors made by Pol\(\alpha\) (Pavlov et al., 2006). However, it is possible that Pol\(\delta\) can partly take over if the polymerase activity of Pol\(\varepsilon\) is absent. Pol\(\delta\) and Pol\(\varepsilon\) are believed to travel together on DNA, bound by separate PCNA molecules held together by Replication factor C (RFC) (figure 5 c).

Summarising the actions on the lagging strand, there is a constant flow of initiation and short DNA elongation by Pol\(\alpha\), a switch to and further elongation by Pol\(\delta\), and maturation and ligation of the Okazaki fragment also by Pol\(\delta\) together with the flap structure-specific endonuclease FEN1 which removes the RNA primer, and DNA ligase which seals the gaps. In addition to the three replicative polymerases \(\alpha\), \(\delta\), and \(\varepsilon\), several additional DNA polymerases have been described in eukaryotes. Pol beta (Pol\(\beta\)) lacks
proofreading exonuclease activity and fills in short gaps (Singhal and Wilson, 1993), and functions in base excision repair (Mullen and Wilson, 1997) and possibly also nucleotide excision repair (Popanda and Thielmann, 1992). Pol gamma (Polγ) has both polymerase and 3’ → 5’ exonuclease activity, and is responsible for replication of mitochondrial DNA. There are also several enzymes with polymerase activity across DNA lesions, so-called translesion synthesis (TLS) polymerases.

**DNA damage tolerance and replication resumption**

Of the thousands of damages induced in a cell every day, some will not be repaired before replication takes place. In addition to the exogenous and endogenous sources of DNA damage, replication forks encounter natural replication pause sites, and fork movement may be slowed down for example by depletion of the nucleotide pool. Several mechanisms have evolved to handle or bypass lesions at the replication fork. A replication fork stalled for a short time may restart after removal of the nucleotide depriving agent (Petermann et al., 2010a). If stalled for a long time, the replication fork collapses into a DNA double-strand break, detectable by pulsed-field gel electrophoresis (Lundin et al., 2005; Petermann et al., 2010a; Saintigny et al., 2001). However, there is also evidence that after a physical block to replication fork progression, replication is resumed on the 5’ side of the lesion, leaving a daughter-strand gap that is later filled in (paper I, (Lopes et al., 2006)).

The mechanisms for continuation of replication despite DNA damage, are often termed DNA damage tolerance. It is important to note that the mechanisms for damage tolerance at replication forks, with the exception of the repair of DNA single-strand breaks, do not remove the damage. Although these processes were earlier referred to as post-replication repair (PRR), it is more accurate to designate them as damage tolerance mechanisms, as repair of the DNA molecule does not take place.
Natural impediments to replication fork progression

The progression of a replication fork may be halted by natural obstructions, such as unusual DNA structures or other proteins (reviewed in (Mirkin and Mirkin, 2007)). A replication fork may also collide with a transcription unit, although this is generally avoided by the cell (Brewer and Fangman, 1988; Deshpande and Newlon, 1996). Head-on collision causes replication fork pausing (Deshpande and Newlon, 1996) and demise, inducing transcription-associated recombination (Prado and Aguilera, 2005). As the DNA polymerase moves about ten times faster than the RNA polymerase, a replication fork may also collide with the rear end of a transcription unit, slowing down the replication fork (Deshpande and Newlon, 1996). It has been suggested that the mRNA may be used as a primer for continued DNA synthesis on the leading strand after in-line collision with transcription (Pomerantz and O'Donnell, 2008).

Figure 6. Homologous recombination repair pathways at replication forks. Only non-crossover products are depicted.
One-ended DNA double-strand breaks

Single-strand breaks are induced by γ-irradiation etc, and are also induced during the nucleotide and base excision repair pathways. A replication fork running into a single-strand break will form a one-ended DNA double-strand break (figure 6 c), a lesion that can also be formed by cleavage of a small lesion blocking the progression of the replication fork (figure 6 b). In yeast, such a structure has been shown to induce break-induced replication (BIR), reviewed by (Kraus et al., 2001), while in mammalian cells, there is little evidence for BIR. Instead, mammalian repair of one-ended DNA double-strand breaks highly resembles the repair of two-ended DNA double-strand breaks by homologous recombination (figure 6, left column). The repair is initiated by the resection of DNA of the single arm, forming a 3’ overhang (figure 6 d) that is immediately covered by RPA. Also, single-stranded gaps of the intact DNA molecule are filled (Hellday, 2003). This enables the single-stranded 3’ overhang to invade the double-stranded template (figure 6 e), allowing DNA synthesis and leading to the formation of a Holliday junction (Zou and Rothstein, 1997) (figure 6 f). This is mostly resolved without crossing-over (figure 6 f, open arrowheads) (Richardson et al., 1998), leading to sister-chromatid exchanges (figure 6 g). Consistent with this, cells defective in single-strand break repair have elevated levels of sister-chromatid exchanges (Caldecott et al., 1992; Thompson et al., 1990).

Resolution of a stalled replication fork

In an intricate pathway to avoid the fork collapse, a stalled replication fork may reverse due to positive torsional strain, forming a structure with four joined double-strands. As this schematically resembles a chicken-foot, it has been named so (figure 6 i). This switching of templates allows bypass of the lesion, and possibly leaves time for repair of the damage (Hanawalt, 2007; Johansson et al., 2004). Alternatively, template switching may occur through invasion of a sister chromatid (Branzei and Foiani, 2007).
Resolution by chicken-foot formation

The chicken-foot structure allows DNA synthesis with the complementary, newly synthesised strand as a template. However, the formation of a chicken-foot involves the formation of an intermediate structure where only the non-damaged, newly synthesised strand has reversed (figure 6 h). This structure, a half chicken-foot, may be cleaved by endonucleases, creating a one-ended DNA double strand break (figure 6 c) repaired as previously described.

Alternatively, if the chicken-foot is formed (figure 6 i), the Holliday junction it constitutes (figure 6 j) may be resolved by two pathways. Either replication is restored before the Holliday junction is resolved, generating a double Holliday junction (figure 6 o), or the Holliday junction is cleaved resulting in a one-ended DNA double-strand break (figure 6 k). As described previously, this one-ended double-strand break will invade the intact template, restoring replication and creating a second Holliday junction (figure 6 l). Cleavage of this (figure 6 m) generates a restored replication fork (figure 6 n). Alternatively, a restored fork where replication can continue may also be obtained dissolution of the double Holliday junction (6 p → 6 q), by the BLM / TopoIII complex (reviewed in Cheok et al roles of the bloom’s syndrome helicase 2005). In line with this, cells deficient in the BLM helicase show elevated levels of sister chromatid exchanges, and BLM suppresses crossing-over in vitro (Wu and Hickson, 2003). However, the regulation between crossing-over and non crossing-over during resolution, and between resolution and dissolution, is not yet fully determined.

The resolution of a stalled replication fork and the repair of one-ended DNA double-strand breaks involve to a large extent the same proteins as for repair of two-ended DNA double-strand breaks by homologous recombination. RAD51 foci can hence be used to measure homologous recombination at replication forks. Consistent with this, depletion of replication protein A (RPA) inhibits the formation of RAD51 foci at replication forks following replication stress (Sleeth et al., 2007).
Template switching by strand invasion

Template switching by strand invasion has mainly been described in yeast (Branzei and Foiani, 2007), although it has been shown to occur also in mammalian cells (Blastyak et al., 2010). Template switching is mainly discussed as a means of sealing gaps left behind a (possibly re-primed) replication fork (figure 7 a, f). Without reversal of the replication fork, the 3’ end next to the lesion invades the double-stranded sister chromatid, using the complementary, newly synthesised strand as a template for DNA synthesis (figure 7 b, g). This creates a pseudo double Holliday junction (figure 7 c, h), that can be dissolved to restore normal replication fork progression (figure 7 d, k). However, the lesion stalling the replication fork will remain unrepaired.

It is unclear to what extent template switching also occurs to allow progression of a stalled replication fork (figure 7 e). In such a case, strand invasion (figure 7 i) creates a pseudo single Holliday junction (figure 7 j) that could be resolved by dissolution to a stabilised replication fork (figure 7 k) still containing the initial damage.

Figure 7. Model for template switching to allow lesion bypass at replication forks
PCNA polyubiquitination

PCNA polyubiquitination on lysine 164 has been implicated in error-free template switching (reviewed in (Ulrich, 2007) and is performed by E2 Ubc13-Mms2 and E3 ligase Rad5 in yeast (Hofmann and Pickart, 1999). Mammalian orthologs of yeast Rad5, HLTF and SHPRH, induce polyubiquitination of PCNA, thereby preventing spontaneous mutations (Motegi et al., 2008; Motegi et al., 2006; Unk et al., 2006). Interestingly, these two enzymes are not essential for PCNA polyubiquitination, indicating the existence of an alternative E3 ligase (Krijger et al., 2011).

Translesion synthesis

In eukaryotes, ten DNA polymerases have been described, reviewed in (Hubscher et al., 2002), out of which five are less stringent than the normal replicative polymerases. These are described to be involved in translesion DNA synthesis (TLS) where the replicative polymerase stalled at the site of a lesion, is exchanged for a DNA polymerase with a more open active site that is able to replicate past the lesion, potentially inducing a mutation but preventing the fork from collapse. Due to low processivity the TLS polymerase quickly falls off the template. The normal replicative polymerase is then restored on DNA and replication continues.

Translesion synthesis can be divided into two separate processes, insertion opposite the damaged base and extension of the DNA strand from the damage. Some TLS polymerases can perform both activities, while others can only do one. Of the five TLS polymerases described in eukaryotes, Pol zeta (Polζ) is the only one that does not belong to the Y family, but is instead part of the B family of DNA polymerases. Polζ is a heterodimer of the catalytic subunit Rev3 and the regulatory subunit Rev7.

The Y family of TLS polymerases consists of polymerases eta (Polη), iota (Polι), kappa (Polκ), and REV1, although the latter differs from the others by not being an actual polymerase, but a dCMP transferase. Due to its structure it is, however, considered to be part of the Y family together with Polη, Polι, and Polκ, all being characterised by a more open structure at their
active site (Trincao et al., 2001) and with a conserved N-terminal catalytic domain and an unconserved C-terminal part (Lehmann et al., 2007).

**Translesion synthesis across UV-induced lesions**

Polη is perhaps the most characterised of the TLS polymerases. This polymerase appears to differ from the other, non-specific low fidelity TLS polymerases in that it has specially evolved to replicate correctly past the most common UV-induced thymine dimer, the cis-syn TT CPD, although it may also bypass other lesions. When the replication fork runs into a DNA lesion, it will initially be stalled while the helicase continues, creating a stretch of ssDNA in front of the stalled fork. This ssDNA is immediately bound by RPA, and is thought to be the signal responsible for ubiquitination of PCNA seen at replication forks stalled at a UV-induced lesion (Kannouche et al., 2004; Lehmann et al., 2007). The ubiquitination mark on K164 on PCNA increases its affinity for Polη, and as the normal replicative polymerase falls off the strand, Polη bypasses the CPD (Masutani et al., 1999a; Masutani et al., 2000), dissociating from DNA after insertion of only a few nucleotides (McCulloch et al., 2004). Deubiquitination of PCNA is performed by USP1, which is degraded following UV-irradiation (Niimi et al., 2008). Since Polη preferentially inserts A, the bypass of a TT-CPD is normally error-free, but Polη has higher error rates at the 3' thymine than at the 5' thymine (McCulloch et al., 2004). Although Polη is required for error-free bypass of CPDs, TLS after UV exposure also involves Polζ (Takezawa et al., 2010), REV1 (Clark et al., 2003; Gibbs et al., 2000; Takezawa et al., 2010), and Polι (Dumstorf et al., 2006; Tissier et al., 2000). Interestingly, depletion of Polκ delayed TLS after UV irradiation (Takezawa et al., 2010).

(6-4)PPs are believed to be bypassed by the combined actions of Polι and Polζ (Johnson et al., 2000; Tissier et al., 2000; Zhang et al., 2001).

In addition to CPDs and (6-4)PPs, UV-irradiation also induces 8-oxoG lesions, which are primarily repaired by short-patch base excision repair. If not repaired before replication of DNA, 8-oxoG has been suggested to be bypassed by replicative polymerases (Tolentino et al., 2008) or by TLS polymerases including Polη (Avkin and Livneh, 2002; Maga et al., 2007). It is possible that Polη is not required for bypass of 8-oxoG but may be
Recent data from yeast indicates that translesion synthesis-dependent filling of gaps after replication on damaged DNA occurs to some extent after chromosomal replication is finished (Daigaku et al., 2010; Karras and Jentsch, 2010). In line with this, there is direct evidence of both REV1 and Polζ being involved not only in bypass during replication but also in mutagenic DNA synthesis during post-replication filling of gaps after S phase (Jansen et al., 2009a; Jansen et al., 2009b; Waters and Walker, 2006).

Individuals mutated in Polη display high sensitivity to UV irradiation, and are categorised as xeroderma pigmentosum variant (XP-V). Unlike patients from the other XP complementation groups, which are deficient in NER, Polη deficient individuals have functioning NER (Hessel et al., 1992), and their UV sensitivity was puzzling for a long time. It was not until 1999 that XP-V patients were found to have a mutated Polη (Johnson et al., 1999; Masutani et al., 1999b).

Following UV exposure, Polη is phosphorylated by ATR (Gohler et al., 2011). This phosphorylation is dependent on physical interaction with RAD18 but is independent of PCNA, and is involved in the checkpoint response after UV exposure (Gohler et al., 2011).

**Re-priming and firing of dormant origins**

A stalled replication fork (figure 8 c) may restart by direct resumption of DNA synthesis if the fork-blocking agent is removed (Petermann et al., 2010a). Also, if the general replication is slowed down, the cell may fire more origins to keep the total replication speed up. To prevent re-replication, only origins licensed before replication, can be fired. Origin firing is suppressed by CHK1 (Petermann et al., 2010b; Zachos et al., 2003), which is phosphorylated by ATR in response to replicative stress (Guo et al., 2000; Liu et al., 2000).

However, if a replication fork becomes completely stalled by a DNA lesion (figure 8 d), general increase of origin firing may not be the ultimate choice. Rather, in yeast cells, when a replication fork encounters a DNA lesion and one strand becomes stalled, leading and lagging strand are uncoupled and replication continues on the undamaged strand (Lopes et al.,
2006), leaving a single-stranded stretch of DNA on the other strand. This ssDNA signals for ubiquitination of PCNA (Kannouche et al., 2004; Lehmann et al., 2007). However replication is resumed on the damaged strand, leaving behind a ssDNA gap opposite the lesion (Lopes et al., 2006). The formation of ssDNA gaps during replication of damaged DNA has also been reported or interpreted previously in bacteria, yeast and mammalian cells (Lehmann, 1972; Prakash, 1981; Rupp and Howard-Flanders, 1968; Svoboda and Vos, 1995), leading to the suggestion of re-priming past the lesion to allow replication to continue (Lopes et al., 2006) (figure 8 e). Indeed, the ssDNA formed after uncoupling of the leading and lagging strands may also be sufficient for Polα to bind and allow replication to resume from a new Okazaki fragment, possibly also on the leading strand. If present, translesion synthesis polymerases fill in the gap, however this activity may be uncoupled from replication (Daigaku et al., 2010; Karras and Jentsch, 2010). It should be noted that although replication origin firing is generally decreased after replicative stress, local activation of dormant origins in close proximity to the damage may occur (Ge and Blow, 2010).

Figure 8. The nature of a replication fork stalling agent affects the outcome. (a, b) Replication fork elongation on unperturbed DNA. (c) Replication fork stalling by nucleotide deprivation causes uncoupling of the helicase. (d) Replication fork stalling by a physical damage initially stalls only one strand. (e) Re-priming may occur to allow continuation of replication past a physical lesion, leaving behind a ssDNA gap.

**Chromatin Remodelling**

Before the findings by Gregor Mendel (1822-1884), traits were believed to be inherited through blood, being diluted as they were passed on. Mendel was able to show that phenotypical attributes are inherited as separate pieces of information, later termed genes. Coinciding in time with Mendel's work, the existence of nucleic acids was shown by Friedrich Miecher (1844-1895).
However, it was not until the early 1900s that Thomas Morgan Hunt (1866-1945) was able to show that inherited traits are coded for by genes inherited on chromosomes. For a long time, the DNA of chromosomes was considered to hold all the vital information needed. However, the field of epigenetics is now growing, as the non-DNA parts of a chromosome, such as nucleosome variants and modifications, have been shown to contain information affecting for example the expression of nearby genes.

**Chromatin**

Chromatin is the complex of DNA and proteins that together form chromosomes. The proteins serve multiple functions in association with DNA: they protect the vulnerable double-helix against ROS, they make condensation of the negatively charged DNA possible, and they are used to organise the genetic material, as a cell only uses a fraction of its entire DNA. This is a complex task involving multiple protein families. In bacteria, mitochondria and chloroplasts, all lacking a nucleus, chromatin is generally found assembled in a certain area, called the nucleoid, reviewed in (Thanbichler et al., 2005). Here, organisation of DNA is achieved by histone-like proteins, which regulate the accessibility of the genetic material. The circular chromosome has been found to be arranged with a central core, and protruding loops, guaranteeing that even in case of a double-strand break, all the energy stored in supercoiling of the molecule won't be lost.

In eukaryotes, chromatin structure requires the histone protein family. Histones are relatively small proteins assembled in octamers, around which DNA is wrapped, forming nucleosomes. Such octamers are built up by so-called core histones; two H2A-H2B heterodimers and an (H3-H4)\textsubscript{2} tetramer. 146 bp of DNA is wrapped around the octamer in less than two turns, entering and leaving the core particle at about the same place. The created structure, captured on an electron micrograph in 1973 by Ada L Olins, is known as beads on a string. In mammals and plants, but not in yeast, linker histone H1 binds between two nucleosomes, stabilising the structure (Robinson and Rhodes, 2006). Scaffold proteins lead to further compaction of chromatin.
In the eukaryotic nucleus, the chromosomes are organised as compartmentalised structures, or territories, reviewed in (Cremer et al., 2000). Within each territory, certain sections of DNA are more accessible, while other parts are more condensed. The less closed parts are designated euchromatin, while the more compacted structures are called heterochromatin. Although euchromatin is often found in the outer boundaries of the domains building up a territory, and the closed heterochromatin is often detected in the less accessible inner parts, there are numerous exceptions from this, reviewed in (Foster and Bridger, 2005). Another level of organisation comes from the positioning of the individual chromosomes. Generally, gene-rich chromosomes are often located in the middle of the nucleus where more transcription factors etc are also found, while gene-poor chromosomes are often seen in the outer parts of the nucleus (Boyle et al., 2001). However, important exceptions to this general rule include transcriptionally active areas located close to nuclear membranes pores (Casolari et al., 2004).

**Chromatin remodelling**

The organisation of chromatin is regularly altered by the cell, due to differences in desired transcription activity, need for DNA repair etc. Such chromatin remodelling can be divided into stable modifications such as general changes in hetero- and euchromatin, and rapid, dynamic changes such as transient, quick signalling for repair where the repair signal has to be removed once repair is finished. Chromatin remodelling involves recruitment of nucleosome remodelling complexes, e.g. SWI/SNF, and post-translational modifications (PTMs) of histone proteins, primarily on the histone tails. These PTMs regulate both histone-histone interactions within and between nucleosomes, and histone-DNA interactions. There are several PTMs such as phosphorylations, acetylations, methylations, ubiquitinations etc. In some cases the modification itself can be a sterical hindrance for other modifications to occur, or change interactions by directly influencing the charge of the modified amino acid, whilst in other cases the modification(s) act as a recruiting platform for other proteins.
Acetylation of lysines by histone acetyl transferases (HATs) is perhaps the most well studied histone modification, and removes the positive charge of the lysine being modified. This reduces the affinity between the histone and the negatively charged DNA, thereby leading to a more open chromatin structure. Consequently, deacetylation by histone deactylases (HDACs) in an area causes a more condensed organisation, leading to decreased accessibility of DNA. The acetylation pattern influences DNA repair (Masumoto et al., 2005), replication (Alexiadis et al., 1997), and transcription (Nightingale et al., 1998; Vettese-Dadey et al., 1996). Methylation of histones by histone methyltransferases does not alter the charge of the modified amino acid, but rather function through the recruitment of downstream effector proteins, such as the H3K9 methylation mark recognised by the chromodomain of heterochromatin protein 1 (Bannister et al., 2001; Lachner et al., 2001). Interestingly, a lysine side chain can be mono-, di-, or trimethylated, and the three methylation states can have different biological outcomes, while methylation of arginine, which can occur once or twice on the side chain, seems to always activate transcription (Berger, 2007).
Present Investigation

Aim

The aim of this thesis was to gain further insight into the cellular mechanisms and signalling pathways induced by damaged replication forks. As a tool for this, much focus has been put on xeroderma pigmentosum variant (XP-V) cells, deficient in the translesion synthesis polymerase Polη, and the corresponding restored cell line. Some focus has also been put on the stabilisation of forks stalling after being slowed down, and the signalling required for stabilisation of forks during unperturbed replication, preventing damage.

The specific aims were
- to study the resumption of DNA synthesis after a physical fork-stalling lesion (paper I)
- to study the role of CHK1 in replication fork stabilisation after induction of physical fork-stalling lesions (paper II)
- to study the role of the MUS81 endonuclease in UV irradiation induced replication fork collapse in human cells (paper III)
- to study the role of SET8 during unperturbed replication (paper IV)
- to study the importance of the RAD51 paralog RAD51D during replication (paper V)

Methodology

Cell lines and cell culturing

Cells were grown in Dulbecco’s minimal essential medium with 10 % foetal calf serum and 1 % streptomycin-penicillin, in an incubator at 37 °C with 5 % CO₂. The medium used for the restored XP30RO+Polη cell line was supplemented with 100 μg/ml of zeocin.

The human Polη deficient fibroblast cell line XP30RO (XP-V), originally derived from a patient and now with an empty pcDNA vector, and
the restored XP30RO+Polη cells stably expressing Polη, were kind gifts from Dr A.R. Lehmann. The Chinese hamster ovary (CHO) cell lines AA8, 51D1, and 51D1.3, were kind gifts from Dr L.H. Thompson.

Table 1. Origin and characteristics of the cell lines used in the study.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin and cell type</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td>Human osteosarcoma</td>
<td>Wild type</td>
<td>(Ponten and Sakela, 1967)</td>
</tr>
<tr>
<td>AA8</td>
<td>Hamster endothelial</td>
<td>Wild type</td>
<td>(Thompson et al., 1980)</td>
</tr>
<tr>
<td>51D1</td>
<td>Derived from AA8</td>
<td>RAD51D knockout</td>
<td>(Hinz et al., 2006)</td>
</tr>
<tr>
<td>51D1.3</td>
<td>Derived from 51D1</td>
<td>RAD51D complemented</td>
<td>(Hinz et al., 2006)</td>
</tr>
<tr>
<td>XP30RO</td>
<td>Human fibroblast</td>
<td>Polη deficient (empty pcDNA vector)</td>
<td>(Stary et al., 2003; Volpe and Cleaver, 1995)</td>
</tr>
<tr>
<td>XP30RO+Polη</td>
<td>Derived from XP30RO</td>
<td>Polη complemented</td>
<td>(Stary et al., 2003)</td>
</tr>
</tbody>
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Replication elongation measuring techniques

Replication fork progression using alkaline DNA unwinding

The alkaline DNA unwinding (ADU) replication fork progression technique utilises the fact that DNA in an alkaline solution will unwind from strand breaks (Erixon and Ahnstrom, 1979). Such strand breaks are present at a replication fork, and by pulse labelling DNA and allowing the replication forks to progress from the labelled area for different times, the average distance that the replication fork has progressed can be measured, as described previously (Johansson et al., 2004). The fraction of labelled single-stranded DNA is greater the slower the replication fork is progressing. However, if replication continues past a DNA damage leaving a daughter strand gap, unwinding will initiate also from this gap.

Briefly, cells are pulse labelled for 30 min with 3H-TdR, exposed to different doses of UVC irradiation, and incubated in the presence or absence of inhibitors. Replication fork progression is terminated by the addition of 0.5 ml ice-cold 0.15 M NaCl and 0.03 M NaOH unwinding solution, and following a regulated incubation unwinding is stopped by the addition of 1 ml 0.02 M NaH₂PO₄. DNA is fragmented to pieces of about 3 kb, and single- and double-stranded DNA is separated on hydroxyapatite columns at 60 °C (Erixon and Ahnstrom, 1979). The fraction of single-stranded DNA is determined in a scintillation counter which converts the radioactive decay to
light flashes. Plotting the fraction of single-stranded DNA against chase time indicates how the labelled area grows into double-stranded DNA as the fork progresses from the pulse labelled area. This is a quick assay for measuring replication fork elongation, although it does not distinguish between different sources of strand breaks.

**Alkaline sucrose gradients**

Alkaline sucrose gradients were used to study the growth of DNA molecules synthesised shortly after UV exposure. Briefly, UVC irradiated cells are chased for 30 min to allow the replication forks to run into a lesion, before pulse labelling with $^3$H-TdR for 30 min. Cells are then incubated in pre-warmed media before harvesting and mild fragmentation of DNA using $\gamma$-irradiation. Irradiated cells were lysed on newly made gradients and centrifuged to allow separation of small and large DNA fragments. The sizes of labelled DNA fragments is determined by dropwise transfer of the gradient to paper strips that are allowed to dry before the radioactive decay is measured, as described previously (Bienko et al., 2010). This system allows us to study the growth of molecules replicated shortly after UVC exposure. It is interesting to note that primary Pol$\eta$ deficient cells have been shown to replicate their DNA in short stretches that later grow into larger molecules (Lehmann et al., 1975), while the short fragments formed in immortalised Pol$\eta$ deficient cells have not been converted to large fragments even at 12 h after labelling (Paper I). In restored cells, the DNA fragments were of a considerable size already one hour after labelling (Paper I), indicating that replication is quicker in immortalised fibroblasts compared to primary cells.

**DNA fibre technique**

The DNA fibre technique is a useful tool to study replication structures after DNA damage induction. In contrast to the ADU replication fork elongation and the sucrose gradient techniques, single molecules are scored, making it vital to analyse coded samples. In addition to the information about replication elongation and replication rate, the DNA fibre technique
can also be used to study changes in origin firing after damage induction or in the presence of inhibitors.

Briefly, cells are labelled with halogenated nucleotides (CldU and IdU), and lysed on glass slides where the DNA is then spread by tilting the slides. Following fixation, CldU and IdU can be visualised by staining the slides with specific antibodies (Groth et al., 2010), yielding fibres that are red and/or green depending on the progression of the fork. Analysing the relative distribution of different fragments allows us to determine changes in new origin firing, active forks, etc. The length of the fibre is converted from μm to kb using the conversion factor $1 \mu m = 2.59$ kb (Henry-Mowatt et al., 2003), which can be used to measure replication speed. The speed of the replication fork is generally lower during the IdU treatment in control samples, possibly because a higher concentration of IdU is used to replace CldU from the nucleotide pool. Incubating the untreated control cells for 2 h in IdU yielded very long fragments, likely due to new origin firing and replicon merging. Such long incubations are not ideal for control samples. In addition to replication speed, the DNA fibre assay can be used to study resumption of DNA synthesis (replication fork restart) after induction of DNA damage.

**DNA damage and signalling measurement techniques**

**Pulsed-field gel electrophoresis**

Pulsed-field gel electrophoresis (PFGE) is a method used to separate large chromosomal fragments on an agarose gel, allowing the visualisation of DNA double-strand breaks. Furthermore, labelling the cells with $^{14}$C-TdR allows quantification of DSBs specifically formed in replicating (pulse labelled) or bulk (homogeneously labelled) DNA. PFGE can be used to study the induction and the repair of DSBs in a large number of cells but is not a very sensitive method, and the migration of replication structures on PFGE is not fully determined.

Briefly, cells are homogeneously (24 h) or pulse (0.5 h) labelled with $^{14}$C-TdR directly before exposure to the DNA damaging agent. At chosen time points, cells are harvested and melted into agarose plugs that are incubated with proteinase K for 48 h in room temperature (Lundin et al.,
2005) before DNA separation on PFGE and staining with ethidium bromide for visualisation and quantification of DSBs. If DNA was labelled with a radioactive nucleotide, the gel can then be dried or the DNA can be transferred onto a membrane to allow quantification of DSBs appearing in the overall genome or specifically at replication forks.

**Immunofluorescence**

Studying the formation or disappearance of repair protein focus formation using immunofluorescence (IF) is a more sensitive method compared to PFGE, and allows us to study co-localisation of protein foci in individual cells. Like the fibre assay, analysis using IF requires coding of all samples to prevent subconscious scoring.

Briefly, cells are grown on cover slips at least 18 hours before induction of DNA damage. siRNA treatments are preferably not done on cover slips, but rather in a well before re-seeding of cells onto cover slips. Following repair, cells are fixed for 20 min in paraformaldehyde of a concentration (1 – 3 %) depending on the cell line. A lower concentration may be required to reduce background but this shortens the life length of the slides.

For replication stress studies it is useful to pulse label cells with CldU directly before exposure to e.g. UV, allowing selective quantification of cells that were in S phase when irradiated, and also to study co-localisation of repair proteins with areas that were replicating when irradiated. The CldU foci pattern depends on the S phase stage of the cells.

γH2AX is a common but rather unspecific marker for DSBs and can be complemented with 53BP1 foci formation quantification for a more accurate measure of DSB induction. RAD51 was used as an indicator of homologous recombination repair, although it is an early HR protein and does not indicate whether the HR event was successful. It should be noted that background levels of repair protein foci, especially γH2AX, may differ greatly between cell lines.
Results and Discussion

UV stalled replication forks restart by re-priming in human fibroblasts (paper I)

After replication fork stalling induced by hydroxyurea, replication forks can actively restart after a short block or use new origin firing to restart after a longer block. After replication fork stalling by physical agents, replication cannot be actively restarted until the damage is removed. However, we show here that replication forks are elongated despite the stalling lesion, despite fork stalling and a decrease in origin firing.

In this study, using Polη deficient and restored cells, we see UV irradiation dependent a general reduction in new origin firing and replication fork movement in the DNA fibre assay, similar in both cell lines. The tiny difference in fork speed between Polη deficient and proficient cells after UV exposure could be explained by efficient bypass by other TLS polymerases in the absence of functional Polη. However, using the alkaline DNA unwinding method we see an apparent stalling of replication forks in UV irradiated Polη deficient cells and they remain stalled for 12 hours, while UV irradiated restored cells display a smaller and only an initial stalling. In addition, using alkaline sucrose gradients, we demonstrate that new DNA is replicated in short fragments after UV exposure and that these fragments are not sealed within 12 hours in the Polη deficient cells. Together, these data suggest that in response to UV exposure, replication forks are initially stalled but re-primed close to the damage, allowing replication to continue. This leaves a gap from which DNA unwinding can start, however the gap is obviously too small to be detected using the DNA fibre assay. To study the fate of these replicative gaps, we used pulsed-field gel electrophoresis, showing an accumulation of DNA double-strand breaks in Polη deficient cells, likely caused by replication fork collapse. We also show co-localisation of the DNA damage marker γH2AX and recombination protein RAD51 to the areas that were replicating when irradiated.

This provides evidence of a restart mechanism that is different from the replication fork restart seen after nucleotide deprivation by hydroxyurea. It is not surprising that a physical block requires replication restart by different means compared to a replication fork slowed down due to shortage of
nucleotides. It is interesting to speculate in what would be needed for re-priming and it is possible that re-priming requires no factors additional to what is already present at the replication machinery, since re-priming essentially happens continuously on the lagging strand. Although it is likely that re-priming occurs also in Polη proficient cells, this remains to be determined.

**CHK1 activity is required for fork elongation but not fork stabilisation after UV irradiation (paper II)**

The ATR-CHK1 signalling pathway is important for replication fork stability after long hydroxyurea treatment and also regulates origin firing. Here, we studied the impact of CHK1 on replication fork stability and replication elongation after exposure to UV. Since the unspecific kinase inhibitor caffeine, inhibiting ATM and ATR, has been shown to sensitise Polη deficient cells to UV, we hypothesised that this might be due to an inability to stabilisation the replication fork via CHK1. To test this, we looked at continuous replication fork elongation using the alkaline DNA unwinding technique. We see that caffeine and CHK1 inhibitor further decrease replication fork speed in UV irradiated Polη deficient and restored cells. We also see that both agents sensitise the cells to UV irradiation. However, by pulse labelling cells directly before UV irradiation and then separating DNA using pulsed-field gel electrophoresis, we can specifically study the induction of replication associated DNA double-strand breaks. Using this setup, we show that UV-induced replication fork collapse in Polη deficient cells is increased by the presence of caffeine, but unaffected by CHK1 inhibitor. This indicates that CHK1 is important for the continuous replication fork elongation, but not replication fork stabilisation after UV exposure.

**UV-induced replication fork collapse in DNA polymerase η deficient cells is independent of the MUS81 endonuclease (paper III)**

The MMS and UV sensitive 81 (MUS81) endonuclease has been implicated in replication fork collapse after treatment with crosslinking
agents. We hypothesised that MUS81 may also be involved in replication fork collapse after exposure to UV. To test this hypothesis, we depleted Polη deficient and restored cells of MUS81 using siRNA. We see that MUS81 depletion sensitises Polη deficient cells to UV, while restored cells are unaffected by the MUS81 status. Interestingly, MUS81 depletion did not affect the formation of replication-associated DNA double-strand breaks visualised by pulsed-field gel electrophoresis in Polη deficient cells pulse labelled directly before irradiation. We also studied the formation of γH2AX and RAD51 foci at replication forks as well as the induction of 53BP1 foci in response to UV irradiation in these cells. MUS81 depletion had no impact on the formation of these foci, clearly showing that replication fork collapse after UV exposure is independent of MUS81.

The histone methyltransferase SET8 is required for S-phase progression (paper IV)

Histone methyltransferase SET8-mediated monomethylation of histone H4-K20, as well as a few other histone post-translational modifications, targets 53BP1 to chromatin after DNA damage. However, the function of SET8 in mammalian cells was poorly understood. Here, we show that siRNA-mediated depletion of SET8 causes an accumulation of cells in S phase, and induction of DNA double-strand breaks. We see that despite the inhibition of SET8 expression, 53BP1 and other repair proteins are recruited to the site of DNA damage.

We found it interesting that DNA damage is induced in unperturbed cell cycles in cells depleted of SET8, and asked what signalling pathways are affected. We see that CHK1 is activated in SET8 depleted cells, and that CHK1 inhibition prevents the S-phase delay induced by siRNA-mediated SET8 depletion. We also see that inhibition of replication proteins or replication fork stalling, prevents the accumulation of DNA damage induced by inhibition of SET8 expression. This is explained by SET8 directly interacting with PCNA, and its requirement for replication fork progression, as visualised by the alkaline DNA unwinding technique.
Uncoupling of RAD51 focus formation and cell survival after replication fork stalling in RAD51D null CHO cells (paper V)

The homologous recombination repair protein RAD51 assembles at collapsed replication forks. Here, we studied the involvement of the RAD51 paralog RAD51D in signalling after replication fork stalling, using RAD51D null cells. We see that RAD51D deficient cells have an increased sensitivity to camptothecin, but the same sensitivity to replication stalling agents hydroxyurea and aphidicolin as restored cells. Using pulsed-field gel electrophoresis, we see no difference in the induction of replication-associated DNA double-strand breaks after hydroxyurea exposure, in RAD51D and wild type cells. Despite the unaffected cell survival after hydroxyurea exposure, RAD51 foci form in control but not in RAD51D deficient cells. Since other cell lines deficient in homologous recombination are highly sensitive against inhibition of PARP, we investigated the response to a PARP inhibitor in RAD51D deficient and restored cells. We see that PARP inhibition causes a dramatic killing of RAD51D deficient cells and no induction of RAD51 foci. In contrast, the survival of control cells is unaffected and RAD51 foci are induced. We found it intriguing that the induction of RAD51 foci is not related to cell survival in these RAD51D deficient cells.
Concluding Remarks and Future Perspectives

Faithful replication and maintenance of replication fork stability is essential in preserving genomic stability and avoiding cancer. In the present thesis we have studied different aspects of replication fork stability and mechanisms for replication restart after damage induction. We have focused on UV irradiation since this is an agent which we are exposed to on an almost-everyday basis, and have also studied other replication-interfering agents and mechanisms involved in replication stability in unperturbed replication.

The DNA fibre technique is an elegant method to study replication elongation and replication restart by analysing single molecules. It gives a lot of information about replication termination and new origin firing. However, it has a fairly low resolution, and cells cannot be labelled for very long, even a 2 hour labelling gives very long fragments in untreated cells. Thus, it has to be combined with other methods to give a better view of replication fork elongation. Here, we chose to combine it with the alkaline DNA unwinding technique and the method of alkaline sucrose gradients, which both measure whole cell populations. None of the methods is sufficient to alone describe the dynamics of replication fork progression on UV-damaged DNA, but the combination gives new views. Pulse labelling the cells to allow separation of replication-associated DNA double-strand breaks on pulsed-field gel electrophoresis provides a system to study replication fork stability. However, it should be kept in mind that replication bubbles migrate differently in the gel compared to linear DNA. Furthermore, pulsed-field gel electrophoresis is not an optimal system to study double-strand breaks after UV exposure since NER induces background levels of DSBs as an artefact of this method. However, combined with each other, these methods enable us to study cellular mechanisms and responses after induction of DNA damage.

The re-priming theory has been suggested before but it has not previously been shown to occur in mammalian cells. It would be of great interest to study in detail the mechanisms and differences on the leading and lagging strands. This might be possible using plasmids where the pyrimidine dimer can only be induced in one place. Furthermore, it remains to be proven
whether re-priming is a mechanism only for lesions that cannot be bypassed, or if it occurs also after induction of damages that can be bypassed by the present translesion synthesis machinery. The slowing of replication forks in UV irradiated control cells seen using the alkaline DNA unwinding technique may represent gaps that are later sealed, or a general reduction of replication speed on a damaged template despite functional translesion synthesis. Also, it is surprising to note that the replicative gaps induced in Polη deficient cells remain unsealed even twelve hours after UV exposure despite the induction of RAD51 foci seen at six hours after irradiation. Also, HR defective cells show no delay in replication elongation and gap closure compared to wildtype cells (Johansson et al., 2004). It remains to be fully determined whether RAD51 plays a role during a late closure of replicative gaps, or if homologous recombination is dispensable for this closure. It would be interesting to immunoprecipitate BrdU-labelled DNA that was UV irradiated before BrdU pulse labelling and sonicated after replication has been allowed to continue, to see what repair proteins are involved in the gap filling after UV exposure. It is possible that combining fluorescent in-situ hybridisation (FISH) with the fibre technique would also help in elucidating the role of recombination or template switching and other mechanisms in the post-replication repair of gaps.

The study of CHK1 signalling indicated a difference in the requirement for CHK1 in replication fork stability and replication fork elongation. As CHK1 is one of the main signal transducers in the ATR pathway, and CHK1 is required for replication fork stability after long hydroxyurea exposures, it may seem surprising that CHK1 activity was not required for stability of replication forks after UV irradiation. However, it is possible that signalling for fork stabilisation differs depending on whether the replication fork was stalled by slowing down, as in the case of nucleotide deprivation, or by a direct physical block that allows fork continuation on the 5' side of the lesion. Really, considering the re-priming theory, it is highly likely that stabilisation of a nucleotide deprivation-stalled replication fork differs from stabilisation of the replicative gap left behind a re-primed fork. It would, however, be of great interest to compare the effect of the CHK1 inhibitor to the effect of inhibitors of ATM, ATR, and other damage signalling proteins. Elucidating the role of caffeine would be very useful considering how often
this unspecific inhibitor is used, however this task is likely to be more complicated than we first believed.

The importance of the MUS81 endonuclease after different damages is interesting, not only to determine the role of this endonuclease, but also to investigate the involvement of different proteins in the physical collapse of a stalled replication fork. Considering the substrate specificity of MUS81, it is not too surprising that it is not required for replication fork collapse after physical blockage, even though the name suggests a role. MUS81 may, however, have a role in repair of collapsed replicative gaps after UV exposure, such as in the resolution of Holliday junctions.

These three projects, although being based on cells from a xeroderma pigmentosum variant patient, has been useful to gain an understanding of the general mechanisms of replication fork maintenance rather than revealing new information about the disease. However, this general understanding is vital to prevent DNA damage and to come up with new cancer treatments, which will benefit a larger group.

The project involving the histone methyl transferase has a slightly different angle of understanding the signalling at replication forks following DNA damage. Post-translational modifications (PTMs) are important not only for the regulation of transcription, but also for repair signalling following induction of DNA damage. This new and exciting field has a lot to reveal about transient modifications working as a local platform for recruitment of repair proteins or initiation of a signalling cascade.

The project regarding RAD51D concluded that formation of RAD51 foci has no obvious connection to cell survival after treatment with recombination-inducing agents. This somewhat unexpected result complicates the studies of cellular responses, and is therefore important for our understanding of fork stability.

This thesis has aimed to contribute to the general understanding of replication fork maintenance in mammalian cells. As always, answering one question enables us to ask ten more.
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