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I. ABSTRACT

ATM (Ataxia telangiectasia mutated) is the protein kinase mutated in the hereditary autosomal disease ataxia telangiectasia and is an important kinase in the DNA damage response following the generation of DNA double-strand breaks (DSBs). Following such lesions, the ATM co-factor NBS1 is crucial for ATM activation, whereas it is dispensable for ATM-mediated signaling following other types of stresses (such as hypotonic stress and DNA alkylation damage).

It has recently been shown that ATM is additionally required to signal low levels of replicative stress. This signaling cascade is required for the recruitment of DNA repair proteins, such as 53BP1, to regions of replicative damage, including fragile sites within the genome. This ATM-mediated pathway therefore shields fragile sites against erosion and degradation.

A novel ATM co-factor, ATMIN (for ATM INteractor), has been found to be required for ATM activation following hypotonic stress and changes in chromatin structure. Here I show that ATMIN is additionally required for ATM activation and substrate phosphorylation following low levels of replicative stress but not after the induction of DSBs. Interestingly, ATMIN is dispensable for ATR-mediated signaling which is known to be the major kinase for signaling replicative stress. As a consequence of defective ATM signaling, I have shown that ATMIN is required for the recruitment of 53BP1 and activated ATM to regions of replicative stress induced damage. This ATMIN-dependent pathway is independent of NBS1.

My findings indicate that ATMIN is crucial for DNA damage repair following replicative stress. This novel role for ATMIN may suggest that the mechanism by which ATMIN functions as a tumor suppressor in a mouse model for B cell lymphoma is by preventing formation of lesions and chromosomal instability within the genome and potentially within fragile sites.

II. KURZBESCHREIBUNG

Die Proteinkinase ATM (Ataxia telangiectasia mutated) ist in der vererblichen autosomalen Krankheit Ataxia Telangiectasia mutiert und eine bedeutende Kinase bei der DNA Schadensantwort auf DNA Doppelstrangbrüche. Bei solchen Läsionen ist der ATM Kofaktor NBS1 essentiell für die Aktivierung von ATM, während er entbehrlich für ATM-vermittelte Signalübertragung bei anderen Arten von Stress (wie zum Beispiel bei hypotonischem Stress und Schäden durch DNA-Alkylierung) ist.

Kürzlich wurde bekannt, dass ATM eine zusätzliche Funktion bei der Signalübertragung nach niedrigen Dosen an Replikationsstress hat. Dieses Signal ist notwendig für die Rekrutierung von DNA Reparaturproteinen, wie zum Beispiel 53BP1, zu gestressten Regionen wie unter anderen auch common fragile sites. Dieser ATM-abhängige Signalweg führt dazu, dass fragile Stellen gegen Erosion und Abbau geschützt sind.

Ein neuer ATM Kofaktor, ATMIN (ATM Interactor), ist essentiell für die Aktivierung von ATM nach hypotonischem Stress und Änderungen der Chromatinstruktur. Ich zeige hier, dass ATMIN zusätzlich für die ATM Aktivierung und die Phosphorylierung von Substraten von ATM notwendig ist als Antwort auf niedrige Pegel von Replikationsstress, aber nicht bei DNA Doppelstrangbrüchen. Interessanterweise ist ATMIN entbehrlich für die Signalübertragung durch ATR, der bedeutendsten Kinase bei der Signalübertragung nach Replikationsstress. Ich konnte zeigen, dass ATMIN, als Konsequenz von fehlerhafter ATM Signalübertragung, essentiell für die Rekrutierung von 53BP1 und aktivem ATM zu Regionen mit Schäden durch replikativen Stress ist. Dieser ATMIN-abhängige Signalweg ist unabhängig von NBS1.

Meine Ergebnisse zeigen, dass ATMIN essentiell für die DNA Reparatur als Antwort auf Replikationsstress ist. Diese neue Rolle von ATMIN könnte der Mechanismus sein, der hinter ATMIN als Tumorsuppressor in einem Mausmodel für B Zell Lymphoma steht und Läsionen und chromosomale Instabilität des Genoms und eventuell von fragilen Stellen der Chromosomen verhindert.

III. INTRODUCTION

III.1 DNA DAMAGE AND GENOMIC INSTABILITY

Cells are constantly exposed to exogenous (e.g. ionizing radiation (IR), ultra violet (UV) light) and endogenous (e.g. reactive oxygen species, ROS) stresses that can cause DNA damage. Among such damage are DNA double strand breaks (DSBs) which are considered to be the most deleterious lesion to DNA, as a single DNA double strand break is sufficient to induce apoptosis (Bensimon, Aebersold et al. 2011). Inefficient repair of such breaks and mutations in genes crucial for DNA damage repair (including those that are often found in a variety of cancers (Ciccia and Elledge 2010) can cause chromosomal rearrangements, cell death and alterations in the normal cell cycle. As a consequence, this can lead to unrestricted or defective proliferation, genome instability and neoplastic transformation (Bensimon, Aebersold et al. 2011). Therefore, mutations in genes controlling cell cycle or the DNA damage response (DDR) lead to diseases with severe phenotypes such as ataxia telangiectasia (A-T) or severe combined immunodeficiency (SCID). Hence, cells have evolved tightly regulated pathways to regulate and monitor DNA integrity and repair, cell cycle progression and cell division (Shiloh 2003).

Checkpoints are signaling pathways that are activated when the cell encounters abnormalities at certain points throughout the cell cycle. Such checkpoints balance cell metabolism and can lead to either cell cycle arrest or apoptosis (Shiloh 2003; Yekezare, Gomez-Gonzalez et al. 2013). Checkpoints monitor for completed replication before the segregation of the chromosomes (DNA replication checkpoint), and also monitor for genome integrity and subsequent faithful replication (DNA damage checkpoints). These DNA damage checkpoints occur at different phases of the cell cycle – in G1 phase before replication starts, in S phase or in G2 phase before the mitosis (Zhao and Piwnica-Worms 2001). Since in eukaryotic cells replication is initiated at different origins, it is further important to control for full replication of the chromosomes while no origin must be fired more than once through this process as this

would lead to irregular repetition of certain areas of the genome (Yekezare, Gomez-Gonzalez et al. 2013). The firing of the origins is regulated by histone modifications and the transcriptional states of the DNA. Origins in areas of euchromatic DNA tend to fire in early S phase whereas the timing is later for origins in heterochromatic or inactive regions of the genome (Shechter and Gautier 2005).

DNA damage checkpoints can be activated following single-strand or double-strand breaks (DSBs), chromatin changes or by stalled replication forks that might collapse into DSBs. Three conserved groups of proteins are involved in these DNA damage signaling and repair pathways – the sensor proteins that recognize damaged DNA, the transducers that pass on and amplify the signal and the effectors that lead to either DNA damage repair, transcriptional regulation, senescence, cell cycle arrest or apoptosis (Shiloh 2003; Falck, Coates et al. 2005). Transducers are usually protein kinases that are recruited to sites of DNA damage where they transduce and relay the DNA damage signal by phoshporylating their substrates. In the DNA damage response, these kinases mostly belong to the family of Phosphatidylinositol 3-kinase-related kinases (PIKKs) (Bakkenist and Kastan 2004; Shechter, Costanzo et al. 2004; Falck, Coates et al. 2005; Bensimon, Aebersold et al. 2011).

III.2 PHOSPHATIDYLINOSITOL 3-KINASE-RELATED KINASES (PIKKS) IN DNA DAMAGE REPAIR

All PIKK family members have shared motifs typical for Phosphoinositide 3-kinases (PI3Ks) incuding their kinase catalytic site. The kinase domains are found in the C-terminus of the proteins and are flanked by two conserved domains termed FAT (FRAP/ATM/TRAPP) and FAT-C (the carboxy-terminal part) (Bakkenist and Kastan 2004). Some of the family members like the serine and threonine protein kinases ataxia telangiectasia mutated (ATM), ataxia telangiectasia mutated and Rad3 related (ATR), autotaxine (ATX, also known as SMG-1) and the DNA protein kinase catalytic subunits (DNA-PKcs) are strongly linked to DNA damage repair whereas other members like mTOR

(mammalian target of rapamycine, also known as FRAP, FKBP-12 Rapamycin Associated Protein) have different roles such as regulating G1 phase progression in correlation with the supply of nutrients and growth factors (Shiloh 2003; Bakkenist and Kastan 2004).

III.2.1 DNA - PROTEIN KINASE CS (DNA-PKCS) – MEDIATED SIGNALING

DNA-PKcs has a molecular weight of about 450 kDa and usually is associated with DSB repair induced by genotoxic stress or during V(D)J recombination via non-homologous end joining (NHEJ) (Bakkenist and Kastan 2004; Bensimon, Aebersold et al. 2011). In undamaged cells, inactive DNA-PKcs is found as a monomer (Bakkenist and Kastan 2004), but after DSB recognition, it is recruited to damage sites by its partner proteins Ku70 and Ku80 that form a heterodimer (Falck, Coates et al. 2005) that binds to DNA ends and translocates to DNA (Bakkenist and Kastan 2004). Here, the C-terminal part of the sensor proteins is crucial for DNA-PKcs recruitment (Falck, Coates et al. 2005). Dimerized DNA-PKcs is consequently associated with the broken DNA ends and is part of a synaptic complex that holds DNA ends together during NHEJ (Shiloh 2003; Bakkenist and Kastan 2004). DNA-PKcs kinase activity is weakly dependent on Mg^{2+} (Bakkenist and Kastan 2004) and the protein kinase targets a variety of substrates including its sensor proteins Ku70 and Ku80 and other proteins that function in NHEJ as for example DNA Ligase IV or Artemis. Furthermore, DNA-PKcs auto-phosphorylates at threonine 2609 after DSBs and is externally phosphorylated by ATM or ATR after UV or IR (Bakkenist and Kastan 2004; Bensimon, Aebersold et al. 2011). There is some evidence that the auto-phosphorylation does not lead to activation of the kinase, but is linked to the dissociation of the protein from DNA ends (Bakkenist and Kastan 2004). Reduced kinase activity leads to impaired NHEJ and the disease severe combined immunodeficiency (SCID) which is associated with hypersensitivity to IR (Shiloh 2003).

III.2.2 Ataxia telangiectasia mutated and Rad3 related (ATR) – mediated signaling

ATR is believed to play a role in the DNA damage response (DDR) after UV, hypoxia and replicative inhibitors such as aphidicolin (Aph) or hydroxyurea (HU) (Shechter, Costanzo et al. 2004). Albeit through different mechanisms – aphidicolin directly inhibits the replicative DNA polymerases α and δ while hydroxyurea is a ribonucleotide reductase inhibitor (Koc, Wheeler et al. 2004) – both treatments lead to replicative stress and ATR activation. ATR signaling is also triggered endogenously by replicative fork stalling or DNA damage during S-phase progression (Shiloh 2003; Shechter, Costanzo et al. 2004). After detection of DNA damage, ATR's role is to inhibit premature condensation of the chromosomes and replication of damaged DNA (Shiloh 2003). This is achieved by prevention of firing of further origins and hindering mitosis entry (Shechter, Costanzo et al. 2004).

ATR, a 350 kDa protein, is found as a heterodimer bound to its partner protein ATRIP (ATR-interacting protein) in undamaged cells (Bakkenist and Kastan 2004). ATRIP has a conserved sequence with other co-factors of PIKKs (such as Ku70-Ku80 for DNA-PKcs or NBS1 for ATM) at its C-terminal end that leads to the interaction with ATR (Falck, Coates et al. 2005). This interaction is maintained when the complex localizes to sites of DNA damage. ATRIP binds directly to RPA (replication protein A) on single stranded DNA (ssDNA) and hence recruits the ATRIP-ATR complex to chromatin and arrested replication forks. During this process, RPA is phosphorylated and remains associated to ssDNA, but there is no evidence that ATR undergoes a stable modification following DNA damage leading to an increase in kinase activity through a conformational change (Bakkenist and Kastan 2004). ATR therefore seems to be activated mainly by ssDNA, a byproduct of resectioned DSBs and collapsed forks. The length of ssDNA and of ssDNA – double stranded DNA junctions are key factors in ATR activation and determine whether the DNA damage response is initiated via ATR or ATM, a protein kinase crucial for DSB repair after IR (Bensimon, Aebersold et al. 2011). The two kinases often function simultaneously and have similar substrates which they phosphorylate at SQ/TQ

sites (serine or threonine followed by Glutamine) (Shechter, Costanzo et al. 2004). After UV damage or replicative stress, ATM is a downstream effector of ATR, but following DSBs induced by IR, ATM is activated first. In this scenario, ATR leads to prolonged phosphorylation of proteins involved in DNA damage repair. Furthermore, ATR activation also triggers checkpoint activation shown by the requirement of ATM and ATR for the activation of checkpoint kinase 1 (CHK1) after IR and UV-mediated damage (Figure 1) (Bensimon, Aebersold et al. 2011).

As ATR and its effector protein CHK1 are master regulators following UV damage and replicative stress, it has been suggested that ATR's function could possibly be limited to the S phase of the cell cycle. However, this has been contradicted by a study showing that hypomorphic mutations in ATR lead to UV hypersensitivity in all cell cycle stages (Bakkenist and Kastan 2004). Interestingly, ATR was further found to retain a low level of activity in undamaged cells in order to autoregulate the time of origin firing. This basal ATR activation in undamaged cells could explain why loss of ATR, but not ATM, is embryonic lethal (de Klein, Muijtjens et al. 2000). Additionally, the protein is also crucial for "fragile site" stabil-

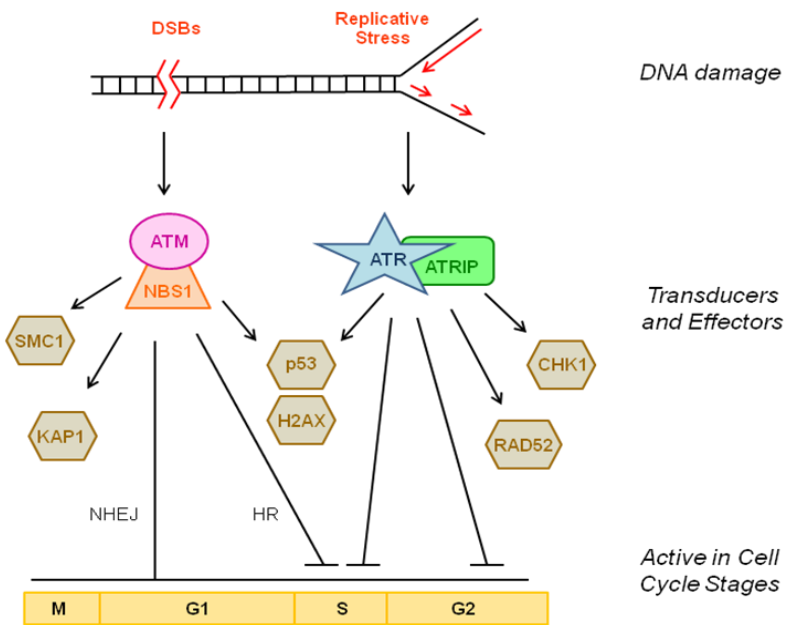


Figure 1. ATM and ATR in the DNA damage response.

ATM is the major kinase after DNA double strand breaks (DSBs) and ATR signals after replicative stress. The ATM co-factor, NBS1, and the ATR co-factor, ATRIP, are crucial for their activation and for subsequent substrate phosphorylation. Some substrates are specific for ATM (SMC1, KAP1) or ATR (CHK1, RAD52), but some are shared targets as for example p53 or H2AX. ATR functions mostly during S and G2 phase of the cell cycle. Since ATM is involved in homologous recombination (HR) and non-homologous end-joining (NHEJ), it can be active throughout the whole cell cycle.

ity (Shechter, Costanzo et al. 2004). Fragile sites are regions of the genome that are prone to DNA damage like single-strand or double-strand breaks and chromosome gaps following replicative stress (Glover, Berger et al. 1984).
Since ATM and ATR have some overlapping functions (Shechter, Costanzo et al. 2004), it is not surprising that an inhibitory kinase-inactive form of ATR leads to sensitivity not only to UV and HU, but also to treatments introducing DSBs like IR or DNA methylating agents such as methyl methanesulfonate (MMS) in mammalian cells (Bakkenist and Kastan 2004).
Taken together, these findings indicate that ATR is weakly active in undamaged cells and is further activated after DNA damage by ssDNA-RPA complexes. ATR is responsible for the regulation of origin firing in undamaged and damaged cells as well as promoting DNA damage repair (Shechter, Costanzo et al. 2004). Furthermore, the protein is not only the major kinase in signaling after replicative stress and stalled replication forks, but also has a broad spectrum of impact on the cell which is demonstrated by the fact that loss of ATR is embryonic lethal (de Klein, Muijtjens et al. 2000).

III.2.3 Ataxia telangiectasia mutated (ATM) – mediated signaling following DSBs

The serine / threonine kinase ATM is critical for the DNA damage response following IR and for normal cell-cycle progression and checkpoint activation (Xu and Baltimore 1996). It further participates in sensing ROS where it controls the cell's response to oxidative stress (Guo, Kozlov et al. 2010; Cosentino, Grieco et al. 2011) and is involved in induction of senescence following various stimuli (Bartkova, Rezaei et al. 2006). ATM's mRNA transcript has a length of about 12 kilobases grouped into 66 exons and translates into a 370 kDa protein (Savitsky, Bar-Shira et al. 1995; Bakkenist and Kastan 2004; McKinnon 2004) where the catalytical domain is found at the C terminus of the kinase (Shechter, Costanzo et al. 2004).
In undamaged cells, inactive ATM is mostly found in a dimeric or multimeric conformation, but after generation of even a single DSB (Bakkenist and Kastan

2003), ATM is activated and auto-phosphorylates at serine 1981 amongst other sites which leads to dimer dissociation (Bakkenist and Kastan 2003). This activation following DSBs is stimulated by the protein complex MRN (MRE11, RAD50 and NBS1) which at the same time serves as an adaptor platform for the assembly and stable binding of ATM's substrates (Uziel, Lerenthal et al. 2003; Lee and Paull 2004). The consequent auto-phosphorylation of ATM does not directly affect the kinase's activity, but is crucial for dimer dissociation (Bakkenist and Kastan 2004). Interestingely, it was found that after oxidative stress ATM is activated and stays bound as a dimer (Guo, Kozlov et al. 2010). Initial activation of ATM occurs at a distance from DSBs and does not depend on direct DNA binding at breaks, but may result from changes in chromatin structure and through sensor proteins that link ATM to DSBs through the nucleoplasm (Bakkenist and Kastan 2003; Bakkenist and Kastan 2004). Activated ATM then accumulates in repair foci at DNA lesions (Kitagawa, Bakkenist et al. 2004). Hence auto-phosphorylation at serine 1981 is found on chromatin-bound, but also soluble ATM (Shiloh 2003). When DNA damage signaling is triggered through treatments that do not lead to DSBs, ATM is still activated, but does not form foci at the breakage sites (Bakkenist and Kastan 2004). In contrast active and hence autophosphorylated ATM is found throughout the nucleus.

Once ATM reaches sites of DNA damage, it phosphorylates the histone variant H2AX (in its phosphorylated form known as y-H2AX) in order to recruit other repair factors. This phosphorylation is not particularly important for ATM's role in inducing cell-cycle checkpoints or NHEJ, but seems crucial for homologous recombination (HR) (Celeste, Petersen et al. 2002). After recruitment of ATM's substrates to DSBs, they are phoshphorylated and either function as adaptor proteins, as further transducers of damage signals or are responsible for cell-cycle checkpoint activation (eg. p53). Some effector proteins that lead to DNA damage repair are connected to HR (eg. BRCA1) in G2 and S phase or NHEJ (eg. 53BP1) all throughout the cell cycle, both of which are dependent on ATM (Yamamoto, Wang et al. 2012).

III.2.4 ATM'S SUBSTRATES AND THEIR RECRUITMENT TO DSBS

One of the first substrates recruited via its BRCT (BRCA1 C terminus) domain and phosphorylated by ATM is MDC1 (mediator of DNA damage checkpoint 1). The recruitment of MDC1 to damage sites promotes the accumulation of the E3 ubiquitin-ligase RNF8 (Ring finger protein 8) and consequently also the recruitment of RNF168 (Mallette, Mattiroli et al. 2012). RNF168 mono-ubiquinates H2A/H2AX on K13-15 and a K63-linked ubiquitin (Ub) chain is then added to these lysines that promotes BRCA1 and 53BP1 binding (Mallette, Mattiroli et al. 2012; Mattiroli, Vissers et al. 2012). Alterations and modifications of chromatin are crucial for recruitment of 53BP1 and BRCA1 that function antagonistically during DNA damage repair after direct or indirect phosphorylation by ATM (Gatei, Scott et al. 2000; Harding, Coackley et al. 2011; Munoz, Laulier et al. 2012). Whereas 53BP1 promotes NHEJ, BRCA1 is essential for HR (Tang, Cho et al. 2013).

The decision of whether 53BP1 or BRCA1 binds to breakage sites is dependent on many different chromatin alterations and proteins. For example, JMJD2A is a direct competitor of 53BP1 for H4K20(me2) binding. In undamaged cells, JMJD2A that has a higher affinity to H4K20(me2) than 53BP1 is stably bound to the chromatin. After IR, RNF8 and RNF168 are recruited to sites of damage and lead to ubiquitin-dependant proteosomal degradation of JMJD2A. H4K20(me2) is consequently available for 53BP1 binding and this promotes NHEJ (Mallette, Mattiroli et al. 2012). This process is counter acted by the proteasomal de-ubiquitinating enzyme POH1 that prevents degradation of JMJD2A by de-ubiquitinating the protein. Hence, BRCA1 accumulates at DSBs and the DNA is repaired by HR (Butler, Densham et al. 2012). 53BP1 accumulation is also regulated via acetylation of H4K16 that reduces the binding affinity of 53BP1 to H4K20(me2) (Tang, Cho et al. 2013). Another factor promoting HR is the E3-ubiquitin ligase RNF169 that functionally competes with 53BP1 by binding to RNF168-modified chromatin via its MIU (motif interacting with ubiquitin). Therefore it negatively regulates and delays 53BP1 recruitment in a non-catalytical manner (Poulsen, Lukas et al. 2012). On the other side, as soon as 53BP1 binds to DSBs, it is phosphorylated by ATM which

leads to the recruitment of its NHEJ-promoting co-factor RIF1. RIF1 antagonizes the accumulation of BRCA1 and its interacting partner CtIP and therefore reduces the HR frequency. This regulation is especially active in G1 phase of the cell cycle (Escribano-Diaz, Orthwein et al. 2013).

Chromatin modifications are also crucial for defining and limiting the area of DNA damage and switching off the signaling pathways after successful repair. For example, RNF4 is recruited to repaired DNA and sumoylates MDC1. This sumoylation leads to degradation and removal of MDC1 which limits accumulation of RNF8/RNF168, ubiquitination of chromatin and consequently prevents further 53BP1 recruitment (Luo, Zhang et al. 2012). Another way for limiting the defective DNA damage sites is by hindering RNF168 from excessively ubiqutinating the area around lesions which would lead to transcriptional silencing. This happens for example through TRIP12 and UBR5, two HECT domain E3 ligases that control accumulation of RNF168 by regulating the size of the RNF168 nuclear pool (Gudjonsson, Altmeyer et al. 2012). Other factors responsible for chromatin dynamics and accumulation of substrates at DNA damage sites are FANCD2 and FANCI (Sato, Ishiai et al. 2012), INT6 (encoding the e subunit of the translation initiation factor eIF3) (Moyal, Lerenthal et al. 2011) and the RNF20-RNF40 heterodimer (Morris, Tomimatsu et al. 2012). Whereas INT6 functions by reducing Ub at DNA lesions, the RNF20-RNF40 heterodimer antagonizes this restriction by catalyzing mono-ubiquitination of H2B, leading to ATM dependent decondensation of chromatin and promoting retention of proteins at DNA damage sites (Moyal, Lerenthal et al. 2011; Morris, Tomimatsu et al. 2012).

Substrates other than BRCA1 and 53BP1 that are recruited to DNA DSBs and phosphorylated by ATM include NBS1, SMC1, KAP1 or p53. NBS1 seems to function in two different ways – it is recruited independently to ATM as a sensor for S-phase checkpoint activation and associates transiently to ATM for optimal ATM activation and enhancement of ATM's catalytic activity (Bakkenist and Kastan 2004). Here, NBS1 is found in a complex together with MRE11 and RAD50 referred to as the MRN complex (D'Amours and Jackson 2002). Interestingly, NBS1 is found upstream and downstream of ATM as it is crucial

for ATM recruitment to DSBs (Uziel, Lerenthal et al. 2003), but is then also phosphorylated by the kinase (Wu, Ranganathan et al. 2000). This phosphorylation is essentiel for the MRN-dependent stimulation of ATM activity towards some of its substrates (eg. CHK2) (Lee and Paull 2004). For the recruitment of ATM to sites of DNA damage by the MRN complex, NBS1 directly binds ATM with its C-terminus. The last 20 residues of the protein are sufficient for a stable binding and contain a conserved motif also found in other co-factors of PIKKs (eg. ATRIP, the co-factor for ATR). The interaction between the kinases and their specific partner molecules differs in the way that these interactions take place. Whereas an ATRIP/ATR complex is already found in undamaged cells, DNA damage is required to trigger the binding of NBS1 and ATM (Falck, Coates et al. 2005).

Apart from its C-terminal ATM-binding domain, NBS1 (also known as Nibrin or p90 due to its size) (Kondratenko, Paschenko et al. 2007) contains a MRE11-binding domain and the N-terminal region harbors a FHA (Forkhead-associated) and a BRCT (BRCA1 C-terminus) domain that enables its interaction with phospho-SQ or phospho-TQ residues on ATM targets. NBS1 is recruited to DSBs via direct interaction of the N terminus with y-H2AX or MDC1. The central region of the protein contains numerous SQ/TQ sites that are partially phosphorylated by ATM and other PIKKs (Difilippantonio and Nussenzweig 2007). In the MRN complex, MRE11 directly and independently binds NBS1 and RAD50. Through this interaction and its consequent stimulated exonuclease and endonuclease activity, the complex partly unwinds and dissociates short DNA duplexes and promotes strand-annealing. The largest protein of the complex, RAD50 (150 kDa), contains an ATP-binding domain where the binding of ATP leads to a conformational change and the consequent induction of MRE11 endonuclease activity. RAD50 is also involved in DNA repair by holding together DNA ends and enhancing the activity of DNA ligases (D'Amours and Jackson 2002). Once activated following DSBs, the complex recruits ATM to sites of damage via NBS1 interaction with ATM and forms DNA repair foci consisting of various proteins. Shortly after exposure to IR, DNA damage foci are small and numerous, but after about

4 hours, larger foci that persist longer appear (D'Amours and Jackson 2002). They are referred to as IRIF (ionizing radiation-induced foci) and are cleared after successful repair. In undamaged cells, spontaneous foci are detected that are sometimes termed PML (progressive multifocal leukoencephalities) bodies (D'Amours and Jackson 2002) or 53BP1-containing nuclear bodies (Lukas, Savic et al. 2011).

The ATM substrates KAP1 and SMC1 are recruited to or accumulate at DNA damage sites where active ATM is located following DNA damage and are consequently phosphorylated. Along with HP1, KAP1 is one of the two main components of heterochromatin and promotes chromosome condensation and transcriptional repression (White, Rafalska-Metcalf et al. 2012). Its function involves recruitment of nucleosome deacetylation, methylation and remodeling activities. KAP1 auto-SUMOylation leads to accumulation of CHD3 (Chromo-domain-helicase-DNA-binding-protein 3) through an interaction via its SIM (SUMO-interaction motif) domain with SUMO1 on KAP1 (Goodarzi, Kurka et al. 2011). CHD3 is part of the nucleosome remodeling and deacetylating (NRD) complex that promotes heterochromatin formation (Tong, Hassig et al. 1998). Following DNA damage via IR, KAP1 is phosphorylated at serine 824 and found at DNA damage sites as IRIFs (White, Rafalska-Metcalf et al. 2012). The phosphorylation perturbs the interaction and recruitment of CHD3 and therefore promotes chromatin relaxation and repair (Goodarzi, Kurka et al. 2011). This ATM-dependent effect of P-S824-KAP1 was also observed after treatment with UV, MMS or HU (Ziv, Bielopolski et al. 2006). Interestingely, as well as the phosphorylation at serine 824 as an early response, KAP1 is also phosphorylated at serine 473 by the protein kinase C delta pathway at later time points (White, Rafalska-Metcalf et al. 2012). Whereas P-S824-KAP1 accumulation is restricted to DNA damage sites, P-S473-KAP1 is found dispersed throughout the nucleus pointing towards a role in a more global event, for example in chromosome organization in the nucleus (Ziv, Bielopolski et al. 2006; White, Rafalska-Metcalf et al. 2012).

The ATM substrate SMC1 is also involved in chromatin organization in the cell. It belongs to the family of SMC (structural maintainance of chromosome)

proteins that has six members that are found in hetero-duplexes to form different cohesion or condensin subunits. Whereas condensins physically compact DNA, cohesions hold sister chromatids together during S/G2 phase by builing ring-like structures around sister chromatids (Losada and Hirano 2005; Wu and Yu 2012). The exact function of SMC1 in different pathways of DNA damage repair is not clear, but it is known that the SMC1-SMC3 complex (also referred to as cohesion) is recruited to DSBs during HR (Losada and Hirano 2005; Wu and Yu 2012). Since HR almost exclusively takes place during S/G2 phase, recruitment of SMC1 is restricted to these cell cycle stages (Wu and Yu 2012). Phosphorylation of SMC1 by ATM at serine 957 is required for proper DNA damage repair, but does not have a particular role in cohesion of sister chromatids (Losada and Hirano 2005; Wu and Yu 2012). After successful DNA repair, IRIFs disappear and SMC1 and KAP1 are desphosphorylated and found throughout the nucleus.

In contrast to the previously described substrates of ATM, p53 is not only phosphorylated and therefore activated following DSB induction, but the level of the protein is also increased. This happens due to a stabilization partly introduced by its ATM- or ATR-dependent phosphorylation at serine 15 (Siliciano, Canman et al. 1997). This phosphorylation leads to a reduced interaction with its negative regulator Mdm2 (Shieh, Ikeda et al. 1997). Furthermore, Mdm2 is a direct target of ATM and its inhibitory phosphorylation at serine 394 decreases its effect on destabilization of p53 (Gannon, Woda et al. 2012). There is evidence that following DNA DSB detection, P-S15-p53 directly binds to sites of DNA breaks where is colocalizes with y-H2AX and P-S1981-ATM (Al Rashid, Dellaire et al. 2005). Moreover, the tumor suppressor p53 is a transcription factor whose activation can lead to checkpoint activation and cell cycle arrest in G1 phase. Here, its transcriptional regulation of p21 plays an important role since p21 (also known as Waf1) is a selective inhibitor for a group of CDKs associated with G1/S cyclins and therefore cell cycle progression (Harper, Elledge et al. 1995). The oscillating p53 response to DNA damage that is strictly regulated by different proteins (Mdm2, WIP1, astrin) can be reversed and cells are competent to recover after a p53-induced cell cycle

arrest. However, sustained p53 activation as a result of ineffective or defective DNA repair can lead to irreversible arrest and apoptosis (Halim, Alvarez-Fernandez et al. 2013).

Due to the fact that cell cycle progressionin the absence of successful DNA repair would be fatal for the cell, various ATM substrates are also involved in checkpoint activation and cell cycle arrest in all cell cycle stages. For example, BRCA1 and NBS1 take part in induction of the intra-S-phase checkpoint and CHK2 partly controls G2 DNA damage-induced checkpoints (McKinnon 2004).

III.3 DSB-RELATED DISORDERS AND DISEASES

Since ATM and ATR are the major kinases in DNA damage response after DNA damage and ATM- and ATR-mediated signaling is crucial for proper DNA repair, deletion or mutation of genes in these pathways lead to diseases with severe phenotypes.

Ataxia telangiectasia (A-T) patients usually suffer from reduced amounts of functional ATM or decreased kinase activity due to truncations of the protein or due to a dominant-negative effect of an ATM allele with a missense mutation (Shiloh 2003; McKinnon 2004). Here, mutations are contributed equally throughout the big kinase without showing mutation “hot spots”. The severe phenotype of the disease includes neurodegeneration, radiosensitivity, immune dysfunction and predisposition to lymphoid cancer and the cause of death is pneumonia, chronic lung disease, leukemia or lymphoma. Throughout life, A-T patients often suffer from atoxic eye movements, ocular telangiectasia, speech defects, sterility and are wheel-chair bound early in life (McKinnon 2004). Mutations in genes belonging to the MRN complex lead to similar but less severe phenotypes. Hypomorphic mutations in MRE11 give rise to ATLD (Atalaxia-telangiectasia-like disorder) with less severe neurodegeneration than observed in A-T patients and a later onset of the disease. Patients with mutations in NBS1 (leading to Nijmegen-breakage-syndrome) experience a very similar phenotype to A-T, but with distinct neurological defects, microcephaly characteristics and a higher cancer predisposition (Shiloh 2003; McKinnon 2004).

In patients, expression of a splicing mutant form of ATR leads to reduced levels of functional protein and consequently to Seckel Syndrome (O'Driscoll, Ruiz-Perez et al. 2003). Clinical features of this disease are intrauterine growth retardation, dwarfism, microcephaly and mental retardation (Bakkenist and Kastan 2004).

III.4 ATM SIGNALING INDUCED BY CHANGES IN CHROMATIN STRUCTURE

It has been commonly known for some time that the ATR protein is majorly responsible for signaling after replicative stress and ATM and its co-factor NBS1 are responsible for DNA damage following DNA DSBs. However, in 2003, Bakkenist and Kastan found an activation of ATM by chromatin-active agents that do not cause any DSBs (Bakkenist and Kastan 2003). Treatment of primary fibroblasts with NaCl (hypotonic conditions), chloroquine or Trichostatin A (a histone deacetylase inhibitor) lead to phosphorylation of ATM at serine 1981 in the absence of DSBs (Bakkenist and Kastan 2003). Furthermore, Difilippantonio et al. showed in 2005 that loss of NBS1 diminished ATM-mediated signaling in B cells following IR (measured by the amount of phosphorylated substrates), but did not affect signaling after treatment with osmotic stress, UV or replicative stress induced by hydroxyurea (HU) or aphidicolin (Aph) (Difilippantonio, Celeste et al. 2005). Taken these results together, it has been proposed that alterations of chromatin structures could lead to an ATM activation independently of the MRN complex and NBS1 (Bakkenist and Kastan 2003).

III.4.1 ATMIN AND ITS ROLE IN DNA DAMAGE RESPONSE

Recently, a novel co-factor of ATM was identified termed ATMIN for ATM INteractor (also referred to as ASCIZ: ATM substrate CHK2-interacting Zn^{2+}-finger) that is required for ATM signaling induced by changes in chromatin structure (Kanu and Behrens 2007; Kanu and Behrens 2008; Zhang, Penicud et al. 2012). There is a stimulus-dependant association of ATMIN with ATM via its C-terminal region that harbors a conserved motif also found in NBS1 (Kanu

and Behrens 2007). Co-locolization of ATM and ATMIN was found after treatment with chloroquine or hypotonic stress, but not after IR (Kanu and Behrens 2007; Kanu and Behrens 2008). Furthermore, knock-down of ATMIN showed reduced phosphorylation of ATM following HU and chloroquine treatment, but not after IR or UV and loss of ATMIN in chicken DT40 B lymphocytes led to increased sensitivity to base damaging agents (MMS and H_2O_2), but had only a minor effect on DSB-inducing treatments (Kanu and Behrens 2007; Heierhorst 2008; Oka, Sakai et al. 2008).

ATMIN has a size of 88.3 kDa and harbors 15-18 (dependent on the species and ortholog) SQ/TQ phosphorylation sites concentrated in a SQ/TQ cluster domain (SCD) which makes it a potential ATM/ATR substrate. On its N terminus there are 4 Zn^{2+}-fingers that could be mediating DNA binding and the C-terminus contains, next to the ATM-binding motif, a PEST domain that leads to proteasome-dependent ATMIN degradation (Kanu and Behrens 2007; Kanu and Behrens 2008).

The conserved ATM interaction motif points towards a potential competition between ATMIN and NBS1 binding. After IR, a reduced interaction between ATM and ATMIN was seen, but the binding was enhanced after chloroquine treatment (Kanu and Behrens 2007; Zhang, Penicud et al. 2012). There is some evidence that ATMIN is found bound to ATM in undamaged cells and that the NBS1 C-terminus contributes to ATMIN/ATM dissociation following DSB detection (Kanu and Behrens 2007; Kanu and Behrens 2008; Zhang, Penicud et al. 2012). Whereas ATMIN forms damage-induced foci after treatment with chloroquine and hypotonic shock that co-localize with ATM, the protein is not recruited to DSBs after IR (Kanu and Behrens 2007). In line with the hypothesis of a direct competition between ATMIN and NBS1, it was found that ATM-mediated signaling is enhanced after IR in cells depleted for ATMIN and that over-expression of ATMIN C-terminus leads to impaired phosphorylation of ATM substrates after IR (Zhang, Penicud et al. 2012).

Despite the competition between NBS1 and ATMIN and their stimulus-dependent activation, there is also evidence that the two proteins have redundant functions since NBS1-deficient cells show residual DSB-induced ATM

signaling (Zhang, Penicud et al. 2012). Furthermore, it was shown that ATMIN functions not exclusively in signaling after chromatin changes, but has various roles. It was proposed that ATMIN functions as a scaffold protein and is crucial for efficient repair of base lesions (Heierhorst 2008). In this regard, ATMIN was found to co-localize with RAD51-containing foci in response to base-modifying DNA methylating agents (McNees, Conlan et al. 2005) and seemed to affect the choice between competing base repair pathways (Oka, Sakai et al. 2008). There is strong evidence that ATMIN is critical for class switch recombination (CSR) in B cells as it is required for the repair of breaks during CSR and hence maturation of B cells and loss of ATMIN leads to B cell lymphoma development in mice as shown by Loizou et al. in 2011 (Loizou, Sancho et al. 2011). NBS1 is also essential during CSR, but in this case ATMIN and NBS1 have non-redundant biological functions and might be crucial at different stages or different aspects of CSR. Furthermore, genomic instability was as well observed on chromosomes not carrying immunoglobulin loci and therefore ATMIN could also function as a tumor suppressor in non-lymphoid tissue (Loizou, Sancho et al. 2011).

In addition, ATMIN seems to be important during development due to the fact that ATMIN-deficient mice are embryonic lethal (Kanu, Penicud et al. 2010). As well as being a co-factor and a scaffold protein, it has been proposed that ATMIN functions as a transcription factor. There is evidence that ATMIN controls survival of developing B cells by regulating DYNLL1 expression through Bim-dependent apoptosis (Jurado, Gleeson et al. 2012). Furthermore, it was also shown that there is a direct binding between ATMIN and DYNLL1 that is mutually affecting their subcellular location and possibly consequently their functions (Rapali, Garcia-Mayoral et al. 2011).

Taken together, it is clear that ATMIN has diverse functions in the DNA damage response and is required for genomic stability and hence tumor suppression (Loizou, Sancho et al. 2011). Furthermore, due to its 4 Zn^{2+}-finger domains that could potentially bind DNA, a function as a transcription factor is likely and has been demonstrated in the case of DYNLL1 (Kanu and Behrens 2008; Jurado, Conlan et al. 2012).

III.4.2 REPLICATIVE STRESS AND 53BP1-CONTAINING NUCLEAR BODIES

ATM function following DSBs has been widely studied, but ATM function after replicative stress has not been examined in depth yet. Since treatment of cells with HU or Aph leads to ATM-mediated signaling which can be measured by looking at the phosphoylation status of SMC1, KAP1 or ATM itself, it seems likely that ATM has a crucial function when cells encounter replicative stress (Bakkenist and Kastan 2003).

Cells naturally experience replicative stress when they come across errors during DNA replication that lead to 53BP1-containing nuclear bodies formed around DNA lesions (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). Interestingly, these large 53BP1 foci are restricted to G0 and G1 phases where DNA is not replicated and the nuclear bodies disappear in S phase. After mitosis, when cells re-enter into G1 phase, 53BP1 nuclear bodies reappear symmetrically in daughter cells (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). Furthermore, recent studies have shown that following low levels of replicative stress induced by Aph or HU treatment that do not primarily lead to DNA DSBs, the number of 53BP1-containing nuclear bodies per cell is significantly increased (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011).

Replicative stress usually affects loci where progression of replication fork is slow or problematic as for example common fragile sites (CFSs), repetitive sequences or telomeres. DNA at CFSs may stay incompletely replicated or have unresolved replication intermediates that can lead to breaks or gaps in mitotic chromosomes (Lukas, Savic et al. 2011). It has been shown that regions encountering replication problems are often bridged during anaphase via so-called ultrafine DNA bridges (UFBs) that are coated by BLM helicase. BLM helicase and its partners are known to catalyze double Holliday junction dissolution and seem to be important for bridge resolution during anaphase (Chan, North et al. 2007).

53BP1-containing nuclear bodies and y-H2AX were found to accumulate at CFSs following replicative stress (Harrigan, Belotserkovskaya et al. 2011;

Lukas, Savic et al. 2011). Apart from co-localizing with y-H2AX, 53BP1 foci strongly co-localize with other components of the DNA damage response as for example MDC1, RNF8, RNF168 or P-S1981-ATM (Lukas, Savic et al. 2011). ATM knock-down or ATM inhibition (Hickson, Zhao et al. 2004) lead to a significant decrease in 53BP1 accumulation into foci (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). ATR depletion synergized with low aphidicolin had the opposite effect where cells showed increased numbers of 53BP1 nuclear bodies (Lukas, Savic et al. 2011).

The proposed model for this phenomenon is that during S/G2 phase there are unresolved replication intermediates and incomplete replication that lead to DNA ruptures or topological chromatin alterations in M phase (Lukas, Savic et al. 2011). In the subsequent G1 phase, once recognized as DNA damage, 53BP1 nuclear bodies are formed (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). These nuclear bodies then shield the unrepaired lesions during G1 phase against excessive chromatin degradation in order to allow repair in S phase when the complex dissociates from chromatin (Lukas, Savic et al. 2011).

IV. AIM OF THE STUDY

Harrigan et al. and Lukas et al. (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011) showed that ATM is crucial for the formation of 53BP1 nuclear bodies following replicative stress, but it is not clear which co-factor is involved. NBS1 is required for ATM-mediated signaling and ATM recruitment to DNA DSBs (Falck, Coates et al. 2005), but low levels of aphidicolin treatment lead to stalled replication forks that only potentially can collapse into DSBs (Carr, Paek et al. 2011). According to Difilippantonio et al., replicative stress is not likely to depend on NBS1 (Difilippantonio, Celeste et al. 2005). Since the co-factor ATMIN is responsible for non-canonical signaling after changes in chromatin (Zhang, Penicud et al. 2012), I propose that ATM functions via ATMIN following replication stress.

It is to be determined whether ATMIN is required for signaling after replicative stress introduced by low levels of aphidicolin and whether ATMIN plays a role in recruitment of 53BP1 into 53BP1 foci at DNA lesions arising from endogenous problems in replication or exogenously induced replicative stress. Furthermore, the question remains if this mechanism functions independently of ATR that is known as the major kinase in replicative stress signaling and repair.

V. MATERIAL AND METHODS

V.1 CELLS AND CELL CULTURE CONDITIONS

HeLa cells, HEK293T cells and MEFs ($ATMIN^{+/+}$, $ATMIN^{\Delta/\Delta}$, $NBS^{+/+}$, $NBS^{-/-}$) were cultured in DMEM (Invitrogen or PAA laboratories) supplemented with 10 % FCS (Invitrogen) and 5 % penicillin / streptomycin (Invitrogen). Trypsin-EDTA at 0.05 % was purchased from Invitrogen or PAA laboratories. The incubator (Galaxy 170 R, New Brunswick) was set to 37 °C with 5 % CO_2 and 3 % O_2.

V.2 THAWING MAMMALIAN CELLS

Frozen vials were taken out of the liquid nitrogen tank and put on ice while 5 ml of pre-heated medium were put into 15 ml falcons. Vials were thawed partly in a 37 °C water bath until there was only a little ice remaining and the cell suspension was then added to the 5 ml medium. Cells were spun down at 1'200 rpm for 5 minutes and the supernatant was removed. Cells were resuspended in 10 ml medium and transferred to a T75 flask or at a density of 40 % confluency to be incubated at 37 °C. Cells were kept in culture for 1 week before any experiment was carried out.

V.3 FREEZING AND STORAGE OF MAMMALIAN CELLS

A confluent T75 flask was washed with PBS (Invitrogen or PAA) and trypsinised (Invitrogen or PAA). Cells were spun down at 1'200 rpm for 5 minutes, supernatant was carefully removed and the pellet was resuspended in 3 ml medium supplemented with 10 % DMSO. Three cryovials (Thermo scientific) were filled with 1 ml cell suspension and put into a "Mr. Frosty" Freezing Container (Thermo Scientific). After keeping the freezing container for 4 days at -80 °C, frozen cryovials were transferred to a liquid nitrogen tank.

V.4 CHEMICALS AND TREATMENTS

Aphidicolin was purchased from Sigma-Aldrich and used at a concentration of 1 µM overnight (immunofluorescence (IF) staining and signaling in MEFs) or for 24 h (signaling in HEK293T cells). Aphidicolin at 4 µM was administered for 24 hours to achieve a block in the cell cycle in the cell release assay. For ionizing radiation, the IR facility at the AKH Vienna was used (Cs 137). Cells were incubated for 30 minutes after ionizing radiation before lysates were prepared.

V.5 REVERSE TRANSFECTION OF MAMMALIAN CELLS

Reverse transfections were either set up in 24-well (IF staining) or 6-well dishes (cell lysates). Volumes of 67 µl (24-well) or 333 µl (6-well) of 375 nM siRNA (all siRNA pools and deconvoluted siRNAs were purchased from Dharmacon) in Opti-MEM (Invitrogen) and the same amount of Lullaby reagent (1:50, Oz Biosciences) in Opti-MEM were added to each well and mixed by tapping. After an incubation of 15-20 minutes at RT, 533 µl (24-well) or 2.66 ml (6-well) of a 5 x 104 cells/ml suspension were added to wells, mixed by tapping and incubated at 37 °C for 48 hours before treatment with aphidicolin or ionizing radiation. Cells were counted via CASY counter (Innovatis) for all experiments. Knock-down efficiency was similarly high in all experiments if not indicated differently.

V.6 IMMUNOFLUORESCENCE (IF) STAINING AND MICROSCOPY

Cells were either plated out directly or reverse transfected onto cover slips (VWR) in a 24-well dish. After 24 or 48 hours, cells were treated with aphidicolin (1 µM, Sigma-Aldrich) overnight. The next day cells were washed twice with ice-cold PBS and fixed with 4 % PFA containing 0.1 % Triton X-100 in PBS for 20 minutes on ice. After washing three times with ice-cold PBS, cells were permeabilized with 0.5 % Triton X-100 in PBS and incubated for 20 minutes at RT. After washing again three times with ice-cold PBS, blocking solution (10 % FCS, 0.1 % Triton X-100 in PBS) was added for 1 hour at RT.

The buffer was then aspirated and primary antibodies (Table 1) diluted in blocking solution were added to wells and incubated for 1 hour at RT. After washing three times with PBS, cells were incubated with secondary antibodies (Table 1) diluted in blocking solution for 1 hour at RT in the dark. Cells were washed again three times with PBS and stained with DAPI (1:1000 in PBS, Sigma-Aldrich) for 20 minutes at RT in the dark.

Cover slips with attached cells were washed three times with PBS, dipped into ddH_2O, dripped off on paper, mounted with mounting medium (Dako) onto adhesion object slides (VWR) and dried overnight at 4 °C. Images of cells co-stained for 53BP1 and P-S1981-ATM were acquired on a Deconvolution microscope (Leica). Cell Profiler cell image analysis software was used for the quantification of 53BP1 focus formation and its significance was calculated using Fisher's exact test.

	Antibody	**Species**	**Company**	**Dilution**
Primary antibodies	53BP1 (H-300)	Rabbit IgG	Santa Cruz	1:600
	Phospho-ATM (Ser1981) (10H11.E12)	Mouse IgG	NEB_Cell Signalling	1:600
Secondary antibodies	Alexa Fluor® 546 goat anti-rabbit		Invitrogen	1:400
	Alexa Fluor® 488 goat anti-mouse		Invitrogen	1:400
	Alexa Fluor® 488 goat anti-rabbit		Invitrogen	1:400
	Alexa Fluor® 568 goat anti-mouse		Invitrogen	1:400

Table 1. Antibody list used for immunofluorescence (IF) staining.
Antibodies were diluted as indicated in blocking buffer.

V.7 WHOLE CELL LYSATE PREPARATION

Cells were plated out in 6-well or 10-cm dishes and treated at a maximum confluency of 80 % with 1 µM aphidicolin overnight (MEFs) or for 24 h (HeLa, HEK293T). Cells were washed with ice-cold PBS on ice and after addition of 1 ml (6-well) or 2 ml (10-cm dish) ice-cold PBS, cells were scraped off using a cell scraper (CytoOne). Cell suspensions were transferred to Eppendorf tubes

and spun down at 2'000 rpm for 8 minutes at 4 °C. HEK293T cells were not scraped off, but trypsinised, spun down at 1'200 rpm for 5 minutes and washed with PBS. Cell pellets were then resuspended in 40 µl (6-well) or 80 µl (10-cm dish) lysis buffer {1 x RIPA buffer, supplemented with 50 mM NaF (Sigma), 1 x protease inhibitor cocktail (Sigma), 1 mM Sodium orthovandate (New England Biolabs)} and incubated on ice for 15 minutes. After three rounds of 5 seconds sonication at 25-30 % (samples kept on ice) with a tip sonicator (Bandelin Sonorex), the suspensions were centrifuged at 13'000 rpm for 15 minutes at 4 °C. Supernatants were collected in new Eppendorf tubes and the concentrations were measured via Bradford Assay (Biorad). Bradford solution was diluted 1:5 with ddH_2O and 1 ml was put into a plastic one-way macro-cuvette (VWR). Sample at a volume of 1 µl was added and mixed by inverting three times. Measurements were taken at 595 nm using a photometer (Amersham Biosciences) and protein concentrations were determined against a BSA standard curve. Lysates were then shock-frozen in liquid nitrogen and transferred to the -80 °C refrigerator.

V.8 SDS-PAGE AND WESTERN BLOTTING

Samples were mixed 1:4 with loading buffer (NuPAGE® LDS Sample Buffer (4 X), Invitrogen) supplemented with 50 mM DL-Dithiothreitol (Sigma-Aldrich) and heated for 10 minutes at 70 °C. Subsequently, samples were loaded onto a NuPAGE® Tris-Acetate 3-8 % gradient gel (Invitrogen) and run with NuPAGE® Tris-Acetate SDS Running Buffer (Invitrogen) for 1 hour and 15 minutes at 140 V. Proteins were then blotted onto a nitrocellulose membrane via wet transfer. NuPAGE® Transfer Buffer was supplemented with 10 % methanol and the transfer was performed at 4 °C for 4 hours at 90 V. Membranes were either left in transfer buffer overnight in the cold-room or probed right away.

Membranes were washed with TBS with 0.1% tween (TBST) for 5 minutes and stained with PONCEAU before being cut with a scalpel. All membrane stripes were washed again with TBST until the PONCEAU staining was gone and were blocked with 5 % milk in TBST for 1 hour at room temperature.

Membranes were then incubated with primary antibodies (Table 2) for either 3 hours at RT or overnight in the cold room. After washing three times for 5 minutes with TBST, membranes were incubated with HRP-conjugated secondary antibodies (Table 2) for 1 hour at RT. Membranes were washed with TBST (three times for 5 minutes) and proteins were detected using chemiluminiscence and ECL reagents (GE healthcare). Films (Amersham Hyperfilm ECL) were purchased from GE healthcare and developed via a Film processor (CAWOMAT 2000 IR).

Membranes were washed with TBST and stored in a plastic wrap at 4 °C.

	Antibody	**Species**	**Company**	**Dilution**
Primary antibodies	Actin	Rabbit IgG	Sigma Aldrich	1:1000
	ATM (2C1)	Mouse IgG	Santa Cruz	1:1000
	ATMIN	Rabbit IgG	Chemicon	1:1000
	CHK1 (DCS-310)	Mouse IgG	Santa Cruz	1:1000
	KAP-1	Rabbit IgG	Bethyl Laboratories	1:1000
	p95/NBS1	Rabbit IgG	NEB_Cell Signalling	1:1000
	SMC1 - ChiP Grade	Rabbit IgG	Abcam	1:1000
	Phospho-ATM (Ser1981) (10H11.E12)	Mouse IgG	NEB_Cell Signalling	1:1000
	Phospho-CHK1 (Ser217)	Rabbit IgG	NEB_Cell Signalling	1:1000
	Phospho KAP-1 (Ser824)	Rabbit IgG	Bethyl Laboratories	1:1000
	Phospho p53 (Ser15) 16G8	Mouse IgG	NEB_Cell Signalling	1:1000
	Phospho-SMC1 (Ser957), clone 5D11G5	Mouse IgG	MILLIPORE	1:400
Secondary antibodies	HRP-conjugated goat anti-rabbit		Jackson Laboratory	1:5000
	HRP-conjugated goat anti-mouse		Jackson Laboratory	1:5000

Table 2. Antibody list used for Western blotting.
Antibodies were diluted as indicated in 5 % milk in TBST.

V.9 STRIPPING OF MEMBRANES

Before stripping, membranes were washed twice for 5 minutes with TBST. Membranes were then either stripped by incubating for 30 minutes at 50 °C with stripping buffer (62.5 mM Tris pH 6.8, 2 % SDS, 0.1 M β-mercaptoethanol) and washed for 1 hour with TBST (4-5 changes) or washed

with ddH_2O for 5 minutes, incubated with 0.2 N NaOH on the shaker for 10 minutes and then washed again with ddH_2O for 5 minutes. After blocking the membranes, primary antibodies were then applied again and Western blotting was performed according to protocol.

V.10 CELL CYCLE RELEASE AND FACS ANALYSIS

HeLa reverse transfections were set up in 6-wells (non-targeting siRNA and siATMIN pool). On Day 3, cells were treated with 4 µM aphidicolin for 24 hours and one sample was left untreated. Blocked cells were then released by changing to normal growth medium without aphidicolin and samples were taken at time points 0 hours (UT and 0 h), 4 hours (4 h), 8 hours (8 h), 12 hours (12 h), 16 hours (16 h) and 24 hours (24 h) after release. One sample was treated with 1 µM aphidicolin over night and taken at the 0 hour release point.
Samples were prepared by aspirating the medium, washing and trypsinising the cells and spinning them down at 1'200 rpm for 5 minutes at 4 °C in a FACS tube. Pellets were washed with PBS, supernatant was discarded fully by pouring out and tapping FACS tubes on paper. EtOH at a volume of 1.5 ml at 70 % EtOH (-20 °C) was added dropwise to pellets while slowly vortexing. Samples were then kept at 4 °C for at least 40 minutes.
Samples were spun down at 1'700 rpm for 5 minutes at 4 °C, EtOH was pipetted off carefully and pellets were washed with 1 ml PBS (2'000 rpm, 6 minutes, 4 °C). Cells were rehydrated in 1 ml PBS for 40 minutes and spun down for 8 minutes at 2'000 rpm at 4 °C. Samples were centrifuged, supernatant discarded and cells stained with 200 µl PI (Propidium Iodide, 1:5 in PBS, 100 µg/ml RNase A). Before cell cycle analysis on a FACScalibur flow cytometer 200 µl PBS was added and the suspension was filtered through a strainer into a 96-well plate. Following cell acquisition analysis was performed using FlowJo software.

Western samples were taken at time points 0 h and 24 h in order to test the knock-down efficiency.

VI. RESULTS

The ATM (Ataxia Telangiectasia Mutated) co-factor NBS1 (Nijmegen Breakage Syndrome 1) has been shown to be necessary for ATM-mediated signaling after DSBs acquired through IR (Difilippantonio, Celeste et al. 2005). However, after other types of DNA damage (including UV irradiation and aphidicolin), depletion of NBS1 does not lead to impaired ATM signaling which points towards the direction that another co-factor of ATM is required to signal certain types of DNA damage (Difilippantonio, Celeste et al. 2005). A recently identified ATM co-factor termed ATMIN (ATM INteractor, also known as ASCIZ) was found to regulate Rad51 focus formation and apoptosis after DNA methylating damage in U2OS cells (McNees, Conlan et al. 2005) and its depletion was shown to impair ATM-mediated signaling after treatment of mouse embryonic fibroblasts (MEFs) with chloroquine, NaCl or hydroxyurea, but did not lead to IR hypersentitivity (Kanu and Behrens 2007). Hence, ATMIN and NBS1 are proposed to function in distinct stimulus-dependent pathways after different kinds of exogenous or endogenous DNA damage.

VI.1 ATMIN IS REQUIRED FOR ATM-MEDIATED SIGNALING AFTER REPLICATIVE STRESS IN MEFS

In order to address whether ATMIN functions in ATM-mediated signaling after replicative stress, phosphorylation statuses of downstream targets of ATM were assessed by Western blotting after treatment with aphidicolin. Aphidicolin is an inhibitor of polymerase α and δ and therefore blocks DNA replication in eukaryotic cells. It has been reported that low levels of aphidicolin, such as 0.4 µM, do not strongly affect cell cycle progression (Harrigan, Belotserkovskaya et al. 2011). Wild-type ($ATMIN^{+/+}$) mouse embryonic fibroblasts (MEFs) were treated at a confluency of about 80 % with 1 µM or 4 µM aphidicolin overnight. While 1 µM treatment led to a partial block at the G1/S boarder, 4 µM aphidicolin led to a complete block at the G1/S boarder (Figure 2).

To determine whether ATMIN is required for signaling replicative stress, wild-type ($ATMIN^{+/+}$) and knock-out ($ATMIN^{\Delta/\Delta}$) immortalized mouse embryonic fibroblasts (MEFs) were treated at a confluency of about 80 % with 1 µM aphidicolin overnight. The next day, Western blotting was performed and activation of ATM-mediated downstream signaling was assessed by using phospho-specific antibodies for ATM's substrates. Induction of signaling after treatment could clearly be seen in wild-type MEFs by activation of ATM (auto-phosphorylation at serine 1981) and phosphorylation of its downstream targets including KAP1 (KRAB domain-associated protein 1) at serine 824 or SMC1 (structural maintenance of chromosomes 1) at serine 957 (Figure 3 A). It was observed that ATMIN depletion resulted in a dramatic decrease in phosphorylation of KAP1 and SMC1 and autophosphorylation of ATM after aphidicolin treatment. To a lesser extent, a reduction of phosphorylation of p53 at serine 15 was observed after aphidicolin treatment (Figure 3 A).

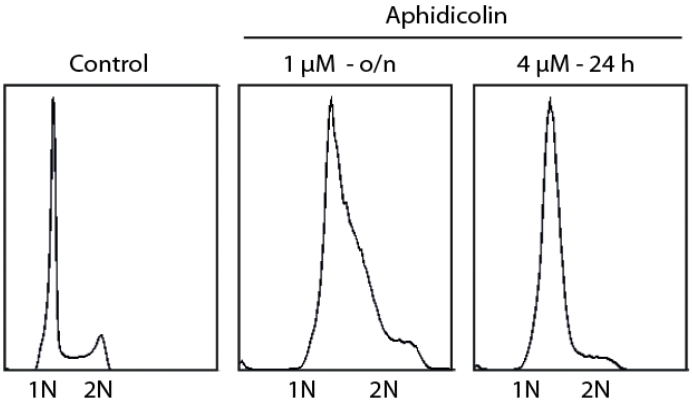


Figure 2. Partial block at G1/S border after overnight treatment with 1 µM aphidicolin.

HeLa cells were untreated, treated with 1 µM aphidicolin overnight (o/n) or 4 µM aphidicolin for 24 hours, then collected the next day, fixed with 70 % ethanol at - 20 °C and analyzed via propidium iodide staining (1:5 in PBS) containing RNase A (100 µg/ml) using flow cytometry.

Thus, ATMIN plays a critical role in ATM-mediated downstream signaling after replicative stress.

VI.2 ATMIN IS NOT REQUIRED FOR INDUCTION OF ATR SIGNALING AFTER LOW LEVELS OF APHIDICOLIN IN MEFS

ATR (Ataxia Telangiectasia and Rad3-related protein) is known to be required for DNA damage responses after UV irradiation and treatment with DNA alka-

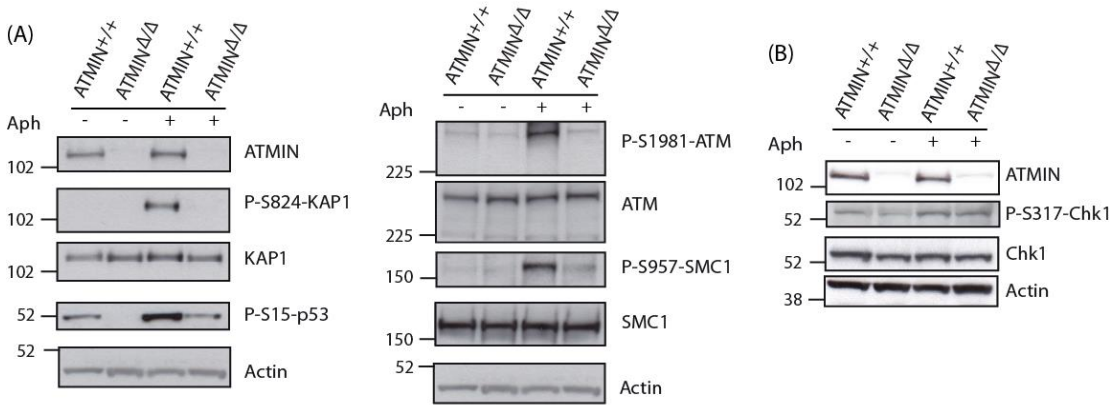


Figure 3. ATMIN is required for ATM-mediated but not ATR-mediated signaling following replicative stress.

(A and B) ATMIN$^{+/+}$ and ATMIN$^{\Delta/\Delta}$ MEFs were either left untreated or treated with 1 µM aphidicolin (Aph) overnight. Whole cell extracts were prepared, resolved on a 3-8 % Tris-Acetate gradient gel and then transferred to a nitrocellulose membrane. The membrane was probed with the indicated antibodies.

lyting agents and aphidicolin that lead to activation of cell cycle checkpoints and halt cell cycle progression (Shechter, Costanzo et al. 2004). Furthermore, the proteins NBS1, ATMIN, Ku and ATRIP use similar motifs in their function as co-factors for the Phosphatidylinositol 3-kinase-related kinases (PIKKs) ATM, ATR and DNA-PKs (Falck, Coates et al. 2005).

In order to determine whether ATMIN specifically affects ATM signaling or also ATR signaling following replicative stress, the phosphorylation of CHK1 (Checkpoint kinase 1) at serine 317 that is exclusively mediated by ATR (Zhao and Piwnica-Worms 2001) was assessed as an indicator for ATR signaling after replicative stress. After treatment of ATMIN$^{+/+}$ and ATMIN$^{\Delta/\Delta}$ MEFs with 1 µM aphidicolin overnight, CHK1 was found to be phosphorylated at serine 317 to an equal extent regardless of ATMIN's presence or absence (Figure 3 B).

This finding points towards the direction that ATMIN is not an ATR co-factor and is therefore not required for ATR signaling after low levels of aphidicolin.

VI.3 Depletion of ATMIN does not affect ATM-mediated signaling after IR in MEFs

It has been proposed that ATMIN and NBS1 have complementary functions in respect to ATM activation (Kanu and Behrens 2007) and that there is a competition between the two co-factors (Zhang, Penicud et al. 2012). Whereas depletion of ATMIN was shown to affect ATM-mediated downstream signaling after treatment with chloroquine, hypotonic stress and HU, but not after IR or UV, overexpression of ATMIN led to impaired signaling after IR by competing with NBS1 for ATM binding (Kanu and Behrens 2007; Zhang, Penicud et al. 2012). Considering these findings, it was important to determine whether ATMIN$^{\Delta/\Delta}$ MEFs, did not alter ATM-mediated signaling after IR (Figure 4). MEFs were treated with different doses of IR (as indicated) followed by an incubation at 37 °C for 30 minutes. It was observed that the phosphorylation of the ATM substrates SMC1 and KAP1 was not affected in ATMIN-deficient cells following IR. Reduced levels of P-S824-KAP1 in ATMIN knock-out cells could be explained by taking into account the total levels of the protein. However, auto-phosphorylation of ATM at serine 1981

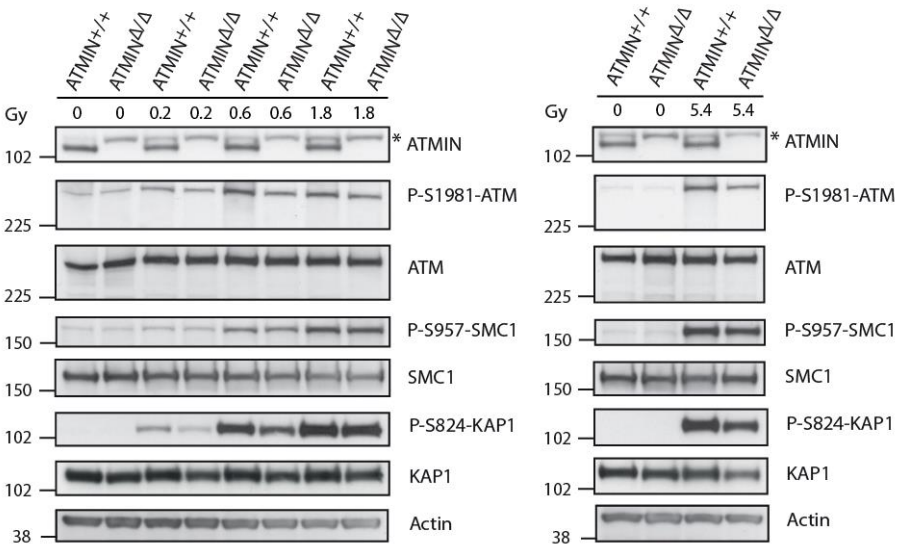


Figure 4. ATMIN is largely dispensible for ATM-mediated signaling after IR.

ATMIN$^{+/+}$ and ATMIN$^{\Delta/\Delta}$ MEFs were either left untreated or treated with different doses of ionizing radiation (as indicated in Gy). Whole cell extracts were prepared, resolved on a 3-8 % Tris-Acetate gradient gel and then transferred to a nitrocellulose membrane. The membrane was probed with the indicated antibodies. * = unspecific band

seemed affected by depletion of ATMIN which is in accordance with previous studies by Kanu and Behrens (Kanu and Behrens 2007).

Taken together, these data indicate that loss of ATMIN does not have a significant influence on ATM-mediated signaling after IR apart from leading to a reduction of P-S1981-ATM.

VI.4 Administration of 1 µM aphidicolin for 24 hours leads to ATM-mediated signaling in HEK293T cells

In order to test ATM-mediated signaling after replicative stress in human cell lines, HeLa cells were treated either for 24 hours or overnight with 1 µM aphidicolin. Induction of signaling was assessed by determining the phosphorylation of ATM substrates. A minor increase in P-S824-KAP1 could be observed, but P-S1981-ATM and P-S957-SMC1 could not be detected (Figure 5 A). Hence, another human cell line, HEK293T, was treated with 1 µM aphidicolin

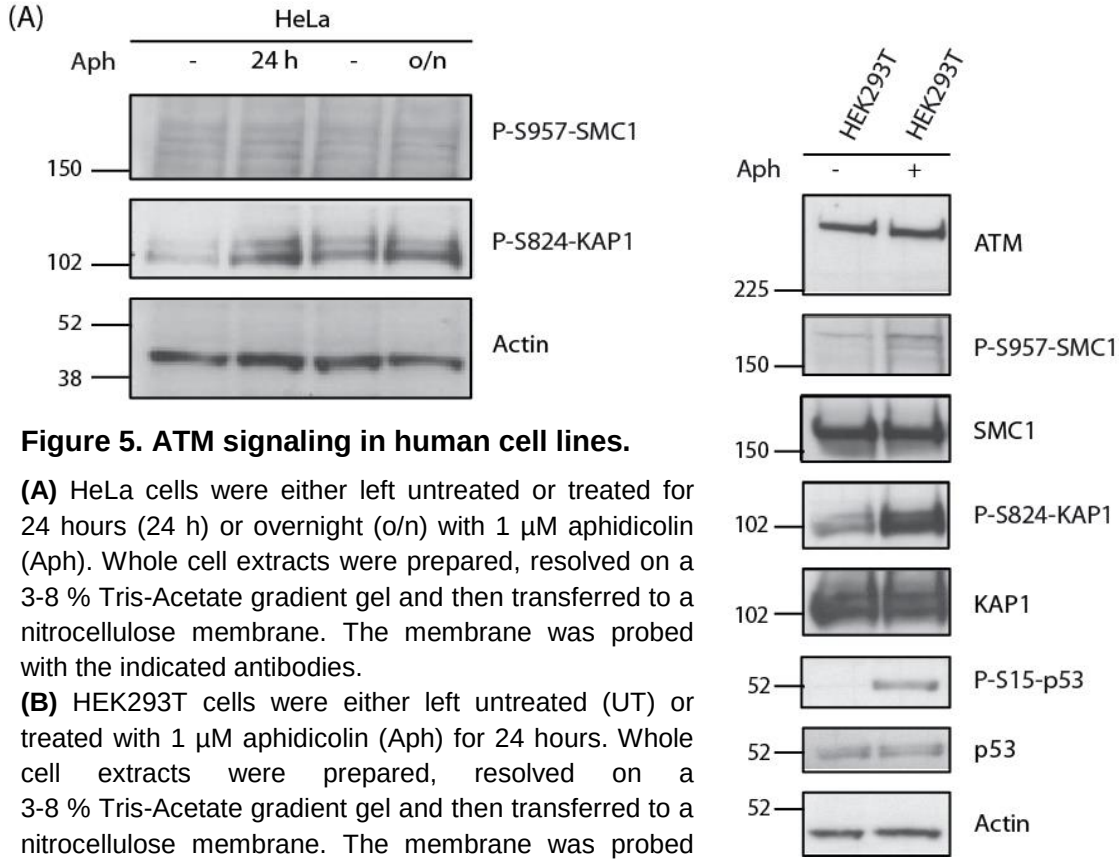


Figure 5. ATM signaling in human cell lines.

(A) HeLa cells were either left untreated or treated for 24 hours (24 h) or overnight (o/n) with 1 µM aphidicolin (Aph). Whole cell extracts were prepared, resolved on a 3-8 % Tris-Acetate gradient gel and then transferred to a nitrocellulose membrane. The membrane was probed with the indicated antibodies.

(B) HEK293T cells were either left untreated (UT) or treated with 1 µM aphidicolin (Aph) for 24 hours. Whole cell extracts were prepared, resolved on a 3-8 % Tris-Acetate gradient gel and then transferred to a nitrocellulose membrane. The membrane was probed with the indicated antibodies.

for 24 hours and ATM-mediated signaling was evaluated. Whereas after replicative stress P-S824-KAP1, P-S957-SMC1 and P-S15-p53 levels were clearly upregulated, P-S1981-ATM could not be detected (Figure 5 B).
According to these results, the HEK293T cell line seems to be a potential candidate for testing the effect of ATMIN depletion on signaling after replicative stress in human cells. Since I could not carry out this experiment during my time at CeMM, it will be followed up by a lab member.

VI.5 ATMIN IS REQUIRED FOR 53BP1 AND P-S1981-ATM FOCUS FORMATION IN UNTREATED MEFS AND AFTER REPLICATIVE STRESS

There is recent evidence that 53BP1-containing complexes are formed at DNA lesions in response to low levels of aphidicolin (0.2 - 0.4 µM) in an ATM-dependent manner (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). These 53BP1 foci were found to strongly co-localize with a subset of DNA damage associated proteins including MDC1, RNF168 and P-S1981-ATM (Lukas, Savic et al. 2011). Since ATMIN appears to be crucial for ATM activation after replicative stress mediated by aphidicolin, the co-factor could potentially play a role in localization of P-S1981-ATM and hence 53BP1 after administration of aphidicolin. Therefore, $ATMIN^{+/+}$ and $ATMIN^{\Delta/\Delta}$ MEFs either untreated or treated with 1 µM aphidicolin overnight were stained for 53BP1 by immunofluorescence. The knock-out was confirmed by checking ATMIN protein levels in $ATMIN^{+/+}$ and $ATMIN^{\Delta/\Delta}$ MEF lysates (Figure 6 A). In untreated wild-type cells, spontaneous 53BP1 foci could be detected in the nucleus and after aphidicolin treatment the number of cells with a higher number of foci increased (Figure 6 B,D). According to the immunofluorescence images, ATMIN depletion led to a strong decrease of 53BP1 focus formation in untreated as well as treated cells (Figure 6 B,D). This could also be shown by quantification of the number of 53BP1 foci per nucleus by Cell Profiler cell image analysis software (Figure 6 C,E). Even though there was always an upregulation of cells with more than two foci after replicative stress regardless of ATMIN's presence or absence, the percentages of cells with less than three

53BP1 foci were always significantly higher in the knock-out cell line (Figure 6 C,E). These results indicate a role for ATMIN in formation and localization of 53BP1 after replicative stress in mouse embryonic fibroblasts.

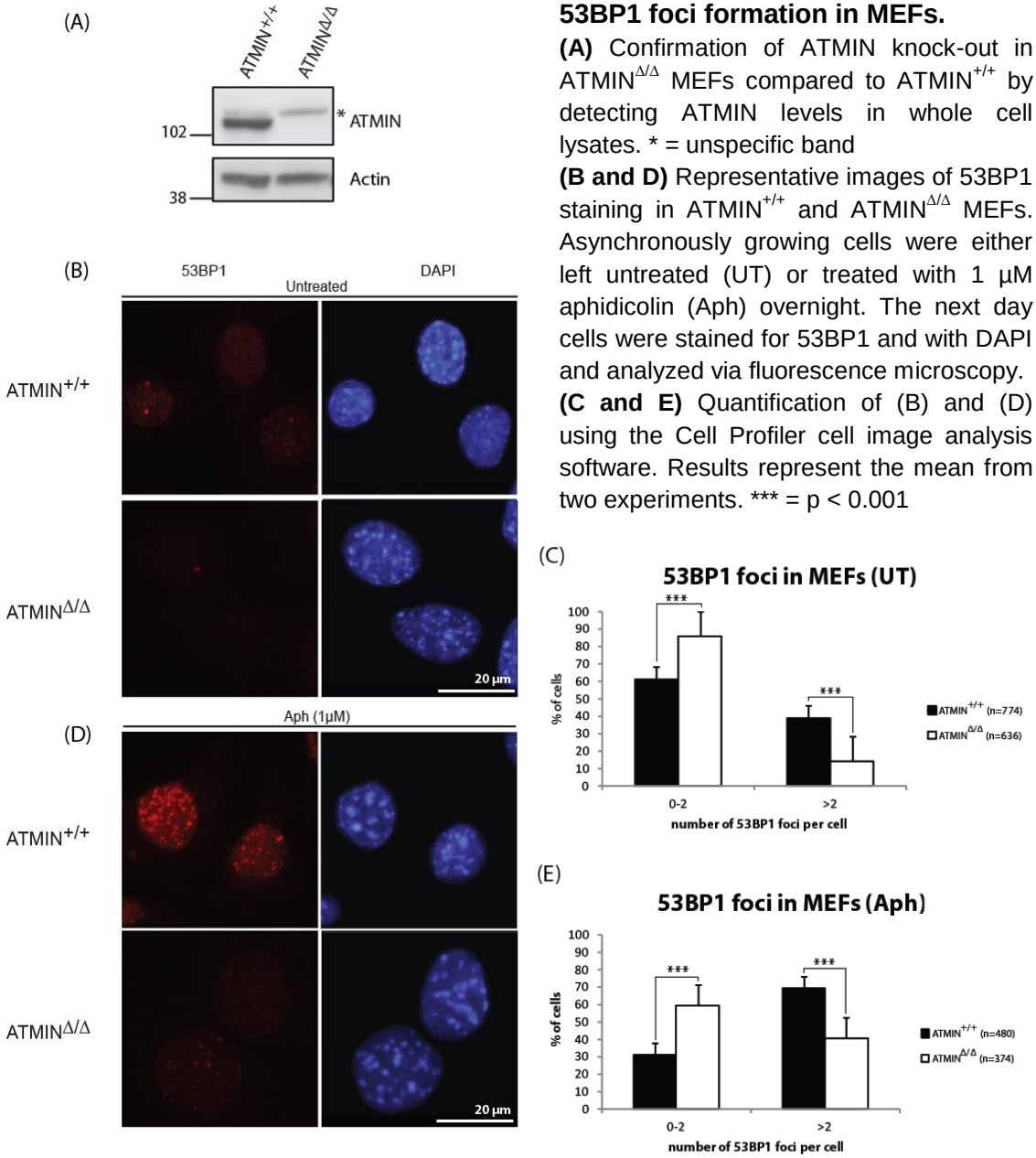


Figure 6. Requirement of ATMIN for 53BP1 foci formation in MEFs.
(A) Confirmation of ATMIN knock-out in ATMIN$^{\Delta/\Delta}$ MEFs compared to ATMIN$^{+/+}$ by detecting ATMIN levels in whole cell lysates. * = unspecific band
(B and D) Representative images of 53BP1 staining in ATMIN$^{+/+}$ and ATMIN$^{\Delta/\Delta}$ MEFs. Asynchronously growing cells were either left untreated (UT) or treated with 1 µM aphidicolin (Aph) overnight. The next day cells were stained for 53BP1 and with DAPI and analyzed via fluorescence microscopy.
(C and E) Quantification of (B) and (D) using the Cell Profiler cell image analysis software. Results represent the mean from two experiments. *** = $p < 0.001$

VI.6 ATMIN IS REQUIRED FOR 53BP1 AND P-S1981-ATM FOCUS FORMATION IN UNTREATED HELA CELLS AND AFTER REPLICATIVE STRESS

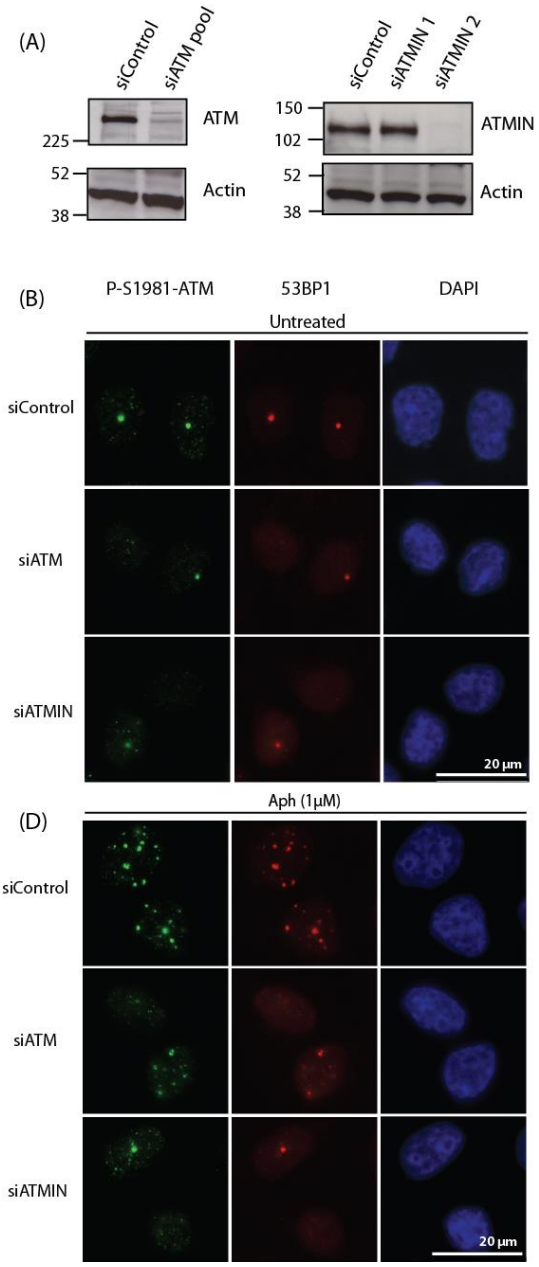
The same experiment was conducted in HeLa cells to test whether this can also be seen in a human cell line. HeLa cells were reverse transfected with non-targeting siRNA, siATMIN and, as a control, siATM and stained for 53BP1 and P-S1981-ATM focus formation in either untreated conditions or after replicative stress induced by aphidicolin treatment. An efficient knock-down could be demonstrated by diminishment of the targeted proteins (Figure 7 A, siATM pool and siATMIN 2). In wild-type HeLas treated with non-targeting siRNA, spontaneous (in untreated conditions, Figure 7 B) and induced (after aphidicolin treatment, Figure 7 D) 53BP1 and P-S1981-ATM focus formation could be detected. In line with already published data (Lukas, Savic et al. 2011), 53BP1 foci strongly co-localized with P-S1981-ATM foci (Figure 7 B,D). Depletion of ATM led to decreased numbers of 53BP1 focus formation in both conditions (Figure 7 B,D). The same effect has already been shown by two research groups after inhibition of ATM (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). Surprisingly, P-S1981-ATM could be detected to the same extend as 53BP1 despite the efficient ATM knock-down. It is possible that there is enough remaining protein to lead to sufficient auto-phosphorylation and focus formation that can be clearly detected by immunofluorescence staining or alternatively the antibody is not specific to the ATM protein and detects other proteins phosphorylated on similar motifs. The immunofluorescence staining and hence DNA damage foci formation in cells treated with siATM and siATMIN were highly comparable (Figure 7 B,D) which was also apparent in the quantification of 53BP1 focus formation (Figure 7 C,E). The percentages of cells with more than two 53BP1 foci in the nucleus were always significantly lower in cells depleted for either ATM or ATMIN (Figure 7 C,E).

Hence, ATMIN is important for 53BP1 and P-S1981-ATM focus formation in untreated conditions and after replicative stress in mouse and human cells.

Figure 7. Requirement of ATMIN for 53BP1 foci formation in HeLa cells.

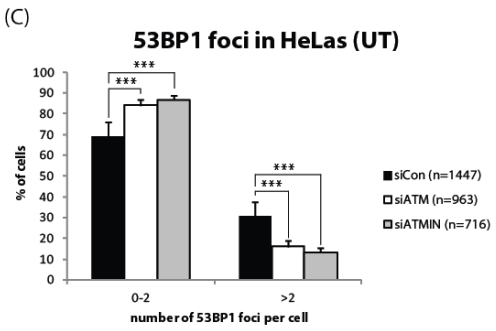
(A, left) HeLa cells were transfected with non-targeting siRNA (siControl) and siRNA against ATM. Whole cell lysates were resolved on a 3-8 % SDS-PAGE, transferred to a nitrocellulose membrane and probed for ATM to confirm ATM knock-down.

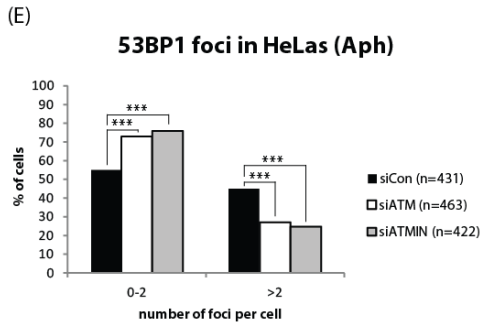
(A, right) HeLa cells were transfected with siRNA against ATMIN. Whole cell lysates were resolved on a 3-8 % SDS-PAGE, transferred to a nitrocellulose membrane and probed for ATMIN to confirm ATMIN knock-down. SiATMIN 1 in lane 2 did not work and was not used for experiments.



(B and D) Representative images of 53BP1 and P-S1981-ATM staining in siControl, siATM and siATMIN HeLa cells. Asynchronously growing cells were either left untreated (UT) or treated with 1 µM aphidicolin (Aph) overnight. The next day cells were stained for 53BP1 and P-S1981-ATM and with DAPI and analyzed via fluorescence microscopy.

(C and E) Quantification of (B) and (D) using the Cell Profiler cell image analysis software. Results represent the mean from three (UT) experiments or one (Aph) experiment. *** = $p < 0.001$





VI.7 NBS1 IS DISPENSIBLE FOR 53BP1 AND P-S1981-ATM FOCUS FORMATION IN UNTREATED HELA CELLS AND AFTER REPLICATIVE STRESS

NBS1 and ATMIN function in a stimulus-dependant manner in response to different DNA damage and do not act simultaneously. Hence, knock-down of NBS1 should not affect focus formation and localization of 53BP1 and P-S1981-ATM after replicative stress. Indeed, NBS1 depletion (Figure 8 A) did not seem to lead to any reduction in 53BP1 and P-S1981-ATM focus formation in untreated and aphidicolin treated HeLas (Figure 8 B,D). On the contrary, quantification of 53BP1 focus formation indicated an increase in the percentage of cells with a higher number of 53BP1 foci after NBS1 knock-down (Figure 8 C,E). This result is in agreement with the published data that ATMIN and NBS1 compete for ATM binding and activation and that loss of NBS1 therefore leads to increased ATMIN-dependent ATM signaling after exposure to e.g. hypotonic stress (Zhang, Penicud et al. 2012).

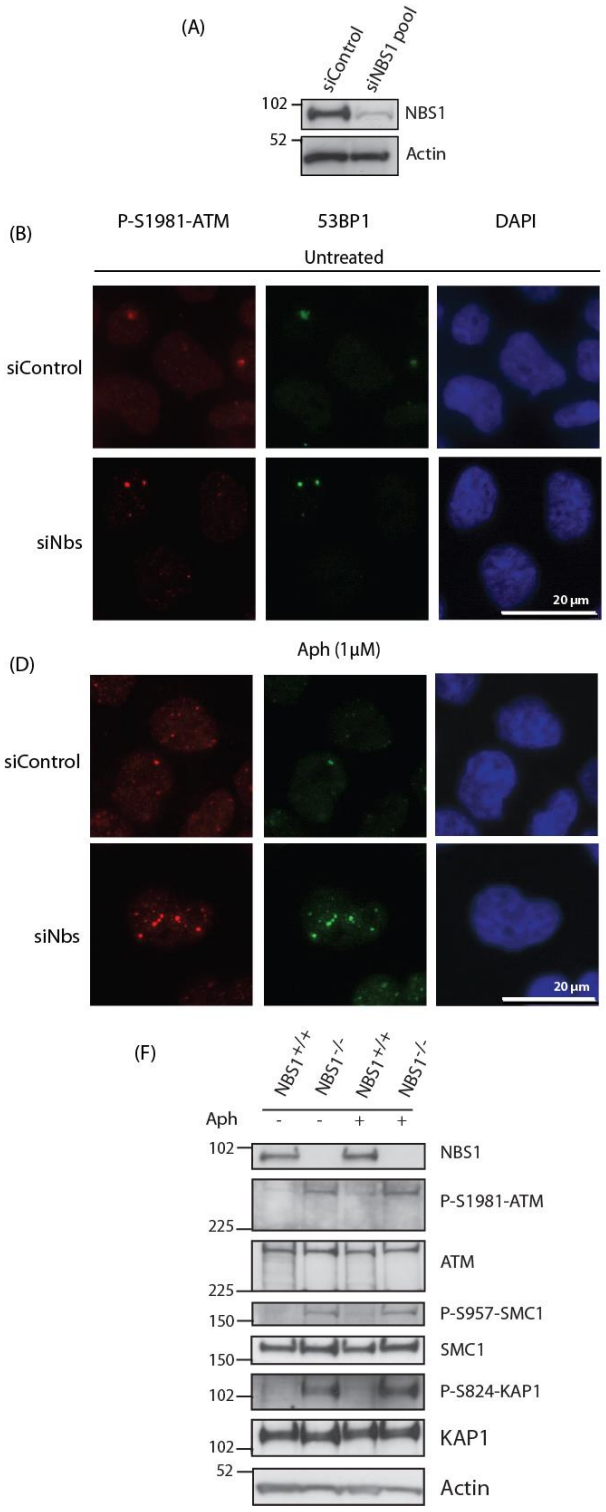
Thus, depletion of NBS1 does not impair 53BP1 and P-S1981-ATM DNA damage focus formation after replicative stress and, to the contrary, appears to increase their recruitment by ATMIN. Alternatively loss of NBS1 could lead to a higher level of basal damage and this is reflected by the increase in 53BP1 and P-S1981-ATM foci. This is supported by the finding that $NBS1^{-/-}$ MEFs have more background damage (Figure 8 F). Treatment with aphidicolin did not further increase the phosphorylation of ATM substrates.

VI.8 ATMIN DOES NOT SEEM TO AFFECT CELL CYCLE PROGRESSION

Since it is proposed that 53BP1 nuclear bodies form around DNA lesions to shield them from excessive DNA degradation during G1 phase until the DNA can be properly resolved and repaired through recombination in S phase (Lukas, Savic et al. 2011), impaired recruitment of 53BP1 due to ATMIN depletion could potentially affect cell cycle progression. To test this hypothesis,

Figure 8. Depletion of NBS1 leads to an increase in 53BP1 foci formation.

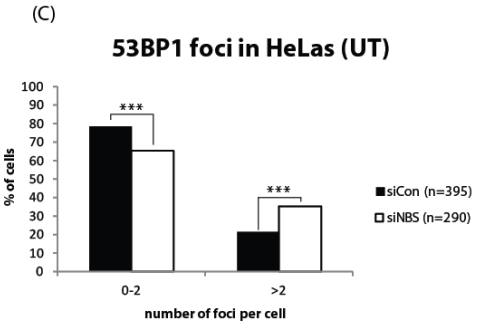
(A) HeLa cells were transfected with non-targeting siRNA (siControl) and siRNA against NBS1. Whole cell lysates were resolved on a 3-8 % SDS-PAGE, transferred to a nitrocellulose membrane and probed for NBS1 to confirm NBS1 knock-down.

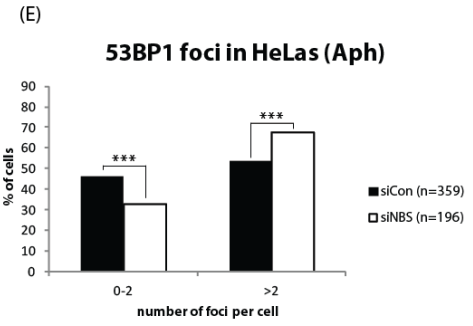


(B and D) Representative images of 53BP1 and P-S1981-ATM staining in siControl and siNBS1 HeLa cells. Asynchronously growing cells were either left untreated (UT) or treated with 1 µM aphidicolin (Aph) overnight. The next day cells were stained for 53BP1 and P-S1981-ATM and with DAPI and analyzed via fluorescence microscopy.

(C and E) Quantification of (B) and (D) using the Cell Profiler cell image analysis software. Results represent one experiment. *** = $p < 0.001$

(F) $NBS1^{+/+}$ and $NBS1^{-/-}$ MEFs were either left untreated or treated with 1 µM aphidicolin (Aph) overnight. Whole cell extracts were prepared, resolved on a 3-8 % Tris-Acetate gradient gel and then transferred to a nitrocellulose membrane. The membrane was probed with the indicated antibodies.





HeLa cells transfected with non-targeting siRNA (siControl) or siATMIN were blocked in S phase by administration of 4 µM aphidicolin for 24 hours and samples were taken at different time points after release. A clear difference between the cell cycle profiles of cells in the presence and absence of ATMIN could not be detected (Figure 9 A). After the treatment and before changing the medium to normal growth medium without drug, most cells were blocked in S-phase (Figure 9 A, 0 h). At the time points 8 hours and 12 hours after release, most cells moved on to G2/M phase (Figure 9 A, 8 h and 12 h) and after 16 hours a large proportion was found in G1 phase (Figure 9 A, 16 h). Cells that had been released for 24 hours showed the same asynchronous cell cycle profile as the untreated sample (Figure 9 A, 24 h and UT). The knock-down efficiency was found to be only partial at the 0 h time point (Figure 9 B) and no knock-down was observed at the 24 h time point. This could be the reason why no difference in cell cycle profiles after release of the S phase block could be observed in HeLa cells either transfected with non-targenting siRNA or siATMIN.

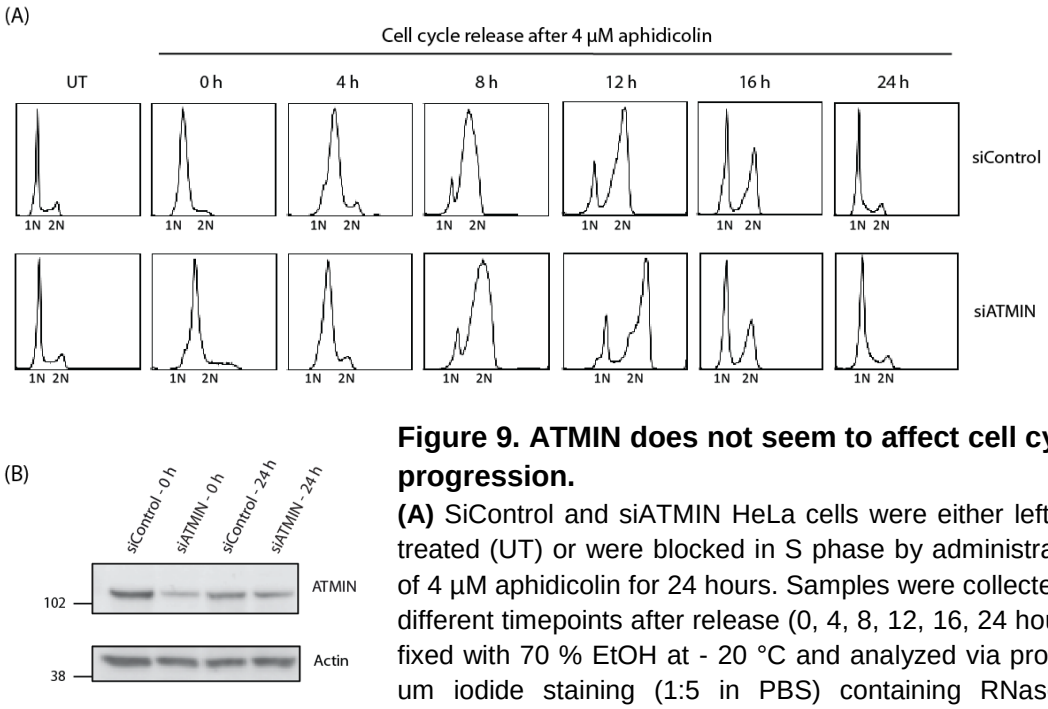


Figure 9. ATMIN does not seem to affect cell cycle progression.

(A) SiControl and siATMIN HeLa cells were either left untreated (UT) or were blocked in S phase by administration of 4 µM aphidicolin for 24 hours. Samples were collected at different timepoints after release (0, 4, 8, 12, 16, 24 hours), fixed with 70 % EtOH at - 20 °C and analyzed via propidium iodide staining (1:5 in PBS) containing RNase A (100 µg/ml) using flow cytometry.

(B) Validation of ATMIN knock-down in HeLa cells by detecting ATMIN levels in whole cell lysates at 0 hours and 24 hours after release

Considering these findings, it seems probable that ATMIN is not crucial for cell cycle progression, but the possibility of its involvement in the cell cycle regulation cannot be entirely excluded. Further experiments have to be conducted before a clear conclusion can be drawn.

VII. DISCUSSION AND CONCLUSION

It is generally considered that ATM or ATR is activated in a distinct and stimulus-dependent matter after DSBs or replicative stress respectively (Figure 1). It has also been shown that there is crosstalk between the two pathways (Stiff, Walker et al. 2006). Furthermore, in recent years it has become more evident that ATM's function is not restricted to signaling after introduction of DSBs, but also after other types of stresses as for example chromatin changes or hypotonic stress (Bakkenist and Kastan 2003; Zhang, Penicud et al. 2012). In this regard, Lukas et al. and Harrigan et al. found that, following low levels of replicative stress, ATM is required for recruitment of DNA damage repair proteins as for example 53BP1 to regions of replicative stress including common fragile sites. These regions are then shielded from degradation and erosion throughout G1 phase of the cell cycle in order to be repaired in S phase (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). My results indicate that the ATM co-factor ATMIN is crucial for ATM-mediated signaling following low levels of replicative stress and that formation of 53BP1-containing nuclear foci is dependent on ATMIN.

In ATMIN knock-out MEFs, ATM-mediated signaling following replicative stress introduced by low levels of the replicative polymerase inhibitor aphidicolin was clearly impaired (Figure 3 A). Phosphorylation of the ATM substrates KAP1 and SMC1 was diminished and auto-phosphorylation of ATM was strongly reduced which led to the conclusion that ATM kinase can only be activated in the presence of ATMIN. Total levels of ATM were not affected by loss of ATMIN despite the fact that a mutual stabilization of ATM and ATMIN has been observed by Kanu and Behrens in 2007 (Kanu and Behrens 2007). A smaller reduction in p53 phosphorylation could also be observed in ATMIN knock-out MEFs (Figure 3 A). The remaining p53 phosphorylation could be ATR dependet as p53 is a common target of the two kinases.

Since ATMIN and the ATM co-factor NBS1 are thought to have distinct and non-redundant functions, it was expected that loss of ATMIN has no effect on

signaling after IR. Indeed, I was able to show that ATM and its downstream substrates were phosphorylated in a dose-dependent manner regardless of the presence of ATMIN (Figure 4). Despite the fact that Zhang et al. were able to show a competition between the two co-factors and an enhancement in ATM signaling after IR in ATMIN$^{\Delta/\Delta}$ MEFs, I could not reproduce this data (Zhang, Penicud et al. 2012). In contrary, there was a minor reduction seen in the activation of ATM substrates in ATMIN knock-out MEFs compared to wild-type cells at all doses tested (Figure 4). Interestingly, even though total ATM and SMC1 levels seemed to stay the same in all samples, total KAP1 was reduced in ATMIN knock-out cells that were treated with IR.

Since ATM-mediated signaling following DSBs was not strongly impaired in ATMIN deficient cells, I expected to see the same for signaling after replicative stress in NBS1 knock-out MEFs. However, loss of NBS1 led to a high level of basal damage and therefore phosphorylation of ATM substrates in untreated conditions that could not be increased by administration of aphidicolin (Figure 8 F). This could be partly due to other yet unknown functions of NBS1 that also lead to embryonic lethality and strong ATM activation in NBS1 knock-out mice (Zhu, Petersen et al. 2001). Furthermore, the same effect seen for ATMIN depleted MEFs on ATM-mediated signaling following replicative stress could not yet be shown in human cell lines because induction of ATM-mediated signaling after replicative stress in wild-type HeLas or HEK293T cells was only achieved by administration of aphidicolin for a longer time period (24 hours instead of overnight, Figure 5) and I was not able to finish these experiments during my time at CeMM. Experiments in knock-down cells will however be followed up by a lab member to determine whether this ATMIN-mediated ATM activation holds true in human cells.

Since ATR is known to be the major kinase in respect to replicative stress and ATM and ATR have some redundant functions and common substrates, it was not clear if loss of ATMIN only affects ATM activation, but not ATR-mediated signaling. Indeed, I was able to show that in ATMIN$^{\Delta/\Delta}$ MEFs treated with aphidicolin phosphorylation of CHK1 at serine 317 (a SQ/TQ site exclusively phosphorylated by ATR (Zhao and Piwnica-Worms 2001)) was not impaired which

points to the fact that the function of ATMIN following replicative stress is exclusively through ATM and independently of ATR (Figure 3 B).

Lukas et al. and Harrigan et al. showed in 2011 that low levels of aphidicolin lead to an increase in 53BP1-containing nuclear focus formation compared to the number of spontaneous foci under basal conditions and that this is ATM dependent (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011). Hence, according to the data mentioned above, it seemed possible that ATMIN is crucial for focus formation of DNA repair proteins at basal levels and following replicative stress and indeed I was able to show that loss of ATMIN significantly reduced the number of cells with higher number of 53BP1-containing nuclear bodies (Figure 6). This effect is not due to reduced levels of total 53BP1 protein since, according to data in the lab, 53BP1 total levels are not affected by ATMIN depletion. Furthermore, I was also able to recapitulate already known data showing that ATM loss leads to reduced numbers of 53BP1 foci (Figure 7, siATM) and could in this regard show that ATMIN knock-down led to a similarily strong reduction on localization of 53BP1 into foci in HeLa cells (Figure 7, siATMIN). I was able to demonstrate this effect in untreated and in aphidicolin treated cells which means that ATMIN is required for 53BP1 foci formation in basal and stressed conditions. Additionally, in line with already published data, I saw a strong co-localization of 53BP1 and P-S1981-ATM nuclear foci (Figure 7 B, D) and ATMIN knock-down affected localization of both proteins. These results lead to the conclusion that ATM is responsible for 53BP1 foci formation through its co-factor ATMIN. This idea could be further bolstered by the fact that NBS1 knock-down did not lead to a decrease in 53BP1 foci compared to non-targeting siRNA (Figure 8). Conversily, NBS1 depletion led to significantly increased foci formation in basal levels and following replicative stress which could be due to the fact that knock-down of NBS1 leads to high levels of DNA damage as demonstrated in NBS1 knock-out MEFs (Figure 8 F).

According to Harrigan et al. (Harrigan, Belotserkovskaya et al. 2011), 53BP1 nuclear bodies are formed in G1 phase of the cell cycle and Lukas et al. (Lukas, Savic et al. 2011) propose that they shield DNA lesions from excessive

DNA degradation until they can be repaired through recombination in S phase. If this was true, ATMIN depletion and consequent reduction of 53BP1 nuclear bodies could have an effect on cell cycle progression. Even though this could not be demonstrated in HeLa cells (Figure 9 A), it cannot be excluded that this finding was due to inefficient knock-down of ATMIN (Figure 9 B) and that the remaining protein is sufficient to hinder the effect on cell cycle progression.

Taken together, I propose a model in which signaling following low levels of aphidicolin is mediated by ATM and its co-factor ATMIN whereas NBS1 is dispensable. Furthermore, out data points to the fact that ATMIN is required for localization of P-S1981-ATM and subsequently 53BP1 into nuclear foci in basal conditions and following replicative stress (Figure 10, left). In my model, ATM activation is exclusively mediated by ATMIN and hence the ATM co-factors NBS1 and ATMIN have non-redundant functions. Consequently, loss of ATMIN leads to impaired signaling and reduced foci formation which causes incomplete and defective DNA damage repair (Figure 10, right). Assuming that the 53BP1 and P-S1981-ATM foci that I observed are the same as those described by Lukas et al. and referred to as 53BP1 nuclear bodies (Lukas, Savic et al. 2011), I would propose that ATMIN leads to an accumulation of these proteins at common fragile sites (CFSs). As a result, I hypothesize that loss of ATMIN would lead to failure in 53BP1 and P-S1981-ATM foci formation resulting in higher number of breaks at common fragile sites and an enhancement of UFBs that bridge regions that encounter replication problems during anaphase (Chan, North et al. 2007) (Figure 10, right).

In order to fully understand ATMIN's function following low levels of replicative stress and to test the model proposed, there are some open questions that have to be addressed in near future. Is ATMIN bound to ATM during the process of P-S1981-ATM and 53BP1 foci formation and DNA damage repair or does it only trigger signaling and soon dissociates from ATM? Do these ATM- and ATMIN-dependent foci form in G1 phase of the cell cycle and are therefore what Lukas et al. and Harrigan et al. refer to as 53BP1 nuclear bodies (Harrigan, Belotserkovskaya et al. 2011; Lukas, Savic et al. 2011)? Is ATMIN found at CFSs within the protein complex and does it directly bind the DNA

with its Zn^{2+}-finger motifs? Does ATMIN function in a complex with other proteins? Does loss of ATMIN elevate the number of UFBs at CFSs?

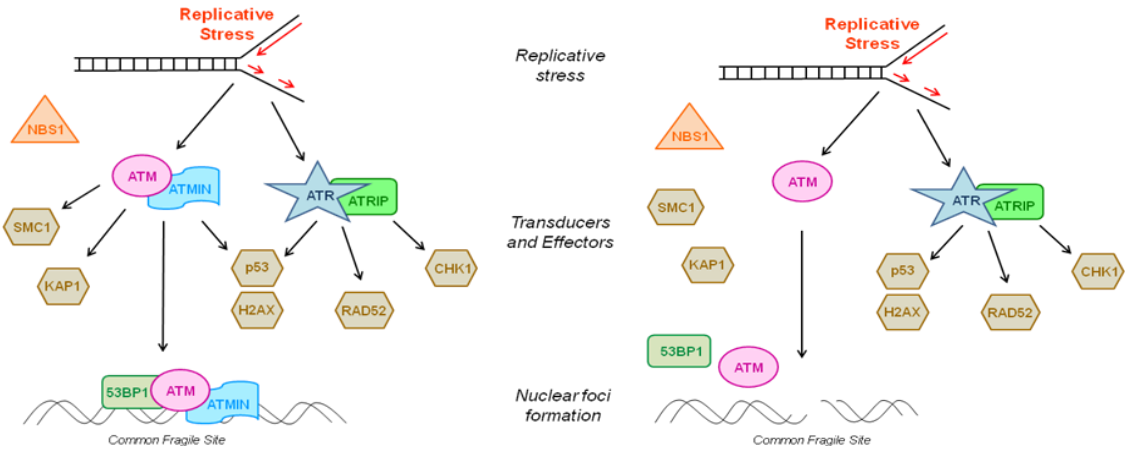


Figure 10. Model for the role of ATMIN following replicative stress.
In the presence of ATMIN (left), replicative stress leads to signaling via ATM/ATMIN or ATR/ATRIP which have unique and common substrates. ATM and ATMIN are also required for assembly of the protein complex containing 53BP1 and P-S1981-ATM and other proteins (possibly also ATMIN) at common fragile sites. In the absence of ATMIN (right), ATM-mediated signaling is impaired and the formation of 53BP1-containing nuclear foci is disturbed which leads to unshielded breaks in the DNA.

Furthermore, the question remains why ATR depletion leads to an increase in 53BP1 nuclear bodies (Lukas, Savic et al. 2011) when ATM and ATMIN depletion lead to a decrease. One possible explanation is that ATM is required for foci formation, but ATR might be crucial for proper repair and clearance of foci. In this regard, ATR could function downstream of ATM following low levels of replicative stress. Also, the role of ATMIN is not totally clear yet – I propose that the protein is required for recruitment of 53BP1 to nuclear foci, but it could possibly also be crucial for retention of 53BP1 at CFSs which would also be in line with my data of reduced 53BP1 foci formation at loss of ATMIN.

Taken together, I was able to show that the ATM co-factor ATMIN is required for ATM-mediated signaling following low levels of replicative stress and for accumulation of 53BP1 and P-S1981-ATM into nuclear foci. However, the exact mechanism is not clear yet. Loss of ATMIN in cells could lead to impaired DNA damage repair and chromosomal breaks giving rise to chromosomal rearrangements, deletions or fusions. Hence, this could potentially be the

mechanism by which ATMIN functions as a tumor suppressor and plays a role in chromosome stability in a mouse model for B cell lymphoma (Loizou, Sancho et al. 2011).

VIII. ABBREVIATION LIST

Aph	……	Aphidicolin
CFS	……	Common Fragile Site
DDR	……	DNA Damage Response
DSB	……	Double-Strand Break
HR	……	Homologous Recombination
HU	……	Hydroxy Urea
IR	……	Ionizing Radiation
MMS	……	Methyl Methanesulfonate
NHEJ	……	Non-Homologous End-Joining
PIKK	……	Phosphatidylinositol 3-Kinase-related Kinase
ROS	……	Reactive Oxygen Species
Ub	……	Ubiquitin
UFB	……	Ultra-Fine Bridge
UV	……	Ultra-Violet

IX. ACKNOWLEDGEMENTS

First of all, I want to thank my supervisor Joanna Loizou – I'm so thankful that I was able to work in her group at CeMM on a very interesting project and that she gave me the chance to work independently. She always believed in my work and skills and I could turn to her for advice any time. Furthermore, she was not only my supervisor, but a person that made me feel appreciated and I was happy to see every day.

In this regard, I also want to thank my collegues Abdel, Jana, Marc, Michel, Sabine and Zhewen. I want to especially thank Sabine and Marc for helping me with my project whenever I needed it and Michel for being always at my side as the second (junior) diploma student in the lab. My work at CeMM would not have been the same without them and their friendship. Likewise, I want to thank all members of the neighboring Nijman and Bergthaler labs for their hospitality and support. Furthermore, I want to thank Sebastian, Stefan and their groups for their input and comments on my work during our lab meetings and Peter for helping me with the statistical analysis of my data.

I am very grateful that I had the opportunity to work in an environment like CeMM where there are so many different departments and specialists that I could turn to for any questions. However, I also appreciate that at CeMM social events are promoted and supported. In this regard, I want to especially thank the Nijman group for all the fun times. I felt very welcomed and comfortable at CeMM and was happy to be a part of the CeMM community.

My thanks also go out to Zhao-Qi Whang from the Fritz Lipmann Institute in Jena, Germany, for providing me the NBS1 knock-out MEFs for my experiment.

Last but not least I want to thank my family and friends for always supporting me and helping me keep the balance between work and leisure time.

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1998 - 2006	Gymnasium – BG13 in Vienna/Austria; graduation *summa cum laude*
2006 - 2013	Diplomstudium Molekulare Biologie – University of Vienna

Scientific Work

July 2007	One month of laboratory work in the Institute of Pharmacology, Medical University of Vienna, Professor Freissmuth's group
August 2008	One month of laboratory work at the Institute of Virology, UCSF
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Colophon

The project described in this thesis was performed at CeMM (Research Center for Molecular Medicine of the Austrian Academy of Sciences) under the supervision of Joanna Loizou.