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# The Functions of Human Dna2 in Mitochondrial and Nuclear DNA Maintenance

Julien Duxin

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**The Functions of Human Dna2 in Mitochondrial and  
Nuclear DNA Maintenance**

By

Julien P. Duxin

A dissertation presented to the  
Graduate School of Arts and Sciences  
of Washington University  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

**August 2011  
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## ABSTRACT OF THE DISSERTATION

The Functions of Human Dna2 in Mitochondrial and Nuclear DNA Maintenance

By

Julien P. Duxin

Doctor of Philosophy in Biology and Biomedical Sciences

(Biochemistry)

Washington University in St. Louis, 2011

Dr. Sheila A. Stewart, Chairperson

Coordination between DNA replication, DNA repair and cell-cycle progression ensures high fidelity DNA replication thus preventing mutations and DNA rearrangements. Interestingly, in addition to nuclear DNA stability, mitochondrial DNA (mtDNA) integrity is also essential for normal development. The current challenge resides in unraveling the different mechanisms that govern nuclear and mtDNA stability and to understand how these two separated genomes have evolved. This work focuses on delineating the biological functions of human Dna2 (hDna2). Dna2 is a highly conserved helicase/nuclease that in yeast participates in DNA replication and Okazaki fragment maturation, DNA repair, and telomere maintenance. Immunofluorescence and biochemical fractionation studies demonstrated that in addition to its nuclear localization, hDna2 is also present inside the mitochondria where it colocalized with a subfraction of DNA-containing mitochondrial nucleoids in unperturbed cells. Upon the expression of disease-associated mutant forms of the mitochondrial Twinkle helicase, which induce DNA replication pausing/stalling, hDna2 accumulated within nucleoids suggesting that it

participates in mtDNA replication/repair. In accordance with these observations, RNA interference-mediated depletion of hDna2 led to a decrease in mtDNA replication intermediates and inefficient repair of damaged mtDNA. I have also investigated the nuclear function of hDna2 and demonstrate that it participates in DNA replication. RNAi-mediated depletion of hDna2 led to nuclear genomic instability that is accompanied by the activation of the replication checkpoint kinase Chk1 in late S/G2 phase. Genetic rescue experiments revealed that both hDna2's nuclease and helicase activities are essential to maintain genomic stability, and suggest that these activities are coupled on long DNA flaps that arise during Okazaki fragments maturation. Furthermore, observations that hDna2 interacts with a member of the replisome, And-1, in a replication dependent manner, suggests that hDna2 is recruited to replication sites and actively participates in DNA replication. In accordance with biochemical and genetic models that predict that Dna2's activity is only required for a small percent of flaps that escape the activities of FEN1, hDna2 depletion did not result in slower maturation of newly synthesized DNA. In contrast, FEN1-depleted cells did result in slower maturation confirming that FEN1 is the main flap endonuclease that processes Okazaki fragments into ligatable nicks. To establish whether hDna2 participates in DNA replication fork progression, we analyzed track length of replicating forks *in vivo* using micro-fluidic-assisted replication track analysis (maRTA). Surprisingly, we did not observe slowing of the replication fork upon hDna2 or FEN1 depletion suggesting that replication fork progression is insensitive to Okazaki fragment maturation. However, maRTA analysis revealed that origin firing events are reduced upon hDna2 depletion suggesting that hDna2 also participates in the firing of replication origins. In agreement with this



hypothesis, chromatin immunoprecipitation (ChIP) analysis revealed that hDna2 specifically localizes to replication origins. Altogether, the work presented here demonstrates that hDna2 is a novel addition to the growing list of proteins that participate in both nuclear and mtDNA maintenance and further suggests that mechanisms of DNA replication/repair are conserved between both organelles. Furthermore, this work increases our understanding of the molecular mechanisms that ensure high fidelity replication and provides novel avenues in our quest to understand human diseases caused by mutations in DNA replication genes.

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As a first year graduate student with no research experience, I walked into Sheila's office to discuss about possible rotation projects. An hour later, I left with a piece of paper filled with undecipherable hieroglyphs resuming what my project would entail; I clearly had no idea of what had just been discussed. Nevertheless, I felt captivated by Sheila's enthusiasm and decided to join her lab for a rotation. This was the beginning of a stimulating, sometimes intense but always honest relationship. I thank Sheila for providing me with the necessary tools to become a scientist. Our numerous arguments about "where will this experiment lead you" have forced me to plan each hypothesis and experiment in a detailed and meticulous manner; she has fostered my intellectual reasoning and ability to ask the "correct" question. I thank her for the hours she spent in perfecting my presentation skills and always encouraging me to communicate with different scientists. I am thankful for the correct balance of guidance but also independence that she provided me throughout the years and for being all around an excellent mentor.

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## **Chapter 1: Background and Significance**



## **1.1 Overview and significance**

Cellular proliferation requires numerous processes that need to be tightly coordinated to ensure the maintenance of the genome (1). Coordination of DNA replication, DNA repair and cell cycle progression ensures high fidelity DNA replication thus preventing mutations and DNA rearrangements. The importance of maintaining genomic stability is best illustrated by human diseases caused by mutations in DNA repair or replication genes; cells from these patients exhibit significant genomic instability predisposing the patients to cancer and/or premature aging (**Table 1.1**). Interestingly, in addition to genomic stability, mitochondrial DNA (mtDNA) integrity is also essential for normal development, and mutations in mitochondrial specific genes are known to cause a variety of human diseases (**Table 1.1**). The current challenge resides in unraveling the mechanisms that govern nuclear and mtDNA stability. Recent discoveries that several nuclear DNA maintenance proteins also participate in mtDNA replication and/or repair suggest that the mechanisms governing DNA maintenance in these distinct organelles have evolved in concert and have more similarities than previously appreciated.

## **1.2 Mechanisms that govern DNA replication**

DNA is particularly vulnerable to mutagenesis during S phase. Indeed the double helix must open to allow the replisome to copy the entire genetic information. During this process, the exposed single strand DNA must be carefully protected to avoid potential detrimental breaks. In addition, the replisome must overcome obstacles such as DNA adducts, single or double stranded breaks (DSBs), or secondary structures that

could lead to severe genomic instability if not resolved correctly. For this reason, cells have evolved checkpoint controls that are continuously measuring the amount of DNA instability that arises during replication, and thereby control the coordination of DNA repair with DNA replication and cell cycle progression (**Figure 1.1**) (1).

### **a. DNA replication**

DNA replication is a conserved process across all kingdoms that ensures faithful duplication of the genome prior to cell division. The challenge resides in making a single precise copy of the chromosomes once per cell cycle. In order to replicate the chromosomes, the DNA double helix unwinds to allow DNA synthesis to copy each strand. The specific locations where the DNA unwinds are known as replication origins and are recognized by a specific protein complex known as the origin-recognition complex (ORC) (59). From this point, replication occurs in a bidirectional manner where both DNA strands are copied simultaneously. Because DNA polymerases always copy DNA in the 5' to 3' directionality, one strand of the DNA known as the leading strand is copied continuously, while the opposite strand is copied discontinuously by short RNA primed DNA fragments known as Okazaki fragments. These Okazaki fragments (100-150 nucleotides long) must be processed and ligated in order to form a continuous DNA strand (**see section 1.4**).

In metazoans, firing of the DNA replication origin proceeds in 3 distinct steps. 1) The DNA replication origin is recognized by ORC; 2) the pre-replication complex (preRC) is assembled at the origin; 3) the replication origin is activated or licensed and replication starts (59). While origins are well defined in *Saccharomyces cerevisiae*

because ORC specifically recognizes a 12 bp consensus sequence, metazoans' ORCs do not exhibit any DNA sequence specificity (79) (6). This has rendered the identification of novel origins difficult, and the mechanisms that govern their placement and timing of firing in mammalian cells are still ambiguous. While a subset of human origins have been well characterized and are known to fire at most rounds of the cell cycle (constitutive origins), it is now well accepted that potential origins are in excess and only a subset fire during each synthesis phase (S-phase) (59). This suggests that replication origins are flexible and fire depending on the nature of the cell and its environmental conditions. For example, some dormant origins can be activated upon replication stress or stalling of a neighboring origin (37). Other origins termed "flexible origins" consist of several potential origin clusters, where one fires at each cell cycle. While origin selection is still unclear, several features can describe them, however they are not present in all origins. For example, at the sequence level, many origins are located in close proximity of AT-rich elements or CpG islands where the chromatin is typically associated with nucleosome free regions close to transcription start sites. Interestingly, transcription has also been shown to control not only replication origin selection, but also the timing of activation. For example, the  $\beta$ -globin origin is known to fire late during S phase in cells not expressing the  $\beta$ -globin gene, but fires in early S phase in erythroid cells expressing the gene (49). In addition, depending on the settings, active transcription either prevents the firing of potential ORIs (negative regulation) or activates them (positive regulation) (59).

In eukaryotic cells, the sequential steps that activate an origin have been well established. ORC, a heterohexamer with ATPase activity, is the first protein complex to

recognize and bind the origin at the beginning of the cell cycle in G1. After ORC binds, two additional factors are recruited, cell division cycle 6 (CDC6) and CDT1, which allow the loading of the replication helicase, minichromosome maintenance protein complex (MCM 2-7), onto replication origins (29) (76) (22). Once this complex is loaded, the origins are “licensed”, marking the end of preRC complex assembly. The MCM replication helicase is a heterohexameric complex that forms a ring around the DNA and unwinds the DNA strands in front of the replication fork (71). Therefore, at least two MCM complexes are loaded per origin to allow replication to initiate in a bidirectional manner. Following preRC licensing, the activations of CDC7-DBF4 and cyclin E-cyclin-dependent kinase 2 (CDK2) protein kinases lead to the melting of the origin and the beginning of replication. MCM-10, which is recruited by the MCM 2-7 complex, is essential for the recruitment of CDC45 and the GINs complex to ensure activation of the MCM helicase complex and replication elongation (84). Similarly, And-1 interacts with MCM10, and is essential for the recruitment of the initiator primase polymerase complex, polymerase  $\alpha$  (pol  $\alpha$ ) (88) (46). MCM10/And1 complex allows for pol  $\alpha$  to bridge with the MCM complex, and travel with the replisome while synthesis of the complementary strands occurs. Two other polymerases are then recruited for the elongation of the leading strand (pol  $\epsilon$ ) and lagging strand (pol  $\delta$ ) (70) (63) (16). As S phase progresses, Geminin, whose expression increases throughout S phase, inactivates CDT1 by blocking CDT1 from binding at replicated origins, ensuring that only one copy of the genetic information is generated per cell cycle (85) (56). While this mechanism of replication initiation and inhibition of re-replication is well established in different organisms, over-replication of short DNA regions was recently observed during S phase at the origin sites of human

cells (39). These re-replicated fragments are double-stranded 100-200 bp long DNA molecules with 5' attached RNA primers. Importantly, their synthesis is dependent on replication and they are generated upon firing of specific origins, suggesting that they correspond to abortive DNA replication products. In addition, the different origins studied in this report were all located adjacent to transcription start sites of expressed genes, and while transcription was not essential for the generation of these over-replicated fragments, it stimulated their formation. These unexpected findings raise intriguing questions about the mechanisms of origin firing that await elucidation; are these over-replicated fragments necessary for an origin to efficiently fire? Does an origin fire multiple times before the “correct” one is elongated? Are there unknown factors required for their formation and/or clearing? What is the role of transcription in this process?

#### **b. Stabilizing replication: the replication checkpoint/repair pathway**

Cell cycle checkpoints and DNA repair are intimately connected with DNA replication to ensure the accurate and timely duplication of the genome (**Figure 1.1**). While extrinsic DNA damaging agents can cause replication dependent damage (i.e., UV or reactive oxygen species (ROS)) the genome contains several intrinsic challenges that can endanger the replication process if not handled properly (9). For example, DNA repeats, such as tri-nucleotide repeats or tandem repeats as found at the telomeres, can generate secondary structures like cruciforms, triplex H-DNA, or G-quadruplexes, which can inhibit replication (61). In addition, unwinding of the DNA during replication induces positive supercoiling in front of the replication fork, which needs to be removed

by specific nucleases called topoisomerases (83). Also, collisions between the replisome and the transcriptional machinery seem inevitable and are reported to slow down the replication fork (68, 69) (60). Other regions of the genome known as replication fork barriers intentionally stall replication forks and have been described at the telomeres, centromeres, and rDNA locus (38). Although their exact purpose is unclear, they have been described as potential replication termination or recombination sites. In any case, the replication checkpoint controls replication forks encountering such difficulties and ensures its stability to complete replication.

Two main parallel signaling pathways become activated upon replication forks encountering DNA damage and ensure the stability of the replication fork (**Figure 1.1**). Stalled replication forks usually result in exposure of single stranded DNA due to the uncoupling of the helicase complex that moves in front of the replisome and activate the ATR pathway which in turn phosphorylates and activates the checkpoint kinase 1 (Chk1) (24) (55) (75). In contrast, replication forks encountering DNA DSBs typically activate the ATM pathway, which phosphorylates and activates the checkpoint kinase 2 (Chk2) (30). Although these pathways are activated by different sources of DNA damage, they are interconnected and a single DNA damage event can lead to the activation of both pathways. ATM, ATR, Chk1 and Chk2 can phosphorylate and stabilize p53, which regulates a plethora of genes involved in DNA repair, and cell cycle progression. Alternatively, Chk1 and Chk2 independently of p53, phosphorylate and inactivate the phosphatase Cdc25 that controls Cdk1 and Cdk2 activities and therefore prevents the completion of replication or entry into mitosis (62). When these pathways are not functional, aberrant DNA structures can accumulate due to misprocessing of the damage

and lead to genomic instability. For example, ATR inhibition can induce common fragile site expression (21) (17). These observations underline the essential role of the replication checkpoint in ensuring efficient chromosomal duplication.

The different repair pathways operating during S phase to promote the repair and/or bypass of different types of DNA lesions are complex and not fully elucidated. For example, damage induced by UV can cause thymine dimers or “bulky DNA lesions” that stall replication forks. If the damage occurs on the leading strand, a gap will form due to a repriming event of the leading strand downstream of the damage. The damage can then be corrected by translesion synthesis (TLS) polymerases, which directly bypass the damage often resulting in DNA mutations. Otherwise, the gap can be repaired by a recombination-mediated mechanism called template switching, which uses the novel synthesized sister chromatid as a template to fill in the gap (41). This complex mechanism occurs through a series of events involving the assembly of Rad51 presynaptic filaments that engage a homology search and ultimately lead to an error-free bypass of the lesion. Alternatively, a wide variety of cancer chemotherapies or cellular metabolites can create covalent links between the DNA strands that would completely block the progression of a replication fork. Interstrand cross links (ICL) repair involves the collaboration between the Fanconi anemia pathway with homologous recombination (HR) or TLS polymerases (58). Fanconi anemia is a rare congenital human disorder characterized by developmental defects, bone marrow failure and predisposition to cancer. Cells from these patients are hypersensitive to ICL inducing reagents and exhibit chromosomal fragility due to the formation of replication dependent DSBs (64). Although the exact function of the different Fanconi anemia proteins in repair of ICLs is

not yet fully elucidated, genetic and biochemical studies suggest that they are responsible for creating an appropriate substrate for HR or TLS. For example one of the 13 known family members, FANCM, was recently shown to localize at a stalled replication fork and dictate the recruitment of the single strand binding protein RPA, therefore promoting the activation of the replication checkpoint. In addition to checkpoint activation, FANCM, an ATPase dependent helicase, plays a role in remodeling stalled replication forks, ensuring their stability during ICL repair and therefore limiting dangerous recombination events (33) (81) (28).

Errors made during DNA replication represent a major source of DNA mutations associated with a variety of genetic disorders and/or predisposition to cancer. Elucidation of the different mechanisms that enable replication fork progression and efficient replication of the genome will be crucial for our understanding of different genetic disorders caused by mutations in DNA repair/or replication genes.

### **1.3 Mechanisms that maintain mitochondrial DNA stability**

Mitochondria are membrane-enclosed organelles found in the cytoplasm of eukaryotic cells often referred to as “power plants” because they synthesize most of the ATP used by the cell. Believed to originally be a free prokaryote organism, mitochondria were engulfed in a symbiotic manner to form the first eukaryotic cells (57). The mitochondrion contains a genome that is maintained separately from nuclear DNA, but completely relies on nuclear-encoded genes for its maintenance and transcription (42). Mitochondrial DNA replication/repair went unexplored for several decades until the recent discoveries of multiple human syndromes caused by mutations in mtDNA



replication genes (**Table 1.1**). In addition, recent research has unraveled the presence of several DNA replication and repair proteins in the mitochondria that were once believed to be exclusively nuclear (**Table 1.2**). These discoveries suggest that the mechanisms of mt DNA and genomic DNA maintenance have evolved together and might have more similarities than previously anticipated, presenting the mitochondrion as an useful tool for the study of genomic replication and repair.

### **a. Mitochondrial DNA**

Mitochondrial DNA in human cells consists of a 16.6 kb double stranded circular genome that encodes for only 37 genes and 13 proteins all contributing to energy production (**Figure 1.2**) (42). The remaining 24 genes encode transfer RNAs (22 genes) and ribosomal RNAs (2 genes) required for mitochondrial protein synthesis. The rest of the original mitochondrial genes have translocated to the nucleus during the course of evolution. mtDNA is arranged into nucleoprotein complexes also known as nucleoids that contain multiple copies of mtDNA and mtDNA maintenance proteins (34) (45) (77). The Twinkle helicase is required for mtDNA replication and was the first identified mitochondrial nucleoid protein (78). Polymerase  $\gamma$  (pol  $\gamma$ ) also found in nucleoid structures, is the only mtDNA polymerase and is responsible for replicating/repairing mtDNA. Other nucleoid-associated proteins are the mitochondrial single strand binding protein (mtSSB), and the transcription factor A (Tfam) (7). In addition to these mitochondrial specific enzymes, proteins that participate in nuclear DNA maintenance have recently been co-localized with nucleoid structures and shown to participate in the replication and repair of mtDNA. These include several DNA glycosylases,

apurinic/aprimidinic endonuclease (APE1), DNA ligase III, PIF1, BRCA1, and RNase H1 among others (27) (54) (42) (52) (31) (53) (19) (**Table 1.2**).

The importance of maintaining mtDNA stability is best illustrated by different mitochondrial diseases caused by genetic defects in mtDNA, or mutations in nuclear genes that encode mtDNA maintenance proteins. For example, mutations in POLG, the gene that encodes the catalytic subunit of pol  $\gamma$ , are associated with a variety of disorders including progressive external ophthalmoplegia (50) and Alpers' syndrome (25). Alpers' syndrome is a rare severe autosomal recessive disease that affects young children who very early experience progressive cerebral degeneration, blindness, deafness, liver failure and death, underlining the importance of maintaining mtDNA (25). Similarly, mtDNA mutations have been linked to several common pathological states, including premature aging, cancer, diabetes, and neurodegenerative disorders such as Parkinson's and Alzheimer's disease. The exact contribution of mtDNA stability to these different human diseases is an active area of investigation.

#### **b. Mitochondrial DNA replication**

Although mtDNA consists of a simple circular plasmid, it has received far less attention than its neighbor in the nucleus, explaining the many uncertainties concerning the mechanisms of its replication and repair (42). While original electron microscopy studies in the early 1970s first suggested the strand displacement model (SDM) to explain how mtDNA is replicated, new findings in this last decade indicate that the story is far more complex than originally thought.

Original electron microscopy images revealed that mtDNA replication involved an unusual mechanism, where replicating molecules had a single stranded branch, implying that leading and lagging strand synthesis were uncoupled (72). Leading strand synthesis starts at a specific region ( $O_H$ ) and advances through approximately two-thirds of the genome before a second strand synthesis is initiated in the opposite direction ( $O_L$ ) (**Figure 1.2**). Nearly three decades later, using two-dimensional agarose gel electrophoresis (2D-AGE), Holt *et al.* demonstrated that replication intermediates behaved in every aspect like products of coupled leading and lagging strand DNA replication as seen in the nuclear genome, and argued that both SDM and conventional coupled DNA synthesis coexisted in the mitochondrion (44). In addition, the recent discoveries that PIF1, flap endonuclease 1 (FEN1), DNA ligase III, and RNase H1 localize to the mitochondrion indicates that it possesses the necessary apparatus for efficient Okazaki fragment processing (54) (20) (31) (52) (53) (**see section 1.4**). Recently, the appearance of RNA/DNA hybrids incorporated throughout the lagging strand (RITOLS) added more complexity to the replication model of mtDNA (86). Interestingly, these observations were reconciled with the SDM model as RNA takes the place of the mitochondrial single strand DNA binding protein which was assumed to coat and protect the single stranded DNA formed in SDM (**Figure 1.2**). Currently, the consensus is that both strand-coupled DNA replication and RITOLS/SDM mechanisms co-exist to replicate mtDNA (42).

Interestingly, mtDNA molecules exhibit a short triple-stranded region called the D-loop, which spans a major part of the non-coding region (NCR) of mtDNA and colocalizes with the  $O_H$  origin. In this region, an arrested nascent-H-strand (7S DNA)

hybridizes to the mitochondrial light strand, displacing the parental H-strand thus creating a displacement loop (D-loop) (**Figure 1.3**). The location of the light strand promoter (LSP) and transcription start sites right at the 5' end of the D-loop suggests a role for this promoter in initiation of H-strand synthesis. Precise mapping of RNA and DNA species in the D-loop region provided evidence that RNA derived from the transcription of LSP serves as a replication primer for H-strand DNA synthesis (35). Interestingly, as observed for nuclear DNA origin regions, 7S DNA is found in over-abundance with respect to the mitochondrial genome, which suggests that it is the product of aborted replication intermediates (43).

### **c. Mitochondrial DNA repair**

While it was originally questioned whether mitochondria had the capacity to repair DNA, it has become clear that many DNA repair proteins are shared between the nucleus and the mitochondrion. This dual localization is achieved via differential splicing (i.e., PIF1 or OGG1 glycosylase), alternative translation initiation site (i.e., DNA ligase III or RNaseH1) or post-translational modification (i.e., APE1 endonuclease) (27). It is now well established that mitochondria are proficient in short-patch base excision repair (SP-BER), long-patch base excision repair (LP-BER), mismatch repair, and recent reports suggest that homologous recombination and non-homologous end-joining also participate in the repair of DSBs in the mitochondrion (40). **Table 1.2** summarizes the different proteins found in both compartments with their respective functions.

#### **1.4 Dna2: a nuclease/helicase that ensures genomic stability**

Dna2 is an essential, highly conserved nuclease-helicase. Originally discovered in *Saccharomyces cerevisiae*, Dna2 orthologs are found in all eukaryotes from yeasts to humans. Based on different genetic and biochemical studies, Dna2 has been proposed to function in lagging strand synthesis during DNA replication. Recent studies also implicate Dna2 in DNA repair and telomere maintenance. Both its nuclease and ATPase-dependent helicase activities have been extensively studied *in vitro*. While originally reported as a 3' to 5' helicase (13), Dna2 was demonstrated to translocate in the 5' to 3' direction (5). In addition, it possesses a strong endonuclease activity that is highly specific to single stranded DNA (4).

##### **a. Dna2 and Okazaki fragment processing: the two-step model**

Originally discovered in a genetic screen for genes involved in DNA replication, Dna2's function in lagging strand synthesis was ascribed shortly thereafter based on its genetic and biochemical interactions with several known Okazaki fragments processing proteins (51) (10). Multicopy expression of *rad27*, the gene encoding for FEN1, the flap endonuclease responsible for processing DNA flaps structures that arise during Okazaki fragment maturation, suppressed the growth defects of *Dna2-1*, a temperature sensitive allele of Dna2 (11). Similarly, overexpression of Dna2 suppressed the temperature sensitive growth defects of *rad27Δ* strains, suggesting that Dna2 compensates for the function of FEN1 in DNA replication (11). These observations were soon supported by *in vitro* experiments that demonstrated that Dna2 could process RNA-DNA flaps more efficiently than DNA-only flaps, making it suitable for processing flaps generated during

Okazaki fragment processing (5). However, the observation that Dna2 would degrade a flap efficiently but not entirely, leaving behind a smaller flap (around 4 nucleotides), suggested that it functions in concert with FEN1 to produce a ligatable nick (3) (2). Interestingly, *dna2-1* is lethal in combination with a mutation in pol  $\delta$  (*pol3-01*) that stimulates strand displacement synthesis. Similarly, deletion of the Pol32 subunit, which limits the strand displacement activity of pol  $\delta$  *in vitro*, suppresses growth defects of *dna2-1* (36) (32) (47) (15). These observations suggested that Dna2 becomes critical under conditions that promote the formations of long flaps. Indeed, elegant *in vitro* studies demonstrated that the coordinated actions of Dna2 and FEN1 on long flaps protected by RPA could produce a ligatable nick (3). This hypothesis is now known as the two-step model, which proposes that under certain unknown conditions, strand displacement activity of pol  $\delta$  forms long DNA flaps that escape the cleavage by FEN1. If these flaps become longer than 27 nucleotides, they are coated by RPA (**Figure 1.4**). RPA inhibits FEN1 endonuclease activity but stimulates Dna2 to track from the 5' end of the flap and cleave in the DNA region leaving behind a shorter RPA free flap of 4-5 nucleotides. FEN1 then processes this shorter flap to produce a nick ligated by DNA ligase I (LigI). While this is an attractive model because it allows for removal of the totality of the DNA synthesized by pol  $\alpha$ , a low fidelity enzyme, it is thought to happen in a minority of the Okazaki fragments, making the one-step model with FEN1 processing the short flap, the most common mechanism of flap removal (**Figure 1.3**) (3) (2) (48). However the fact that Dna2 is essential *in vivo*, whereas FEN1 is dispensable under certain growth conditions, suggests that Dna2 is the only enzyme able to process long flaps while the processing of short flaps can occur through multiple redundant

pathways. Further work done in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* demonstrated a genetic interaction between PIF1 and Dna2 (74) (14). PIF1 is a 5' to 3' helicase involved in telomere maintenance and mtDNA replication. The observation that the selective inactivation of the nuclear PIF1 isoform suppressed the lethality of *dna2Δ* suggests that PIF1 could play a role in Okazaki fragment maturation by promoting long flaps formation (14) (80). Recently, it was shown *in vitro* that PIF1 helicase directly stimulates the strand displacement synthesis of pol  $\delta$ , demonstrating that the DNA flaps can become long in the presence of PIF1 (73) (67).

#### **b. Dna2 and double strand break repair**

Flap structures are not exclusive to Okazaki fragment maturation and can arise from several DNA repair processes, suggesting that Dna2 could play an active role in DNA repair. Involvement of Dna2 in double strand break (DSB) repair was first proposed by Budd and Campbell who demonstrated that Dna2 mutant strains are hyper-sensitive to X-ray damage (12). Later, Dna2 was shown to localize to sites of damage after treatment with bleomycin, a DSB inducing agent (23). DSBs are particularly deleterious to the cell because they can compromise genetic integrity if incorrectly repaired. Cells have evolved different pathways to repair DSBs depending on the nature of the DSB and the cell cycle phase in which the damage has occurred. HR is considered a high fidelity repair mechanism because it uses a homologous sequence as template DNA to repair the break. The initial 5' end nucleolytic degradation is an essential event for a break to be repaired by HR. The 3' overhang that results from this resection will ultimately invade a homologous template initiating the recombination process. Dna2 was recently

demonstrated to play an active role in resecting the 5' end. Using genetic *in vivo* models, Zhou *et al.* were the first to demonstrate that the MRX (Mre11/Rad50/Xrs2) complex in conjunction with Sae2, are essential components that initiate 5' end resection (87). This initial resection is then further processed by the RecQ helicase Sgs1 in collaboration with Dna2. However, while Dna2 is essential for viability in yeast, its role in 5' end resection can be compensated by another nuclease, Exo1 (**Figure 1.4**). Indeed, in order to detect significant reduction in 5' end resection following DSB induction, Zhou *et al.* needed to delete both Exo1 and Dna2 or Exo1 and Sgs1 (87). These original observations were soon confirmed by *in vitro* studies using purified yeast or human proteins (18) (66) (65). Interestingly, Dna2's helicase activity is dispensable for 5' end resection while this activity is essential for viability in yeast. These observations suggest that the essential function of Dna2 does not come from its resection activity that can be compensated by Exo1, but rather from the loss of its function in removing long flaps during Okazaki fragment maturation.

### **c. Dna2 and telomeres**

Telomeres are specialized chromatin structures that form a protective cap at the ends of eukaryotic chromosomes. This protective structure is achieved through a coordinated action of telomeric DNA and telomere-associated proteins. In mammalian cells, the DNA component of the telomere consists of TTAGGG tandem repeats of double stranded DNA ending in a 3' single stranded overhang of the G-rich strand. The 3' overhang is postulated to loop back and invade the double stranded region forming a displacement loop referred as the telomeric loop (T-loop) (26). Through this mechanism,

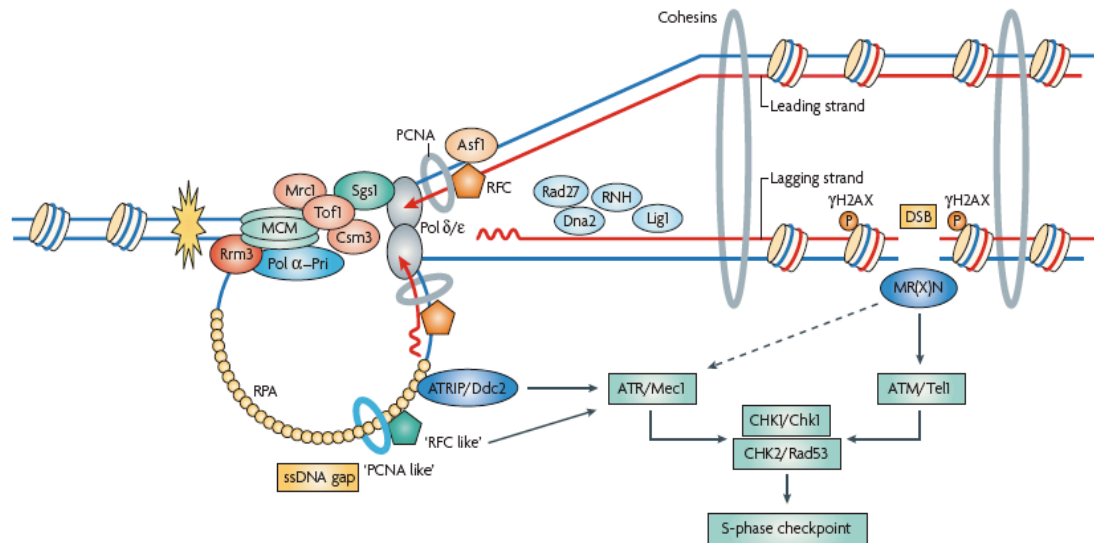


the 3' tail is hidden from DNA repair enzymes, suggesting that the T-loop plays a protective function (**Figure 1.4**). Interesting studies in *Saccharomyces cerevisiae* indicate that in addition to its role in Okazaki fragment processing and DNA repair, Dna2 also localizes to the telomeres. Notably, in this report, Dna2 was shown to localize to telomeres in G1 and late S/G2 phases of the cell cycle while relocating to internal genomic sites in S phase to participate in DNA replication (23). These observations suggest that Dna2 carries two separate functions at the telomere; the first in G1, where it may function in protecting the end of the chromosome, and a second in late S early G2 phase where it could participate in telomere replication and telomerase dependent telomere elongation. In addition, early work done in *Schizosaccharomyces pombe* implicates Dna2 in the formation of the 3' overhang and the maintenance of telomere length (82). Only recently was the detailed mechanism of the 5' end telomeric resection elucidated; the proteins responsible for generating the 3' overhang at a DSB are equally responsible for generating the 3' telomeric overhang. MRX in conjunction with Sae2 initiate the resection, while either Exo1 or Sgs1 acting in conjunction with Dna2, further process the 5' end (8). As observed upon DSB formation, a lack of Sgs1, Exo1 or Dna2 alone does not affect the resection process, indicating that both nucleolytic pathways can compensate for each other.

## **1.5 Summary**

Elucidating the mechanisms that ensure efficient replication of the genome is essential for our understanding of the causes of the genomic instability observed in a variety of human diseases including cancer. This work focuses on characterizing the

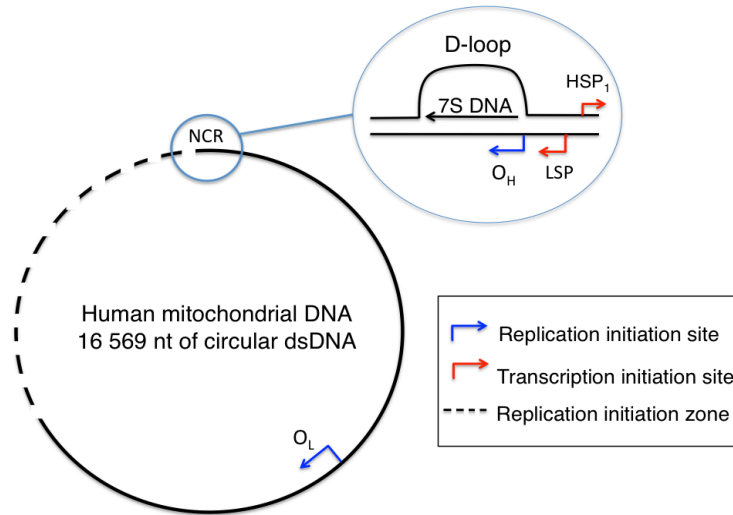
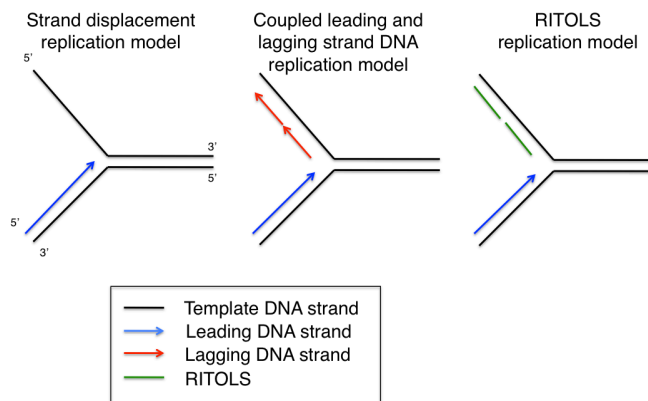
functions of the well-conserved human replication helicase/nuclease Dna2 (hDna2). In chapter 2, I demonstrate that hDna2 is a genuine nuclear and mtDNA maintenance protein, while chapter 3 focuses on elucidating its function in DNA replication. My last chapter highlights the significance of this work and the intriguing questions that it raises.



### Figure 1.1 Coordinating DNA replication

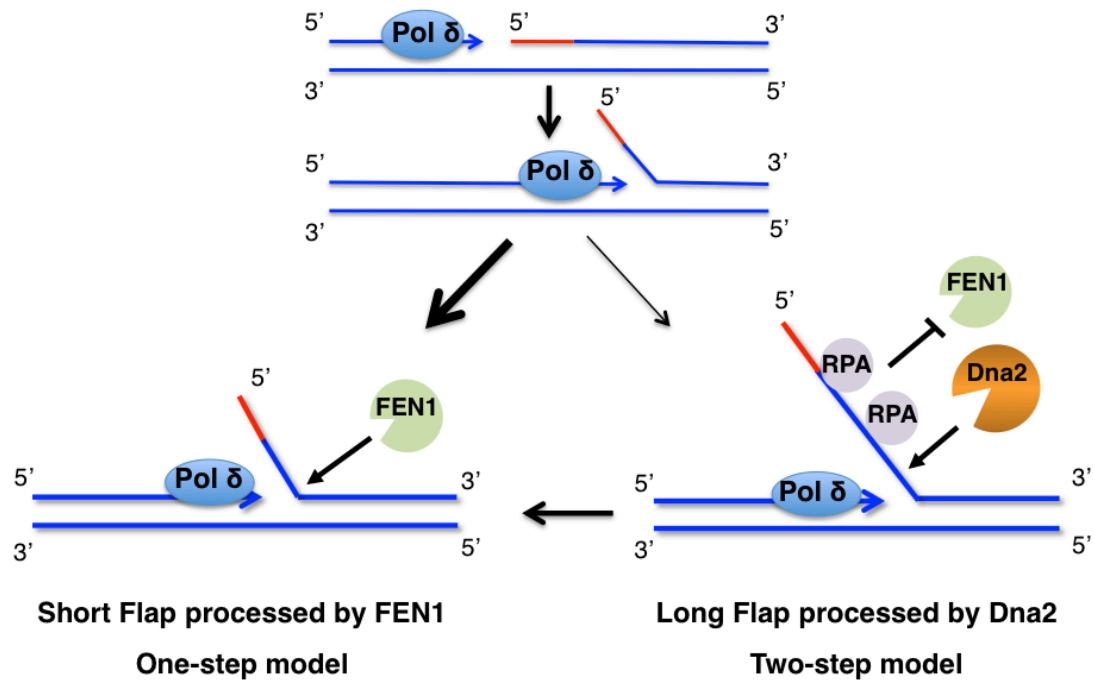
Synchronization of DNA replication, DNA repair, and cell cycle control ensures genomic stability. On the left, a replication fork stalling leads to the appearance of single strand DNA which activates the ATR replication checkpoint pathway and blocks cells in S phase until the damage is repaired or bypassed. Alternatively, double stranded breaks classically activate the ATM pathway essential to repair the break (1).

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**A****B**

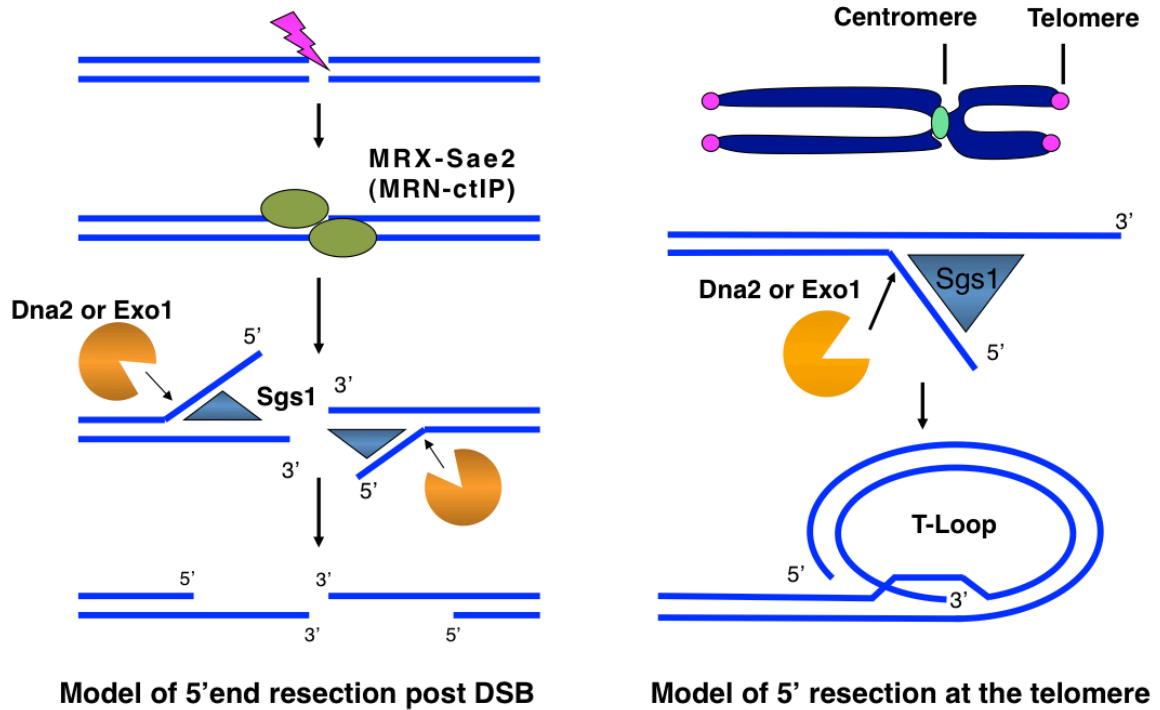
### Figure 1.2 Mitochondrial DNA replication

**A.** Schematic of the mitochondrial double stranded circular genome. DNA replication was at first thought to only initiate in a unidirectional manner at the  $O_H$  site in the major non-coding region (NCR) while initiation of the complementary strand occurs at the  $O_L$  site once replication has copied two-third of the genome (strand displacement model). Recently, bidirectional replication from dispersed sites has been mapped across a broad region of the genome called Ori-z and represented by a dashed line. **B.** Schematic representing the three models of mitochondrial DNA replication. On the left is depicted the strand displacement models (SDM) where the single strand DNA is protected by mtSSB protein while on the right, RNAs incorporated throughout the lagging strand (RITOLS) protect the ssDNA. In the middle is represented the conventional coupled DNA replication with leading and lagging strand synthesis. This figure was adapted from (42).



**Figure 1.3 Dna2 and Okazaki fragment processing: the two-step model**

Strand displacement activity of pol  $\delta$  on the previous Okazaki fragment creates short flaps that are efficiently processed by FEN1 to generate a ligatable nick by DNA ligase I (one-step model). However under certain conditions, flaps escape the cleavage by FEN1 and when becoming longer than 27 nt, they are coated by RPA which inhibits FEN1 endonuclease activity. Dna2 uses both its helicase and nuclease activities to process a long DNA flap and cleaves in the single strand DNA region 4-5 nucleotides away from the base. FEN1 then processes this shorter flap to produce a nick ligated by DNA ligase I (two-step model). Blue lines represent DNA while red lines represent the RNA primer layed down by pol  $\alpha$ . This figure was adapted from (3) (2).



**Figure 1.4 Dna2 and 5' end DNA resection**

On the left is represented a model of 5' end resection post double strand break (DSB) formation. The MRX complex in conjunction with Sae2 initiate the resection at the 5' end. This initial resection is then further processed by Exo1, or by the collaboration of the RecQ helicase Sgs1 with Dna2. On the right is depicted the mechanism of 3' overhang formation at the telomere. Telomeres are DNA protein structures that cap or protect the end of the linear chromosomes. The T-loop is a predicted structure postulated to protect the end of the telomere from potential DNA repair enzymes. The formation of a 3' overhang is an essential step for the generation of the T-loop. Recently it was demonstrated that the same proteins involved in the 5' end resection post DSB, are equally responsible for the formation of the 3' telomeric overhang. For both processes, it was demonstrated that Dna2's nuclease activity is essential while its helicase is dispensable. This figure was adapted from research conducted in different studies (89) (8) (66) (18) (65).

<b>Mamalian gene</b>	<b>Function</b>	<b>Human disease</b>
<b><i>Checkpoint</i></b>		
ATM	transducer Kinase	Ataxia telangiectasia; cancer predisposition
ATR	transducer Kinase	Seckel syndrome
CHK2	checkpoint kinase	Li-Fraumeni Syndrome variant; cancer predisposition
<b><i>Replication</i></b>		
MCM4	replicative helicase subunit	Cancer predisposition
RPA	replication factor A	Cancer predisposition
BLM	RecQ helicase	Bloom syndrome; Cancer predistosition
WRN	RecQ helicase	Werner syndrome; Cancer predisposition
Twinkle	mitochondrial DNA helicase	Progressive External Ophtamoplegia
Pol Gamma	mitochondrial DNA polymerase	Alper syndrome
<b><i>DNA Repair</i></b>		
MRE11	HR + NHEJ	Ataxia telangiectasia like syndrome
NBS1	HR + NHEJ	Nijmegen breakage syndrome; cancer predisposition
BRCA1	checkpoint mediator	Familial breast and ovarian cancer
BRCA2	crosslinkage repair + HR	Familial breast cancer
Lig4	NHEJ	Lig4 syndrome
FANC	crosslinkage repair	Fanconi Anemia; Cancer predisposition

**Table 1.1 DNA maintenance genes mutated in human disease**

<b>Mamalian protein</b>	<b>Enzymatic activity</b>	<b>Function</b>
PIF1	helicase	Okazaki fragment maturation
FEN1	flap endonuclease	Okazaki fragment maturation/BER
RNAseH1	ribonuclease	Okazaki fragment maturation/BER
DNA ligase III	DNA ligase	BER/DNA replication?
APE1	endonuclease	BER
BRCA1	?	HR/checkpoint mediator
OGG1	DNA glycosylase	BER
MYH	DNA glycosylase	BER
UNG1/2	Uracil DNA glycosylase	BER
RECQL4	RecQ helicase	DNA replication initiation/elongation?
hTert	reverse transcriptase	Telomere maintenance/ROS response

**Table 1.2 DNA maintenance proteins found in the nucleus and mitochondria**



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## **Chapter 2: Human Dna2 is a Nuclear and Mitochondrial DNA Maintenance Protein**

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## **Abstract**

Dna2 is a highly conserved helicase/nuclease that in yeast participates in Okazaki fragment processing, DNA repair, and telomere maintenance. Here, we investigated the biological function of human Dna2 (hDna2). Immunofluorescence and biochemical fractionation studies demonstrated that hDna2 was present in both the nucleus and cytoplasm. Analysis of cytoplasmic hDna2 revealed that it co-localized with a subfraction of DNA-containing mitochondrial nucleoids in unperturbed cells. Upon expression of disease-associated mutants of the mitochondrial Twinkle helicase, which induce DNA replication pausing/stalling, hDna2 accumulated within nucleoids. RNAi mediated depletion of hDna2 led to a modest decrease in mitochondrial DNA replication intermediates and inefficient repair of damaged mitochondrial DNA (mtDNA). Importantly, hDna2 depletion also resulted in the appearance of aneuploid cells and the formation of internuclei chromatin bridges, indicating that nuclear hDna2 plays a role in genomic DNA stability. Together, our data indicate that hDna2 is similar to its yeast counterpart and is a new addition to the growing list of proteins that participate in both nuclear and mitochondrial DNA maintenance.

## **Introduction**

DNA damage arises from errors in the replication process as well as a myriad of intrinsic and extrinsic DNA damaging agents that continually assault cells. Failure to efficiently repair DNA lesions leads to accumulation of mutations that contribute to

numerous pathologies including carcinogenesis. In addition to the genomic DNA, mitochondrial DNA (mtDNA) is subject to damage that requires repair to maintain integrity. For these reasons, it is not surprising that DNA replication and repair proteins display significant plasticity that allow participation in several, divergent replication and repair processes. In addition, numerous mechanisms including alternative splicing, posttranslational modifications or utilization of alternative translation initiation start sites allow DNA replication and repair proteins such as Pif1, DNA ligase III, and APE1 to localize to the nucleus and the mitochondrion and participate in DNA replication and/or repair (9, 16, 24), thus ensuring genomic and mitochondrial DNA integrity.

Dna2 is an evolutionarily conserved helicase/nuclease enzyme. Originally discovered in *Saccharomyces cerevisiae*, Dna2 orthologs are found throughout the animal kingdom including humans (5, 21, 27). Early studies demonstrated that Dna2 functions in concert with Flap endonuclease 1 (FEN1) to remove long DNA flaps that form upon lagging strand DNA replication (6). However, in contrast to FEN1, Dna2 is an essential gene in yeast, suggesting that other proteins including FEN1 cannot compensate for its loss in DNA replication or that it possesses functions beyond its role in Okazaki fragment processing. In agreement with this, genetic and biochemical studies have implicated Dna2 in DNA double strand break (DSB) repair, telomere regulation, and mitochondrial function (2, 8, 10, 14, 25, 36, 42, 43).

Analysis of Dna2 in yeast revealed that it undergoes dynamic cell cycle localization. During G1, Dna2 localizes to telomeres, relocalizes throughout the genome in S phase and moves back to the telomere during late S/G2 where it participates in telomere replication and telomerase dependent telomere elongation (10). Dna2 also

leaves the telomere following treatment with bleomycin and localizes to sites of DNA double strand breaks (10). In addition, *dna2* mutants are sensitive to DNA damage induced by  $\gamma$ -radiation and methanesulfonic acid methyl ester (MMS) (7, 14). These phenotypes may be explained by recent work demonstrating that Dna2 plays an important role in 5' end resection following DSBs. Indeed, upon induction of DSBs and initiation of 5' end resection by the Mre11-Rad50-Xrs2 (MRX) complex, Dna2 and Sgs1 cooperate to further degrade the 5' end, creating long 3' strands essential for homologous recombination (25, 43). Finally, while *dna2 $\Delta$*  mutants are lethal in budding yeast, *dna2 $\Delta$  pif1-m2* (nuclear PIF1) double mutants rescue *dna2 $\Delta$*  lethality but display a petite phenotype, suggesting that Dna2 is also involved in mtDNA maintenance (8).

Recently the human ortholog of Dna2 was cloned and characterized (22, 28). Biochemical analysis revealed that similar to its yeast counterpart, the human Dna2 (hDna2) protein possesses nuclease, ATPase, and limited helicase activity (22, 28), suggesting that it carries out analogous functions in yeast and mammalian cells. However, hDna2's putative role in genomic DNA repair and replication was called into question by a recent study suggesting that hDna2 is absent from the nucleus and found exclusively within the mitochondria where it participates in mtDNA repair (42). Further *in vitro* biochemical studies suggested that hDna2 also participates in mtDNA replication (42). Here, we confirm that hDna2 localizes to the mitochondria and demonstrate that hDna2 participates in mtDNA replication and repair. However, our studies go further by uncovering a nuclear form of hDna2 that plays an important role in genomic stability. Indeed, we demonstrate that depletion of hDna2 leads to the appearance of aneuploid

cells and the formation of internuclei chromatin bridges, indicating that hDna2 like its yeast counterpart, is essential for nuclear DNA stability.

## **Materials and Methods**

### **Cell Culture**

Unless otherwise indicated, cells were incubated at 37 °C in 5% CO<sub>2</sub>. HeLa and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep). Primary human BJ fibroblasts were cultured in DMEM and M199 (4:1, Sigma, St. Louis, MO) containing 15% FBS and 1% pen/strep. Stable cell lines expressing wild type and various inducible Twinkle mutants were created as described (38) using the Flp-In<sup>TM</sup> T-Rex<sup>TM</sup> 293 host cell line (Invitrogen, Carlsbad, CA). The resulting cells were grown in DMEM medium (Sigma, St. Louis, MO) supplemented with 10% FBS (Sigma, St. Louis, MO), 2 mM L-Glutamine, 150 µg/ml hygromycin and 15 µg/ml blasticidin in a 37 °C incubator at 8.5% CO<sub>2</sub>. U2OS and 143B osteosarcoma cell lines were similarly maintained but in the absence of selection antibiotics.

### **Virus Production and Infection**

Viral production and infections were carried out as described (31). Briefly, 293T cells were transfected using TransIT-LT1 (Mirus, Madison, WI). Virus was collected 48 hr post transfection, and infections were carried out in the presence of 10 µg/ml of protamine sulfate. 48 hr post infection, target cells were selected with 2 µg/ml of puromycin. The pLKO.1 shDna2, pResQ shDna2', pLKO.1 shSCR lentiviruses were

produced by cotransfection with pCMV $\Delta$ R8.2 and pCMV-VSV-G (8:1 ratio). The sequence used for the hairpins was 5'-CATAGCCAGTAGTATTCGATG-3' for shDna2, 5'-GCAGTATCTCCTCTAGCTAGT-3' for shDna2', and for shSCR as previously reported (31). For fractionation experiments, HEK293 and HeLa 1.2.11 cells were transfected on 10 cm plates with a mixture of three Stealth<sup>TM</sup> siRNA duplex oligonucleotides (DNA2HSS 141856, 141857, 141858, Invitrogen) against hDna2, at a concentration of 800 pmol each, using Lipofectamine<sup>TM</sup>2000 according the manufacturer's protocol. As a negative control we used a Stealth<sup>TM</sup> Universal negative control (12935-200, Invitrogen). Cells were isolated and processed for sucellular fractionation 48 hours following transfection.

### **Immunoprecipitation and Western Blot Analysis**

Cells were washed in phosphate buffered saline (PBS), lysed in Buffer H-500 (50 mM Hepes pH 7.4, 500 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.5% nonidet P-40, aprotinin, leupeptin, pepstatin and PMSF), and sonicated (three cycles of a 30 s pulse and 30 s cooling interval) (22). Cell extracts were dialyzed overnight with H-100 (50 mM Hepes pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.5% nonidet P-40) and 1 mg of lysate was immunoprecipitated. Cellular extracts were precleared for 2 hr with 20  $\mu$ l of protein A beads (Amersham, City, State) and incubated with 5  $\mu$ g of hDna2 antibody (42439, ABCAM, Cambridge, MA) or IgG (Sigma, St Louis, MO), overnight. Bound protein was eluted and analyzed by 8% SDS-PAGE and western blot analysis carried using the hDna2 antibody (42439, ABCAM, Cambridge, MA). All protein concentrations were measured using the Bradford assay (3).

### **Mitochondrial and subcellular fractionations**

Crude mitochondrial fractions of all cell lines were obtained and validated as previously described (11). In brief, cells were homogenized using hypotonic swelling and dounce homogenization. The homogenized sample was centrifuged at 800xg at 4°C for 5 min to pellet the nuclear fraction. The centrifugation was repeated and the resulting supernatant centrifuged at 12000xg at 4°C for 10 min to obtain a mitochondrial pellet. The mitochondrial pellet was again centrifuged with fresh homogenization buffer and lysed either directly in sample buffer or with the addition of 50 mM TrisHCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X100 in the presence of protease inhibitors (Roche). The crude nuclear pellet obtained from the initial cell lysis was further purified using an Optiprep gradient and processed as previously described (11). All protein concentrations were measured using the Bradford assay (3).

### **Proteinase K protection assay**

We used Proteinase K protection assays to more precisely determine the localization of hDna2 within mitochondria. For this purpose, HEK293 mitochondria were isolated as described above. Mitoplasts were made by permeabilizing the outer mitochondrial membrane (OM) with digitonin (50% HPLC pure) solution using a fixed final ratio ( $\mu\text{g}$  digitonin:  $\mu\text{g}$  mitochondria = 0.4). Digitonin was made fresh prior to each experiment by dissolving 50% HPLC pure digitonin (Sigma, St. Louis, MO), in PBS (with protease inhibitors). The degree of permeabilization and more particularly lack of inner-membrane permeabilization during mitoplast isolation was verified by immunoblotting



the final submitochondrial fractions with antibodies against known endogenous proteins including CoxII and TFAM. Mitochondria and mitoplasts were washed once with protease-inhibitor free PBS and treated with proteinase K (100 µg/ml) in the absence or presence of 0.5% Triton X100, for 15 min at 4°C. The reaction was terminated by the addition of 10 mM (final) PMSF (in ethanol), an equal volume of 2X sample buffer was added and the sample immediately placed at 95°C for heat denaturation prior to Western blot analysis. Western blot analyses of cell and mitochondrial subfractions and proteinase K protection assays used antibodies against hDna2 (42439, ABCAM, Cambridge, MA),  $\gamma$ -actin (NB 600 533, Novus Biologicals, Littleton, CO), nucleophosmin (32-5200, Invitrogen) and TFAM (kind gift of Dr. Rudolf Wiesner).

### **Immunofluorescence microscopy**

HeLa, HEK293, U2OS, 143B and BJ cells were grown for 1-2 days on coverslips and Flp-In<sup>TM</sup> T-Rex<sup>TM</sup> 293 were grown on poly-L-lysine coated coverslips. Cells were then washed in PBS and fixed in 3.7% paraformaldehyde, permeabilized in 0.5% Triton X 100, and treated with blocking buffer (10% FBS, 2% goat serum and 0.2% Tween 20) at room temperature. Antibodies were diluted in blocking buffer and incubated with cells for 1 hr at room temperature. Cells were washed in PBS containing 0.02% Tween 20 and mounted with 4', 6-diamidino-2-phenylindole (DAPI). For transient transfection of Twinkle expression constructs TransIT-LT1 (Mirus, Madison, WI) was used. Induction of expression of Twinkle variants using Flp-In<sup>TM</sup> T-Rex<sup>TM</sup> 293 cells was done 2 days prior to immunofluorescence detection with the indicated amounts of doxycycline. Immunofluorescence detection was carried out with a polyclonal hDna2 antibody (42439,

ABCCAM, Cambridge, MA) or monoclonal Alexa 488 conjugated BrdU antibody (A21303, Invitrogen, Carlsbad, CA) as primary antibodies. Secondary antibodies were anti-rabbit IgG-Alexa-Fluor<sup>®</sup> 488 or 546 (Invitrogen, Carlsbad, CA). Immunofluorescent detection of nucleoids was otherwise done as described previously (11) using a monoclonal IgM anti-DNA antibody AC-30-10 (PROGEN, Shingle Springs, CA, USA), a monoclonal c-myc IgG antibody (Roche, Indianapolis, IN) or a polyclonal hDna2 antibody (42439, ABCAM, Cambridge, MA) as primary antibodies. Secondary antibodies were anti-mouse IgG-Alexa-Fluor<sup>®</sup>488 or 568 (Invitrogen, Carlsbad, CA, myc), anti-mouse IgM-Alexa Fluor<sup>®</sup> 488 or 568 (DNA) and anti-rabbit IgG -Alexa Fluor<sup>®</sup> 488 or 568 (hDna2). The images were acquired using a LSM510 Confocal microscope (Carl Zeiss, Thornwood, NY). Images were processed using Photoshop 7.0 (Adobe, San Jose, CA). Co-localization images were generated using the calculator function in PhotoshopCS2 on the RGB merged color image. Similar images were also obtained using a co-localization plugin in ImageJ (not shown). Specificity of the hDna2 antibody in BJ cells was confirmed by co-incubating hDna2 antibody with a specific blocking peptide (42548 ABCAM, Cambridge, MA) at 1:500 dilution.

### **Two Dimensional agarose gel electrophoresis**

Mitochondrial nucleic acids were extracted as previously described (40) from shRNA infected HEK293 cells. Purified mtDNA was digested with *HincII* and separated by 2-dimensional-neutral/neutral-agarose electrophoresis (2DNAGE) as previously described (4, 15, 38). Equal loading was verified by ethidium bromide staining. Gels were transferred to a nucleic acid membrane and hybridized with a <sup>32</sup>P-labeled probe for

human mtDNA (nucleotides 14983-15593). DNA replication intermediates were quantitated by scanning the 2DNAGE gels and normalizing them to the 1n spot of a shorter exposure using the measuring function in ImageJ. Briefly, boxes were drawn around RITOLS, X spikes, y arcs, bubble arcs (long exposure) and 1n spots (shorter exposure). Each value was first normalized to the 1n spot, added, and then normalized to the shSCR samples. An overall 20% reduction in mitochondrial replication intermediates was observed upon hDna2 depletion averaging 2 different independent experiments.

### **Mitochondrial DNA damage assay**

shRNA infected HeLa cells were seeded at  $10^6$  cells per 10 mm culture plate and incubated overnight at 37 °C. The next morning, cells were washed once with PBS and treated with 2 mM H<sub>2</sub>O<sub>2</sub> in medium at 37°C for 30 min. Cells were either immediately harvested, or allowed to recover in DMEM plus 10% FBS medium for 1, 2, or 24 hr. Total cellular DNA was extracted using a genomic DNA extraction kit (Qiagen, Valencia, CA).

Quantification of mitochondrial DNA damage was done using quantitative PCR (qPCR) that amplifies long DNA targets as previously described (32). Briefly, the following primers were used to amplify a 8.9 kb fragment of mtDNA: 5'-TCTAAGCCTCCTTATTCGAGCCGA-3' (sense) and 5'-TTTCATCATGCGGAGATGTTGGA-3' (antisense). A 221-bp fragment of mtDNA was also amplified to normalize to the copy number of the mitochondrial genome present: 5'-CCCCACAAACCCATTACTAAACCCA-3' (sense) and 5'-TTTCATCATGCGGAGATGTTGGA-3' (antisense). The relative PCR product was

calculated by dividing the fluorescence value of the treated samples by the untreated sample. Quantification of genomic DNA damage was done using qPCR that amplifies a 13.5 kb fragment of the  $\beta$ -globin gene. Primers used to amplify the  $\beta$ -globin gene: 5'-CGAGTAAGAGACCATTGTGGCAG-3' (sense) and 5'-GCACTGGCTTTAGGAGTTGGACT-3' (antisense). The relative PCR product was normalized to the copy number of the mitochondrial genome.

### **Internuclei chromatin bridges assay**

Knockdown hDna2 and control U2OS and HeLa cells were seeded in a 12 well plate at  $5 \times 10^4$  cells per well 5 days post infection. 24 hours later, cells were fixed and stained with DAPI. The number of internuclei bridges was quantified in relation to the total nuclei number. At least 1000 nuclei were counted per well. Images were processed using Photoshop 7.0 using the gray scale and invert function (Adobe, San Jose, CA)

### **Growth curve**

Knockdown hDna2 and control U2OS cells were seeded in 6 well plates at  $7.5 \times 10^4$  cells per well, in triplicate, 5 days post infection. Cells were lifted and counted 24, 48, and 72 hours after seeding.

### **Flow activated cell sorting (FACS) analysis**

U2OS and HeLa cells were seeded at  $1 \times 10^6$  cells per 10 cm plate, 5 days post infection. 24 to 48 hours later, cells were lifted and stained with hypotonic propidium iodide, and subjected to FACS analysis to determine DNA content as previously described (35).

## Results

### Human Dna2 localizes to the nucleus and cytoplasm

Yeast Dna2 is found at telomeres during the G1 phase of the cell cycle and relocalizes to sites of DNA replication during S phase (10). In addition, when cells are treated with DNA damaging agents such as bleomycin, Dna2 transits from telomeres to sites of DNA damage (7, 10). This dramatic redistribution of Dna2 suggests that in yeast, Dna2 plays roles in both DNA replication and repair. To characterize the biological functions of Dna2 in human cells, we first set out to establish whether it displayed a similar dynamic subcellular localization. As expected, we noted the presence of hDna2 in the nucleus of a variety of cell types (including BJ, HeLa, HEK293, U2OS and 143B cells) albeit to varying degrees (**Figure 2.1A**). Primary human BJ fibroblasts displayed the most robust hDna2 nuclear staining of the cell lines analyzed (**Figure 2.1A**). In addition to the nuclear staining, we also observed a robust punctate hDna2 pattern within the cytoplasm (**Figure 2.1A**). To control for the specificity of our antibody and demonstrate that the cytoplasmic and nuclear hDna2 signals were real, we depleted hDna2 from HeLa cells by RNAi. Upon depletion of hDna2, both the nuclear and cytoplasmic hDna2 foci were significantly reduced (**Figure 2.1B**). Because we were unable to achieve significant hDna2 knockdown in BJ fibroblasts, which displayed the most robust nuclear hDna2 signal, we utilized blocking peptides to demonstrate that the cytoplasmic and nuclear hDna2 signal in BJ cells was lost, indicating that the

immunofluorescence signal was specific (**Supplementary Figure 2.1**). Taken together, our data indicate that cytoplasmic and nuclear forms of hDna2 exist in the cell.

In primary BJ fibroblasts we observed more robust nuclear hDna2, while analysis of HeLa cells revealed that hDna2 was predominantly cytoplasmic. To determine whether the majority of hDna2 was retained in the cytoplasm during interphase and localized to the nucleus upon entrance into S phase, we incubated HeLa cells with BrdU for one hour. As shown in **Figure 2.1C**, hDna2 did not re-localize to the nucleus of HeLa cells actively undergoing DNA replication, suggesting that the limited nuclear fraction of hDna2 is sufficient to participate in replication (**Figure 2.1A & 2.2B**).

### **Human Dna2 localizes to the mitochondria**

In yeast, Dna2 is a nuclease/helicase enzyme that functions in the nucleus but is also implicated in mtDNA maintenance (8). The existence of cytoplasmic Dna2 raised the possibility that it localized to the mitochondria and participated in mtDNA replication and/or repair. To determine whether hDna2 localized to the mitochondria, we utilized a mitochondrial specific dye, mitotracker. The osteosarcoma cell line U2OS was utilized for these studies because they are flat and contain large cytoplasm and thus allow high resolution of the mitochondria. Upon immunofluorescence analysis of hDna2 in U2OS cells, we observed significant co-localization with mitotracker (**Figure 2.2A**), indicating that a substantial fraction of cytoplasmic hDna2 is present in the mitochondria. Similar results were obtained in HeLa and HEK293 cells (**Figure 2.4 & data not shown**).

We next utilized subcellular fractionations to confirm our immunofluorescence findings. Analysis of a crude mitochondrial fraction and a highly pure nuclear fraction of HEK293, HeLa, 143B and U2OS cells revealed the presence of a 120 kDa species of hDna2 within the mitochondria and nucleus (**Figure 2.2B** & data not shown). Importantly, depletion of hDna2 in HEK293 and HeLa cells led to a reduction in both the mitochondrial and nuclear bands, indicating that the ~120 kDa band is hDna2 (**Figure 2.2B** & data not shown). It is unlikely that the nuclear form is the result of cytosolic contamination of the nuclear fraction because we did not detect cytoplasmic  $\gamma$ -actin in the nuclear fraction, whereas we did observe it in the crude mitochondrial fractions (**Figure 2.2B**) and cytosolic fractions (data not shown). Similarly, we did not observe any appreciable mitochondrial contamination in our nuclear prep (see TFAM control). Together, these results support our immunofluorescent analysis and indicate that hDna2 localizes to both the nucleus and the mitochondria.

hDna2's presence in the mitochondria raised the possibility that it played a role in replication and/or repair of mitochondrial DNA (mtDNA). Therefore, we next sought to determine whether hDna2 co-localized with mtDNA. Immunofluorescent analysis of hDna2 and mtDNA revealed that they were often in close juxtaposition and frequently showed overlap in their signals (**Figure 2.3A**). This result was confirmed by further co-immunofluorescence experiments with the mitochondrial helicase Twinkle (**Figure 2.3B**). Mitochondrial DNA is packed into nucleoprotein complexes referred to as nucleoids and the Twinkle helicase localizes to these structures (17, 34). Co-localization experiments using cells transduced with Twinkle-EGFP revealed a modest degree of

overlap in the Twinkle and hDna2 signals (**Figure 2.3B**), further demonstrating that hDna2 localizes to nucleoids in a subset of mitochondria.

The limited co-localization of hDNA2 with mtDNA raised the possibility that hDNA2 localizes within the mitochondria but is excluded from the majority of nucleoids or that the majority of hDNA2 is situated in the outer membrane or inter-membrane space of mitochondria. To distinguish between these possibilities, we carried a proteinase K protection assay (**Figure 2.3C**). These results showed that both in mitochondria and mitoplasts (with the outer mitochondrial membrane ruptured by digitonin treatment), a small fraction of hDna2 was protected from proteinase K degradation. However, hDna2 was degraded in the presence of the detergent Triton X100, illustrating the efficiency of the proteinase K treatment. As expected, TFAM, also a nucleoid protein, showed a similar pattern of proteinase K protection as the hDna2, but more of the protein was protected. These data indicate that at least a subfraction of hDna2 is found in the same mitochondrial compartment (inner membrane and/or matrix) as other nucleoid proteins.

### **Human Dna2 associates with mitochondrial DNA in the presence of mutant Twinkle proteins that induce replication stalling and nucleoid aggregation**

Our initial immunofluorescence analysis of hDna2 subcellular localization indicated that only a fraction of hDna2 co-localized with DNA containing nucleoids. We next questioned whether DNA damage resulting from physiological sources altered hDna2 subcellular localization. Twinkle is a mitochondrial specific helicase. Point mutations in Twinkle have been identified in patients with progressive external



ophthamoplegia (PEO), a mitochondrial disorder associated with accumulation of mtDNA deletions (34, 41). Importantly, expression of Twinkle mutants results in stalling of mitochondrial DNA replication forks and loss of mtDNA (18, 38). To address how hDna2 responded to Twinkle mutant expression, we utilized inducible HEK293 cell lines expressing either wildtype or mutant Twinkle proteins. Importantly, the inducible cell lines allowed us to examine the impact of acute protein expression at near physiological levels of Twinkle that have been shown to be sufficient to induce stalled replication forks within mitochondrial DNA (18, 38). Induction of wildtype Twinkle expression had little to no detectable impact of hDna2 localization (**Figure 2.4**). In contrast, expression of Twinkle mutants had variable effects on hDna2 localization. The G575D mutant failed to alter hDna2's subcellular localization (not shown), while both K421A (not shown) and K319E had only a modest effect, showing some nucleoid aggregation and accumulation of hDna2 in a subset of nucleoids. In contrast, expression of mutants R374Q and W474C resulted in a dramatic relocalization of hDna2 to mtDNA nucleoids, indicating that under some conditions hDna2 is more permanently associated with nucleoids and illustrating that its partial co-localization under normal conditions is not by chance. This altered hDna2 localization is exemplified by the W474C mutant, which showed a faint uniform mitochondrial staining, illustrating that the W474C mutant did not result in a collapse or fractionation of the mitochondrial network that might otherwise also explain the hDna2 accumulation with W474C containing nucleoids.

## **Human Dna2 depletion abrogates mitochondrial DNA repair**

Expression of Twinkle mutants known to cause mitochondrial replication fork stalling led to a more pronounced hDna2 nucleoid localization. This observation raised the possibility that hDna2 contributes to mitochondrial replication and/or repair. To address the role hDna2 plays in mitochondrial function, we utilized viral-mediated RNAi to target hDna2. Introduction of a hDna2 viral RNAi construct into HeLa cells resulted in a ~ 80% reduction in mRNA and protein levels (**Figure 2.5A & 2.5B**). To determine whether hDna2 impacted mtDNA repair, we analyzed the kinetics of mitochondrial DNA repair upon hDna2 depletion. Cells were treated with 2 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 30 minutes to induce oxidative DNA lesions. Because base lesions, abasic sites and strand breaks interfere with amplification of DNA, long range PCR can be used to measure the kinetics of mtDNA repair (32). Therefore, to assess mitochondrial DNA repair, DNA from cells expressing a control RNAi hairpin or cells expressing a hairpin targeting hDna2 were isolated following H<sub>2</sub>O<sub>2</sub> treatment. Lesions in mitochondrial DNA were assessed by PCR amplification of an 8.9 kb fragment of mitochondrial DNA and normalized to mitochondrial DNA copy number using a shorter mitochondrial DNA PCR product (221 bp). As an additional control, a DNA fragment from the nuclear  $\beta$ -globin gene (13.5 kb) was also amplified.

Analysis of mtDNA 0, 1, 2 and 24 hours after H<sub>2</sub>O<sub>2</sub> treatment revealed that hDna2 depletion resulted in a significant reduction in repair of oxidative lesions within the mtDNA (**Figure 2.5C**). In contrast, analysis of the nuclear  $\beta$ -globin gene revealed no significant differences in the repair of nuclear DNA (**Figure 2.5D**). However, given the

higher degree of protection and the faster kinetics of repair of nuclear DNA after H<sub>2</sub>O<sub>2</sub> treatment, (compare mtDNA and  $\beta$ -globin for shSCR sample), this result does not exclude a putative role for hDna2 in nuclear DNA repair (**Figure 2.2B & Figure 2.7**). Together, these experiments indicate that hDna2 plays an active role in the repair of mitochondrial DNA.

### **Human Dna2 depletion impacts the fidelity of mitochondrial DNA replication**

In order to determine whether hDna2 participates in mitochondrial DNA replication, we utilized two-dimensional neutral/neutral electrophoresis (2DNAGE) to analyze mitochondrial replication intermediates in cells depleted of hDna2. Mitochondrial DNA was purified from HEK293 cells and digested with *HincII* to release a 3.9 kb DNA fragment. Digested mtDNA was run in two dimensions and hybridized with a mitochondria-specific cytochrome *b* probe (**Figure 2.6A**). As shown in **Figure 2.6B**, depletion of hDna2 led to a moderate yet reproducible decrease in replication intermediates (right panel). Indeed, we found a 20% reduction in  $\gamma$ -arcs, bubble arcs and ‘ribonucleotide incorporation throughout the lagging strand’ (RITOLS) structures in cells depleted of hDna2 (**Figure 2.6B, right panel**) compared to the control cells (shSCR) (**Figure 2.6B, left panel**). This observation indicates that hDna2 depletion leads to reduced mtDNA replication, indicating that hDna2 participates in the replication of mtDNA.

## **Human Dna2 is important to maintain nuclear DNA stability**

Our original sub-cellular fractionation and immunofluorescence studies indicated that hDna2 was present in the mitochondria and nucleus. This raised the possibility that hDna2, like its yeast counterpart is important to maintain both mitochondrial and nuclear DNA stability (2, 8, 10, 14, 25, 36, 43). We next sought to determine if hDna2 participated in genomic DNA maintenance. To address this possibility, we depleted hDna2 from several cell lines and obtained varied degrees of knockdown of hDna2 mRNA (~95% knockdown in U2OS cells, 80% in HeLa, 65% in HEK293 cells and 20% in BJ cells). Given the significant knockdown obtained in U2OS and HeLa cells, we analyzed the impact of hDna2 depletion in these cells. Upon hDna2 depletion, U2OS cells showed a dramatic growth defect compared to control cells (**Figure 2.7B**). Analysis of DNA content revealed that depletion of hDna2 resulted in an accumulation of cells in the G2/M phase of the cell cycle, and the appearance of aneuploid cells (**Figure 2.7C**). Furthermore, hDna2-depleted cells displayed a significant increase in the appearance of internuclei chromatin bridges (**Figure 2.7D**), indicative of genomic instability. Similar results were obtained with a second independent hDna2 construct (shDNA2'), indicating that neither phenotype was due to an RNAi off-target effect (**Figure 2.7A-D**). In addition, hDna2 depletion in HeLa cells led to similar results albeit to a lesser extent consistent with the reduced hDna2 depletion observed in these cells (**Figure 2.7E**). Together, these observations demonstrate that hDna2 plays a role in nuclear DNA stability.

## Discussion

In this study, we present immunofluorescence and biochemical evidence for the existence of both a nuclear and mitochondrial form of hDna2. RNAi-directed depletion of hDna2 leads to growth defects, accumulation of aneuploid cells, and the appearance of internuclei chromatin bridges at high frequency, demonstrating a role for hDna2 in genome stability. Moreover, we demonstrate that hDna2 localizes within the mitochondria where a fraction associates with the mitochondrial inner membrane/matrix. In addition, hDna2 partially co-localizes with the mitochondrial specific helicase Twinkle, which plays an important role in mtDNA replication. Upon expression of Twinkle mutants known to cause replication fork stalling and DNA damage, hDna2 accumulates within mtDNA-containing foci, indicating that hDna2 co-localization with mtDNA is not by chance but occurs in a temporal/transient fashion to participate in mtDNA replication and/or repair. In support of this, we show that depletion of hDna2 leads to reduced mtDNA repair and replication. Together, these results indicate that hDna2 participates in DNA repair, likely via long patch excision repair, (1, 13, 26) and DNA replication within mitochondria.

Our findings that hDna2 participates in mitochondrial DNA stability are in agreement with a recent report showing that hDna2 is a mitochondrial repair and replication protein (42). However, our findings differ significantly from this report, which failed to identify nuclear hDna2 and thus suggested that hDna2 differs from its yeast counterpart in regards to a role in nuclear DNA stability. Instead, our findings are in agreement with results from yeast, which indicate that Dna2 plays an important role in

genomic and mitochondrial DNA stability. Indeed, Budd et al. demonstrated that while a *pif1-m2* mutant grows normally on glycerol, a *dna2Δ pif1-m2* double mutant displays a petite phenotype indicative of mitochondrial dysfunction (8). Furthermore, Dna2 mutants in yeast exhibit defects in telomere stability and DNA repair (10, 36). Again, our studies are in agreement with those in yeast by showing that hDna2 localizes to the nucleus and that hDna2 depletion results in nuclear DNA dysfunction. While we cannot explain the discordance between the studies in regard to nuclear hDna2, it is important to note that the amount of hDna2 detected by immunofluorescence inside the nucleus fluctuates from cell line to cell line. In addition, the antibodies used in the two studies differ, which may explain the varied localization results.

Our basic understanding of the replication and repair mechanisms that act on mitochondrial DNA are rudimentary but a multitude of proteins localize to the mitochondria and participate in DNA replication and repair. For example, the mitochondrial-specific polymerase  $\gamma$  and helicase Twinkle are important in mitochondrial DNA replication and when reconstituted *in vitro* together with the mitochondrial single-stranded DNA-binding protein functions as a minimal mtDNA replisome (23). The importance of Twinkle has been underscored by the discovery of human mutations that lead to the mitochondrial diseases including progressive external ophthalmoplegia (PEO) (34), mtDNA depletion syndrome (20, 33), and infantile onset spinocerebellar ataxia (19, 29). In this report, we provide *in vivo* evidence that hDna2 accumulates within nucleoid structures upon expression of Twinkle mutants known to induce mitochondrial DNA stalling/pausing. Furthermore, we demonstrate that hDna2 is important to ensure

efficient mtDNA replication. These observations corroborate results from Zheng et al., which demonstrate that hDna2 directly interacts with polymerase  $\gamma$  and stimulates its polymerase activity *in vitro*. The reduction in mitochondrial replication intermediates in hDna2-depleted cells could indicate that hDna2 is important for initiating mtDNA replication and that the hDna2 helicase activity plays an essential role in this process. Elucidating which hDna2 activity (helicase and/or nuclease) is necessary for efficient mtDNA replication will provide a better understanding of the molecular mechanisms that govern mtDNA replication.

While the Twinkle and polymerase  $\gamma$  proteins localize exclusively to the mitochondria, other proteins with known nuclear functions have also been found within the mitochondria. For instance, Flap endonuclease 1 (FEN1), Ligase III and the helicase Pif1 function within the nucleus and mitochondria (16, 24, 26, 30). Investigation of the mechanisms that control the subcellular localization of these proteins revealed that the Pif1 mRNA undergoes differential splicing, producing a nuclear and mitochondrial isoform (16) while Ligase III utilizes an alternative start site to produce a protein with and without a mitochondrial targeting signal (24). Studies from Zheng et al., suggest that the hDna2 mitochondrial localization signal is present in the C terminus of the protein and that no canonical nuclear localization signal is present (42). Therefore, we do not know how hDna2 is alternatively targeted to the mitochondria and the nucleus. Although in our biochemical fractionations both hDna2 species correspond to an approximate 120 kDa protein, we can not assume that the mitochondrial and nuclear variants are identical gene-products. The observation that the consensus hDna2 full-length GFP-tagged protein

seems to be exclusively mitochondrially targeted (40) does not rule out the presence of a distinct but similar sized isoform that would show a robust nuclear targeting. It does however strengthen the notion that hDna2 is a *bona fide* mitochondrial protein. Nevertheless, careful biochemical fractionation with appropriate controls in our hands clearly demonstrate the existence of an endogenous nuclear hDna2 protein, and RNAi-mediated knockdown resulted in a clear nuclear phenotype leaving no doubt that hDna2 is present both in the mitochondrial and nuclear compartment.

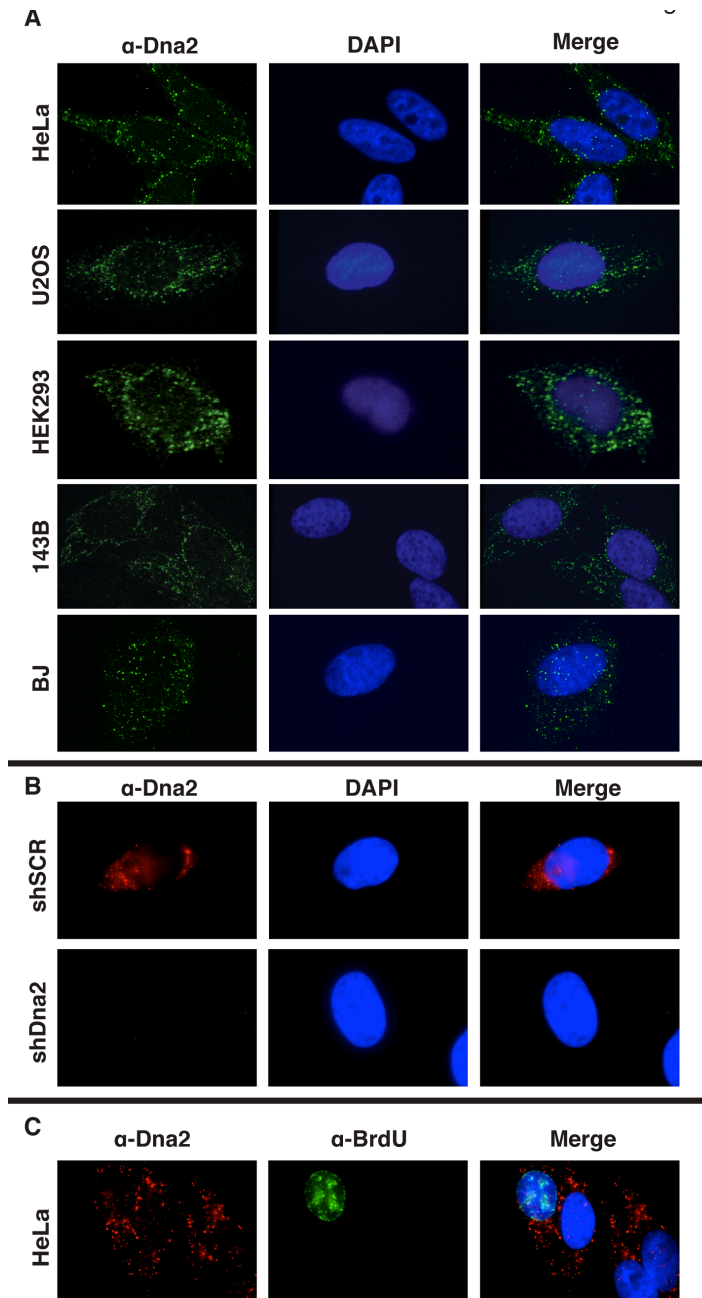
Importantly, we now provide direct evidence that hDna2 functions analogously to its yeast and *Xenopus* counterparts where it participates in the maintenance of nuclear DNA stability. Here we demonstrate that hDna2 depletion leads to reduced cell growth, accumulation of aneuploid cells, and an increase in internuclei bridges. Interestingly, the appearance of aneuploid cells is reminiscent of the “cut” phenotype observed in *Schizosaccharomyces pombe* mutants defective in DNA replication and the S phase/DNA damage checkpoint, where cells with incompletely replicated genomes fail to respond to the cell cycle checkpoint and enter a defective mitosis, resulting in aberrant DNA content (39). The appearance of internuclei bridges in hDna2-depleted cells may be ascribed to hDna2’s putative role DNA repair or telomere stability. In yeast, Dna2 is important for telomere maintenance (10, 36), and in mammalian cells loss of proteins essential in telomere stability, including the single strand telomere binding protein Pot1, can lead to the formation of such structures (12, 37). Alternatively the internuclei bridges that we observe could be the result of defective DNA repair. Indeed, in yeast it was shown that Dna2 plays an important role in 5’ end resection and subsequent DNA repair (25, 43).



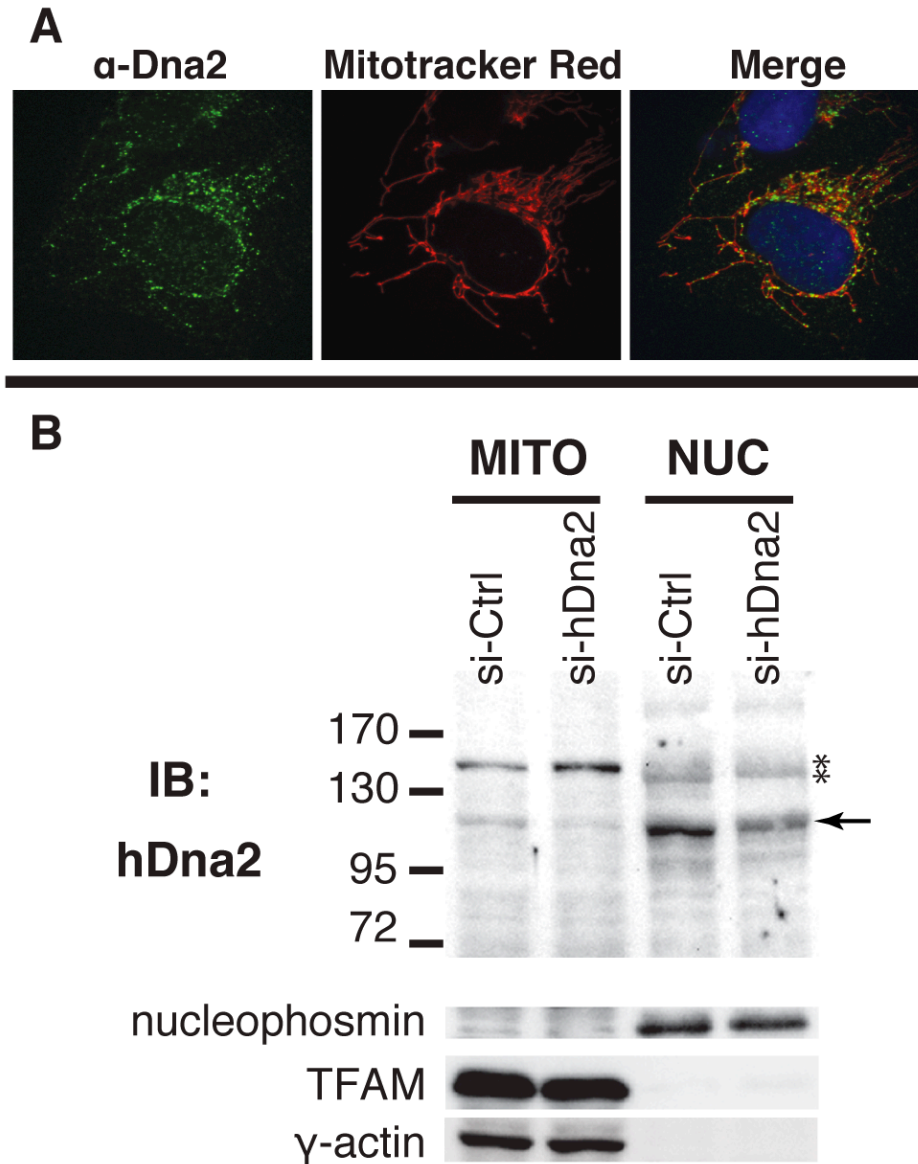
Further work will focus on determining whether hDna2 plays an important role in telomere maintenance, DNA replication and/or DNA repair like its yeast counterpart.

### **Acknowledgements**

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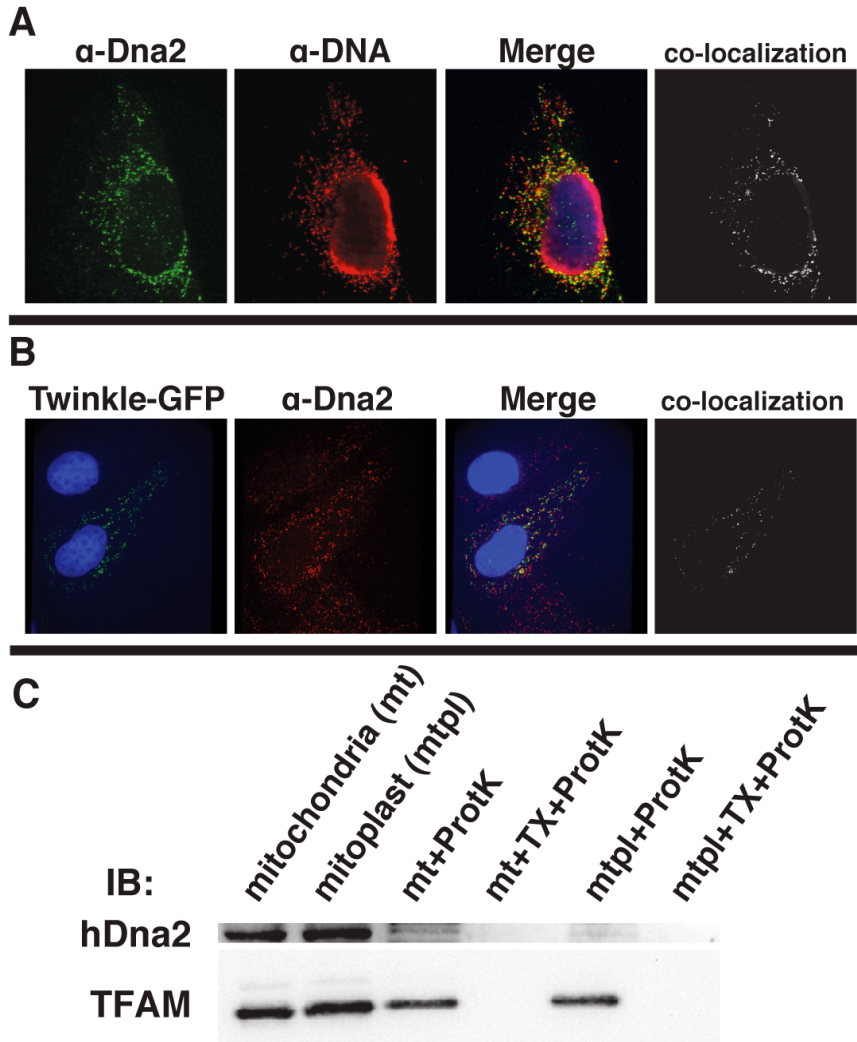
**Figure 2.1 hDna2 localizes within the cytoplasm and nucleus**  
**A.** Representative image of confocal immunofluorescence staining of hDna2 in HeLa, U2OS, HEK293, 143B cells, and primary BJ fibroblasts. hDna2 was stained with a polyclonal antibody targeting the hDna2 C-terminus (green) while nuclei were stained with DAPI (blue). **B.** Representative image of immunofluorescence staining of hDna2 in HeLa cells infected with a short hairpin targeting hDna2 (shDna2, bottom panel) versus a control hairpin (shSCR, top panel). **C.** Immunofluorescence images of cells stained for hDna2 in replicating HeLa cells. Cells were grown in the presence of BrdU for 1 hour and stained for hDna2 (red) and BrdU (green).



**Figure 2.2 hDna2 localizes to the mitochondria and nucleus**

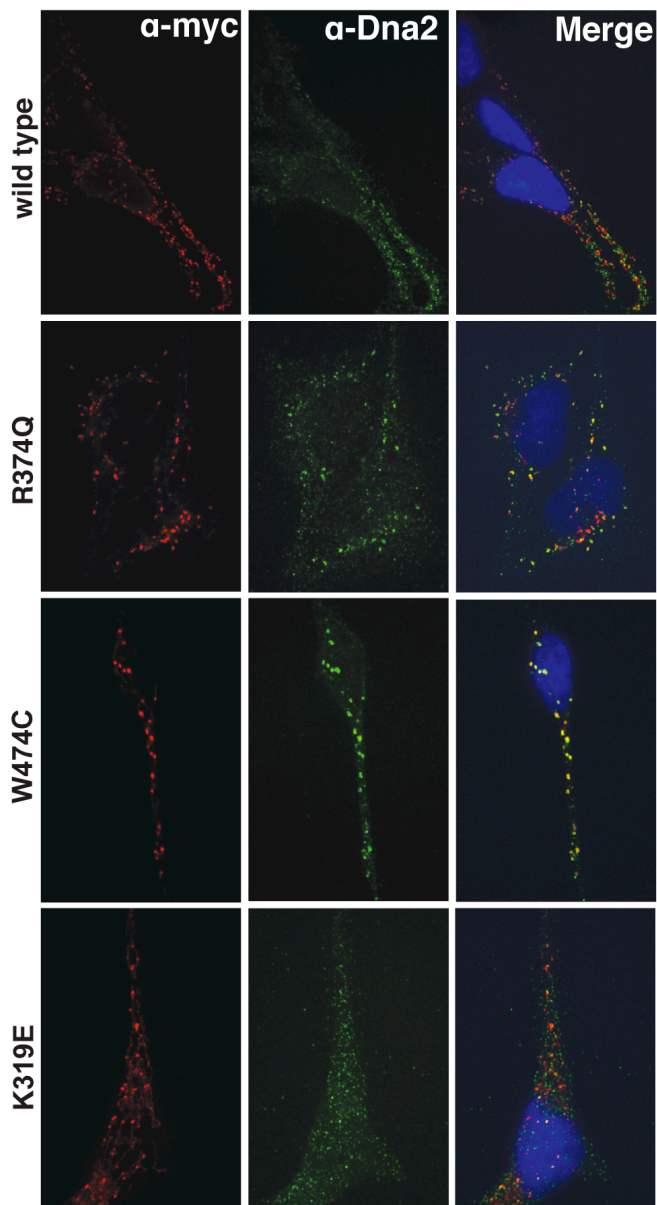
**A.** Confocal immunofluorescence images of U2OS cells showing hDna2 (green) and mitochondria (red) labeled with mitotracker. Note: punctate nuclear staining of hDna2.

**B.** Subcellular fractionation of HEK293 cells following transduction with synthetic RNAs targeting hDna2 mRNA. Mitochondrial (MITO) and nuclear (NUC) fractions were resolved on a 7.5% SDS-PAGE gel and probed with the hDna2 antibody. Controls for the mitochondrial and nuclear fractions were TFAM and nucleophosmin, respectively. Arrow indicates hDna2 and \* denotes nonspecific bands.



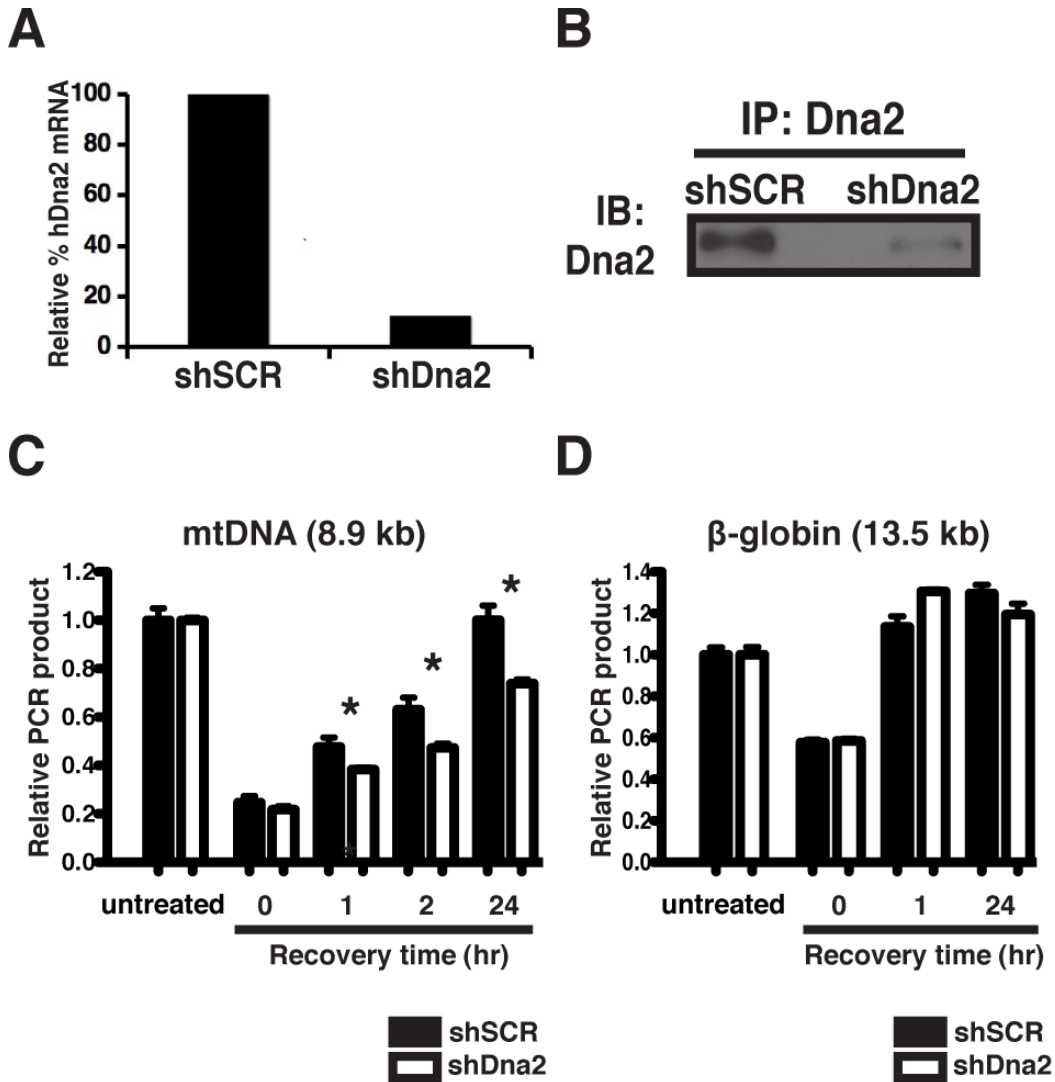
**Figure 2.3 hDna2 colocalizes with mitochondrial DNA**

**A.** Confocal images showing colocalization of hDna2 (green, left panel) with mitochondrial DNA (red) in U2OS cells. Mitochondrial DNA was stained with a specific DNA antibody ( $\alpha$ DNA). Colocalization is shown in black and white (right panel). **B.** Representative confocal images of U2OS cells transiently transfected with Twinkle-GFP (green, left panel) and stained for hDna2 (red). Right panel shows colocalization depicted in black and white. **C.** Western blot analysis of hDna2 following mitochondrial subfractionation and proteinase K treatment reveal the presence of hDna2 species within the mitochondria. HEK293 mitochondria were isolated and subfractionated as described in the material and methods section. Samples were analyzed by immunoblot (IB) analysis using an antibody against hDna2 and reprobbed with an antibody against the mitochondrial nucleoid protein TFAM. Mitochondria (mt) were loaded at a concentration of approximately 20  $\mu$ g, typically 3-5 % of the starting mitochondrial pellet. Mitoplasts (mtpl) were loaded at an equivalent volume of the total. Mitochondria (mt) and mitoplasts were either untreated, treated with 100  $\mu$ g/ml proteinase K (+ProtK) for 15 min at 4°C or treated with ProtK and Triton X100 (+TX+ ProtK), as indicated.



**Figure 2.4 Co-localization of hDna2 with wildtype and mutant Twinkle proteins**

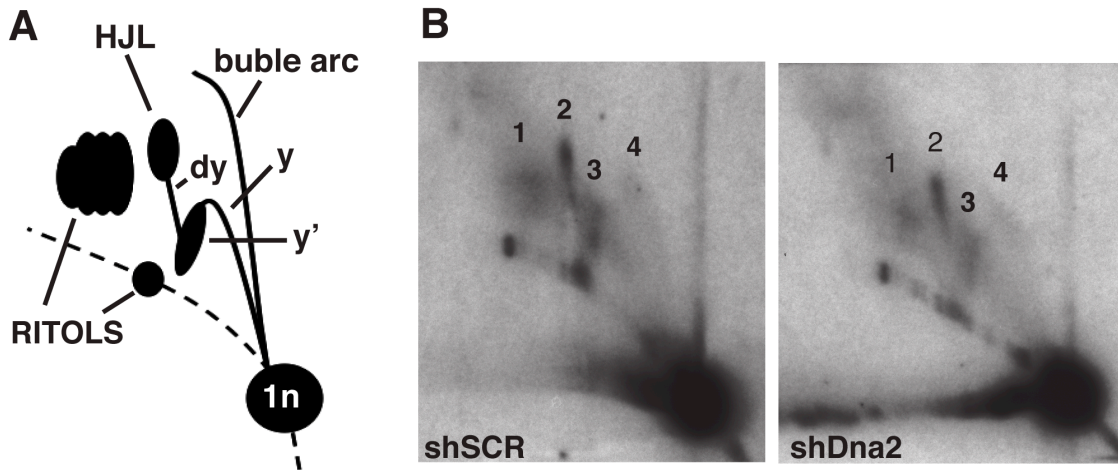
Expression of wild-type Twinkle with a myc epitope tag and the Twinkle R374Q, W474C and K319E mutants with similar tag was induced in Flp-In<sup>TM</sup> T-Rex<sup>TM</sup> 293 cells for two or three days with 3 ng/ml doxycycline. Twinkle proteins were detected by immunofluorescence using a myc monoclonal antibody (red) while hDna2 was detected using a rabbit polyclonal antibody (green). Merged images (right panels) include the signal detected from staining with DAPI.



**Figure 2.5 hDna2 depletion abrogates mitochondrial DNA repair**

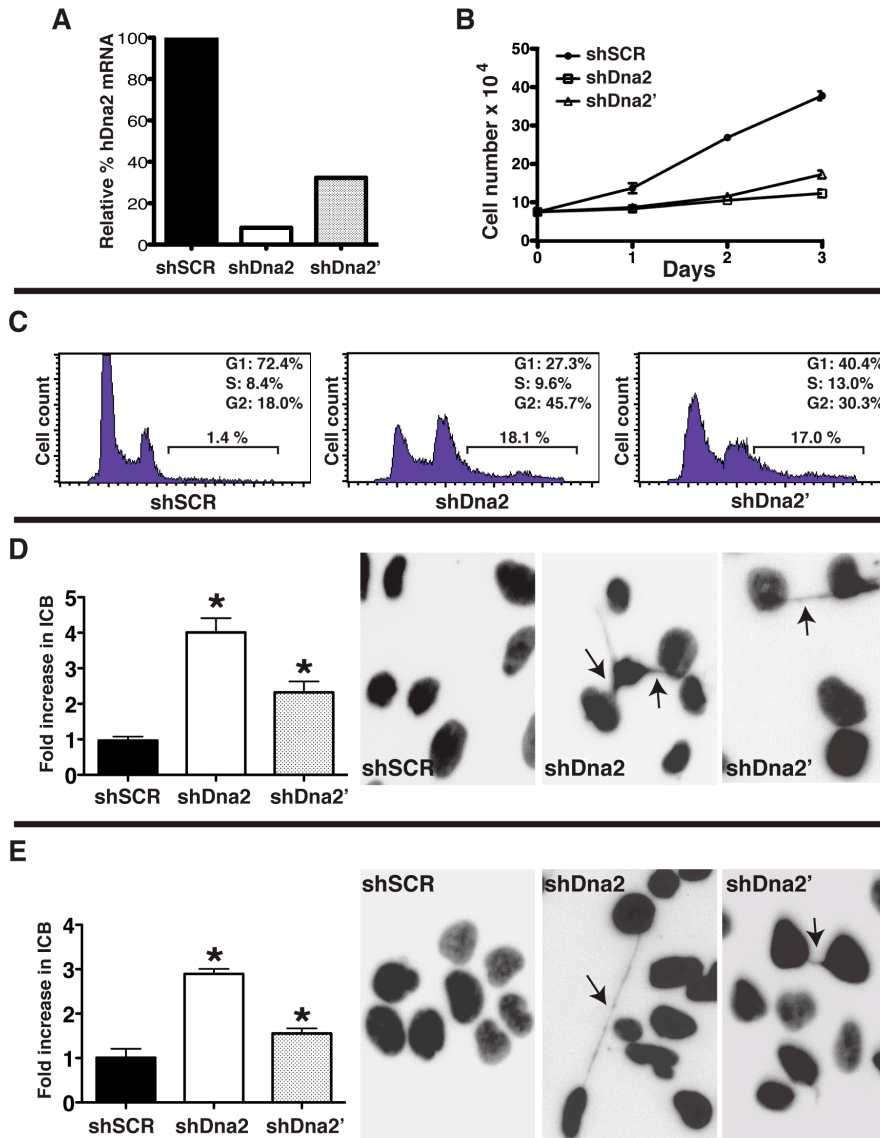
**A.** Stable knockdown of hDna2 by viral-based RNAi determined by qRT-PCR in HeLa cells. Results are normalized to shSCR control cell line. **B.** Immunoblot analysis (IB) of hDna2 immunoprecipitations (IP) from HeLa cells, stably expressing shSCR (control) or shDna2 hairpins. **C.** Histogram representing mitochondrial DNA amplification by qPCR of DNA extracted from HeLa cells expressing shSCR or shDna2. Cells were treated with 2 mM H<sub>2</sub>O<sub>2</sub> and allowed to recover for 0, 1, 2, and 24 hours. An 8.9 kb mitochondrial PCR product was quantified by picogreen and normalized to untreated samples and to mitochondrial DNA copy number determined by amplification of a 221 bp short mitochondrial fragment. Error bars represent standard errors of PCR reactions run in quadruplicate for each condition. \*p<0.05. **D.** Histogram representing amplification of a 13.5 kb region of the  $\beta$ -globin gene representing repair of genomic DNA in untreated cells or 0, 1, or 24 hours following H<sub>2</sub>O<sub>2</sub> treatment.





**Figure 2.6 hDna2 depletion impacts the fidelity of mitochondrial DNA replication**

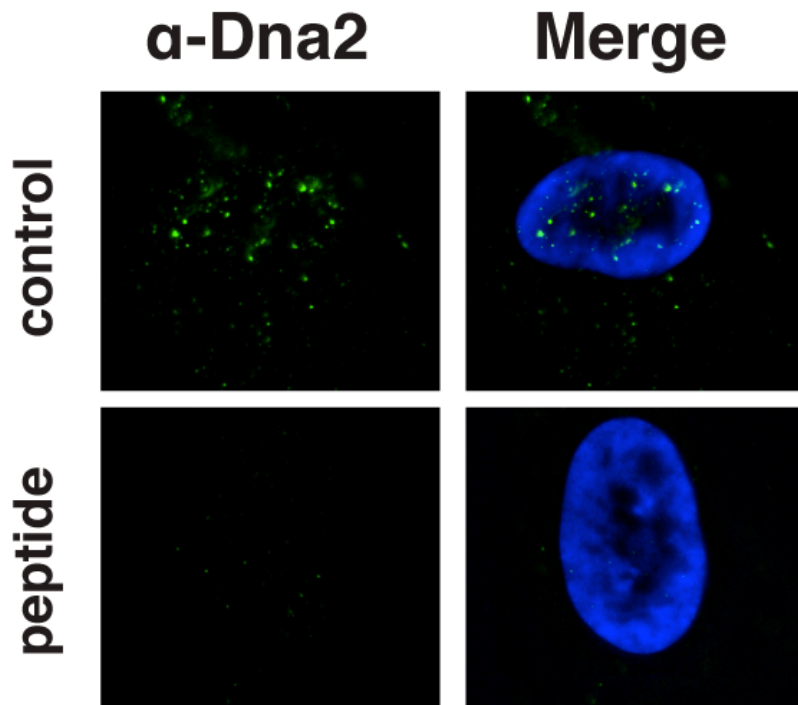
Two dimensional neutral/neutral agarose gel electrophoresis (2DNAGE) analysis of purified mitochondrial DNA digested with *HincII* and probed with a cytochrome b-specific fragment. Panel **A**. Schematic of 2DNAGE. Abbreviations: 1n, 3.9kb non-replicating *HincII* fragment; RITOLS, ribonucleotide incorporation throughout the lagging strand; y and y' indicate ascending and descending parts of the y arc; and (dy) indicates double-Y structures. These will eventually form resolution intermediates resembling Holliday junctions (HJL-Holliday junction like molecules). Panel **B**. 2DNAGE of mitochondrial DNA isolated from control cells (left panel) or cells depleted of hDna2 (65% knockdown of hDna2 mRNA, right panel). Numbers on the 2D gels represent (1) RITOLS; (2) HJL; (3) y'; and (4) bubble arc, respectively.



### Figure 2.7 Depletion of hDna2 leads to nuclear DNA instability

**A.** Stable knockdown of hDna2 determined by qRT-PCR in U2OS cells. Results of two independent hairpins (shDna2 & shDna2') are normalized to the shSCR control cell line. **B.** Control U2OS cells or cells depleted of hDna2 were seeded in 6 well plate at  $7.5 \times 10^4$  cells per well in triplicate. Cells were lifted and counted 24, 48 and 72 hours after seeding. Error bars represent standard error between the triplicate wells. **C.** Flow activated cytometric analysis of the cell cycle of U2OS cells expressing shDna2, shDna2' and shSCR. *x*-axis, cell count and *y*-axis, DNA content. **D.** Relative internuclei chromatin bridge levels (ICB) and representative bridges (arrowhead) following hDna2 depletion in U2OS cells. Results were normalized to shSCR. Note: hDna2 depletion was higher in shDna2 versus shDna2' cells. Error bars represent standard error and \* denotes  $p < 0.05$ . **E.** Relative internuclei chromatin bridge levels (ICB) and representation of HeLa cells following hDna2 depletion. Arrowhead indicates chromatin bridges. Note: hDna2 depletion in HeLa was lower than in U2OS cells.





**Supplemental Figure 2.1 The hDna2 antibody is specific for both nuclear and cytoplasmic hDna2**

Representative image of immunofluorescence staining of hDna2 in primary BJ fibroblasts. hDna2 was stained with a polyclonal antibody targeting the hDna2 C-terminus in the presence or absence of a blocking peptide. Nuclei were stained with DAPI (blue). Note: loss of specific hDna2 staining in both the nucleus and cytoplasm.

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**Chapter 3: Human Dna2 participates in DNA replication  
and ensures genomic stability**

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## Abstract

Dna2 is a conserved helicase/nuclease that in yeast participates in DNA replication and Okazaki fragment maturation, DNA repair, and telomere maintenance. We previously demonstrated that the human homologue (hDna2) participates in the maintenance of mitochondrial and nuclear DNA. Here we investigated the nuclear function of hDna2 and demonstrate that it plays an active role in DNA replication. RNAi-mediated depletion of hDna2 led to genomic instability accompanied by the activation of the replication checkpoint kinase Chk1 in late S/G2 phase. Genetic rescue experiments revealed that both hDna2's nuclease and helicase activities are essential to maintain genomic stability, supporting a model established in yeast where the helicase activity allows processing of long flaps that arise during Okazaki fragment processing by facilitating hDna2's ability to track down the flap where its nuclease cleaves. Furthermore, our observation that hDna2 interacts with And-1, a member of the replisome, in a replication dependent manner, suggests that hDna2 is recruited to replication sites and actively participates in DNA replication. Biochemical and genetic models predict that Dna2's activity is only required for a small percent of flaps that escape the activities of FEN1. In agreement with this prediction, hDna2 depletion did not result in slower maturation of newly synthesized DNA. In contrast, FEN1 or DNA ligase I depleted cells did result in slower maturation confirming that FEN1 is the main flap endonuclease that processes Okazaki fragments into ligatable nicks sealed by DNA ligase I. To establish whether hDna2 participates in DNA replication fork progression, we analyzed the track lengths of replicating forks *in vivo* using micro-fluidic-assisted replication track analysis (maRTA). Surprisingly, we did not observe slowing of the

replication fork upon hDna2 or FEN1 depletion suggesting that replication fork progression is insensitive to Okazaki fragment maturation. Taken together, this work provides clear evidence that hDna2, like its yeast homologue, plays an essential role in ensuring high fidelity DNA replication.

## **Introduction**

Human diseases that are caused by mutations in DNA repair or replication genes best illustrate the importance of maintaining genomic stability. Indeed, cells from patients harboring such mutations are characterized by significant genomic instability and individuals are predisposed to a variety of diseases, including cancer and/or premature aging syndromes. For example, mutations in the DNA repair proteins BRCA1 or BRCA2 cause familial breast and ovarian cancer (1). Alternatively, mutation in the RecQ helicase WRN causes Werner's syndrome, a premature aging syndrome with an increase in cancer susceptibility (16). Given the importance of maintaining DNA stability, it is not surprising that DNA replication and repair proteins have evolved multiple functions and display a significant degree of plasticity allowing a single protein to participate in several replication and repair processes.

Previously, we demonstrated that hDna2 localizes to the nucleus and mitochondria and participates in DNA maintenance in both compartments (18). Dna2 is a highly conserved helicase/nuclease, originally discovered in *Saccharomyces cerevisiae* and found in all organisms from yeast to humans (9) (29) (22) (32) (26). It possesses a 5' to 3' ATP-dependent helicase activity and a flap endonuclease activity (23). Genetic and biochemical experiments conducted in yeast support a model in which yeast Dna2 (yDna2) participates in lagging strand DNA replication through its role in Okazaki



fragment maturation (4) (3). Flap endonuclease 1 (FEN1) is postulated to function as the major endonuclease that removes flaps that arise from DNA polymerase  $\delta$  (Pol  $\delta$ ) strand displacement activity on the previous Okazaki fragment. However, under certain conditions, flaps can escape FEN1 cleavage and become longer than 27 nucleotides. The single strand DNA binding protein replication protein A (RPA) binds long flaps and inhibits FEN1 nuclease activity (4) (25). The two-step Okazaki fragment processing model proposes that Dna2 is the only enzyme able to process these long flaps. In addition, Dna2 is postulated to utilize both its helicase and nuclease activities to cleave the long RNA-DNA flap, leaving a short 5-6 nucleotide DNA flap which is further processed by FEN1 to produce a ligatable nick that is then sealed by DNA ligase I (LigI) (24) (6) (3). Supporting this model, both PIF1 helicase and Pol  $\delta$  subunit (Pol 32) promote long flap formation *in vitro*, and their deletion rescues the lethality associated with yDna2 loss (11) (48) (41) (40). Therefore, it is proposed that without PIF1 and/or Pol 32, long flaps no longer form *in vivo*, alleviating the need for Dna2.

In addition to its role in Okazaki fragment processing, Dna2 functions in DNA repair and telomere maintenance through its DNA resection activity. In yeast, Dna2 undergoes a dynamic localization where it is present at telomeres in G1, relocalizes throughout the genome in S phase, and moves back to the telomeres in late S/G2 phase (15). In addition, upon bleomycin treatment, yDna2 leaves the telomere and localizes to sites of DSBs (15). Recent studies have demonstrated that yDna2 endonuclease activity participates in the formation of a 3' single strand DNA overhang essential to initiate the homologous recombination process or to maintain telomeric stability (53) (28) (49) (8) (38) (13). In addition, Nimonkar et al. elegantly reconstituted DNA end resection *in vitro*

using purified human proteins and demonstrated that hDna2 physically interacts with BLM to resect 5' end DNA in a process that is dependent on hDna2's nuclease activity, while its helicase activity is dispensable (37). However, *in vivo* studies in yeast, as well as *in vitro* studies in yeast or human, indicate that Exo1 can compensate for Dna2's nuclease activity in this process (37) (13) (53). Indeed, to observe significant reduction in 5' end resection following DSB induction, both Exo1 and Dna2 need to be simultaneously deleted (53) (8). These observations suggest that the essential function of Dna2 is not its resection activity during double strand break repair but rather its function in removing long flaps during the processing of Okazaki fragments.

Our previous work demonstrating that hDna2 localizes to both the nucleus and the mitochondria and is required to maintain genomic stability support data established in yeast (18). Here we further investigate the nuclear function of hDna2 and demonstrate that hDna2 ensures high fidelity replication of the genome.

## **Materials and Methods**

### **Cell Culture**

U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep), and incubated at 37 °C in 5% CO<sub>2</sub>.

### **Virus Production and Infection**

Viral production and infections were carried out as described previously (42) (18). Briefly, 293T cells were transfected using TransIT-LT1 (Mirus, Madison, WI). Virus

was collected 48 hours post transfection, and infections were carried out in the presence of 10 µg/ml of protamine sulfate. 48 hours post infection, transduced cells were selected with 2 µg/ml of puromycin. The pLKO.1 shDna2, pResQ shDna2' and pLKO.1 shSCR lentiviruses were produced by cotransfection with pCMV8.2ΔR and pCMV-VSV-G (8:1 ratio). The sequences used for the hairpins were 5'-CATAGCCAGTAGTATTCGATG-3' for shDna2, 5'-GCAGTATCTCCTCTAGCTAGT-3' for shDna2' and for shSCR as previously reported (18). The pBabe-Hygro-3xFlag-Dna2 wild type, D294A and K671E and double mutant (DM) were cloned from pFastBACHTc-hDna2-Flag (33) and the sequences confirmed by DNA sequencing. Briefly, knock down rescue experiments were carried out by producing pBabe-Hygro-3XFlag-Dna2s or pBabe-hygro-3xFlag control constructs in 293Ts cells. U2OS cells were transduced with the different constructs and selected in the presence of 200 µg/ml of hygromycin B (Sigma, St Louis, MO). Once selection was completed, and overexpression was confirmed by western blotting, cells were transduced with shDna2' and selected with puromycin as described above. Five days post infection, cells were analyzed for DNA content by FACS analysis.

### **Immunoprecipitation and Western Blot Analysis**

For co-immunoprecipitation studies cells were washed in PBS and lysed in TBS-Tx buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X100 and protease and phosphatase inhibitors. 2 mg of lysates were immunoprecipitated with 2.4 mg of anti-hDna2 or IgG control antibodies using protein A beads overnight at 4 °C. The following morning, the beads were wash 3 times in 1 mL of TBS-Tx buffer before eluating the proteins in 2X laemmli buffer boiled for 5 min at 95 °C.

For Western blotting, cells were washed in phosphate buffered saline (PBS), lysed in MCLB (50 mM Tris pH 8.0, 5 mM EDTA, 0.5% NP40, 100 mM NaCl, 2 mM DTT, and protease and phosphatase inhibitors), sonicated (6 cycles of a 30 s pulse and 30 s cooling interval), and spun down for 20 min at 4 °C. Western blot analysis was carried out with anti-hDna2 antibody (ab96488, Abcam, Cambridge, MA), anti-FEN1 (BL586, Bethyl laboratories, Montgomery, TX), anti-DNA Ligase I (ab615, Abcam, Cambridge, MA), anti-phospho-Histone H2A.X (Ser139) (clone JBW301, #05-636, Millpore, Temecula, CA), anti-Chk1 (clone 2G1D5, #2360, Cell Signaling), anti-phospho-Chk1 (Ser 317) (17, Cell Signaling), anti- $\beta$ -Catenin (#610153, BD Biosciences), anti-Gamma1 Actin (NB600-533, Novus Biological, Littleton, CO), anti-And-1 (A-301-141A, Bethyl laboratories, Montgomery, TX) and anti-phospho-Histone H3 (Ser 10) (#9701, Cell Signaling). All protein concentrations were measured using the Bradford assay.

### **Immunofluorescence microscopy**

U2OS were grown for 1-2 days on coverslips. Cells were then washed in PBS and fixed in 3.7% paraformaldehyde, permeabilized in 0.5% Triton X 100, and treated with blocking buffer (10% FBS, 2% goat serum and 0.2% Tween 20) at room temperature. Antibodies were diluted in blocking buffer and incubated with cells for 1 hr at room temperature. Cells were washed in PBS containing 0.02% Tween 20 and mounted with 4', 6-diamidino-2-phenylindole (DAPI). Immunofluorescence detection was carried out with anti-phospho-Histone H2A.X (Ser139) (clone JBW301, #05-636, Millpore, Temecula, CA) or anti-phospho-ATM (Ser1981) (clone 10H11.E12, #4526, Cell

Signaling) as primary antibodies. Secondary antibodies were anti-mouse IgG-Alexa-Fluor<sup>®</sup> 488 or 546 (Invitrogen, Carlsbad, CA).

For RPA immunofluorescence, cells were pre-extracted before fixing according to Shi et al. (45). Briefly, U2OS cells were grown for 1-2 days on coverslips. Cells were then washed in ice cold cytoskeleton buffer (CSK) (10 mM HEPES/KOH pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl<sub>2</sub>), extracted for 6 min on ice with 0.5% Triton X-100 in CSK buffer supplemented with protease and phosphatase inhibitors. Cells were then fixed in 3.7% formaldehyde at room temperature for 25 min, followed by immunofluorescence using anti-Replication Protein A antibody (RPA-70-9, #NA13, Calbiochem, La Jolla, CA).

### **Internuclei chromatin bridges and micronuclei counts**

Knockdown hDna2 (shDna2 and shDna2') and control (shSCR) U2OS cells were seeded in a 12 well plate at  $5 \times 10^4$  cells per well five days post infection. 24-36 hours later, cells were fixed and stained with DAPI. The number of internuclei bridges (ICB) or micronuclei was quantified in relation to the total number of nuclei. At least 1000 nuclei were counted per well, and 6 wells were quantified per experiments. Two independent experiments were quantified for micronuclei counts in **Figure 3.1C** and ICB counts in **Figure 3.3D**. Images were processed using Photoshop 7.0 using the gray scale and invert function (Adobe, San Jose, CA).

### **Fluorescence-activated cell sorting (FACS) analysis**

U2OS cells were seeded at  $0.8 \times 10^6$  cells per 10 cm plate five days post infection. 24 to 48 hours later, cells were lifted and stained with hypotonic propidium iodide buffer (0.1 % sodium citrate tribasic, 0.3% triton X-100, 0.01 % propidium iodide, 50  $\mu\text{g/ml}$  RNase A (Sigma, St Louis, MO)) and subjected to FACS analysis to determine DNA content as previously described (18).

### **S phase progression Assay**

U2OS cells were cultured for 30 min in the presence of 20  $\mu\text{M}$  bromo-2-deoxyuridine (BrdU) in the dark as previously described (44). Briefly, cells were washed, cultured in fresh medium and harvest by trypsinization at the indicated times. The harvested cells were washed in PBS, and fixed in 4% paraformaldehyde and 0.1% Triton X-100 in PBS for 20 min at room temperature. The DNA was denatured with 30  $\mu\text{g}$  of DNase I (Sigma, St. Louis, MO) at 37 °C for 1 hour. BrdU was detected with an Alexa Fluor 488-conjugated anti-BrdU antibody (A21303, Invitrogen, Carlsbad, CA) and the DNA content of cells was determined by propidium iodide staining. The stained cells were analyzed by FACS.

### **Metaphase preparation and Chromosome FISH**

Subconfluent U2OS cells were incubated for 4 hr with 0.1  $\mu\text{g/ml}$  of colcemid and mitotic cells isolated by mitotic shakes. After hypotonic swelling in 75 mM KCl for 10 min at 37 °C, cells were fixed in methanol/acetic acid (3:1) and then dropped onto glass slides and aged at room temperature for 3 days. FISH was performed as previously described (42). Briefly, slides were rehydrated for 10 min in PBS and fixed with 4% paraformaldehyde

in PBS for 2 min. Hybridization was performed with 0.3  $\mu\text{g/ml}$  PNA probes targeted to the telomere, Cy3-(CCCTAA)<sub>3</sub>, and the centromere (FFLU-OO-CTTCGTTGGAAACGGGA) in 70% formamide, 10 mM Tris HCl (pH 7.2) and blocking reagent (Roche Applied Science, Indianapolis, IN). DNA was denatured for 3 min at 80 °C and hybridization was carried out at 37 °C for 4 hours in a moist chamber. The slides were subsequently washed, dehydrated and mount using VectaShield (Vector Labs, Burlingame, CA) containing DAPI. Images were taken using a Nikon 90i microscope and analyzed using the ISIS FISH imaging software (Metasystems, Altusheim, Germany).

#### **BrdU Comet assay**

The BrdU comet assay was performed as previously described (47). Briefly, U2OS cells were plated at  $5 \times 10^5$  cells per 6 cm plate and grown for 36 hours at 37 °C. Cells were then pulsed for 30 min with 100  $\mu\text{M}$  of BrdU (Sigma), and chased for 1 hour, 5 hours, or 8 hours by replacing with fresh medium. Cells were lifted by trypsinization, combined with low-melting-point agarose at 37°C and spread onto comet slides (Trevigen, Gaithersburg, MD). Cells were then lysed, denatured and run through electrophoresis under denaturing conditions (200 mM NaOH, 1 mM EDTA). After electrophoresis, gels were incubated with anti-BrdU antibody (#555627, BD Biosciences) in the dark for 1 hour. The primary antibody was detected using anti-mouse IgG-Alexa-Fluor<sup>®</sup> 488 (Invitrogen, Carlsbad, CA), and cells counterstained with DAPI. At least 40 comets were quantified per sample, per experiment, per time point using CometScore<sup>™</sup> software. The sequence used for the FEN1 short hairpins RNA were shFEN1 5'-

GGAGATCGTGCGGCGACTTGA-3' (pLKO.1 shFEN1), or shFEN3 5'-TTAAGAGCTACAGCTAGAGAA-3' (pLKO.1 shFEN3) (42); for shLigI 5'-GCTCAAGCTGAAGAAGGACTA-3' (pLKO1 shLigI).

### **maRTA assay**

Replication fork progression rates were determined using microfluidic-assisted replication track analysis protocol (maRTA) (46). Briefly, hDna2-depleted (shDna2) or control (shSCR) U2OS cells were labeled for 30 min each with 50  $\mu$ M IdU followed by 50  $\mu$ M CldU. For FEN1-depleted cells, U2OS-hTert cells were used to avoid potential telomeric defects induced by FEN1 depletion (43). Cells were then collected by trypsinization and used to prepare agarose plugs as previously described (46). High-molecular-weight DNA was isolated from cells embedded in agarose by brief heating to 75°C to melt the agarose, followed by agarose digestion. The resulting high-molecular-weight DNA was then loaded by capillary tension into microchannels to uniformly stretch and capture DNA on glass coverslips for immunostaining and fluorescence microscopy. Origin firing efficiency was determined by counting the fraction of origin firing events among all active replication events (i.e., ongoing forks and converging forks). Replication elongation efficiency was determined by measuring the mean length of first-label replication tracks in double-labeled tracks in order to unambiguously analyze active/ongoing fork rates. Track lengths were measured in digital images of tracks by using the AxioVision software package (Carl Zeiss). Three replicate samples of hDna2-depleted U2OS cells or mock-depleted U2OS cells (hDna2 experiment), or FEN1-depleted U2OS-hTert or mock-depleted U2OS-hTert (FEN1 experiment) were analyzed



for each determination, where 250 to 450 replication tracks were measured in each sample.

## **Results**

### **hDna2 contributes to genomic maintenance**

We previously reported that hDna2 depletion leads to genomic instability, characterized by the appearance of aneuploid cells and inter-nuclei chromatin bridges (ICBs) (18). Here we further report that hDna2 depletion results in an increase expression of phosphorylated H2A.X ( $\gamma$ -H2AX), a well-characterized marker of DNA breaks and the appearance of micronuclei. U2OS cells were infected with one of two shRNA hairpins that led to greater than 80% reduction in hDna2 protein levels (**Figure 3.1A & 3.1B**). Analysis of hDna2-depleted cells revealed a two-fold increase in micronuclei compared to cells expressing a control shRNA (**Figure 3.1C**). Furthermore, hDna2-depleted cells displayed an increase in  $\gamma$ -H2AX foci, consistent with the increase in expression observed by western blot analysis (**Figure 3.1D & 3.1E**). In addition to the appearance of  $\gamma$ -H2AX foci we also observed phosphorylated-ATM foci in hDna2-depleted cells, confirming that DNA breaks form upon depletion of hDna2 (**Figure 3.1D**). Together these observations demonstrate that hDna2 is needed to maintain genomic stability.

### **hDna2's nuclease and helicase activities are essential to maintain genomic stability**

Dna2 is a highly conserved enzyme that possesses nuclease and helicase/ATPase activities that are postulated to contribute to its function *in vivo*. While both activities are

essential for viability in *Saccharomyces cerevisiae*, recent *in vitro* biochemical studies have called into question the importance of the helicase activity in the human protein (26) (32). Therefore, we next addressed whether hDna2's nuclease and/or helicase activities contribute to genomic stability in mammalian cells. To assess the role of these activities, we carried out a series of genetic knockdown-rescue experiments utilizing an shRNA targeting the 3'UTR of endogenous hDna2 while expressing FLAG-tagged hDna2 cDNAs insensitive to the shRNA. We expressed either a control vector (ctrl), wild-type hDna2 (WT), nuclease-deficient (D294A), or helicase/ATPase-deficient (K671E) hDna2 cDNAs. To allow for the differentiation of the endogenous and exogenous hDna2, we designed specific RT-PCR primers that amplify a DNA fragment covering a region starting at the last exon (exon 21) and finishing at the 3' UTR of the hDna2 gene that is not present in the cDNA constructs (see Materials and Methods). This allowed us to confirm the knock down of endogenous hDna2 while verifying exogenous overexpression by western blotting using a FLAG antibody (**Figure 3.2A**). We have previously demonstrated that depletion of endogenous hDna2 results in a reduced G1 population, a late S/G2 cell cycle arrest and appearance of aneuploid cells (18). Therefore, to assess the role of the helicase and nuclease activities of hDna2 we utilized FACS analysis to determine the cell cycle profile and DNA content. Expression of the wild-type allele partially rescued the cell cycle defects observed upon depletion of hDna2 demonstrating that the cell cycle arrest was specific to hDna2 loss (**Figure 3.2B**). In contrast, expression of the nuclease-deficient or helicase-deficient alleles failed to rescue the cell cycle abnormalities indicating that both activities of hDna2 are essential to maintain genomic stability (**Figure 3.2B**). Furthermore, the nuclease-deficient protein,

which was expressed at lower levels than the wild-type or helicase-deficient proteins, displayed more severe cell cycle anomalies than cells depleted of endogenous hDna2 alone (compare G1:(S+G2) ratio, and cells with >4N DNA content) (**Figure 3.2A & 3.2B**). These results suggest that the nuclease deficient protein functions in a dominant-negative fashion, and clones expressing low levels of the nuclease-deficient allele are selected over the course of the experiment. This finding supports both biochemical and genetic data obtained in yeast suggesting that Dna2's helicase activity is important for the protein to track down long flaps that arise during Okazaki fragment processing and subsequently enables Dna2's nuclease activity to act on these flaps (10) (5). To address this model, we also expressed a double mutant (DM) allele lacking both nuclease and helicase activities. This mutant allowed us to address whether the helicase activity is important for the nuclease deficient protein to act in a dominant negative fashion. Interestingly, while expression of the nuclease-deficient protein drastically decreased over two weeks in culture, DM protein expression was maintained at similar levels to the helicase-deficient protein (**Figure 3.2C**). Together, these results demonstrate that the nuclease and helicase activities of hDna2 are essential to maintain genomic stability. In addition, they support a model established in yeast where the helicase activity of hDna2 stimulates its nuclease to process long DNA flaps.

### **Interchromatin bridges that form upon depletion of hDna2 are not the result of telomere fusions**

Interchromatin bridges arise from unresolved replication intermediates, defective mitosis, and/or telomere fusions that form upon loss of telomeric integrity (31). Because

Dna2 plays an important role in telomere stability in yeast, we investigated whether the ICBs observed upon hDna2 depletion were the result of telomeric fusions. To address this possibility, we collected metaphases from control or hDna2-depleted cells. No increase in chromosomal end-to-end fusion was observed in cells expressing shRNAs targeting hDna2, suggesting that telomere dysfunction is not responsible for the formation of ICBs in these cells (**Supplementary Figure 3.S1A**). Interestingly, we observed the appearance of metaphases with pulverized chromosomes (**Supplementary Figure 3.S1B**). These metaphases are reminiscent of chromosomes that enter mitosis with unfinished replication, and could ultimately lead to ICBs and aneuploidy (36).

### **hDna2 depletion leads to replication checkpoint activation**

Genetic and *in vitro* biochemical studies in yeast have demonstrated that Dna2 is an essential replication enzyme that participates in the processing of long flaps that arise during lagging strand DNA synthesis. Therefore, it is not surprising that loss of yDna2 results in replication defects and loss of cellular viability. Furthermore, genetic experiments in yeast have shown that hDna2 can rescue *Dna2-1* replication mutant defects, suggesting that Dna2's role in DNA replication is conserved in higher organisms (21).

To determine whether hDna2 depletion impacted DNA replication we examined the phosphorylation status of the replication checkpoint kinase 1 (Chk1). Western blot analysis of hDna2-depleted cells revealed a significant increase in phosphorylated Chk1 at serine 317 compared to control cells, indicating that cells depleted of hDna2 experience DNA replication stress (**Figure 3.3A** top panel). In agreement with this

finding, immunofluorescence analysis of hDna2-depleted cells revealed a significant increase in RPA foci compared to control cells (**Figure 3.3B**). The increase in RPA foci indicates an accumulation of single stranded DNA, which would be expected to activate the replication checkpoint. Interestingly, further western blot analysis of hDna2-depleted cells revealed reduced phosphorylation of the mitotic marker Histone H3 at serine 10 (**Figure 3.3A** bottom panel). This observation indicates that the cell cycle accumulation observed in hDna2-depleted cells corresponds to a late S/G2 arrest prior to mitosis, further supporting that it arises from a replication defect.

To address whether the G2 arrest observed upon hDna2 depletion was blocking cells from entering mitosis with unresolved damage, we treated cells with one of two Chk1 inhibitors, Gö6976 or AZD7762. Cells depleted of hDna2 were grown for 8 hours in the presence of the Chk1 inhibitor. FACS analysis revealed a drastic alteration in the cell cycle profile of hDna2-depleted cells treated with a Chk1 inhibitor as compared to untreated cells (**Figure 3.3C**). hDna2-depleted treated cells were released from the G2 arrest and underwent mitosis. Chk1 activation is proposed to prevent cells from moving into mitosis without first completing DNA replication (39). Therefore, if Chk1 is inhibited in cells undergoing replication stress, they would move into mitosis with incompletely replicated DNA and unresolved replication intermediates, ultimately leading to aberrant mitosis or cell death. hDna2-depleted cells treated with a Chk1 inhibitor were also examined for ICBs formation. While a significant number of cells underwent mitotic catastrophe (data not shown), hDna2-depleted cells displayed a net increase in ICBs, indicating that cells released from the G2 block underwent aberrant mitosis (**Figure 3.3D**). Together these experiments provide strong evidence that cells

depleted of hDna2 arrest in late S/G2 due to a replication defect; if this block is bypassed, cells with uncompleted replication undergo aberrant mitosis.

### **hDna2 interacts with And-1 in a replication dependent manner**

Above, we demonstrated that hDna2 depletion leads to replication dependent damage. Furthermore Wawrousek *et al.* recently demonstrated that *Xenopus laevis* Dna2 (xDna2) is recruited to DNA shortly after replication licensing where it interacts with the replisome proteins And-1 and Mcm10 (51). Therefore, to address whether hDna2 actively participates in DNA replication we investigated the association of hDna2 with And-1. We found that endogenous And-1 co-immunoprecipitated with hDna2 from asynchronous cells (**Figure 3.4A**). To address whether this interaction was replication dependent, we compared cells arrested in G1-G0 by serum starvation to cells blocked at the G1-S border by a double thymidine treatment. While And-1 co-immunoprecipitated with hDna2 in cells blocked at the G1-S transition, no interaction was observed between hDna2 and And-1 in cells arrested in G0-G1 (**Figure 3.4B**). These observations mimic *Xenopus* observations and suggest that hDna2 interacts with And-1 shortly after the licensing of the pre-replication complex (Pre-RC). Furthermore, they demonstrate that hDna2 is recruited to the replisome in a replication dependent manner, suggesting that it actively participates in DNA replication.

## **hDna2 depletion does not lead to detectable defects in maturation of newly synthesized DNA**

A model proposed from yeast studies suggests that hDna2 participates in lagging strand maturation through its role in processing long flaps. Furthermore, it is postulated that long flaps only arise in a small percentage of Okazaki fragments in specific regions of the genome (48) (23). Therefore, the need for Dna2 should be dispensable for processing the vast majority of Okazaki fragments, wherein short flaps are cleaved by FEN1. To address whether hDna2 functions in Okazaki fragment processing, we measured the maturation kinetics of newly replicated DNA using a BrdU/comet assay. We reasoned that if hDna2 was necessary to process a minority of the flaps, it would be difficult to observe significant maturation differences in hDna2-depleted cells. Therefore, we knocked down FEN1 in addition to hDna2, to increase the accumulation of long flaps requiring Dna2's activity. Additionally, because this assay was previously used to demonstrate that DNA ligase I (LigI) is important to ligate Okazaki fragments *in vivo*, we also depleted cells of LigI (47). Western blotting analysis demonstrated that we successfully knocked down FEN1, LigI or hDna2 (**Figure 3.5B**). Interestingly, FEN1 knockdown cells did not display major signs of DNA damage monitored by  $\gamma$ -H2AX levels nor cell cycle defects by FACS analysis (**Figure 3.5B** and data not shown). However, FEN1-hDna2 co-depletion resulted in a net increase in  $\gamma$ -H2AX levels and cell cycle alteration as compared to cells depleted of hDna2 alone. These observations suggest that hDna2 and FEN1 can partially compensate for each other.

To assess maturation of newly replicated DNA, control cells and cells depleted of hDna2, FEN1, LigI, or hDna2 and FEN1 were pulsed with BrdU and analyzed by comet

assay in alkaline conditions. Immunofluorescence against BrdU assessed the integrity of newly replicated DNA by measuring its migration from the tail (unligated DNA fragments) to the head (ligated DNA) of the comet (**Figure 3.5A** compare 1 hour to 8 hour images). While LigI-depleted cells displayed slower migration of DNA from the tail to the head as previously reported (47), no significant difference was observed in the absence of hDna2 compared to control cells (**Figure 3.5C**). Furthermore, while FEN1 knockdown cells displayed similar kinetics as LigI-depleted cells, FEN1-hDna2 co-depleted cells were not significantly different from FEN1-depleted cells (**Figure 3.5C**). These results indicate that FEN1 is the major flap endonuclease responsible for processing Okazaki fragments in lagging strand replication, and that hDna2 is not able to compensate for FEN1 depletion in this process. Additionally, it suggests that if hDna2 is important for processing long flaps, these represent a minor portion of the flaps generated, rendering this assay insensitive to hDna2 depletion.

### **Defects in Okazaki fragment processing do not impact replication fork progression**

Genetic evidence in yeast indicates that Dna2 plays a critical role in Okazaki fragment processing. Despite this, Dna2 mutant cells arrest in late S phase and do not display defects in bulk DNA synthesis (22). This observation suggests that the rate of replication is unaffected by aberrant Okazaki fragment processing. In other words, replication fork movement appears to be insensitive to unligated Okazaki fragments that persist behind the replication fork. To address whether hDna2 impacts replication kinetics, we first assessed S phase progression in cells depleted of hDna2. Control or hDna2-depleted U2OS cells were pulsed with BrdU for 30 minutes and chased for 12



hours. While hDna2-depleted cells incorporated BrdU at the same rate as control cells, they displayed a marked delay in completing the S-G2 phase and consequently took longer to appear in the next G1 (**Figure 3.6A and 3.6B**) (compare 8, 10 and 12 hour time points). These observations support the original cell cycle profiling data and suggest that hDna2 depletion does not impact the rate of bulk DNA synthesis, but rather creates damage that slows cell cycle progression in late S or G2 before cells enter mitosis.

Because subtle differences in the kinetics of replication fork progression could be missed in an S phase progression assay, we next measured the replication fork kinetics of single DNA molecules upon hDna2 depletion using micro-fluidic-assisted replication track analysis (maRTA), wherein DNA is sequentially labeled *in vivo* with short pulses of the base analogues IdU and CIdU (46). Following labeling, DNA is isolated, stretched on coverslips, and IdU and CIdU are detected by immunostaining. By measuring the mean length of first-label replication tracks in double-labeled tracks, DNA replication elongation efficiency can be determined. Using this method we found that depletion of hDna2 had no discernable impact on replication fork velocity. Indeed, we found that DNA track lengths were similar in control and hDna2-depleted cells (**Figure 3.7A**). This finding supports our S phase progression observations and suggests that damage that arises behind the fork due to unprocessed DNA fragments does not impede the progression of replication forks.

To address this hypothesis we also depleted cells of FEN1, the flap endonuclease responsible for processing of the majority of Okazaki fragment flaps (3). Strikingly we found that FEN1 depletion did not slow replication fork progression. Indeed, we found that DNA track lengths were slightly longer in FEN1-depleted cells than in control cells

(Figure 3.7B & 3.7C). Together these observations indicate that damage arising behind the replication fork due to an inability to ligate Okazaki fragments is not sufficient to slow replication forks and DNA synthesis *in vivo*.

## Discussion

In this study, we provide evidence that hDna2 is essential to ensure high fidelity DNA replication. RNAi-directed depletion of hDna2 leads to genomic instability characterized by the appearance of  $\gamma$ -H2AX and phospho-ATM foci. Furthermore, cells depleted of hDna2 arrest in late S/G2 phase in a Chk1-dependent manner thus preventing cells from entering mitosis with incomplete DNA replication. Interestingly, hDna2's nuclease and helicase activities are essential to maintain genomic stability, and hDna2 interacts with the replisome protein AND-1 in a replication dependent manner, indicating that hDna2 actively participates in DNA replication like its yeast homologue. Consistent with the model proposing that long flaps are formed at a minority of Okazaki fragments, we were unable to detect defects in maturation of newly replicated DNA. Furthermore, replication fork kinetics were unaffected by the depletion of hDna2 or FEN1, indicating that the inability to ligate Okazaki fragments behind the replication fork does not impact its progression.

Our findings that hDna2 participates in DNA replication are in agreement with Dna2's known functions in yeast. In *Saccharomyces cerevisiae* temperature sensitive Dna2 mutant alleles arrest cells in G2/M with a 2C DNA content when shifted to the restrictive temperature (19). Furthermore, expression of hDna2 suppresses the growth

defects of the replication mutant *dna2-1*, suggesting hDna2 is a functional homolog of its yeast counterpart. In *Schizosaccharomyces pombe*, temperature sensitive mutants also arrest in late S phase and display no defects in bulk DNA synthesis (22). When combined with a checkpoint inhibitor, these cells bypass the arrest and undergo aberrant mitosis. Here we demonstrate that hDna2-depleted cells arrest in late S/G2 phase due to an inability to complete DNA replication. Upon Chk1 inhibition, cells bypass the replication checkpoint and progress through mitosis, displaying aberrant mitotic structures. These phenotypes recapitulate observations collected in yeast and strongly suggest that hDna2's function in DNA replication is conserved in humans.

The requirement for Dna2's helicase activity for yeast viability in *Saccharomyces cerevisiae* indicates that this function is necessary for Dna2's physiological role *in vivo* (10). Furthermore, different studies have demonstrated that Dna2's helicase activity is essential for the protein to efficiently cleave long flaps that form secondary structures (5) (23). Interestingly, mixing of Dna2 ATPase/helicase deficient (K1080E) and Dna2 nuclease deficient (D657A) mutants failed to process these long flap substrates, indicating that both activities have to be coupled to the same protein in order to efficiently cleave long DNA flaps (5). Supporting these observations, simultaneous expression of both mutants failed to rescue *dna2Δ* lethality (5). However, recent studies observed that the helicase activity of its human homologue was weak or undetectable *in vitro*, calling into question its *in vivo* function (26) (32). Our observation that nuclease or helicase-deficient hDna2 failed to rescue hDna2 depletion clearly demonstrates that both activities are essential to maintain genomic stability. Furthermore, the nuclease-deficient protein was consistently expressed at lower levels than the helicase-deficient protein,

suggesting it functions in a dominant negative fashion and cells with low levels of expression are selected rapidly in culture. Curiously, studies in yeast have also demonstrated that the nuclease-deficient mutant acts in a dominant negative manner (10) (5). These observations support a model in which Dna2's helicase activity is important to load the protein on long flaps and subsequently enables Dna2's nuclease activity to act on these flaps (5) (7). Our results that the DM protein expression was maintained at similar levels than the helicase-deficient protein indicate that a nuclease deficient mutant without its helicase activity can no longer act in a dominant negative fashion, suggesting that both activities are coupled within a single Dna2 molecule to act on its substrates *in vivo*.

Our observations that hDna2 interacts with And-1 in a replication dependent manner are in accordance with yeast and *Xenopus* observations. Ctf4, the homologue of And-1 in *Saccharomyces cerevisiae*, has been characterized as a member of the replication progression complex and responsible for recruiting pol  $\alpha$  to replication forks (34) (52). Ctf4 also interacts with MCM10 with which it forms a complex that couples the lagging strand polymerase with the replicative MCM helicase complex (zhu and dutta 2007). Interestingly, yDna2 genetically interacts with both Ctf4 and MCM10 and was recently found in *Xenopus* to form a complex with their respective homologues And-1 and MCM10 (20) (12) (2) (51). Our observation that hDna2 interacts with And-1 in a replication dependent manner demonstrates that hDna2 actively participates in DNA replication, and is consistent with a conserved role of hDna2 in lagging strand synthesis.

While ligating Okazaki fragments is an essential event that ensures a continuous lagging DNA strand and avoids single and double strand break formation, several studies suggest that defects in this process do not impact the rate of DNA synthesis. Indeed, we

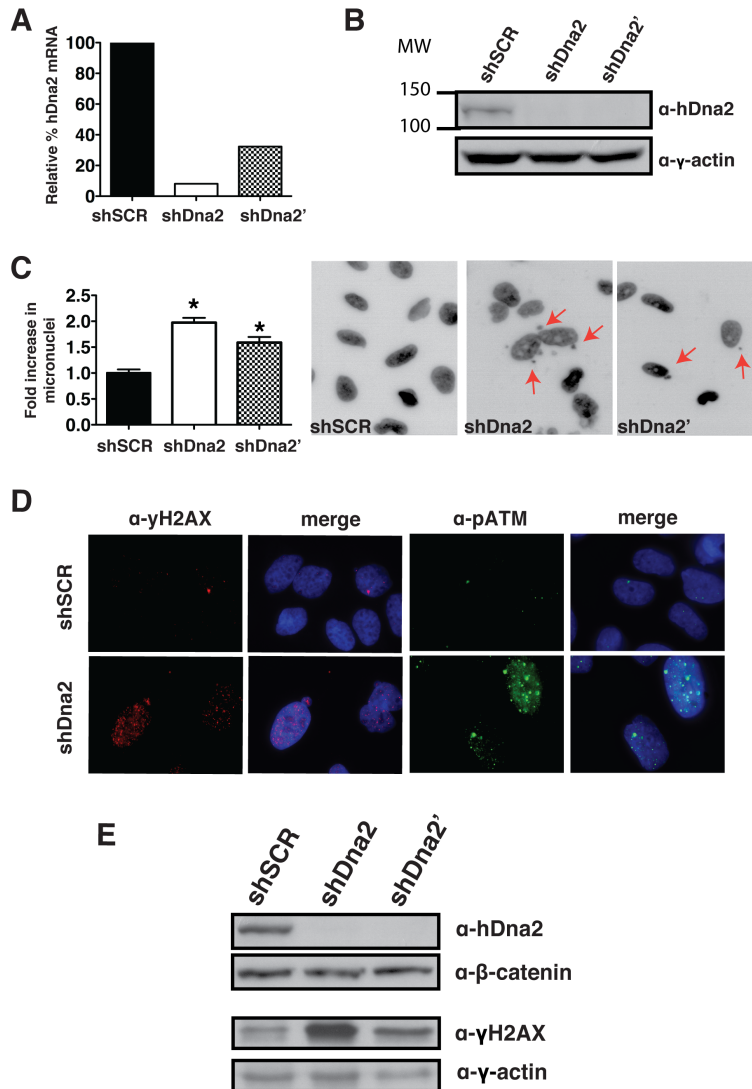
have previously demonstrated that FEN1-depletion neither impacts S-phase progression nor *in vitro* SV40 LargeT antigen-dependent supported DNA replication (44). Similarly, LigI mutant cells (46BR.1G1) that exhibit low LigI activity do not activate the intra-S phase checkpoint (47). While protein extracts from these cells are deficient in Okazaki fragment ligation during *in vitro* SV40 DNA replication, they support incorporation of [ $\alpha$ -<sup>32</sup>P] dATP into plasmid DNA with similar kinetics to extracts from control fibroblasts, indicating that replication progression is unperturbed in the absence of LigI (30). Here, using the sensitive maRTA technique, we demonstrate for the first time that depletion of essential Okazaki fragment maturation proteins like FEN1 or hDna2 does not impact replication fork progression *in vivo*. These observations strongly suggest that flaps that persist behind the replication fork do not affect the synthesis rate of DNA during replication. These unprocessed Okazaki fragments are recognized as damaged DNA later in S or G2 phase.

FEN1 is suggested to be the main endonuclease that processes flaps that are formed *in vivo* during lagging strand synthesis. However for unknown reasons, a small fraction of the flaps escape FEN1 processing and exceeds 27 nt in length. These longer flaps require Dna2's activity to cleave them into shorter flaps which are then processed by FEN1 to produce a ligatable nick. To address this model in human cells we depleted FEN1 and hDna2 separately or together. Our observation that maturation of nascent DNA occurs with slower kinetics in FEN1-depleted cells confirms that FEN1 is the primary endonuclease responsible for processing Okazaki fragments. Furthermore, hDna2-depleted cells did not display defects in this process, suggesting that if hDna2 is indeed responsible for processing long flaps, they form in a minority of Okazaki fragments.

Furthermore, our results that FEN1-Dna2 co-depletion did not result in significant maturation rate differences compared to FEN1 depletion alone, indicate that hDna2 is unable to compensate for FEN1 depletion and suggest that flaps do not become long in the absence of FEN1. In yeast, other nucleases such as RNAseH2 or Exo1 can compensate for FEN1 loss by processing short flaps, suggesting that they their function could be conserved in humans (35) (27) (48). However, these results are intriguing because hDna2-depleted cells display more DNA damage than FEN1-depleted cells alone (H2AX levels **Figure 3.5B**), suggesting that the few unprocessed long flaps left in the absence of hDna2 are extremely dangerous to the cell, and DNA repair pathways are unable to efficiently resolve them. Alternatively, these findings may also suggest that hDna2 participates in additional DNA replication or replication/repair processes that remain to be elucidated.

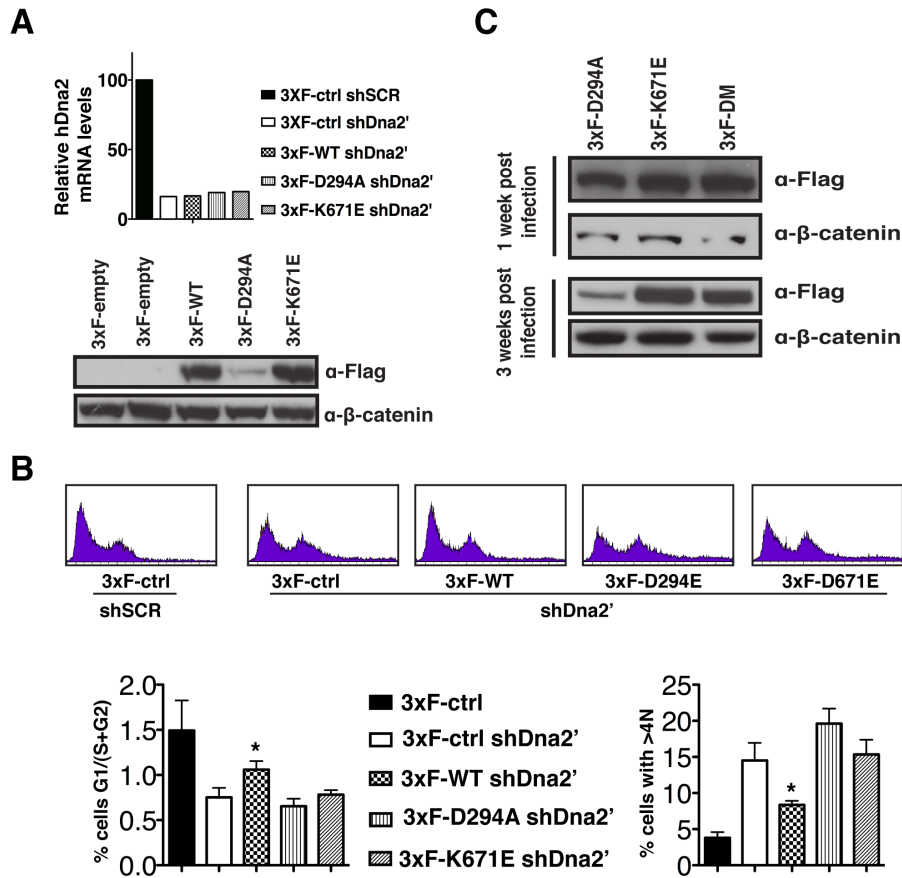
### **Acknowledgements**

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### Figure 3.1 hDna2 contributes to genomic maintenance

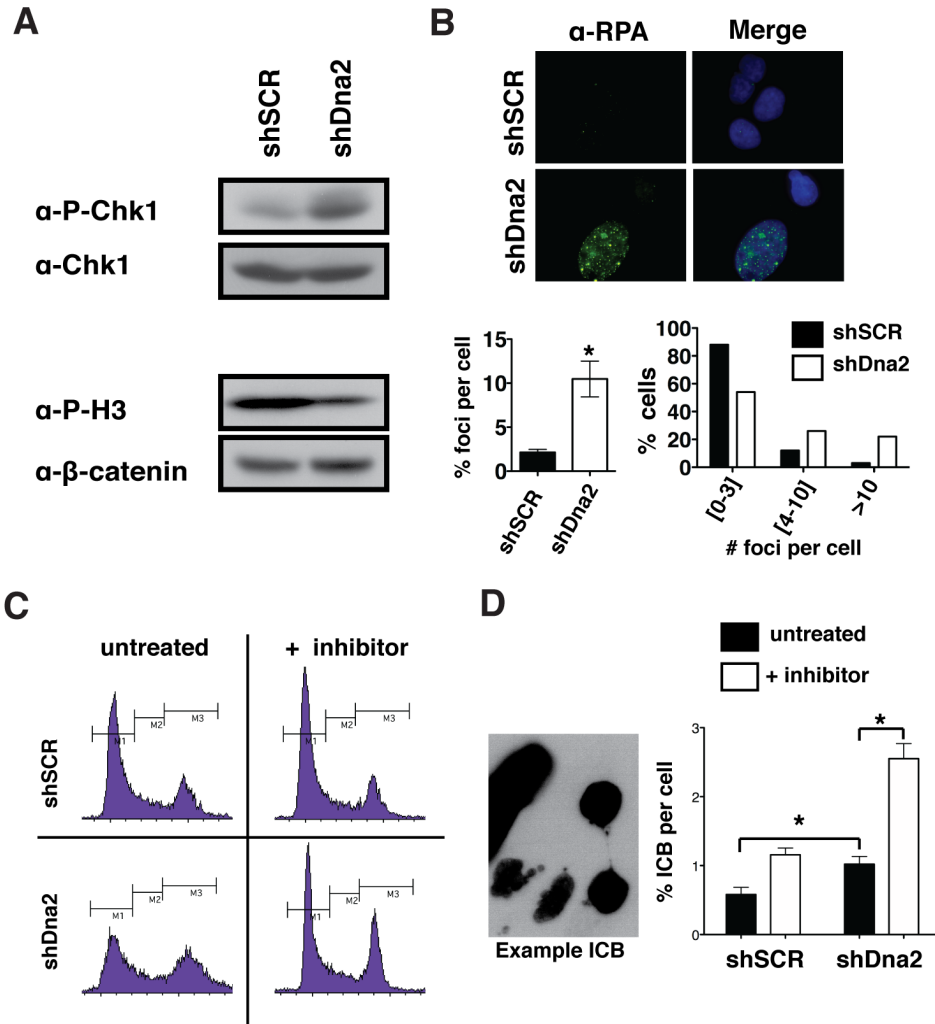
**A.** Knockdown of hDna2 in U2OS cells determined by quantitative RT-PCR. Results of two independent hairpins (shDna2 & shDna2') are normalized to shSCR control cell line. **B.** hDna2 knock down determined by immunoblot analysis probed with a specific antibody against hDna2.  $\gamma$ -Actin is used as a loading control. **C.** Relative micronuclei counts and representative images depicting micronuclei (50) following hDna2 depletion in U2OS cells. Results were normalized to shSCR cells. Error bars represent standard error and \* denotes  $p < 0.01$  compared to shSCR cells. **D.** Representative image of immunofluorescence staining of  $\gamma$ -H2AX and phosphorylated-ATM in U2OS cells infected with a short hairpin targeting hDna2 (shDna2, bottom panel) versus a control hairpin (shSCR, top panel). **E.** Immunoblot analysis of control (shSCR) versus hDna2-depleted cells (shDna2 and shDna2'). Lysates were probed for hDna2 and  $\gamma$ -H2AX. Loading was controlled with  $\beta$ -catenin and  $\gamma$ -actin.



**Figure 3.2 hDna2's nuclease and helicase activities are essential to maintain genomic stability**

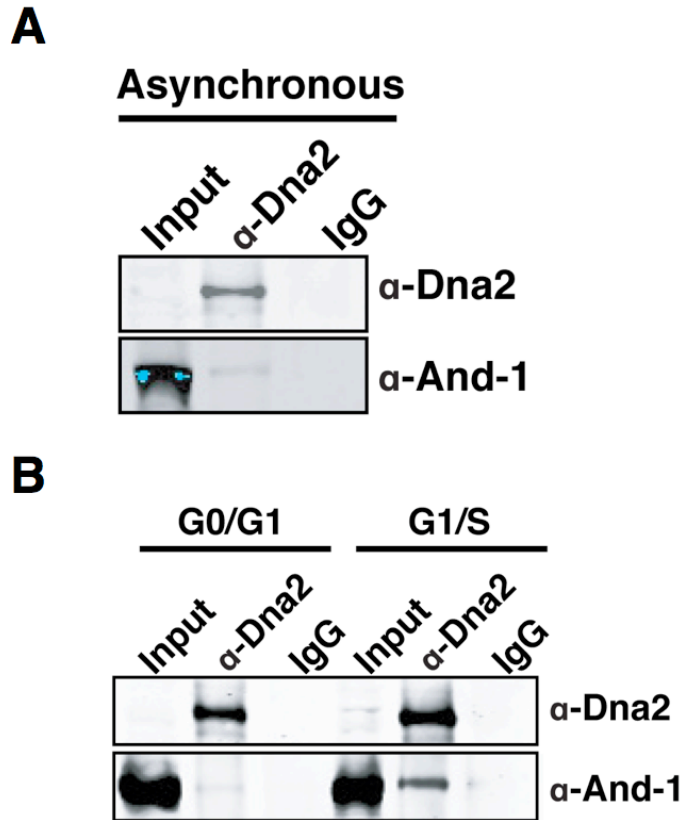
**A.** Relative knockdown of hDna2 in U2OS cells determined by quantitative RT-PCR (top panel). Results are normalized to the 3xF-ctrl shSCR control cells. Specific RT-PCR primers were designed to only detect the endogenous hDna2. The bottom panel is an immunoblot probed with a FLAG antibody to detect overexpressed cDNA constructs. Note that the 3xF-D294E (nuclease deficient) protein expresses at lower levels compared to the 3xF-WT or 3xF-K671E (helicase deficient) **B.** Flow cytometric analysis of the cell cycle and DNA content of the same U2OS cells. Cell count is plotted on the y-axis while DNA content is on the x-axis. Quantification of 4 independent experiments are quantified in the 2 bar graphs. The left graph represents the ration between the percent of cells in G1 versus the percent of cells in S+G2. In the right graph is depicted the same 4 independent experiments quantified for the percent of aneuploid cells containing abnormally high DNA content (>4N). Error bars represent standard errors of the 4 independent experiments and \* denotes  $p < 0.05$  compared to 3xF-ctrl shDna2'. **C.** Western blot analysis of 3xF-D294A, 3xF-K671E, and 3xF-DM (double mutant-helicase/nuclease deficient) expressing U2OS cells. Western blots were conducted on lysates prepared 1 week and 3 weeks post-infection. Note that the 3xF-D294A expression goes down with respect to the 3xF-K671E and 3xF-DM.





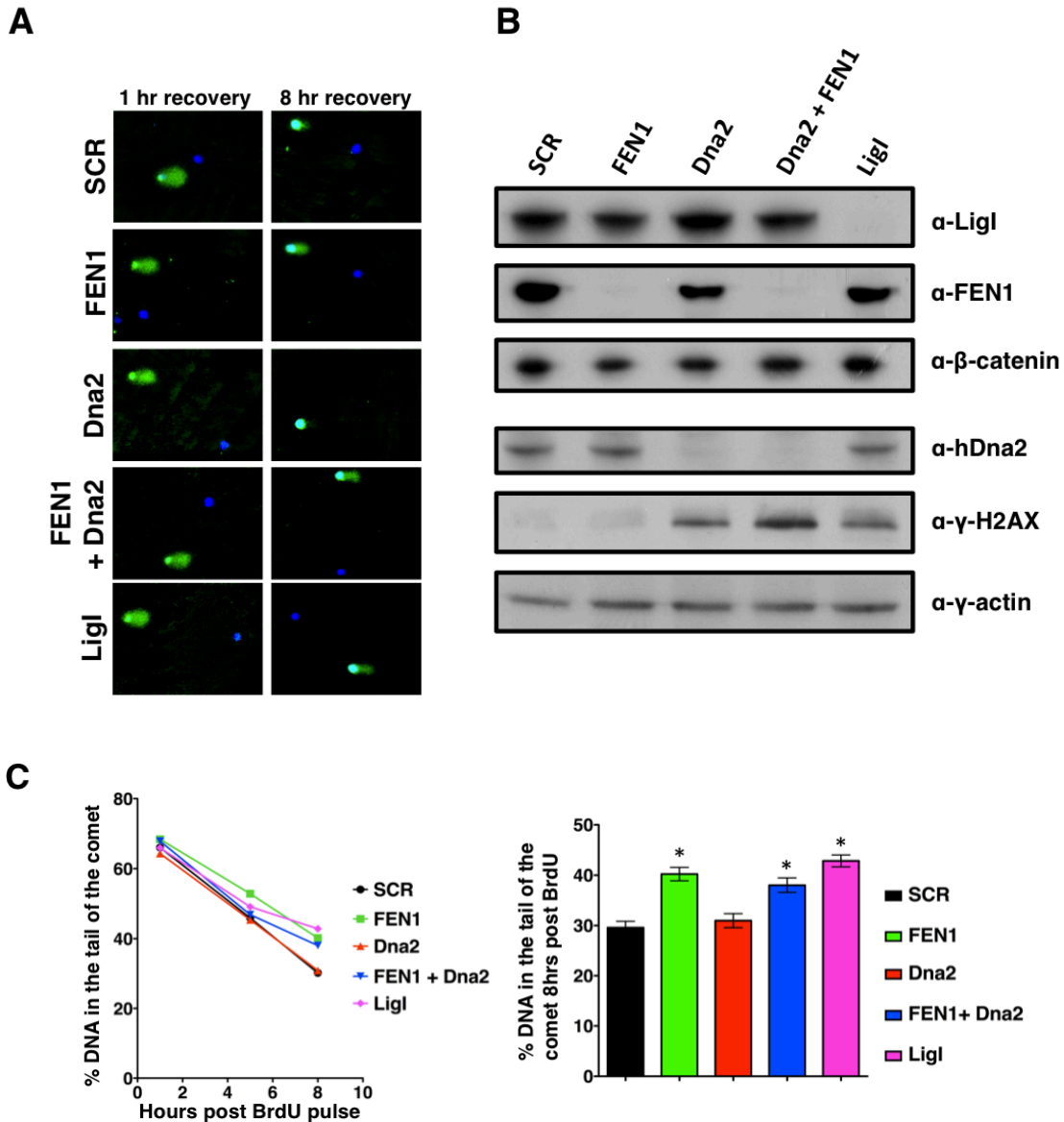
**Figure 3.3 hDna2 depletion leads to the activation of the replication checkpoint**

**A.** Immunoblot analysis of control (shSCR) and hDna2-depleted (shDna2) U2OS cells. Whole cell lysates were probed for total versus phosphorylated Chk1 (serine 317) (top panels). Samples were also probed for phosphorylated-Histone H3 (serine 10); β-catenin is used as a loading control. **B.** Representative image of immunofluorescence staining of RPA-70 in U2OS shSCR and shDna2 cells (top images). Cells were pre-extracted before fixing according to Shi *et al.* (45). On the bottom are shown the quantification of RPA foci per cells. \* denotes  $p < 0.01$ . **C.** Flow activated cytometric analysis of the cell cycle and DNA content of shSCR and shDna2 untreated U2OS cells or treated with a Chk1 inhibitor. **D.** Control (shSCR) or hDna2-depleted (shDna2) U2OS cells +/- Chk1 inhibitor were quantified for inter-nuclei chromatin bridge (ICBs) (31) (right histogram). Representative image of hDna2-depleted cells + Chk1 inhibitor is shown on the left. \* denotes  $p < 0.01$ .



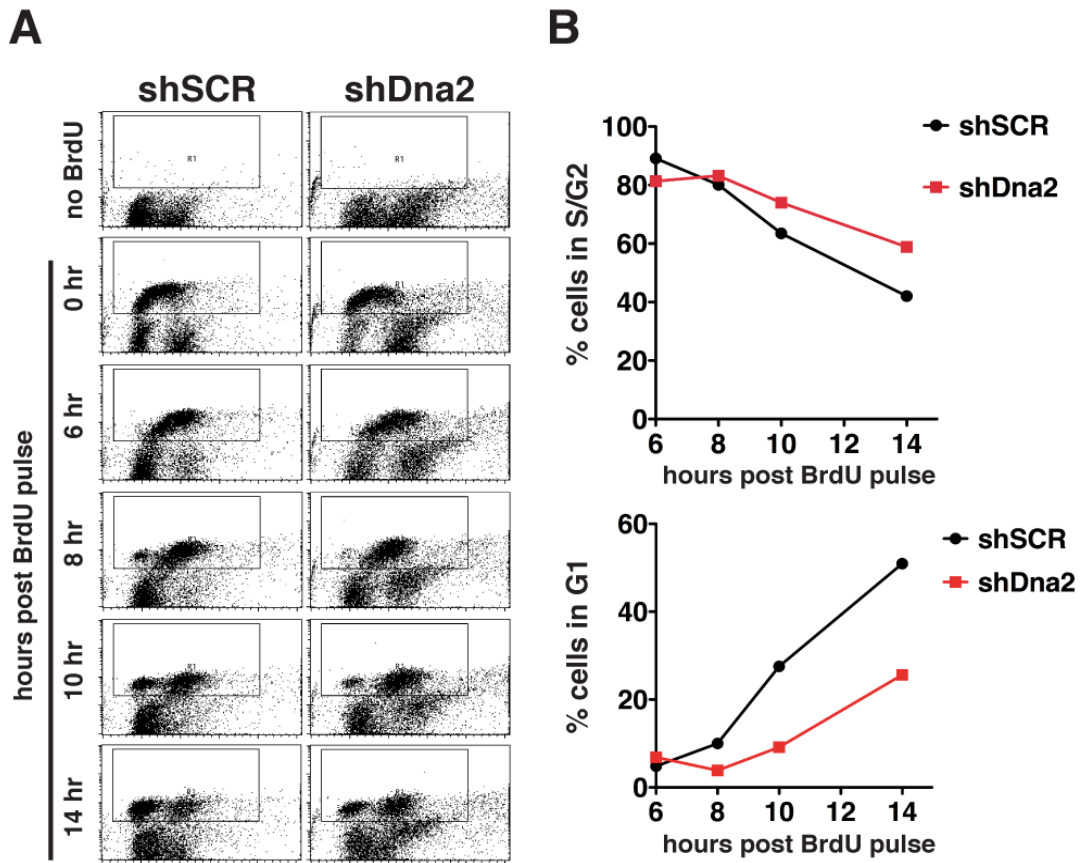
**Figure 3.4 hDna2 interacts with And-1 in a replication dependent manner**

**A.** Interaction between hDna2 and And-1 in asynchronous cells. hDna2 was immunoprecipitated from asynchronous HeLa cells followed by western blot analysis probing for And-1 or hDna2 as indicated. **B.** Interaction between hDna2 and And-1 occurs during G<sub>1</sub>/S transition. HeLa cells were arrested in G<sub>0</sub>/G<sub>1</sub> by serum starvation or during G<sub>1</sub>/S by double thymidine block. hDna2 was immunoprecipitated from the arrested cells and western blots were performed to probe for And-1 or hDna2.



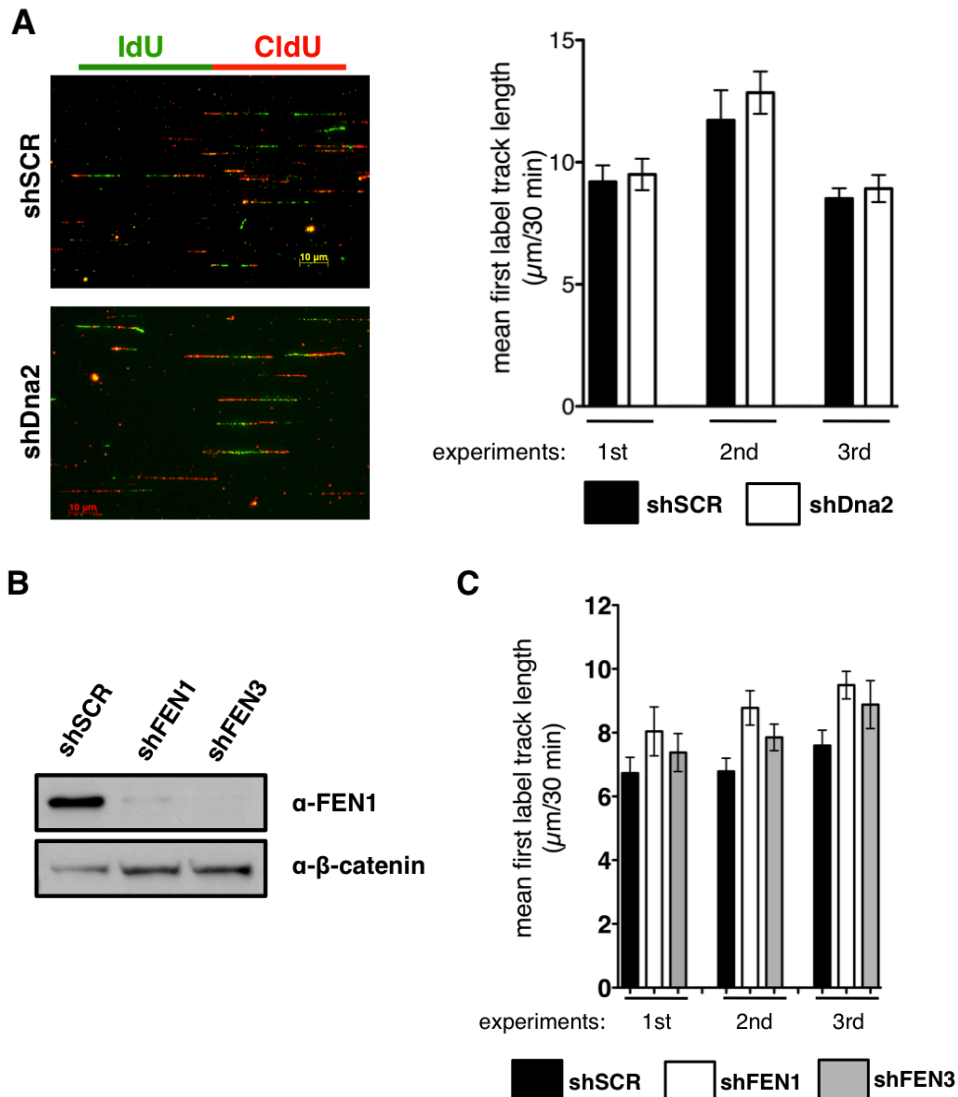
**Figure 3.5 hDna2 depletion does not lead to detectable defects in the maturation of newly synthesized DNA**

BrdU comet analysis of U2OS cells depleted of FEN1, hDna2, LigI or hDna2 + FEN1. Cells were pulsed for 15 min with BrdU and chased for 1, 5 or 8 hours prior to comet processing. **A.** Representative images of the different samples at 1 and 8 hours post BrdU pulse. Cells were immunostained against BrdU (green) or DAPI (blue) **B.** Immunoblot analysis of the different cell lines probed for LigI, FEN1, hDna2, and  $\gamma$ -H2AX.  $\gamma$ -actin was used as a loading control. **C.** Quantification of the BrdU comets at 3 different time points (1, 5 and 8 hours post BrdU). The percent DNA in the tail versus the head of the comet is plotted on the y-axis; the hours post BrdU pulse on the x-axis. Results are based on the analysis of 140 to 180 comets analysed in a total of three independent experiments. On the right is shown the quantification of the 8 hours post BrdU pulse. The error bars correspond to the standard error of the mean and \* denotes  $p < 0.01$  comparing to shSCR.



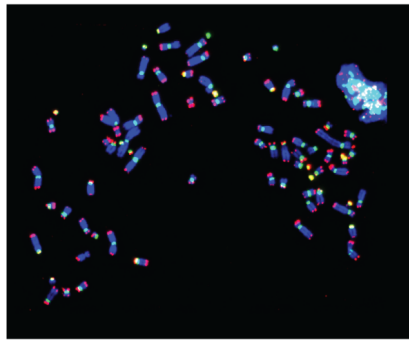
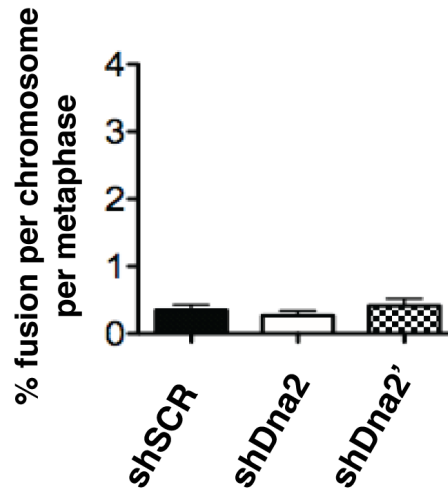
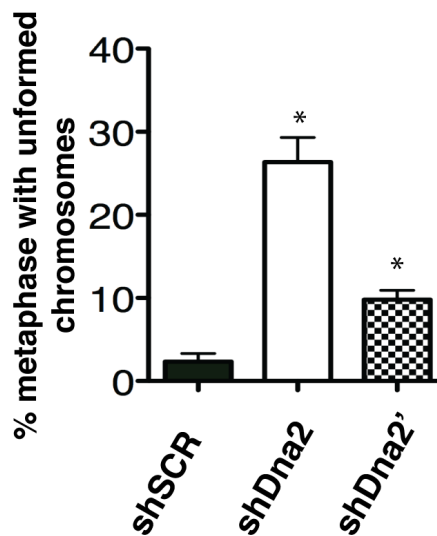
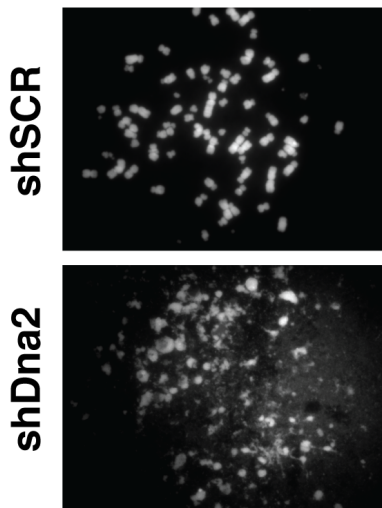
**Figure 3.6 hDna2 depletion slows cell cycle progression in late S/G2 phase**

**A.** Progression of cells through the different phases of the cell cycle. U2OS cells expressing shSCR or shDna2 were pulsed with BrdU for one hour and analyzed at the indicated times by FACS using the anti-BrdU antibody (FITC-conjugated) (*y*-axis) or propidium iodide to mark DNA content (*x*-axis). **B.** Quantification of the number of BrdU-positive cells in S/G2 phase (top graph) or BrdU-positive cells appearing in G1 (bottom graph). This experiment was conducted twice and a representative experiment is shown.



**Figure 3.7 Defects in Okazaki fragment processing do not impact replication fork progression**

**A.** U2OS shSCR or U2OS shDna2 cells were labeled consecutively with IdU (green) and then with CldU (red) for 30 min each prior to isolating and stretching DNA for immunostaining. Representative images of replication tracks in control (shSCR) or hDna2-depleted cells are shown on the left. Quantification of three independent experiments is shown on the right. The bar graph summarizes mean lengths of first-label segments labeled for 30 min in two-segment tracks to ensure that fork rate measurements were made from active replication forks. Error bars show 95% confidence intervals for sample means. **B.** Immunoblot analysis of U2OS cells depleted of FEN1 (shFEN1 or shFEN3) compared to a control cell line (shSCR). Whole cell lysates were probed for FEN1 or  $\beta$ -catenin for loading control. **C.** Quantification of three independent experiments summarizing mean track length of the first label in U2OS cells depleted of FEN1 (shFEN1 or shFEN3) versus a control cell (shSCR). Note that the shFEN1 tracks are slightly longer than shSCR tracks.

**A****Example of metaphase****B**

**Supplementary Figure 3.1 Interchromatin bridges that form upon hDna2 depletion are not the result of telomere fusions**

**A.** Quantification of chromosomes end-to-end fusion following shRNA expression against hDna2 (shDna2 or shDna2') or control (shSCR) in U2OS cells. 80 metaphases, from two independent experiments were quantified in a blinded fashion. On the left is a representative metaphase showing chromosomes in blue (DAPI) telomeres in red (PNA-FISH probe) and centromeres in green (PNA-FISH probe). **B.** Quantification of metaphases with pulverized chromosomes. On the left is shown representative metaphases of control cells (shSCR) or hDna2-depleted cells (shDna2) with pulverized chromosomes. 200 metaphases in 2 independent experiments are quantified on the right bar graph. Error bars represent standard error of the mean and \* denotes  $p < 0.01$  compared to shSCR.

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## **Chapter 4: Conclusions, Work in Progress, and Future Directions**

## 4.1 Summary

Genomic maintenance is a complicated task that requires coordination of different processes such as replication, repair, transcription and cell cycle progression. In addition to nuclear DNA maintenance, mitochondrial DNA (mtDNA) stability is also essential, and mutations in mtDNA maintenance genes lead to a variety of different human diseases. Given the importance of maintaining mitochondrial and nuclear genomic stability, DNA maintenance proteins have evolved a broad degree of flexibility and are involved in multiple DNA processes both in the nucleus and the mitochondrion. Recently, several studies found that numerous nuclear DNA maintenance proteins can also be found in the mitochondrion suggesting that these distinct organelles possess more similarities than previously appreciated. These findings raise the exciting possibility that the mitochondria could serve as a novel and exciting model for the study of DNA maintenance. In chapter 2, I present a novel addition to the growing list of proteins found in both organelles. I demonstrate that hDna2 localizes to the nucleus and mitochondria, and that it colocalizes with a subfraction of DNA-containing mitochondrial nucleoids in unperturbed cells. Upon expression of disease-associated Twinkle mutants known to induce replication fork stalling, hDna2 accumulates within nucleoids, suggesting that it participates in mtDNA maintenance. In accordance with these observations, RNA interference-mediated depletion of hDna2 leads to a decrease in mtDNA replication intermediates and inefficient repair of damaged mtDNA. These observations demonstrate that hDna2 participates in the maintenance of mitochondrial DNA. Given the essential role Dna2 plays in the nucleus in yeast, I investigate the role of hDna2 in nuclear DNA maintenance in chapter 3. RNAi-directed depletion of hDna2 leads to replication-

dependent damage that arrests cells in late S/G2 in a Chk1 dependent manner. Bypass of the checkpoint by treatment with a Chk1 inhibitor allows cells to proceed through mitosis with incomplete DNA replication leading to aberrant mitotic structures (micronuclei and inter-nuclei chromatin bridges). Furthermore, work that I present in chapter 3 demonstrates that hDna2 interacts with a member of the replisome, And-1, in a replication dependent manner, suggesting that hDna2 participates in Okazaki fragment maturation like its yeast homologue. Supporting this hypothesis, I demonstrate that the nuclease and the helicase activities of hDna2 are essential to maintain genomic stability, supporting a model established in yeast wherein both activities are coupled to efficiently process long flaps that arise during Okazaki fragment maturation. Finally, I demonstrate that depletion of FEN1 or hDna2 does not affect replication fork velocity, suggesting that unligated Okazaki fragments that arise behind replication forks do not impact fork progression. Below, I discuss the implications of my thesis work in the context of mitochondrial and nuclear DNA replication. Additionally, I deliberate on the outstanding questions and present preliminary studies suggestive of additional unexpected functions of hDna2 in DNA replication.

#### **4.2 hDna2 and mitochondrial DNA repair and replication**

While the mechanisms that direct mitochondrial DNA repair and replication were unknown for several decades, recent research has shown that several nuclear DNA maintenance proteins are also found in the mitochondria. These discoveries suggest that the mechanisms of mitochondrial and genomic DNA maintenance have evolved in concert, presenting the mitochondria as a prospective tool for the study of genomic

replication and repair (19). Indeed, FEN1, RNaseH1, PIF1, DNA ligase III, APE1, BRCA1, OGG1, MYH, UNG1/2, and hTERT are all DNA maintenance proteins found in both organelles (**Table 1.2**) (27) (24) (14) (11) (9) (25) (38). The work presented here demonstrates that hDna2 is a novel addition to this growing list.

While mitochondria were historically thought to repair their damaged DNA exclusively by single nucleotide base excision repair (SN-BER) (10), recent work using highly purified HeLa mitochondrial extracts observed the generation and removal of 5' DNA flaps that are a hallmark of long patch base excision repair (LP-BER) (1). Using similar mitochondrial extracts, it was later demonstrated that FEN1 participates in the removal of these flaps (27). However, FEN1 nuclease activity is not essential, and long flap processing activity was still detected in the absence of FEN1 suggesting the involvement of an additional nuclease (41) (27). My observations that hDna2 colocalized with mitochondria and mtDNA suggested that the additional nuclease could be hDna2. Indeed, biochemical studies performed with hDna2 had demonstrated its ability to process DNA flaps (23) (30). Furthermore, my observations that hDna2-depleted cells inefficiently repair mtDNA upon hydrogen peroxide treatment indicated that hDna2 participates in mtDNA repair, most likely through a role in LP-BER. These observations were confirmed by a different study, which demonstrated that hDna2 associates with DNA polymerase  $\gamma$  (Poly) and stimulates its primer extension activity (44). Importantly, mitochondrial hDna2 cooperates with mitochondrial FEN1 to process long flaps into ligatable nicks sealed by DNA ligase III. These experiments recapitulate the two-step model of Okazaki fragment processing (**Figure 1.3**) and suggest that mechanisms of flap removal are conserved between the nucleus and the mitochondrion. Additionally, these



observations raise the possibility that flap-removal processes also participate in the replication of mtDNA. While current models of mtDNA replication are yet to be fully elucidated, recent experiments have demonstrated the coexistence of a strand-displacement model (SDM) and a strand-coupled bidirectional model for replicating mtDNA (7) (19). The known function of hDna2 in removing long RNA-DNA flaps could be relevant to both models. Interestingly, in chapter 2, I demonstrate that hDna2-depleted cells have reduced mtDNA replication intermediates, suggesting that hDna2 actively participates in mtDNA replication. However, this reduction is difficult to reconcile with the known function of hDna2 removing long flaps, but rather suggests that mitochondrial replication initiation is inhibited in hDna2-depleted cells. This suggests that hDna2 depletion creates mtDNA-specific damage that inhibits mtDNA origin firing. Alternatively, it may also suggest that hDna2 plays an essential role in origin firing (see work in progress **section 4.4**). Elucidating which of hDna2's enzymatic activities (helicase and/or nuclease) is necessary for efficient mtDNA replication will provide a better understanding of its molecular mechanisms acting on mtDNA replication and repair. In addition, chromatin immunoprecipitation (ChIP) analysis would reveal whether it specifically localizes to mtDNA origins.

### **4.3 hDna2 and nuclear DNA replication**

Although Zheng *et al.* argued for an exclusive role of hDna2 in mitochondrial DNA maintenance, work presented in chapter 2 and 3 demonstrates that hDna2 is also found in the nucleus and participates in nuclear DNA replication like its yeast homologues. Using highly purified nuclear fraction we demonstrate that hDna2 is found

inside the nucleus where it interacts with the replication factor And-1 (Ctf4 in yeast) in a replication dependent manner. Ctf4/And-1, originally found to interact with pol  $\alpha$  in *Saccharomyces cerevesiae*, was recently reported to interact with MCM10 and pol  $\alpha$  in yeast and human (45) (21). This report further demonstrated that MCM10-And-1 interaction is required for the loading of DNA pol  $\alpha$  onto chromatin and subsequent DNA synthesis (45). This indicates that And-1 is essential for the recruitment of pol  $\alpha$  primase to the replication origins and subsequent origin firing. In addition, because pol  $\alpha$  is responsible for laying down multiple primers throughout the lagging strand template, its interaction with MCM10 and And-1 ensures that the lagging strand elongation complex is coupled to the replicative helicase complex MCM2-7 moving in front of the replication fork. Our finding that hDna2 interacts with And-1 in replicating cells is in accordance with previous studies performed in *Xenopus laevi* (43). Along with the accepted function of Dna2 in lagging strand processing, this finding suggests that hDna2 is recruited to the replication fork by And-1 to process long RNA-DNA flaps that arise during Okazaki fragment maturation.

While FEN1 is known as the primary flap endonuclease acting on Okazaki fragments (one-step model), a few of these flaps can escape FEN1 cleavage and require Dna2's nuclease and helicase activities to be processed (two-step model) (4) (3) (22) (**Figure 1.3**). Our genetic experiments presented in chapter 3 demonstrate that both enzymatic activities of hDna2 are essential for maintaining genomic stability and suggest that these activities are coupled to act on long DNA flaps. These results are in accordance with previous yeast studies that demonstrated the helicase activity of Dna2 is important for the nuclease to efficiently process long flaps that form secondary structures (5), and

suggest that these long flaps do occur *in vivo*. While the one-step model (FEN1 only) and the two-step model (Dna2 and FEN1) have been extensively studied *in vitro*, it remains elusive when, where and why long flaps form *in vivo*. Pol  $\alpha$  is a low fidelity polymerase that lacks exonuclease activity. It synthesizes the original 20-30 RNA/DNA nucleotides of each Okazaki fragment before handing off the synthesis to the high fidelity polymerase  $\delta$  (pol  $\delta$ ). A recent report proposed that strand displacement synthesis by pol  $\delta$  and long flap formation allows for removal of mismatched base pairs that are laid down by pol  $\alpha$  (6). Therefore, promoting long flap formation would ensure a higher fidelity of DNA replication. Interestingly, both FEN1 and hDna2 are acetylated *in vivo* by p300, and while acetylation of FEN1 inhibits its endonuclease activity, it stimulates hDna2's activity on long flaps (18) (13) (6). p300 acetyltransferase activity on FEN1 and hDna2 could be critical in active transcribed chromatin regions to ensure complete removal of RNA/DNA primers thus protecting genetic information. In accordance with this model, in my hands, overexpression of FEN1 does not rescue the cell cycle defects observed in hDna2-depleted cells (**Figure 4.1A & 4.1B**). This observation suggests that FEN1 is inhibited in specific regions of the genome, and long flaps form independently of the amount of FEN1 residing in the cell. Overexpressing a FEN1 mutant that cannot be acetylated by p300 in hDna2-depleted cells would address whether this model holds true. Alternatively, highly repetitive regions, as such as those found in the rDNA locus or telomeres, have the propensity to form flaps with secondary structures that could inhibit FEN1 endonuclease activity. In addition, repetitive sequences enhance polymerase slippage, which can stimulate pol  $\delta$  strand displacement activity and longer flap formation. In any case, a detailed analysis of the location of the DNA damage observed

in hDna2-depleted cells would address whether specific regions of the genome are prone to long flap formation and would greatly enhance our understanding of the different mechanisms of Okazaki fragment processing.

My observations that maturation of nascent DNA occurs with slower kinetics in FEN1-depleted cells confirm that the one-step model is the primary mechanism of flap removal during Okazaki fragment maturation in human cells. Furthermore, the observations that hDna2-depleted cells do not display detectable defects in this process suggest that the two-step model is utilized in a minority of Okazaki fragments. Lastly, FEN1-Dna2 co-depletion do not present significant rate differences compared to FEN1-depleted cells, indicating that hDna2 is not able to compensate for FEN1 depletion and that flaps do not become long in the absence of FEN1. In yeast, other nucleases such as RNAseH2 or Exo1 can compensate for FEN1 loss by processing short flaps (31) (26) (40). My results suggest that their function is conserved in humans. However, these results are intriguing because hDna2-depleted cells display more DNA damage and cell cycle abnormalities than FEN1-depleted cells alone (H2AX levels **Figure 3.5B**), suggesting that the few unprocessed long flaps left in the absence of hDna2 are extremely dangerous to the cell and repair processes are inefficiently dealing with them. Alternatively, these findings may also suggest that hDna2 participates in additional DNA replication or replication/repair processes independently of its Okazaki fragment function.

#### 4.4 hDna2's additional functions in genome maintenance: work in progress

Several reports have demonstrated that Dna2 functions in DNA double strand break (DSB) repair and telomere maintenance by participating in resection of the 5' end (46). While *in vitro* studies suggest that these mechanisms are conserved in humans, several observations support that defects in these pathways are not responsible for the genomic instability observed in hDna2-depleted cells. First, yeast studies *in vivo* as well as human studies *in vitro* demonstrated that another nuclease, Exo1, compensates for loss of hDna2's nuclease activity (46) (33) (8) (32). Therefore, to observe defects in 5' end resection and homologous recombination, both Exo1 and Dna2 need to be deleted. We have addressed hDna2's role in homologous recombination by measuring GFP fluorescence upon specific introduction of DSBs by I-SceI endonuclease as previously described (35) (**Figure 4.2A**). hDna2-depleted cells were more efficient than control cells in repairing DSBs by homologous recombination (**Figure 4.2B**). These results are in accordance with our analysis of metaphases from hDna2-depleted-cells that show a small increase in sister chromatid exchange (SCE) versus control cells (**Figure 4.2C**). While these observations are preliminary and further work is needed to address whether hDna2 participates in 5' end resection, they support observations in yeast and suggest that Exo1 compensates for hDna2's nuclease activity in this process. Furthermore, while Dna2's helicase activity is dispensable for 5' end resection, our results indicate that it is essential to maintain genomic stability. Together, these observations do not negate a role for hDna2 in 5' end resection, but suggest that defects in this process are not responsible for the genomic instability observed in hDna2-depleted cells.

Interesting work done in *Xenopus laevis* suggests that Dna2 also participates in early steps of DNA replication. Immunodepletion of xDna2 in interphase egg extracts inhibited DNA replication (28), hinting that Dna2 is also involved in the initiation of DNA replication through its interaction with And-1. Our results demonstrating that hDna2 interacts with And-1 in a replication dependent manner suggests that hDna2 is involved in early processes of DNA replication. Interestingly, using DNA micro-fluidic-assisted replication track analysis (maRTA) one can also determine the percent of DNA origin firing events present among all tracks labeled with CldU and IdU. Our preliminary data suggests that origin firing is reduced in hDna2-depleted cells (**Figure 4.3A**). While Chk1 activation is known to block origin firing, these results may also suggest that hDna2 actively participates in origin firing. Further experiments +/- a Chk1 inhibitor will address whether hDna2 actively participates in origin firing.

Supporting a putative role of hDna2 in early fork firing, preliminary findings demonstrate that hDna2 localizes specifically to an origin of replication. We performed chromatin immunoprecipitation (ChIP) followed by real-time quantitative PCR with primers that specifically amplify the Lamin B2 (B48) origin and compared it with an adjacent region approximately 5kb downstream that does not contain the origin (B13) (**Figure 4.3B**) (42). With an antibody against hDna2, origin DNA was enriched 3 fold over IgG control, while no enrichment was detected for the control DNA region. Furthermore, this enrichment at the origin was specific to hDna2; no enrichment over IgG control was observed in hDna2-depleted cells (**Figure 4.3C**). While further experiments are needed using different human origins to confirm the genuine association of hDna2 with DNA origins, these preliminary results confirm that hDna2 is an integral member of

the replisome and further suggest that it participates in early replication processes. Interestingly, while no nuclease has yet been demonstrated to participate in origin firing, over-replication of short DNA regions was recently observed during S phase at the origin sites in human cells (17). These re-replicated fragments are double-stranded 100-200 bp long DNA molecules with 5' attached RNA primers. Notably, their synthesis is dependent on replication, and they are generated upon firing of specific origins, suggesting that they correspond to abortive DNA replication products. These unexpected findings raise intriguing questions about the mechanism of origin firing and suggest that additional unknown factors participate in the clearing or production of over-replicated DNA to enable replication elongation. Re-replicated short DNA fragments could be generated by repeated extrusion of newly synthesized DNA strands by fork reversal, or by repeated re-initiation resulting in replication forks running into previous ones (16). Interestingly, while only two MCM2-7 complexes should be needed to initiate replication in a bidirectional manner, between 5 and 40 of these complexes bind to each replication origin and could account for multiple firing events occurring at each origin (34) (2). hDna2 could play a role in origin firing by clearing the way for newly fired origins to correctly elongate. This hypothesis is further supported by our mitochondrial 2D replication analysis that shows a reduction of replication intermediates, suggesting that mitochondrial replication initiation is inhibited in hDna2-depleted cells. Interestingly, mtDNA molecules exhibit a short triple-stranded region called the D-loop, which spans a major part of the non-coding region of mtDNA (NCR) and colocalizes with the O<sub>H</sub> origin (also known as 7S DNA). As observed in genomic origin regions, 7S DNA is found in over-abundance with respect to the mitochondrial genome, suggesting that it is the

product of aborted replication intermediates (15) (20). The ability of hDna2 to cleave long RNA-DNA flaps could allow abortive replication forks to be extruded from the DNA to allow a new replication fork to fire and elongate properly. Therefore, it is exciting to postulate that in addition to its role in processing long flaps, hDna2 participates in DNA replication by promoting origin firing both in the nucleus and the mitochondrion.

#### **4.5 Conclusions**

Originally believed to be an independent prokaryotic organism, mitochondria were engulfed in a symbiotic manner to form the first eukaryotic cells (29). Like its neighbor the nucleus, mitochondria contain their own DNA. These separated organelles have concurrently evolved and present today notable similarities in the maintenance of their respective genomes. The work presented here demonstrates that hDna2 is a novel addition to the growing list of proteins that participate in both nuclear and mtDNA maintenance. These results further support the notion that mechanisms governing DNA maintenance in these distinct organelles have evolved in concert, presenting the mitochondrion as an attractive tool for the study of DNA replication and/or repair processes. Furthermore, this work increases our understanding of the molecular mechanisms that ensure high fidelity replication and provides new avenues in our quest to understand human diseases caused by mutations in DNA replication genes.



## **Material and methods**

### **FEN1 complementary experiment**

For adenovirus production, FEN1 cDNA was cloned into the pShuttle vector (Stratagene, La Jolla, CA) at the EcoRV site (37). After subcloning, the FEN1 or GFP cDNAs were recombined into the pAdEasy-1 plasmid (Stratagene), and the resultant DNA was transfected into HEK293 cells to produce infectious adenovirus. Adenovirus production and concentration were carried out according to the manufacturer's protocol using the AdEasy XL Adenoviral Vector System (Stratagene). Adenovirus was titered before use with the AdEasy Viral Titer kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

Viral production and infections were carried out as described previously (36) (12). U2OS cells were infected with pLKO.1 shSCR or pLKO.1 shDna2 lentiviruses for 5 hours. Cells were then lifted and counted and seeded in the presence of media containing Adeno-FEN1 or Adeno-GFP. 48 hours post infection, cells were lifted and plated at  $0.8 \times 10^6$  cells per 10-cm plate. 24 to 48 hours later, cells were lifted and stained with hypotonic propidium iodide, and subjected to FACS analysis to determine DNA content as previously described (12).

### **DR-GFP homologous recombination assay**

U2OS DR-GFP cells were stably infected with pLKO.1 shSCR or pLKO.1 shDna2 lentiviruses. 48 hours post infection, cells lifted and plated with media containing adeno-I-SceI. 48 hours post adenovirus infection, GFP expressing cells were detected by FACS analysis. 10 000 events were quantified for U2OS infected with I-SceI while 100 000

events for – I-SceI control cells.

### **Sister chromatid Exchange analysis**

hDna2-depleted cells (shDna2 or shDna2') or control cells (shSCR) were grown for 2 replication doubling in the presence of BrdU at 10 µg/ml (36 hours). Metaphases were prepared as previously described and dropped on microscope slides. Metaphases were dried a room temperature for 2 days and then stained in 50 ml PBS containing 10 µl of 5 mg/ml of Hoechst 33258 (Sigma, St Louis, Mo) for 10 minutes at room temperature. Metaphases were then washed in Gurr's buffer for 1 minute and mounted in Gurr's buffer and visualized by microscopy using a 100X magnification lens.

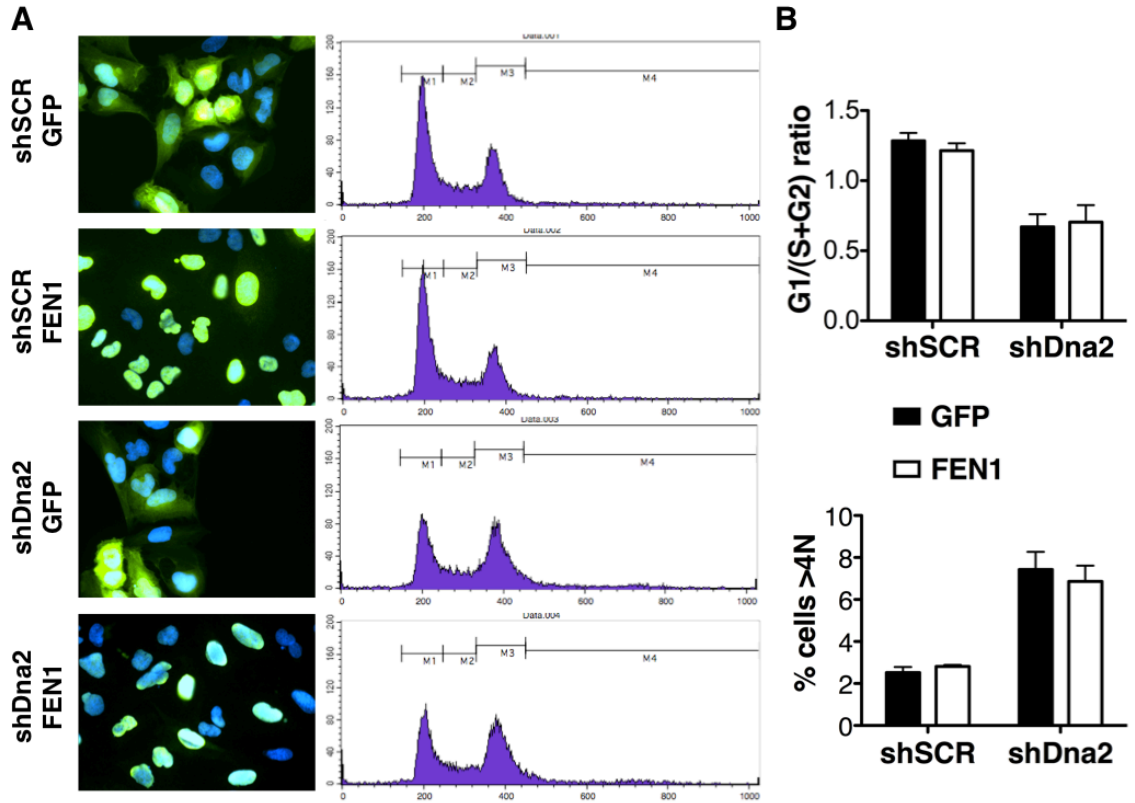
### **Microfluidic-assisted replication track analysis**

Origin firing frequency were determined by using a recently described microfluidic-assisted replication track analysis protocol (maRTA) (39). In brief, Dna2-depleted (shDna2 or shDna2') or control (shSCR) U2OS cells were labeled for 30 min each with 50 µM IdU followed by 50 µM CldU and then collected by trypsinization and used to prepare agarose plugs as previously described. High-molecular-weight DNA was isolated from cells embedded in agarose by brief heating to 75°C to melt the agarose, followed by agarose digestion. The resulting high-molecular-weight DNA was then loaded by capillary tension into microchannels to uniformly stretch and capture DNA on glass coverslips for immunostaining and fluorescence microscopy (55). Origin firing efficiency was determined by counting the fraction of origin firing events among all active replication events (i.e., ongoing forks and converging forks). Track lengths were measured in digital images of tracks by using the AxioVision software package (Carl

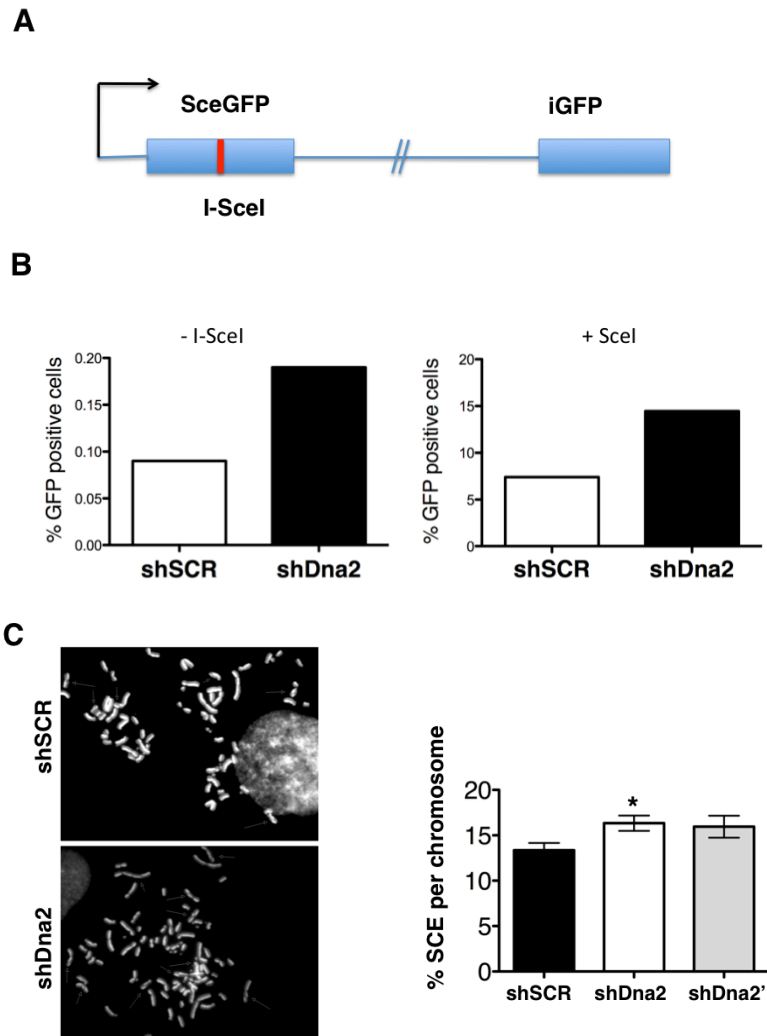
Zeiss). Two replicate samples of shDna2, shDna2' or shSCR cells were analyzed for each determination, where 250 to 450 replication tracks were measured in each sample.

### **Chromatin Immunoprecipitation (ChIP)**

ChIP was conducted as previously described (37). Briefly, U2OS cells were fixed in 1% formaldehyde for 1 hour, washed with PBS and lysed in 1% SDS, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA plus protease inhibitors. Lysates were sonicated, and equal quantities of protein lysates were immunoprecipitated using 8  $\mu$ g of hDna2 antibody (abcam) or control IgG antibody (sigma) and 40  $\mu$ l of Protein A/G-PLUS Sepharose beads (Santa Cruz, sc-2003) that were preblocked as previously mentioned (37). The beads were washed and eluted in 70 mM Tris (pH 8.0), 1 mM EDTA, and 1.5% SDS at 65 °C for 4 hours. The eluate was treated with 40  $\mu$ g of Proteinase K treatment for an hour at 37 °C. DNA was isolated using PCR purification kit (Quiagen) and eluted in 100  $\mu$ l of ddH<sub>2</sub>O. Real-time quantitative PCR was performed using previously described B48 primers amplifying the lamin B origin and non-origin control sequence B13 (42).

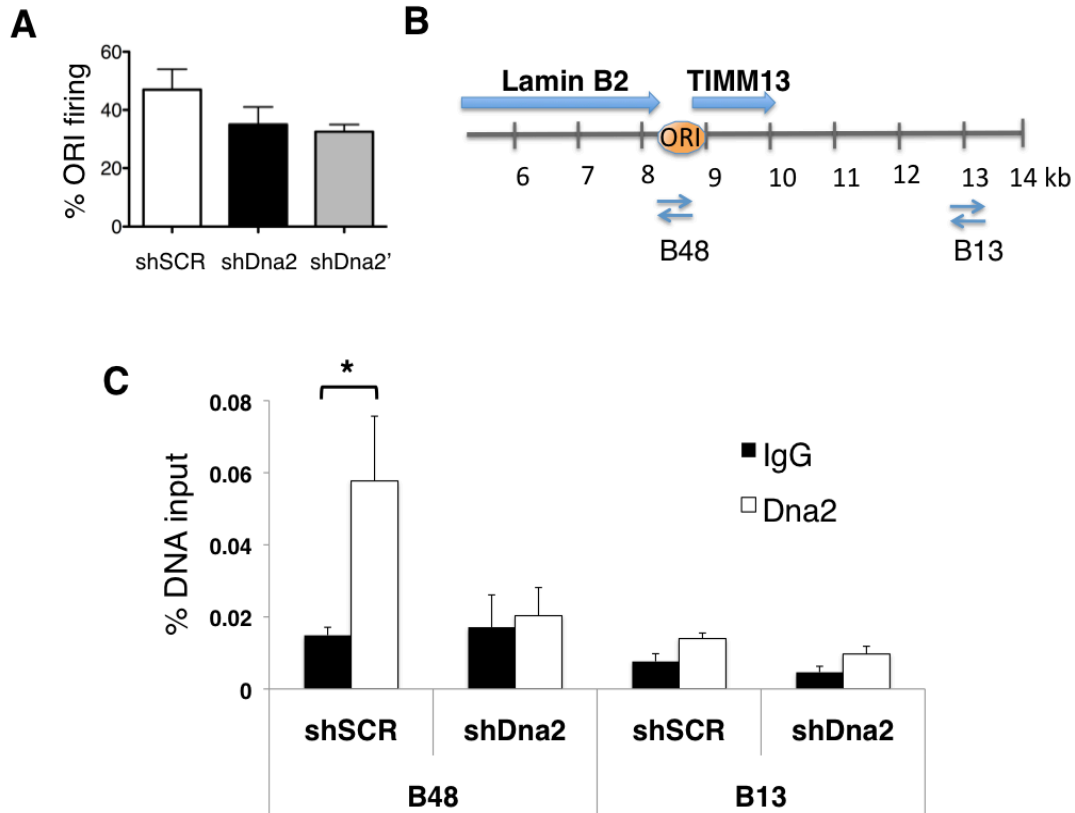


**Figure 4.1 FEN1 overexpression does not compensate for hDna2 depletion**  
**A.** U2OS cells expressing GFP (ctrl) or FEN1 were knocked down of hDna2 by virus based RNAi targeting hDna2 (shDna2) or control (shSCR). Left panels are representative immunofluorescence images of FEN1 or GFP expressing cells. Cells overexpressing FEN1 or GFP were >60% of the population. Right panels represent flow activated cytometric analysis of the cell cycle of the respective cell lines. Cell count is represent on the  $y$ -axis while DNA content on the  $x$ -axis. **B.** Histograms representing quantification of 2 independent experiments. The top histogram represents the ratio of the percent of cells in G1 versus the percent of cells in S+G2. In the bottom histogram is represented the percent of cells containing abnormally high DNA content (>4N). Error bars represent standard errors of the mean between the 2 independent experiments.



**Figure 4.2 hDna2 depletion does not inhibit homologous recombination**

**A.** After expression of I-SceI, U2OS cells containing DR-GFP can repair the DSB through HR and become GFP+. **B.** Histogram representing GFP fluorescence in hDna2-depleted U2OS cells versus control cells (shSCR) +/- I-SceI induction. Note that without I-SceI expression we observe more GFP + cells in hDna2-depleted cells than control cells suggestive of a small hyper-recombination phenotype. Quantification represents a single experiment. **C.** Sister chromatid exchange (SCE) quantification in hDna2-depleted (shDna2 and shDna2') versus control cells (shSCR). On the left are shown representative metaphases for shSCR and shDna2 U2OS cells. On the right the percent SCE/chromosome is plotted for each cell line. 60 metaphases were quantified for shSCR and shDna2 cells while 30 metaphases were quantified for shDna2' cells. \* denotes  $p=0.01$ .



### Figure 4.3 hDna2 specifically localizes to a replication origin

**A.** hDna2 depletion reduces the probability of origin firing in stretched DNA samples. Origin firing events among all tracks labeled were identified as CldU-only (red only) or CldU-IdU-CldU (red-green-red) triple-segment tracks. The mean percentages of new origin firing events defined by these two track types among all labeled tracks are shown for two independent experiments in which 200 to 450 tracks/experiment were analyzed for control shSCR, shDna2 or shDna2'. Error bars show standard deviations of the means between the two experiments **B.** Genomic region containing the lamin B2 origin is shown together with the set of primers used for quantitative real-time PCR analysis. Primer set B48 amplifies the origin while B13 corresponds to a control set of primers 4.5 kb downstream of the origin. **C.** Quantification of three independent ChIPs from U2OS cells using antibodies against hDna2 (white bars) or IgG (ctrl) (black bars). ChIP experiments were done using shSCR cells and shDna2' cells to confirm for the specificity of the antibody. \* denotes  $p=0.07$  using a student t-test.

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