PhD degree in Molecular Medicine

SEMM - European School of Molecular Medicine

University of Milan, Faculty of Medicine

NOTCH1 inhibits the DNA damage response by impairing the formation of the ATM-FOXO3a-KAT5 complex

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Academic year 2015-2016

Some Figures presented in this dissertation are already part of published reports. I have listed them below:

Figures: 7C; 8A-C; 9; 10A-B; 13A-C; 14; 15A-C; 16A-C; 17A and C; 18; 19A-B; 20A-C; 21A-B are part of Vermezovic et al. report (Vermezovic et al., 2015).

Figures: 22; 23A-B; 24A-B; 25A-E; 26A-C; 27A-B; 28A-B; 29A-B; 30A-D; 31A-D; 32A-C; 33A-C; 34A-F; 35; 36A-B; 37A-D; 38A-E; 43; Table1; Table2 are part of Adamowicz et al. report (Adamowicz et al., 2016).

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Abstract

The DNA damage response is a pathway responsible for the maintenance of genome integrity. In my thesis I focused on the investigation of the modulation of ATM activity, a DNA damage response master kinase, by NOTCH1 receptor.

Here I show that NOTCH1 inhibits DNA damage response activation. This inhibitory effect of NOTCH1 is not mediated by its transcriptional activity, but it is the result of direct binding between NOTCH1 and ATM kinase. I show that NOTCH1 binds to the FATC domain of ATM, and this results in an inhibition of ATM kinase activity. Furthermore, I provide evidence that NOTCH1-mediated ATM inhibition does not result from the impairment of ATM recruitment to DNA double-strand breaks. Rather, I show that NOTCH1 competes with FOXO3a transcription factor for the binding to the FATC domain of ATM and that over-expression of FOXO3a prevents NOTCH1-mediated ATM inhibition. As the exact function of FOXO3a in ATM activation was unclear, I sought to understand molecular mechanisms underlying NOTCH1-mediated ATM inactivation and the role of FOXO3a as an opposing factor in this process. I discovered that FOXO3a forms a direct complex with KAT5 lysine acetyl transferase that is critical for ATM activation upon DNA damage. Moreover, I observed that FOXO3a was necessary for the formation of a complex between ATM and KAT5. Surprisingly, I observed that NOTCH1 was not only impairing ATM-KAT5 interaction, but also FOXO3a-KAT5 one. This unexpected observation led me to the discovery that FOXO3a-KAT5 interaction is restricted to the formation of this three-protein complex together with the ATM kinase. Next, I demonstrated that induction of FOXO3a nuclear localization as well as inhibition of NOTCH1 increases ATM activation in NOTCH1-driven cancer cells, which leads to augmented DNA damage-induced cell death.

Finally, I show that, in addition to ATM, NOTCH1 interacts also with other PI3K-like kinases: DNA-PKcs and ATR. Although I did not observe a significant impact of NOTCH1 on ATR kinase activation in the experimental settings I used, I observed an

impaired activation of DNA-PKcs, which however did not result in a significant reduction of DNA damage repair in NOTCH1-expressing cells.

Introduction

DNA damage response

The DNA damage response (DDR) is a signaling pathway, which is responsible for sensing discontinuities in the DNA and transmitting this signal further into the cell. Outcomes of this signaling will depend on the amount of DNA damage that cells need to cope with as well as genomic location of this damage itself, therefore DDR activation might result in the activation of different cellular processes and pathways. Upon DNA damage DDR activation will results in cell cycle checkpoint activation and induction of the DNA damage repair pathways. If the amount of the DNA damage is too big or damage is impossible to repair, cells will induce programed cell death (apoptosis) or will enter an irreversible cell-cycle arrest described as cellular senescence.

DDR is a complex pathway that involves many factors, which often act on many different levels of the response. Below I will try to discuss what we know about the DDR and more specifically about its most important player that is the ataxia-telangiectasia mutated kinase (ATM).

An overview of the DDR

Upon DNA damage, ATM is recruited to the DNA double-strand breaks (DSBs) by its interaction with the MRN complex (MRE11, RAD50, NBS1)(Nakada et al., 2003), which is thought to be one of the first factors that recognize DSBs (Polo and Jackson, 2011)(Figure 1). MRN complex is composed of three proteins: Nijmegen breakage syndrome 1 (NBS1), meiotic recombination 11 (MRE11) and RAD50. NBS1 does not posses any enzymatic activity, but it stimulates activities of MRE11 and RAD50 (Paull and Gellert, 1999). Additionally, NBS1 mediates interaction between ATM and MRN complex (Falck et al., 2005). RAD50 is a DNA helicase, which is necessary for DNA unwinding and tethering together DNA ends (Paull and Gellert, 1999). MRE11 is both an endo- and exonuclease, whose activity and DNA processing is necessary for the DSB repair (Paull and Gellert, 1999; Shibata et al., 2014)(for more details see the ”DNA damage repair”

chapter in the introduction). Once ATM-MRN complex is associated with DSB, ATM undergoes the complicated and yet not entirely elucidated process of activation. First ATM is acetylated by lysine acetyl transferase 5 (KAT5)(Sun et al., 2005). This process favors ATM autophosphorylation and monomerization (Bakkenist and Kastan, 2003; Sun et al., 2005). At this stage ATM is considered as fully activated. Activated ATM starts phosphorylating its substrates, which will result in the DDR activation, initiation of the repair and checkpoint activation to prevent cell from entering S or M phase with damaged DNA.

ATM-mediated DDR activation starts with the phosphorylation of histone H2AX at Serine 139 (γH2AX)(Burma et al., 2001)(Figure 1). This phosphorylation event is a key step in the DDR cascade, as it is recognized by other DDR factors and primes their accumulation at the site of DNA damage. Indeed it has been shown that cells lacking H2AX have impaired accumulation of the DDR factor at DSBs (Celeste et al., 2003). γH2AX is recognized by the mediator of DNA damage checkpoint 1 (MDC1), which recruits more MRN-ATM complexes to the DSBs in a positive feedback loop (Stucki et al., 2005). MDC1 is further phosphorylated by ATM, which results in the recruitment of ring finger protein 8 (RNF8), a E3 ubiquitin ligase, which will ubiquitinate histone H1. This ubiquitination will result in the recruitment of yet another E3 ubiquitin ligase, RNF168 that will ubiquitinate histone H2A (Doil et al., 2009; Mailand et al., 2007; Thorslund et al., 2015). Ubiquitination of H2A, methylation of H4 (carried out by MMSET methyl transferase) and phosphorylation of histone H2AX are a scaffold for the recruitment of p53 binding protein 1 (53BP1) (Doil et al., 2009; Kleiner et al., 2015; Pei et al., 2011), which will promote accumulation of additional factors necessary for DNA damage repair like: Rap1 interacting factor 1 (RIF1) and Melanoma associated antigen mutated 1 (MUM1) as well, which will impact on the DNA repair pathway choice between non-homologous end joining and homology directed repair, by regulating DSB resection (for more details see

the ”DNA damage repair” chapter in the introduction) (Chapman et al., 2013; Huen et al., 2010).

One of the most important roles of the DDR is to spread information about DNA damage throughout the cell, which will result in cell cycle checkpoint activation. G1/S phase cell cycle checkpoint activation will prevent cells from initiating DNA replication with damaged DNA. The G2/M phase cell cycle checkpoint on the other hand will prevent cells form entering mitosis until damaged DNA is not fully repaired. ATM activates G1/S phase checkpoint by impacting on the stability of p53. Upon DNA damage, activated ATM will phosphorylate checkpoint kinase 2 (CHK2) (Matsuoka et al., 2000), which in turn will phosphorylate p53 on Serine 20 leading to an increase of its stability (Shieh et al., 2000). ATM by itself also phosphorylates p53 on the Serine 15, which leads to an increase of p53 transcriptional activity (Dumaz and Meek, 1999). This will result in the accumulation of p21 (a transcription target of p53). p21 binds to the cyclinE/CDK2 (cyclin dependent kinase) complex and inhibits it, which results in the G1 phase cell cycle arrest (Harper et al., 1995).

G2/M cell cycle phase checkpoint regulation as G1/S is regulated by DNA damage checkpoint kinases 1 and 2 (CHK1 and 2). Upon DNA damage ATR and ATM phosphorylate CHK1 and 2 kinases respectively. Activated CHK1 and CHK2 phosphorylate cdc25 phosphatase (Chen et al., 2003; Falck et al., 2001). This leads to the nuclear re-localization of cdc25 to the cytosol in 14-3-3-dependent manner (Peng et al., 1997). The role of cdc25 is to dephosphorylate the CDK1 kinase, which will result in CDK1 activation and cell cycle progression into mitosis (Nilsson and Hoffmann, 2000).

DNA damage repair activation upon DSB formation will be described in a separate chapter. Here I would like to briefly discuss the role of ATM kinase in DNA damage repair. Although ATM is a DDR master kinase, it seems that the role of ATM in the DNA damage repair is fairly limited. It has been shown that ATM knockout cells repair DSBs almost as efficiently as wild type cells (Riballo et al., 2004). It has been reported that ATM

is strictly necessary for the repair of no more then 10% of induced DNA DSBs (amount varies depending on the type of DNA damaging agent used). Those DSBs have been identified to be preferentially localized in heterochromatic regions. In this context, ATM activation leads to the phosphorylation of KRAB associated protein 1 (KAP1), which leads to the dissociation of KAP1 together with other heterochromatic markers like heterochromatin protein 1 (HP1) and suppressor of variegation 3-9 homolog 1 (Suv39h1) methyl transferase from the chromatin resulting in chromatin relaxation necessary for repair factors to access DNA (Ayrapetov et al., 2014; Goodarzi et al., 2008). Moreover, it has been recently suggested that ATM is required for the repair of blocked DSBs. Interestingly, it has been shown that this mechanism of ATM action is independent from the chromatin compaction status (Álvarez-Quilón et al., 2014).

Apart form the role of ATM in the repair of this small (10%) amount of DSBs, it has been reported that ATM kinase activity is necessary for the control of DNA end resection by phosphorylation of MRE11 and CTIP (Jazayeri et al., 2006; Kijas et al., 2015; Peterson et al., 2013), regulation of the KU70/80 heterodimer ubiqutnation by RNF138 and its removal from the DSBs (Ismail et al., 2015). Additionally it was suggested that ATM may play a role in the homology directed repair downstream of RAD51 nucleofilament formation (Bakr et al., 2015; Koecher et al., 2012), although this role could be compensated (to some extent) by ATR activity in ATM absence.

Over all, it is difficult to pinpoint a single function of ATM in the DNA DSB repair, and still its impact is not huge. As most of the DNA breaks are repair by the non homologous end joining repair pathway (NHEJ), it has been shown that ATM is necessary only for the repair of the non-compatible DNA ends by NHEJ and that the repair of compatible DNA ends is independent from ATM activity necessary for end processing (Zhu and Peng, 2016).

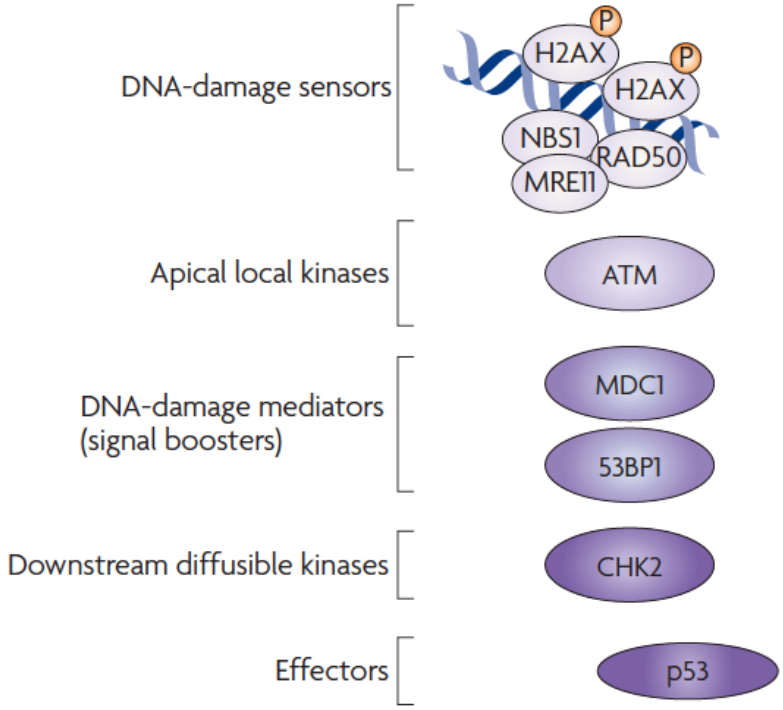


Figure 1. DDR cascade. Scheme of DDR. In short: MRN complex recognizes the discontinuity in the DNA in the form of a DSB and brings ATM to the site of damage. ATM phosphorylates its substrates, mainly histone H2AX, which will scaffold the recruitment of further DDR proteins like 53BP1 and MDC1, responsible for DDR signal propagation. Adapted from (d'Adda di Fagagna, 2008).

PI3K-like kinases

Phosphatidylinositol 3 kinase-like kinases (PI3K-like kinases) have a structure similar to PI3 kinases. PI3K-like kinases are involved in DNA damage signaling, repair, RNA biology and energy sensing and they posses catalytic activity to phosphorylate their protein substrates on Serine or Threonine residues.

Six members of this family of kinases have been characterized: ATM, ATM and Rad3-related protein (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), SMG1, mammalian target of rapamycin (mTOR) and transformation/transcription domain-associated protein (TRRAP). ATM, ATR and DNA-PKcs are master kinases involved in

DDR and repair. mTOR is responsible for sensing of the energy status and regulating protein synthesis. SMG1 has been described to play role in the processing of RNA. TRRAP, although it does not posses any detectable kinase activity (Vassilev et al., 1998), plays a role in DDR as a part of the NuA4 complex.

ATM - PI3K like kinase - structure

We can distinguish five different domains in the ATM structure (Figure 2). α-helical repeats called HEAT repeats (Huntingtin, elongation factor 3 protein phosphatase 2A TOR1 repeats) extend from the N-terminal part of ATM throughout most of the ATM structure. It has been shown that many ATM interacting proteins like NBS1, hMOF or cABL bind within the N-terminal region covered by the HEAT repeats (Gupta et al., 2005; Shafman et al., 1997; You et al., 2005). N-terminal region has been recently implicated to the formation of the ATM dimer structure (Lau et al., 2016; Sawicka et al., 2016). FRAP, ATR, TRRAP domain (FAT) and the FAT C-terminal domain (FATC) surround the ATM kinase domain. FATC domain has been shown to play a critical role in the ATM activation process, as it is a binding site for the KAT5 acetyl transferase, which acetylates ATM in the PI3K-like kinase regulatory domain (PRD), which is necessary for ATM kinase activation upon DNA damage (Sun et al., 2007). Interestingly, it has been shown that the FATC domains of ATM, ATR and DNA-PKcs are functionally equivalent (Jiang et al., 2006). The ATM kinase domain is located between the FAT and the FATC domains at the C-terminal end of the protein. It is worth to note that in the recently published structures of ATM and ATR dimers (Lau et al., 2016; Sawicka et al., 2016), it has been suggested that although ATM kinase domains are positioned in front of each other (in the dimer state) there is a possibility for substrates to be phosphorylated as there is enough free and accessible space between such kinase domains in this conformation (Sawicka et al., 2016). This kind of feature has not been suggested for the ATR kinase. ATR kinase domains have

been reported to be tightly packed with each other with no possibility for substrate to access and be phosphorylated by ATR dimer (Sawicka et al., 2016). Among all of PI3K like kinases, only TRRAP has been shown to not posses any kinase activity (Vassilev et al., 1998).

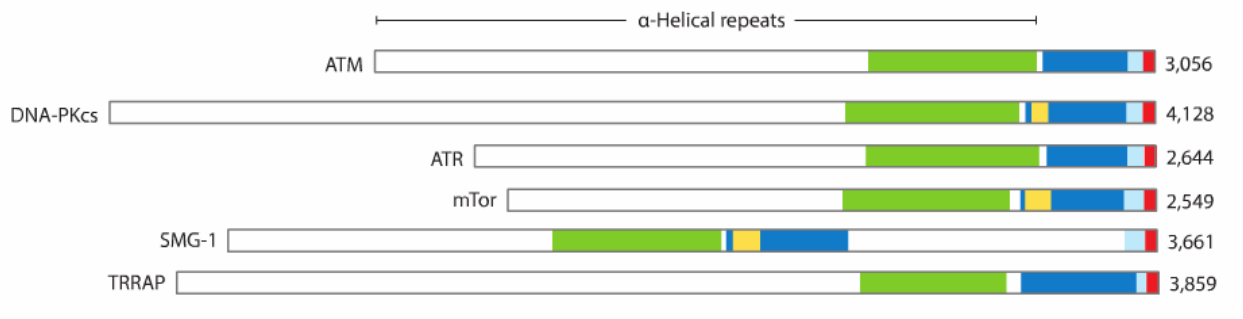


Figure 2. Structure of the PI3K-like kinases. Scheme of the structure of: ATM, ATR, DNA-PKcs, mTOR, SMG-1 and TRRAP. Color code: green - FAT domain; blue - kinase domain; light blue - PRG domain; red - FATC domain. For explanations of the abbreviations please see text. Numbers on the left represent length of each of the kinases [aa]. Adapted from (Paull, 2015).

Ataxia telangiectasia disorder

Mutations in the ATM kinase gene that result in the impairment of ATM function, decrease or ablation of its expression cause ataxia telangiectasia disorder (A-T). A-T had been discovered and characterized before ATM gene was identified in 1995 (Boder and Sedgwick, 1958; Savitsky et al., 1995).

Frequency of A-T occurrence in the population spans between 1 in 50.000 to 1 in 100.000. Although, once diagnosed, A-T patients are monitored with a special care which results in an increase of their life span, on average they do not live longer then 25 years. A-T patients suffer from severe ataxia, which results in mobility problems. Neurodegeneration is another characteristic feature of A-T patients, which results in the degeneration of cerebral neurons that is responsible for the observed ataxia symptoms. The other main feature of A-T is telangiectasia. It is characterized by the presence of the wide blood vessels in the eyes

and skin. From the molecular point of view, deletion or lack of functional ATM kinase results in DNA damage hypersensitivity, which is the main feature of A-T patients. Cells from A-T patients have been characterized to be hypersensitive to DNA damaging agents and, as a result, they display elevated levels of genome instability. This leads to an increased risk of cancer in A-T patients. A-T patients suffer form many types of cancer, with particular susceptibility to the development of leukemias and lymphomas (Chun and Gatti, 2004; Pagon et al., 1993; Shiloh, 1997; Shiloh and Lederman, 2016).

Interestingly, two syndromes have been characterized to recapitulate some symptoms of A-T patients. Those syndromes are: ataxia telangiectasia like disorder (ATLD) and Nijmegen breakage syndrome (NBS), which result from mutations in the *MRE11* and *NBS1* genes, respectively. As mentioned above, NBS1 and MRE11 are a part of MRN complex, which is necessary for ATM recruitment to DNA damage sites. This explains similarities between A-T, ATLD and NBS patients. Similar to A-T cells, cells from ATLD or NBS patients display increased genome instability and hypersensitivity to DNA damage and increased cancer risk (Shiloh, 1997; Stewart et al., 1999; Taylor et al., 2004; Varon et al., 1998).

KAT5

KAT5 is a Lysine acetyl transferase 5 (KAT5), known also as Tip60 - Tat interacting protein 60 kDa. KAT5 is a very conserved protein. Its orthologues are expressed in yeast (*Saccharomyces cerevisiae* - Esa1) and lower organisms (*Drosophila melanogaster* - DmeI). KAT5 is a Lysine acetyl transferase that can perform acetylation of not only histones but also other proteins, therefore it is involved in many cellular processes such as DDR, apoptosis, chromatin modifications and transcription.

Roles of KAT5 in the DDR can be at least two: one related to ATM functions, the other to the NuA4 complex. The ATM-related role of KAT5 is linked to its involvement in ATM activation and initiation of DDR (Sun et al., 2007)(for more details about DDR please see "An overview of the DDR" chapter of the introduction). In addition, KAT5 has been

shown to be part of the chromatin remodeling complex called NuA4. NuA4-KAT5 complex has been shown to be involved in the acetylation of histone H4, thus regulating DDR and repair at the chromatin level (Murr et al., 2006). Relevant to my work, below I will focus on these two aspects of KAT5 function.

KAT5 was reported to interact with the FATC domain of ATM. It has been shown that this interaction results in ATM acetylation (Jiang et al., 2006), which is indispensable for ATM activation. It has been suggested that KAT5-mediated ATM acetylation is one of the first steps involved in the process of ATM activation at DSBs, upon MRN-mediated recruitment (Sun et al., 2007). To activate ATM, KAT5 has to be stimulated by the interaction with tri-methylated histone H3 at the Lysine 9 residue (H3K9m3). Upon MRN-mediated recruitment to the DSBs together with ATM, KAT5 binds to H3K9m3 through is chromodomain. Methylation of H3K9 at the site of damage is mediated by SUV39h1 methyl transferase in a complex with HP1 and KAP1 proteins (Sun et al., 2009). Indeed it has recently been shown that upon DNA damage repressive chromatin is formed around DSB and it is necessary for KAT5-mediated ATM activation, which in a negative feedback loop will phosphorylate KAP1 leading to the removal the KAP1, HP1, SUV39h1 complex from the DSBs, facilitating chromatin relaxation (Ayrapetov et al., 2014).

Although it has been shown that H3K9m3 chromatin mark is specifically loaded at the DSB surroundings, it is worth remembering that it is also a transcriptional repressive mark that is distributed throughout the genome. Therefore, likely as a fail safe mechanism, KAT5 needs to undergo additional post transcription modification to be able to acetylate ATM. Indeed it has been recently reported that upon DNA damage KAT5 is phosphorylated on a Tyrosine residue (Tyr44) by the cAbl kinase. This phosphorylation event has been shown to facilitate interaction between KAT5 and H3K9m3 which is necessary for KAT5-mediated ATM acetylation and consequent activation. (Kaidi and Jackson, 2013). It is worth remembering that ATM was shown to interact with cAbl, to mediate its phosphorylation upon DNA damage and to stimulate its kinase activity

(Baskaran et al., 1997). This suggests a possible positive feedback loop mechanism resulting in the boosting of ATM activation. Upon DNA damage induction cAbl mediates KAT5 phosphorylation, which leads to the activation of KAT5 and KAT5-mediated ATM acetylation. Acetylated and activated ATM in return phosphorylates cAbl leading to the increase of cAbl kinase activity that will boost further KAT5 phosphorylation.

NuA4 is a large multisubunits protein complex involved in histone acetylation. KAT5 has been described as a key component of the NuA4 complex. The role of NuA4-KAT5 complex in the DDR is complex and not yet fully understood. It has been shown that NuA4-KAT5 complex plays a role in the DDR by facilitating fast chromatin compaction changes at the DSB site. First, p400, a component of NuA4-KAT5 complex, facilitates exchange of histone H2A for H2A.Z at the site of damage (Xu et al., 2012). Next, the ANP32E histone chaperone and INO80 remodeling complex remove H2A.Z accumulated around the DSB. This is a key step that allows the acetylation of histone H4 by the NuA4-KAT5 complex, which promotes chromatin relaxation (Alatwi and Downs, 2015; Gursoy-Yuzugullu et al., 2015). It has been proposed (consistent with the previously-mentioned chromatin methylation on H3K9 around the DSB) that, due to the exchange of H2A with H2A.Z a transient compaction of the chromatin that occurs around the DSBs is necessary for the initiation of the DDR. After that, the histone H2A.Z is removed allowing for the acetylation of H4 by NuA4-KAT5 (carried out by KAT5) complex, which leads to chromatin relaxation necessary for DNA damage repair (Gursoy-Yuzugullu et al., 2016).

The structure of KAT5 is typical for an acetyl transferase. Human KAT5 is composed out of 513 amino acids. We can distinguish four characteristic domains of KAT5. On the N-terminal part there is a chromodomain that allows KAT5 to recognize and bind to chromatin marks like H3K9m3 or other proteins like methylated p53 (Kurash et al., 2008). At the center of the protein there is the Acetylo-CoA binding domain and Zinc finger domain. Acetylo-CoA binding domain is necessary for the binding of Acetylo Coenzyme A, which is a source of the acetyl groups in KAT5-mediated acetylation reactions. In

addition, Zinc Finger domain that promotes interaction of KAT5 with other proteins, like for example NOTCH1 (Kim et al., 2007a). At the very C-terminal region of KAT5 there is a nuclear receptor binding domain that has been characterized to be necessary for the interaction between KAT5 and nuclear receptors (Gaughan et al., 2001).

DNA damage repair

DNA damage needs to be repaired as fast as possible and as accurately as possible to avoid introduction of mutations in the genome. We can distinguish several DNA repair pathways in the cell: base excision repair, mismatch repair, nucleotide excision repair, non homologous end joining and homology-directed repair.

In the chapters below I will described more in the details the two main pathways necessary for the repair of the DSBs: non homologous end joining and homology directed repair. Non homologous end joining (NHEJ) is a repair pathway active throughout the cell cycle and it is responsible for the repair of the vast majority of the DSBs. NHEJ is characterized by the direct ligation of the broken DNA ends. When DNA ends are not compatible, small end processing by removing or adding nucleotides occur. As a result, NHEJ leads to the introduction or deletion of nucleotides therefore it is considered to be an error prone repair mechanism. When NHEJ pathway is perturbed, DNA DSBs can be repaired by an alternative pathway that involves microhomology search between two DNA ends.

Differently from the previously described NHEJ, homology directed repair (HDR) is an error-free DNA repair mechanism. The mechanism standing behind it depends on the utilization of the sequence of sister chromatid as a template for the recovery of the correct nucleotide sequence of a broken DNA. As this repair pathway requires a sister chromatid, its usage is limited to the S/G2 phase of the cell cycle.

Homology directed repair

As mentioned above, homology directed repair (HDR) is a DSB repair pathway that is error-free. Upon DNA damage induction, MRN complex is recruited to the DSB. MRE11 together with the help of the C-terminal binding protein (CtIP), exonuclease 1 (EXO1) and DNA nuclease/helicase (DNA2) resects double stranded DNA to produce 3' overhanging single stranded DNA ends (Nimonkar et al., 2011). CtIP has been shown to stimulate MRE11 endonuclease activity, which is necessary for the processing of blocked DNA ends (Cannavo and Cejka, 2014; Sartori et al., 2007). Additionally, MRN complex has been shown to be necessary for the processivity and recruitment of both: DNA2 and EXO1 nucleases (Nicolette et al., 2010; Nimonkar et al., 2011). The 3' single stranded DNA ends are promptly bound by the replication protein A (RPA) that protects it from degradation (Wold, 1997). RPA recruitment brings ATR kinase to the resected DSB through ATR interacting protein (ATRIP), which results in its activation (Jazayeri et al., 2006). DNA ends resection prevents KU70/80 heterodimer from binding to the DNA ends and therefore prevents non homologous end joining repair (Foster et al., 2011).

Once the resection is finished and single stranded DNA covered with RPA, BRCA2-RAD51 complex is recruited to DNA ends. Breast cancer type 2 (BRCA2) initiates RAD51 loading and stabilization on single stranded DNA (Carreira et al., 2009; Esashi et al., 2007), by exchanging RPA. RAD51 binding to the single stranded DNA forms a nucleofilament that will perform an invasion to the sister chromatid and search for the homology sequence. RAD51 interaction with the single stranded DNA is indispensable for the homology search (Sung et al., 2003). After finding of matching sequence, RAD54 promotes disassembly of RAD51 that binds to the double stranded DNA molecules. This step is believed to be necessary for the DNA polymerase processing, which will synthetize the DNA lost due to the damage. In this process, DNA polymerase utilizes 3' end of the invading strand as a primer and sister chromatid sequence as a template (Mazin et al., 2010). Once synthesis is finished synapsis is resolved and DNA break repaired.

Non-homologous end joining

Non homologous end joining repair (NHEJ) is the main repair mechanism of the DSBs and it is composed from several steps that have been depicted below in the Figure 3. For simplicity steps: 1-7 presented in the Figure 3 has been also indicated in the text below.

NHEJ repair is initiated by the recruitment of the KU70/80 heterodimer to the DSB within seconds after DSB formation (1) (Uematsu et al., 2007). KU70/80 heterodimer binds to DNA ends (2). Upon formation of the complex between KU70/80 heterodimer and the DNA end, DNA-PKcs is recruited to the DSB (3) (Gottlieb and Jackson, 1993). As result of that KU70/80 heterodimer slides inside to the DSB, which triggers DNA-PKcs kinase activation (Yoo and Dynan, 1999). In addition to DNA-PKcs recruitment, KU70/80 plays a scaffold role for the association of X-ray repair cross-complementing protein 4 (XRCC4), LIGASE IV and XRCC4 like factor (XLF) complex (Nick McElhinny et al., 2000). At this stage of NHEJ presence of DNA-PKcs at DNA ends is believed to inhibit DNA ends ligation by the LIGASE IV complex probably due to the DNA ends masking function, which allows for DNA end processing to occur first (3)(Jiang et al., 2015). DNA-PKcs undergoes ATM-mediated transphosphorylation and/or autophosphorylaton, which is necessary for the recruitment of ARTEMIS nuclease that will induce DNA end processing if ends are not compatible (4)(Goodarzi et al., 2006; Jiang et al., 2015). Although DNA-PKcs kinase activity is strictly indispensable for the NHEJ repair, the direct substrates of DNA-PKcs phosphorylation that are critical for repair are yet poorly known. Unfortunately most of DNA-PKcs-mediated phosphorylation sites on its substrates have been characterized to be dispensable for repair, like: ARTEMIS nuclease (Goodarzi et al., 2006; Ma et al., 2002), LIGASE IV (Kim et al., 1999; Wang et al., 2004) or XRCC4 (Matsumoto et al., 2000; Sharma et al., 2016; Yu et al., 2003). What is known is that DNA-PKcs autophosphorylation, together with the ATM-mediated transphosphorylation, has been shown to regulate the kinetic of DNA-PKcs disassociation from DSBs (5-6). DNA-PKcs

mutants that cannot be phosphorylated neither by itself nor by ATM display delayed disassociation from the DNA break (Uematsu et al., 2007). Once DNA end processing is finished, DNA-PKcs disassociates from DNA ends allowing for the LIGASE IV-mediated ligation (7).

It is necessary to mention that apart from the already mentioned NHEJ repair core components, there are yet many more proteins and enzymes that assemble at DNA repair foci. Those are mostly DNA processing enzymes like: werner syndrome nuclease (WRN) or recently discovered paralog of XRCC4 and XLF (PAXX), which has been shown to be necessary for the stabilization of the NHEJ factors at DNA ends (Ochi et al., 2015). Recruitment of those factors is mediated by KU70/80 heterodimer (Li and Comai, 2001; Ochi et al., 2015)

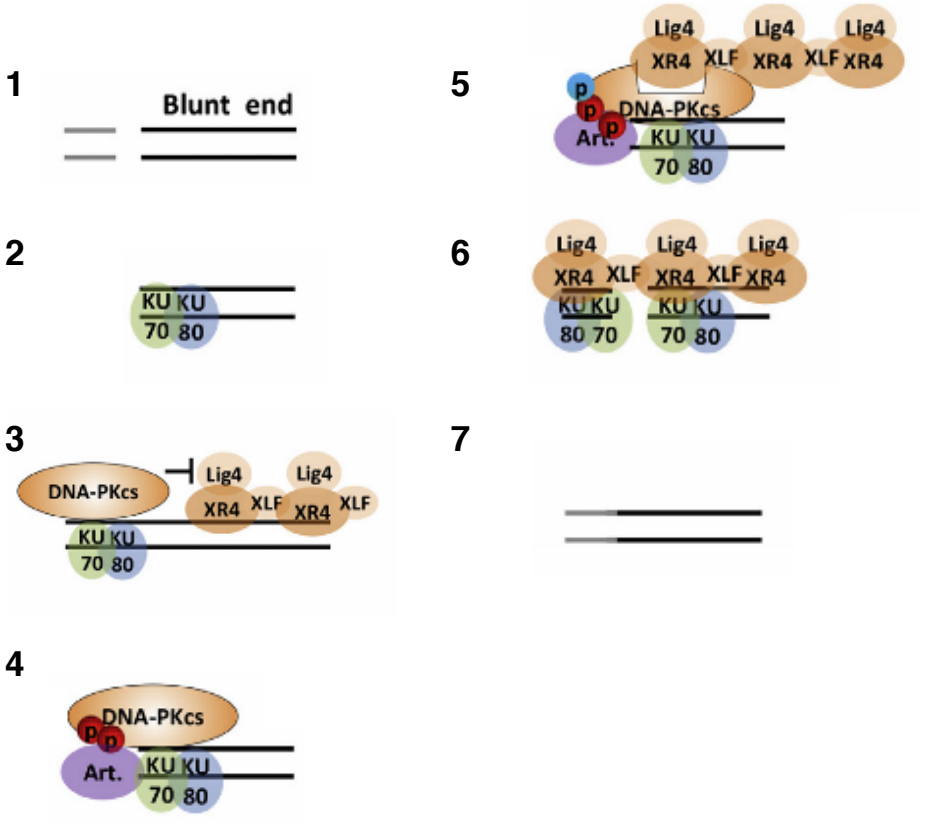


Figure 3. Sequence of events of NHEJ repair pathway. In brief: upon DNA damage induction, KU70/80-DNA-PKcs heterodimer is recruited to the DSB. DNA-PKcs kinase undergoes activation, which is necessary for the processing of DNA ends by the Artemis nuclease (Art.), followed by the disassociation of DNA-PKcs and break ligation by the Ligase IV (Lig4) and XRCC4 (XR4) complex.

Color code: red “P” - ATM or DNA-PKcs transphosphorylation of DNA-PKcs; blue “P” - autophosphorylation of DNA-PKcs. For more details please see the text. Adapted form (Jiang et al., 2015).

Notch family of transcription factors

The mammalian Notch family of proteins consists of four different transmembrane transcription factors, NOTCH1-4. Notch receptors are conserved among other species, from the *Caenorhabditis elegans* (*Glp1; Lin12*) through *Drosophila melanogaster* (*dNotch*) and mammals (*NOTCH1, NOTCH2, NOTCH3 and NOTCH4*). Notch receptors play crucial roles in many cellular processes like: embryogenesis, neurogenesis, hematopoiesis, differentiation of T-cells, neurons, keratinocytes and more (Andersson and Lendahl, 2014). Therefore it is not surprising that Notch receptors are being expresseed in many tissues: skin, reproductive system, nervous system, cardiovascular, connective tissue etc. (for more details regarding Notch expression see (Baldi et al., 2004)).

Notch was first discovered in *Drosophila melanogaster* as the gene in which a mutation leads to the formation of abnormal wings. Later on Notch was linked to the formation of T-cell acute lymphoblastic leukemias (T-ALL)(Pear et al., 1996). More then fifty percent of all T-ALL tumors show mutations in the *NOTCH1* gene or other genes that regulate functions of NOTCH1 (Weng et al., 2004). Indeed it has been shown that the majority of *NOTCH1* mutations detected in T-ALL cells were NOTCH1 activating mutations resulting in: decreased degradation of NOTCH1, ligand independent receptor activation and increased stabilization of the intracellular part of NOTCH1 (Weng et al., 2004). Additionally, NOTCH1 has also been shown to drive tumorgenesis in the breast tissue, where NUMB, a negative regulator of NOTCH1, is frequently mutated (Pece et al., 2004).

Structure of the mammalian Notch receptors

Since all Notch receptors have similar structure I will use NOTCH1 as an example while describing the individual domains of Notch receptors. NOTCH1 is a large transmembrane protein composed of 2555 aminoacids (aa). We can distinguish eight domains, each with its own separate function (Figure 4). From the structural point of view, we can distinguish the N-terminal part (extracellular part) of NOTCH1 composed of EGF like repeats, Lin-like repeats and a heterodimerization domain. The C-terminal part (intracellular part) consists out of: RBP-Jκ binding site (RAM), Ankyrin repeats, transcription activation domain (TAD) and Proline, Glutamic acid, Serine and Threonine rich domain (PEST). C- and N-terminal portions are connected through the transmembrane domain.

EGF like repeats are responsible for the interaction with the ligand. The number of repeats differs among different species and different Notch receptors. Heterodimerization domain is processed by the furine convertase, which will result in the formation of the heterodimer between NOTCH1 N- and C-terminal part (Logeat et al., 1998). This is an important step of NOTCH1 activation process since the heterodimerization domain and Lin-like repeats domain will mask the S2 cleavage site. This will protect NOTCH1 from further processing and activation in the absence of the ligand. Interaction between a ligand and Notch receptor will result in structural changes of the extracellular part of NOTCH1, which will lead to the unmasking of S2 cleavage site, that will become accessible for the processing enzymes (Gordon et al., 2007). Transmembrane domain is a single-membrane transpassing domain that hooks NOTCH1 on the cellular membrane. Ankyrin repeats (seven repeats) together with the RAM domain have been shown to be responsible for its interaction with CBF1 (Recombining binding protein suppressor of hairless), a NOTCH1 coactivator and DNA binding protein (Nam et al., 2007; Tamura et al., 1995). Ankyrin repeats are also responsible for the binding with mastermind like protein 1 (MAML1) coactivator (Wu et al., 2000). TAD domain is present in NOTCH1 and 2, NOTCH3 seems to bear only a partial fragment of the TAD domain and NOTCH4 does not have it at all. TAD domain has

been shown to interact with the transcription coactivators in order to facilitate transcription and increase the stability of the complex between NOTCH1, CBF1 and MAML1 (Gerhardt et al., 2014). PEST domain plays a role in the control of NOTCH1 stability as it is a target for post translational modifications that induce NOTCH1 degradation after transcription initiation (Oberg et al., 2001).

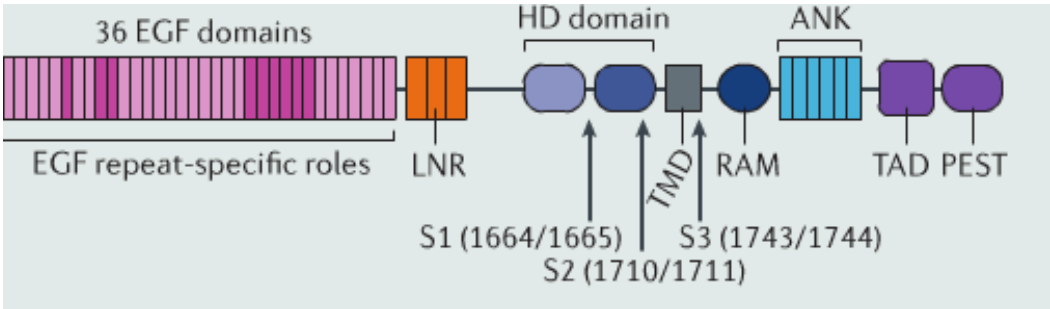


Figure 4. Structure of NOTCH1 receptor. Abbreviations: EGF - epidermal growth factor like repeats; LRN - Lin like repeats; HD - heterodimerization domain TMD - transmembrane domain; S1-3 - cleavage sites for proteases; RAM - RAM domain ARK - Ankyrin repeats; TAD - Transactivation domain; PEST - Pest domain. Adapted from (Andersson and Lendahl, 2014)

NOTCH1 signaling

NOTCH1 signaling consists of two different signaling pathways: canonical and non-canonical. Canonical signaling of NOTCH1 is connected with its role as the transmembrane receptor and it is dependent on the interaction with a specific ligand that will result in the translocation of NOTCH1 from the membrane to the nucleus in order to induce transcription of its target genes.

NOTCH1 interacts with one of its ligands that belongs to the DSL family of trans-membrane ligands: Delta like (Delta like 1; Delta like 3; Delta like 4), Jagged1 and Jagged2 (D'Souza et al., 2010). Upon interaction with the ligand NOTCH1 undergoes sequential cleavage processes, but first before NOTCH1 will interact with its ligand, it undergoes proteolytic cleavage by the Furine convertase at the S1 site (Figure 4). This

cleavage occurs in the Golgi apparatus and will result in the expression of inactive form of NOTCH1 receptor on the plasma membrane (Logeat et al., 1998). Next, after activation of NOTCH1 by the interaction with its ligand, a disintegrin and metalloprotease (ADAM) cleaves NOTCH1 at the S2 site in the intracellular space (Figure 5). This cleavage results in the formation of NOTCH1 with a truncated extracellular domain (Brou et al., 2000). NOTCH1 processed in this way is next cleaved again by γ-secretase, a trans-membrane protein complex (hooked at the intracellular part of the plasma membrane, opposite to ADAM protease (Figure 5)) composed of several proteins: PRESSELIN1, PRESSELIN ENCHANCER2, NICASTRIN and APH-1. At this stage, intracellular part of NOTCH1 is released from the plasma membrane and translocates to the nucleus (Strooper et al., 1999). This is a key step of NOTCH1 activation and it has become a target for many inhibitory drugs, which prevent NOTCH1 processing at this step. As these drugs act directly on γ-secretase, inducing its inhibition, they have been called γ-secretase inhibitors (GSIs).

In the nucleus, intracellular part of NOTCH1 forms a protein complex with CBF1, a DNA binding transcriptional repressor and mastermind like coactivator (MAML1)(Hsieh et al., 1996). This complex initiate transcription of NOTCH1 target genes such as hairy enhancer of split (*HES1*) and many more.

Recently it is has been revealed that apart from CBF1, MAML1 and NOTCH1 more proteins take part in the formation of this transcriptional active complex. These include chromatin remodelers like LSD1 and PHF8 demethylases, p300 acetyl-transferase and nucleosome remodeling complex PBAF (Yatim et al., 2012). It is important here to underline a newly discovered role of LSD1 in the regulation of NOTCH1 transcription. LSD1 forms a transcriptional repressive complex together with the CBF1 by demethylating histone H3K4me2, but upon interaction with the intracellular part of NOTCH1 it becomes indispensable for transcription activation by mediating demethylation of the histone H3K9me2. A NOTCH1-mediated change in LSD1 substrate recognition is an example that

shows how NOTCH1 just by its interaction with the other protein can affect their substrate specificity (Yatim et al., 2012).

After transcription initiation, NOTCH1 is phosphorylated by cyclinC:cdk8, which binds to the transcriptionally active complex though MAML1 (Fryer et al., 2004). This results in the phosphorylation of NOTCH1 at the PEST and TAD domains. This modification of NOTCH1 is recognized by F-box and WD repeat domain containing 7 (FBW7) ubiquitin ligase that will ubiquitinate NOTCH1 at the PEST domain, resulting in the prompt proteasomal degradation of NOTCH1 and transcriptional inhibition (Wu et al., 2001). Apart from FBW7-mediated ubiquitination, another NOTCH1-regulatory ubiquitination has been described. ITCH is an ubiqutine ligase, which has been reported to ubiqutinate NOTCH1 outside the nucleus (McGill and McGlade, 2003; Qiu et al., 2000). Regulation of NOTCH1 activity by the ITCH mediated degradation is very important. Since T-ALL leukemias have been frequently observed in A-T patients (Takeuchi et al., 1998) and recently it has been shown that ATM regulates ITCH activity by its phosphorylation, this suggests a potential possible role of ATM in the suppression of NOTCH1 activation and T-ALL development (Santini et al., 2014). A part form ubiqitination, other post transcriptional modifications like methylation and acetylation have been shown to regulate NOTCH1 stability and connected with that its transcriptional ability (Guarani et al., 2011; Hein et al., 2015).

As described above, mechanism of NOTCH1-mediated transcription regulation by fast degradation of NOTCH1 complex is very important, therefore it is not a surprise that in many tumors even in the absence of a direct NOTCH1 mutations, mutations in the NOTCH1 regulators like NUMB or FBW7 have been frequently found (Neumann et al., 2014; Pece et al., 2004).

For simplicity, steps of NOTCH1 activation upon interaction with its ligand have been depicted in the Figure 5.

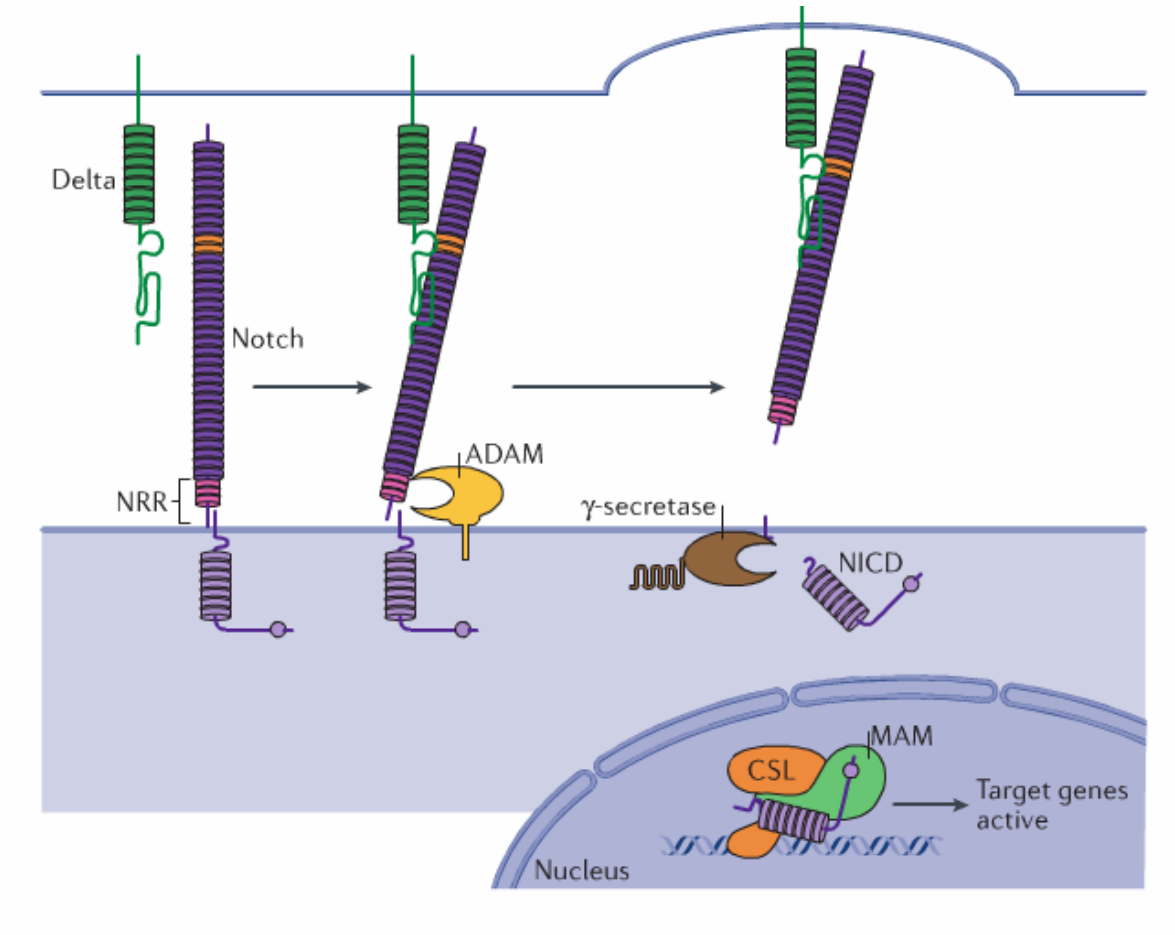


Figure 5. NOTCH1 signaling. Abbreviations: NRR - Negative regulator region a domain composed from Lin like repeats and heterodimerization domain (for more details please see text); NICD - NOTCH1 intracellular domain; MAM - MAML1 transcription activator. CLS - CBF transcriptional coactivator. Adapted form (Bray, 2016).

FOXO3a transcription factor

Like Notch, FOX transcription factors were first discovered in *Drosophila melanogaster*. FOX transcription factors family is characterized by the presence of the specific DNA binding domain called Forkhead box. FOXO1, FOXO3a FOXO4 belong to the FOX-O transcription factors subfamily. The FOX-O subfamily of transcription factors is one among many others that have been described, ranging from A to S, each of them playing different roles in many cellular processes, from differentiation, stress response, cell death, proliferation and longevity (Greer and Brunet, 2005).

Apart from FOXO1/3a/4 that were characterized initially, FOXO6 was then discovered (Jacobs et al., 2003). FOXO6 possesses structural similarities to the other FOX-O members but due to its specific expression (mainly in the brain tissue) and different regulation I will not be describing it in more details. FOXO3a is uniformly expressed among different tissues. FOXO1 although is expressed in many tissues its high levels were identified in ovaries. FOXO4 seems to be very highly expressed in muscle cells (for more details see the reference) (Biggs et al., 2001).

FOX-O family members are conserved among different species. In *Caenorhabditis elegans* there is just one FOX-O family member called *daf-16*. The same is true in the *Drosophila melanogaster*, which expresses only *dFOXO* gene. As mentioned above, in mammals there are four FOX-O members: FOXO1/3a/4/6. A lot has been learnt about the roles of FOX-O proteins through the studies in knockout mice performed in Ronald DePinho's as well as in other laboratories. Considering that FOX-O proteins are involved in many cellular processes vital for the organism results of the knockout experiments in mice were very surprising. FOXO4 knockout mice are born alive and more unexpectedly with no obvious defects (Hosaka et al., 2004). FOXO3a knock-out mice are also born alive and healthy, with only female littermates developing sterility connected with the impairment in the growth of the ovarian follicles (Castrillon et al., 2003). Differently, mice missing FOXO1 die before birth (Furuyama et al., 2004). This dramatic phenotype was connected with problems in the vascular development of embryos. Interestingly, triple inducible knockout of FOXO1/3a/4 revealed the great importance of FOX-O factors. Mice missing FOXO1/3a/4 displayed increased tumorigenesis, decreased survival and impairment in vasculogeneisis (in agreement with the results of single FOXO1 knockout). These results proved the tumor suppressive roles of FOX-O factors (Paik et al., 2007). Lack of differences between FOXO3a and FOXO4 knockout mice as well as the dramatic effects of the triple FOXO1/3a/4 depletion in mice suggest that there might be a possible redundancy between those transcription factors. This is a big problem for researchers,

which may impact proper understanding the physiological function of each single FOX-O transcription factor.

FOXO3a structure and regulation

FOXO3a is a tightly regulated protein. Regulation of the FOXO3a transcription activity is connected with the regulation of its cellular localization. The main player in this process is protein kinase B (PKB/AKT). Under normal conditions (absence of the stress signals, presence of growth factors, insulin) FOXO3a is localized in the cytosol were it is subjected to proteasomal degradation. This process is regulated by AKT kinase, which under those conditions phosphorylates FOXO3a at three residues: Threonine 32; Serine 252 and 315. These phosphorylations result in the recognition of FOXO3a and binding by the chaperone protein 14-3-3 (Brunet et al., 1999). Binding of 14-3-3 also induces conformational changes of FOXO3a resulting in the inhibition of its DNA binding abilities and unveils its nuclear export signal. Binding of 14-3-3 directly masks the nuclear localization signal of FOXO3a, which results in the export of FOXO3a to the cytosol and its degradation (Vogt et al., 2005).

In the presence of stress conditions (for example: oxidative stress) or absence of grow factors, ATK-mediated FOXO3a phosphorylation is suppressed, allowing for the nuclear accumulation of FOXO3a. In has been shown that apart from AKT-mediated phosphorylations, also other post transcriptional modifications like acetylation or dephosphorylation can regulate localization and function of FOX-O factors (Matsuzaki et al., 2005; Singh et al., 2010). Once FOXO3a localizes in the nucleus, it induces transcription of its target genes: *GADD45α, p21, BIM, Bcl-2* etc. to contribute to the stress response of the cell.

The structure of FOX-O factors (FOXO1/3a/4/6) is very similar to each other (Figure 6). The DNA binding domain, called Forkhead domain is a key characteristic domain of FOX family members. In all of FOX-O factors it is localized in the N-terminal part of protein,

but in some cases it can be also in the C-terminal part (egz. FOX-P family). Forkhead domain allows for the recognition of a specific DNA binding site. For all of FOX-O members it is TTGTTTAC (Furuyama et al., 2000). This suggests a possible overlap in the transcriptional regulation between different FOX-O members. Moreover we can additionally distinguish three conserved regions (1-3) localized in the very N-terminal, middle and very C-terminal parts respectively. Conserved region 3 is known as a transactivation domain (TAD) that was shown to mediate FOX-O interaction with other transcription factors, and more recently also with ATM kinase (Nasrin et al., 2000; Tsai et al., 2008). The conserved regions (1-2) are targets of posttranscriptional modifications that regulate FOXO3a activity (like AKT-mediated phosphorylation).

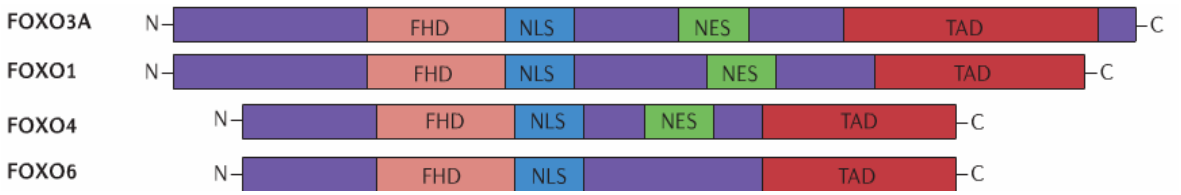


Figure 6. Structure of FOXO3a transcription factor. Abbreviations: FHD - conserved regions; FHD - Forkhead domain/DNA binding domain; NLS - nuclear localization signal; NES - nuclear exporting signal; TAD - transactivation domain. Adapted form (Lam et al., 2013)

FOXO3a in the DDR

Among many cellular processes, recently FOXO3a has been implied to play an active role in the DDR by regulation of ATM kinase activation. In its role as a transcription factor FOXO3a has already been shown to facilitated transcription of many genes that are known for their role in preventing DNA damage induction or involved in DNA damage repair, like manganese superoxide dismutase (Kops et al., 2002), preventing oxidative DNA damage by scavenging reactive oxygen species or GADD45α that has been shown to be involved in basic excision and nucleotide excision repair (Tran et al., 2002).

Few years ago a Mikey Hu group published two seminal studies showing a novel function of FOXO3a: ATM kinase regulation. In the presented results authors demonstrated that upon DNA damage ATM forms a direct protein complex with FOXO3a. Formation of this complex is necessary for ATM activation, its autophosphorylation and phosphorylation of its substrates (Tsai et al., 2008). Lack of ATM-mediated phosphorylation of CHK2 and p53 in FOXO3a deficient cells results in impaired DNA damage-induced apoptosis (Chung et al., 2012a). Additionally, a report has been published showing that overexpression of FOXO3a in cells not exposed to any DNA damaging treatment, was able to induce ATM activation as assessed by ATM autophosphorylation and phosphorylation of its substrate (cAMP response element binding protein - CREB)(Geiger et al., 2012).

It is possible that FOXO3a as well as other FOX-O family members may play additional role in the DDR as well as repair independently form the described above mechanism, but this will need further elucidation.

Materials and Methods

Cell culture and treatments

In my project I used a number of human and mouse cell lines to study NOTCH1-mediated DNA damage response inhibition. All cell lines were grown at 37°C and 5%CO_2 conditions. HEK293T cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine. CUTLL1 cells were kind gift of A. Ferrando (Columbia University)(Palomero et al., 2006) and were cultured in the Roswell park Memorial Institute medium (RPMI 1640) supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. TALL-1 cells from German collection of microorganisms and cell cultures (DSMZ) were grown in the RPMI 1640 medium supplemented with 15% FBS, penicillin/streptomycin and L-glutamine. HeLa cells from American Type Culture Collection (ATCC) were cultured in the DMEM Glutamax medium supplemented with 10% FBS, penicillin/streptomycin and non-essential aminoacids. MCF10a cells (ATCC) were grown in the DMEM/F12(1:1) medium supplemented with 5% horse serum, 0,5mg/ml hydrocortisone, 10μg/ml human insulin, 100ng/ml cholera toxin and 20ng/mlEGF. Mouse embryonic stem cells (mESC) were grown in the DMEM Glutamax medium supplemented with 15% FBS, penicillin/streptomycin, non-essential aminoacids, sodium piruvate, leukemia inhibitory factor (LIF) and β-mercaptoethanol.

To inhibit NOTCH1 translocation to the nucleus I used different gamma-secretase inhibitors (GSI). GSI I (t-3,5-DMC-IL-CHO or N-trans-3,5-dimethoxycinnamoyl)-Ile-leucinal)(Merck Chemicals) was dissolved in dimethyl sulfoxide (DMSO) and used at 10μM concentration 3h prior to the experiments. Compound E (N-[(1S)-2-[[(3S)-2,3-Dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]amino]-1-methyl-2-oxoethyl]-3,5 difluorobenzeneacetamide) (Tocris) was dissolved in DMSO and used at 1μM concentration 24h prior to the experiment. DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester)(Sigma) was dissolved in DMSO and used at the 10μM concentration 16h (HeLa cells) or 4h (TALL-1 cells) prior to the experiment.

To check the dependency of DNA damage induced cell death on ATM activation, I treated cells with an ATM inhibitor (ATMi) (Kudos60019)(Selleck Chemicals). ATMi was dissolved in DMSO and used at the 10μM concentration, 16h prior to the experiment.

To induce DNA damage (DNA double-strand breaks) in my work I used two tools: X-ray generating machine (Faxitron X-Ray Corporation) generating 2 Grays/minute or neocarzinostatin (NCS). Cells were irradiated with the amount of Greys (G) indicated in the figure legends. In case of NCS cells were treated with 50 ng/ml for 10 minutes and then left for 50 minutes for the recovery.

To induce FOXO3a nuclear localization, I treated TALL-1 cells with Metformin, which is an AMP-activated protein kinase (AMPK) activator at the concentration 500nM (dissolved in water) or SB203580 a p38 mitogen activated protein kinases (p38) inhibitor at the concentration of 10μM (dissolved in DMSO). In both cases TALL-1 cells were pretreated with the drugs 24h before induction of DNA damage.

Calcium phosphate transfection method (for HEK293T and Phoenix cells)

I mixed 439μl of water with 10ug of DNA and subsequently with the 61μl of $CaCl_2$ (2M). Next, I added this solution into 500μl of 2xHBS, constantly mixing. After 10 minutes incubation at room temperature, I added the mixture to the cells.

Lipofectamine (for HeLa cells)

For this transfections I mixed: 10μl of Lipofectamine (Invitrogen) and 3μg of DNA in 500μl of OPTIMEM (Invitrogen), and incubated for 20 minutes at the room temperature before adding the mixture to the cells's medium.

Lipofectamine 2000 (for the mESC)

For this transfections I incubated separately: 6µl of the Lipofectamine 2000 (Invitrogen) in 500µl of OPTIMEM (Invitrogen) medium and 3µg of DNA for 5 minutes. Next, I mixed together both solutions and after 20 minutes at the room temperature I added the transfection mixture to the cells.

RNAiMAX (for the siRNA transfections)

The procedure of the transfection was similar to that of Lipofectamine 2000 with the exception of the initial mixtures preparation: I mixed 4µl of lipofectamine RNAiMAX (Invitrogen) and used 20nM of final concentration of siRNA targeting the indicated genes (Dharmacon).

Plasmids

Human N1ΔE-Flag construct was kindly provided by of P.P. Di Fiore (Istituto Europeo di Oncologia). Human N1ΔEΔANK-Flag construct was cloned by Jelena Vermezovic (Vermezovic et al., 2015). Human N1ΔE-Myc construct was obtained from G. Del Sal (University of Trieste). Human N1IC in MIGR1 construct was a kind gift of J. Aster (Brigham and Women's Hospital). Human GFP-N1IC-NLS, CD8-N1IC-GFP, GFP-N1IC-NES and EGFP constructs were a gift of A. Sarin (National Centre for Biological Sciences). Human Myc-ATM (2-797, 798-1964, 1965-3056aa) fragments were a kind gift of S. J. Kim (CHA University)(Park et al., 2015b). Human Flag-ATM construct was a kind gift of M. Kastan (Duke cancer institute) (Bakkenist and Kastan, 2003). Flag-ATMΔFATC (1-2992aa) construct, was generated by the IFOM Biochemistry unit. GST-ATM fragments (1-247, 250-522, 523-769, 772-1102, 1098-1371, 1245-1435, 1439-1770, 1764-2138, 2141-2428, 2427-2841, 2842-3056, 2682-3012aa) were kindly provided by A. Behrens (Francis Crick Institute)(Khanna et al., 1998). Mouse 3xFlag-KAT5 (1-258, 69-290, 158-395, 285-513aa) fragments were obtained from H. S. Park (Chonnam National

University)(Kim et al., 2007a). Human Flag-KAT5 construct was a generous gift of S.P. Jackson (Gurdon Institute)(Kaidi and Jackson, 2013). Myc-KAT5 (450-513aa KAT5 fragment) was PCR-amplified using primers described in the Table 2 and cloned into the BamHI and XhoI restriction sites of Myc-pcDNA3 plasmid (Wang et al., 2008). GST-KAT5 as well as GST-FOXO3a were PCR-amplified using primers described in the Table 2 and cloned into the BamHI and SalI restriction sites of pGEX2rbs. Human Myc-FOXO3a construct was obtained from K. Yamamoto (Nagasaki University)(Wang et al., 2008). Myc-FOXO3a (1-300, 1-500, 500-673, 500-650, 500-620aa) fragments were PCR-amplified using primers described in the Table 2 and cloned into the BamHI and XhoI restriction sites of Myc-pcDNA3 plasmid (Wang et al., 2008). Human N1IC-Flag construct for expression in insect cells was obtained by PCR amplification of the intracellular part of NOTCH1 from N1ΔE-Flag construct and cloned into the BamHI and SalI sites of pFastBac1 (Invitrogen)(Vermezovic et al., 2015).

Fluorescence-activated cell sorting of transfected cells

In order to obtain homogenous population of the cells transiently transfected with GFP-tagged human N1IC, I performed Fluorescence-activated cell sorting (FACS). For this purpose I transfected HEK293T cells with different constructs carrying EGFP or N1IC-GFP-NLS (see section "Plasmids and constructs"). 24h after transfection cells were FACS-sorted, plated and left for 8h for recovery. After this time cells were subjected to irradiation, collected and lysed according to the experimental setup.

Virus infections

For the purpose of some experiments to obtain homogenous population of cells expressing the indicated constructs, I infected HeLa or MCF10a cells with the virus carrying specific

constructs: human N1IC or the empty vector (MIGR1), which allows to sort infected cells according to GFP expression.

First, I transfected the indicated constructs into amphotrophic Phoenix cells. One day after transfection, I replaced the medium and added half of the volume of medium to concentrate the virus. 2 days after transfection, I collected the medium from transfected Phoenix cells, filtered it through the 0,45μm filter, add polybryne (8mg/ml) and added it to the targeted cells. After 4h incubation I repeated the procedure adding freshly collected supernatant from Phoenix cells to the targeted cells. After another 4h I replaced virus medium with normal medium. 3 days post transfection I repeated all of the steps from the 2^{nd} day. On the 4^{th} day post transfection, FACS sorting was performed after which cells were re-plated and kept in the culture for no more then 3 days.

RNA extraction and revers transcription

For RNA extraction cells were harvested in 350μl of RNeasy lysis buffer (RLT)(Qiagen) supplemented with 1% β-mecaptoethanol. Next, I added 350μl of 70% ethanol to the sample, mixed it and spun ($8x10^3$g for 15 seconds) on the RNeasy Mini spin column. Next, samples were washed with 350μl of RW1 buffer (Qiagen) followed by DNase treatment (Qiagen) on the column for 15 minutes at the room temperature followed by a short spin, one wash with 350μl of RW1 buffer (Qiagen), two consecutive washes with the 500μl RPE buffer (Qiagen) and one empty spin. At the end samples were resuspended in 50μl of water. RNA was quantified by nanodrop (GE Healthcare) and equal amounts of RNA were used for the revers transcription step.

Reverse transcription reactions

To allow the amplification of the signal with the use of DNA primers in the quantitative real time PCR (qRT-PCR) reaction, I first reverse-transcribe 200-1000ng of mRNA. I

incubated it in the SuperScript™ III Reverse Transcriptase (Invitrogen) mix for 1h at 42°C in the VILO™ Reaction Mix (Invitrogen). The reaction was stopped by the incubation of the samples at 85°C for 5 minutes. As a negative control for qRT-PCR analyses, I performed always one reaction in the absence of the Reverse Transcriptase. cDNA was next used in the qRT-PCR analysis.

qRT-PCR

The qRT-PCR analysis is a method that allows to detect expression levels of the transcripts in a very precise and quantitative way with the use of the fluorescent dyes that intercalate into the newly synthetized DNA double helix in the PCR reaction, measured and acquired in real time. For my qRT-PCR analyses, I used the LightCycler® 480 SYBR Green I Master kit, which contained SYBR Green I dye. Reactions were performed in the Light Cycler 480 Roche machine with the use of indicated primers (Table 2)(500nM) in 20μl volume. qRT-PCR reaction was set as follows:

1) Pre-incubation for 5 min at 95°C

2) 50 cycles of the 3 steps: denaturation for 10 seconds at 95°C; annealing for 10 seconds at 65°C; amplification for 1 second at 72°C.

3) Melting curves - to visualize quality of the amplified product

Western blot analysis

To analyze protein levels and modifications in my experiments I performed a number of western blot analyses. In these experiments cells were lysed in TEB150 lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA pH 8, 1 mM dithiothreitol (DTT), 0.5% Triton X-100, 10% glycerol, protease inhibitor cocktail set III (Calbiochem) and Benzonase 1:1000 (Sigma)) for 45 minutes at 4°C, followed by spinning for 15 minutes at $16x10^3$ g at 4°C. Concentration of the protein lysates obtained in this way

was measured with the use of Bradford dye-binding method (Bio-rad). Next, equal amounts of protein lysate of each sample were taken and incubated with 4x sample buffer (final concentrations: 50mM Tris-HCl pH 6,8, 2%SDS, 10% glycerol, 100μM DTT, 12,5mM EDTA and 0,02% bromophenol blue) for 10 minutes at 95°C.

Samples prepared in this way were resolved by SDS-PAGE followed by protein transfer (400mA; 1h) on Nitrocellulose membrane (0,45mm) followed by blocking the membrane with the 5% milk solution in TBS-Tween (0,1%) for 1h at room temperature. Membranes were further incubated with the primary antibody at the indicated concentrations (Table 1) overnight at 4°C. Next, membranes were washed with TBS-Tween (0,1%) 3 times for 10 minutes and incubated with secondary HRP-conjugated antibodies (Bio-rad). Membranes were washed 3 times for 10 minutes with TBS-Tween (0,1%) and subjected to chemiluminescent reaction with the ECL (GE Healthcare). Chemiluminescent signals were acquired with the use of chemiluminescence sensitive films (Sigma).

Protein lysate fractionation

To obtain different cellular protein fractions from cell lysates, I first trypsinized and pelleted cells. After one wash with PBS, I resuspended cells in nuclear isolation buffer (0.25M Sucrose, 10mM Tris HCl pH 7.4, 5mM $MgCl_2$ supplemented with protease inhibitor cocktail set III (Calbiochem)) and incubated them for 20 minutes at 4^oC followed by centrifugation of the samples at $5x10^3g$ at 4°C for 20 minutes. I took the supernatant and marked it as the cytosolic fraction. Pelleted nuclei were resuspended in nuclear lysis buffer (NLB) (150mM KCl, 25mM Tris HCl pH 7.4, 5mM $MgCl_2$ and 0.5% NP40, protease inhibitor cocktail set III (Calbiochem)) and incubated for 30 minutes at 4^oC followed by centrifugation of the samples at $16x10^3g$ at 4°C for 30 minutes. I took such supernatant and marked it as the nuclear soluble fraction. Next, I resuspended the remaining pellet in NLB with Benzonase nuclease (1:300)(Sigma) and incubated it for 30

minutes at 4°C followed by centrifugation of the samples at $16x10^3$g at 4°C for 30 minutes. I took the supernatant and marked it as the chromatin fraction.

***In vitro* ATM kinase assay**

For that I harvested and lysed HeLa cells in TEB 150 (without phosphatase inhibitors) for 30 minutes on ice followed by spinning for 15 minutes at $16x10^3$g at 4°C. 0.5mg of protein lysate was pre-cleared with 30µl of Protein G beads (Zymed Laboratories)(1.5h) followed by the incubation with anti ATM antibody (Sigma)(0.5µg in 500µl volume, 1.5h) and then with 30µl of protein G beads (1.5h). Beads were pulled down and washed sequentially: once with TEB150, once with TEB150 supplemented with 0.5M LiCl, once with 0.5M NaCl TEB150, twice with TEB150 and at the end with a kinase buffer (without ATP) - 5 minutes each wash. Next, beads were incubated with the recombinant NIC-Flag or ATMi for 2h on ice in kinase buffer (without ATP) followed by the addition of 50nM GST-p53, 10ng of DNA 1kb ladder (NEB) and ATP, all in 20µl volume (kinase buffer: 50mM Hepes pH=7.5; 50mM KCl; 5mM $MgCl_2$; 1mM ATP; 1mM DTT; 10% glycerol). Beads were incubated for 90 minutes at 30°C. Reactions were stopped by the addition of sample buffer.

Immunoprecipitation

HEK293T cells were harvested and lysed in the TEB150 lysis buffer for 45 minutes followed by spinning for 15 minutes at $16x10^3$g at 4°C. Next, supernatant was collected and protein concentration was assessed by spectrophotometer (595nm) with use of Bradford dye-binding method (Bio-rad). 1mg or more of the protein lysate was used per each immunoprecipitation in a reaction volume of 500µl (equalized for each sample). At the same time 1% of the immnoprecipitation reaction was collected and denatured in the sample buffer (50 mM Tris-HCl pH 6.8; 2% SDS; 10% glycerol; 12.5 mM EDTA; 0,02 %

bromophenol blue; 100μM DTT) for 10 minutes at 95°C, as the loading/expression control. To reduce the background from unspecific binding of proteins to the beads, samples were pre-cleared with Protein G beads (50μl)(Zymed Laboratories) for 1h at 4°C. Followed by the short spinning (1,5 minute at 370g) and supernatant removal, samples were incubated with the indicated antibodies (Table 1) overnight at 4°C. Next, 40μl of the Protein G beads was added to the samples and incubated for 2h followed by 3-6 washes with lysis buffer. Immunoprecipitated proteins were released by the addition of sample buffer and incubation at 95°C for 10 minutes.

Protein purification

Bacterial expression constructs of GST-ATM (pGEX5X-1), GST-KAT (pGEX2rbs), GST-FOXO3a (pGEX2rbs) and p53 (pGex4T3) were transformed into *Esherichia Coli* BL21 bacteria. Expression of the proteins was induced when growing bacteria reached optical density of 0,6-0,8 (at 600nm) with the use of Isopropyl β-D-1-thiogalactopyranoside (IPTG)(0,4mM). After induction, bacteria were incubated overnight at 20°C.

Recombinant GST-ATM fragments were purified as follow. Bacteria were spun for 25 minutes at 4°C and resuspended in the lysis buffer (50mM Tris-HCl pH 7,9, 300mM KCl, 1% Triton X-100, 2mM DTT and protease inhibitor cocktail set III (Calbiochem) 1:200). Next, cells were sonicated 3 times for 20 seconds at the amplitude of 5 impulses and power of 75% followed by centrifugation of the samples at $20x10^3$g at 4°C for 90 minutes. Supernatant was next incubated with the Gluthatione sepharose beads (GE Healthcare) for 2h at 4°C, followed by 3 consecutive washes with three different buffers (30 minutes at 4^oC): Buffer I (PBS, 1% Triton X-100, 2mM DTT and protease inhibitor cocktail set III (Calbiochem) 1:200); Buffer II (300mM KCl, 50mM Tris-HCl pH 8, 2mM DTT and protease inhibitor cocktail set III (Calbiochem) 1:200); Buffer III (100mM KCl, 50mM Tris-HCl pH 8, 2mM DTT, 10% glycerol and protease inhibitor cocktail set III

(Calbiochem) 1:200). Next captured proteins were flash-frozen in liquid nitrogen and stored until use.

Recombinant GST-KAT, GST-p53 and GST-FOXO3a proteins were purified in a similar way to the GST-ATM fragments with few exceptions. For this purification I used different a lysis buffer (2xTBS, 10% glycerol, 1mM EDTA, 1mM DTT and protease inhibitor cocktail set III (Calbiochem) 1:200). The three post-capturing washes were performed with: one wash with the lysis buffer containing 1M NaCl and 2 sequential washes just with the lysis buffer. For the purpose of some experiments, GST-tagged proteins were eluted as described above but for other experiments GST tag was removed by the incubation with PreScission protease for 2h at 4°C. Next, supernatants were taken and concentrated on the Millipore Centrifugal Filter (10kDa), which was combined with the buffer exchange to the PBS supplemented with the 10% Glycerol.

Recombinant human N1IC-Flag was purified by IFOM crystallography unit from Hi5 insect cells (Vermezovic et al., 2015).

Immunofluorescence

To visualize the activation and accumulation of DNA damage response factors at sites of DNA double-strand breaks, I performed a number of immunofluorescence stainings. To do this I fixed cells grown on coverslips in 4% solution of paraformaldehyde (PFA) in PBS for 10 minutes at room temperature or in Methanol/Acetone (1:1) solution for 2 minutes at room temperature. Next, in case of PFA fixation, cells were subsequently permeabilized with 0,2% solution of Triton X-100 for 10 minutes. To decrease unspecific binding of the antibodies, cells were incubated 1h in PBG solution (solution of 0,5% BSA and 0,2% Cold-water-fish gelatin in PBS) followed by 1h incubation with indicated primary antibody (Table 1) in PBG at room temperature. After washing out primary antibody (3 times for 5 minutes) with PBG, coverslips were incubated with the secondary antibody conjugated with a fluorophore (Alexa488, Alexa555 or Alexa647) for 1h at room temperature. At the

end coverslips were washed 3 times with the PBG solution, cells nuclei were stained with DAPI for 5 minutes and coverslips were mounted on slides with Moviol.

Proximity ligation assay (PLA)

Cells were fixed in 4% solution of PFA in PBS. Next, cells were permeabilized with the use of 0,2% solution of Triton X-100 for 10 minutes, blocked for 1h with PBG solution and incubated with the primary antibodies of different species for 1h. After 3 washes with PBG, coverslips were incubated with the secondary antibodies containing oligonucleotide probes (plus and minus) for 1h at 37°C, followed by 2 washes for 5 minutes with buffer A (Sigma) and 30 minutes ligation of the connector oligonucleotides at 37°C. Next, coverslips were washed twice for 2 minutes in buffer A and incubated for 90 minutes at 37°C with DNA polymerase which performs a rolling circle amplification (RCA) allowing for the hybridization of detection probes to the RCA products. After that, coverslips were washed twice with buffer B (Sigma) for 10 minutes and once with 1% solution of buffer B for 1 minute. At the end cell nuclei were stained with DAPI and coverslips were mounted on slides in Moviol.

Image analysis

PLA signals as well as quantification of the number of phosphorylated histone γH2AX foci were analyzed with the use of the Cell Profiler software (2.1.0). For this purpose raw images were uploaded to the software. Signal threshold was constant within each experimental replicate – due to stainings variation it might be slightly different between different replicas. PLA signals were always normalized to the untreated cell.

Immunofluorescence analysis of the activation of DNA damage response factors were performed manually and expressed as a percentage of DNA damage response positive cells. Cells with more then four foci per nucleus were counted as a positive.

Statistical analysis

Statistical analysis of the immunoblot and immunofluorescence quantifications as well as PLA analysis were performed with the use of two tailed Student's t-test and represented as a mean ±SEM. Asterisk in the figures indicates p value <0.05.

Antibodies

Antibodies used together with the concentration at which they were used is described in the table below.

Table 1. Antibody information.

Antibody:	Provider:	Use:	Cat. No.:	Species:
ATM	Abcam	WB 1:6000 5% Milk	ab32420	Rabbit
ATM	Sigma	WB 1:2000 5% Milk IP 1: 500 IF 1:200	A1106	Mouse
DNA-PKcs	Millipore	WB 1:6000 5% Milk	04-1024	Rabbit
pT2609DNA-PKcs	Biolegend	WB 1:3000 5% Milk	612902	mouse
pT2047DNA-PKcs	Abcam	WB 1:3000 5% Milk	ab61045-100	Rabbit
pS2056DNA-PKcs	Abcam	WB 1:3000 5% Milk	ab18192	Rabbit
Celavaded	Cell Signaling	WB: 1:1000 5% BSA	9661	Rabbit

CASPASE 3				
pS1981ATM	Rockland	WB 1:3000 5%Milk IF 1:200	200-301-400	Mouse
NOTCH1	Santa Cruz	WB 1:1000 5% Milk	sc-6014	Goat
N1IC	Cell Signaling	WB 1:2000 5% BSA	4147	Rabbit
GFP	Santa Cruz	WB 1:6000 5% Milk	sc-9996	Mouse
Flag	Sigma	WB 1:6000 5% Milk IP 1:500	F1804	Mouse
Flag	Cell Signaling	WB 1:6000 5% Milk	2368	Rabbit
Myc	Santa Cruz	WB 1:6000 5% Milk IP 1:500	sc-40	Mouse
Myc	Cell Signaling	WB 1:6000 5% Milk	2272	Rabbit
FOXO3a	Cell Signaling	IF 1:200	2497	Rabbit
FOXO3a	Abcam	WB 1:6000 5% Milk	ab109629	Rabbit
FOXO3a	Santa Cruz	IP 1:50	sc-11351	Rabbit
FOXO1	LifeSpan BioSciences	WB 1:8000 5% Milk IP 1:250	LS-C287207	Rabbit
FOXO4	Abcam	WB 1:8000 5% Milk IP 1:500	Ab128908	Rabbit
KAT5	LifeSpan BioSciences	WB 1:5000 5% Milk	LS-C109474	Rabbit
KAT5	Santa Cruz	WB 1:1000 5% Milk IP 1:30	sc-5725	Goat
KAT5	Kind gift of B. Amati (Frank et al., 2003)	WB 1:1000 5% Milk	x	Rabbit

pS15p53	Cell Signaling	WB 1:10000 5% BSA	16G8	Rabbit
H2AX	Abcam	WB 1:6000 5% Milk	ab11175	Rabbit
pS139H2AX	Millipore	WB 1:6000 5% Milk	05-636	Mouse
SMC1	Bethyl	WB 1:8000 5% Milk	A303-834A	Goat
pS966SMC1	Bethyl	WB 1:6000 5% Milk	A300-050A	Rabbit
KAP1	Abcam	WB 1:8000 5% Milk	ab10484	Rabbit
pS842KAP1	Bethyl	WB 1:6000 5% Milk	A300-767A	Rabbit
CHK2	Millipore	WB 1:6000 5% Milk	05-649	Mouse
pT68CHK2	Cell Signaling	WB 1:1000 5% BSA	2661	Rabbit
pS317CHK1	Cell Signaling	WB 1:6000 5% BSA	2344	Rabbit
pS345CHK1	Cell Signaling	WB 1:6000 5% BSA	2341	Rabbit
CHK1	Santa Cruz	WB 1:2000 5% Milk	sc-8408	Mouse
53BP1	Novus	IF 1:2000	NB100-304	Rabbit
MEIS	Santa Cruz	WB 1:6000 5% Milk	Sc-10599	Goat
H3K9m3	Abcam	WB 1:6000 5% Milk	8898	Rabbit
GST	Biochemistry Facility, IFOM	WB 1:8000 5% Milk	x	Rabbit
NBS1	Novus	WB 1:1000 5% Milk IP 1:500	NB 100-143	Rabbit
TUBULIN	Sigma	WB 1:8000 5% Milk	T6074	Mouse
VINCULIN	Sigma	WB 1:8000 5% Milk	V9131	Mouse
H3	Abcam	WB 1:8000 5% Milk	ab10799	Mouse
H4	Abcam	WB 1:8000 5% Milk	ab10158	Rabbit
NUCLEOLIN	Novus	WB 1:8000 5% Milk	NB 600-241	Rabbit

WB - Westen blot; IF - immunofluoresncece; IP - immunoprecipitation

Primers

Primers used together with their sequences are described in the table below.

Table 2. Primers information.

Primers used:	Sequence (5'->3'):
qRT-PCR:	
GADD45α	Fr TTTGCAATATGACTTTGGAGGA
	Rv CATCCCCCACCTTATCCAT
HES1	Fr GAAGCACCTCCGGAACCT
	Rv GTCACCTCGTTCATGCACTC
B2M	Fr TTCTGGCCTGGAGGCTATC
	Rv TCAGGAAATTTGACTTTCCATTC
Cloning:	
GST-KAT5	Fr GATCGGATCCATGGCGGAGG
	Rv GATCGTCGACTCACCACTTCCC
Myc-FOXO3a 1-300	Fr GATCGGATCCATGGCAGAGGCACCGG
	Rv GATCCTCGAGTCAACTGCTGCGTGACGTGGG
Myc-FOXO3a 1-500	Fr GATCGGATCCATGGCAGAGGCACCGG
	Rv GATCCTCGAGTCACACAGCGGTGCTGGCC
Myc-FOXO3a 500-673	Fr GATCGGATCCATGTCTGCCCAGAATTCCC
	Rv GATCCTCGAGTCAGCCTGGCACCCAG
Myc-FOXO3a 500-650	Fr GATCGGATCCATGTCTGCCCAGAATTCCC
	Rv GATCCTCGAGTCAATTCTGTGTGGAGATGAGGG

Myc-FOXO3a 500-620	Fr GATCGGATCCATGTCTGCCCAGAATTCCC
	Rv GATCCTCGAGTCACAAGCTCCCATTGAAC
Myc-KAT5 450-513	Fr GATCGGATCCAAGAAGGAGGATG
	Rv GATCCTCGAGTCACCACTTCCC
GST-FOXO3a	Fr GATCGGATCCATGGCAGAGGCAC
	Rv GATCGTCGACTCAGCCTGGCAC

Results

Here I will present results that I have obtained during my PhD program on NOTCH1-mediated regulation of the DNA damage response (DDR). Presented in this section data include some unpublished results, as well as parts of already published articles (Adamowicz et al., 2016; Vermezovic et al., 2015).

NOTCH1 inhibits DDR activation.

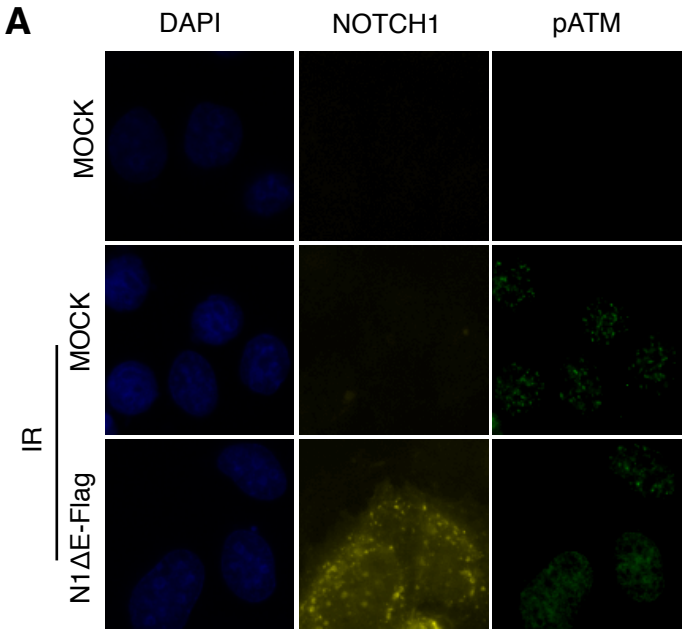
It has been suggested that in the stem cell compartment (cells expressing high levels of NOTCH1) of the *Caenorhabditis elegans* gonad activation of ATM kinase is perturbed (Vermezovic et al., 2012). Additionally, it has been shown that expression of NOTCH1, in a transcriptional independent manner, can affect the phosphorylation of p53, and impact on the transcription of its downstream targets upon induction of DNA damage in colon cancer cells (Kim et al., 2007b). On the basis of this observations, I decided to investigate whether NOTCH1 can impact on the DDR upstream of p53 in mammalian cells and, if so, by which mechanism.

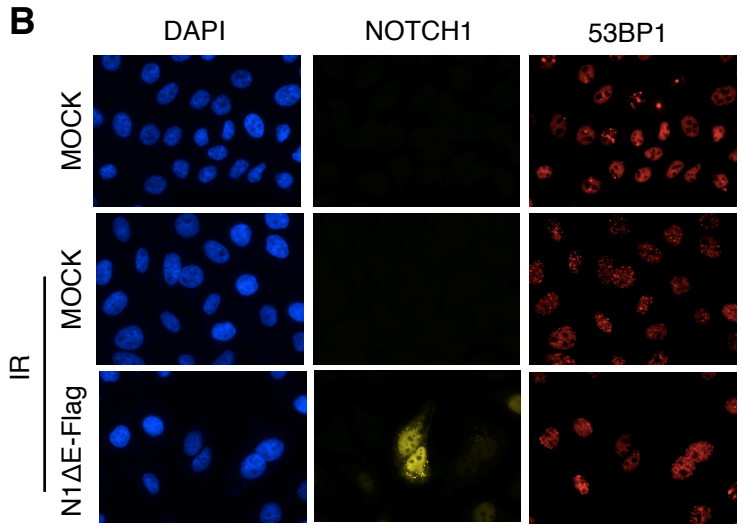
In order to be activated NOTCH1, as a trans-membrane receptor, requires an interaction with one of its ligands. To study the role of NOTCH1 in mammalian cells, I decided to express a constitutive activate form of the NOTCH1 (N1ΔE) (Rustighi et al., 2009) or the intracellular part of NOTCH1 (N1IC) (Perumalsamy et al., 2009) in the commonly used cell lines HeLa and HEK293T.

N1ΔE is NOTCH1 protein missing the extra-cellular part of NOTCH1, although it still posses the transmembrane region. Therefore this form of NOTCH1 (N1ΔE) still needs to be processed by endogenous cellular gamma secretase proteinase in order to be translocated to the nucleus, it does not need any interaction with NOTCH1 ligand to be activated. N1IC construct instead encodes just for the intracellular part of NOTCH1 that does not need to be processed by gamma secretase or to interact with NOTCH1 ligand, and therefore it directly localizes to the nucleus (for more details see the “NOTCH1 signaling” chapter of the introduction)

Thus, to study the impact of Notch1 on DDR activation, I expressed Flag-tagged N1ΔE construct in HeLa cells that I exposed to ionizing radiation (IR) to induce DNA double strand-breaks (DSBs). As shown in Figure 7A and B, I was able to observe an activation of the DDR in HeLa cells upon treatment with IR as measured by the formation of foci of the phosphorylated form of ATM (pATM) and 53BP1, which indicates the recruitment of such factors to DSBs. In HeLa cells that had been successfully transfected with the N1ΔE-Flag construct, I observed a reduction of foci formation by both pATM and 53BP1 (Figure 7A and B). Next, I checked if the observed inhibition of the DDR by expression of NOTCH1 can be overcome by exposing cells to higher doses of IR. I subjected HeLa cells to different doses of IR: 1, 3 and 10G. As shown in Figure 7C, expression of NOTCH1 impaired formation of 53BP1 foci formation despise higher levels of DNA damage.

To analyze more broadly the DDR factors affected by NOTCH1 expression, I decided to perform an immunoblot analysis of HeLa cells infected with the N1IC-expressing construct tagged with the GFP. Thus, I sorted infected cells to obtain homogenous cell population that I subsequently exposed to IR, lysed and analyzed by western blot.


A
DAPI
NOTCH1
pATM
MOCK
IR
MOCK
N1ΔE-Flag


B
DAPI
NOTCH1
53BP1
MOCK
IR
MOCK
N1ΔE-Flag

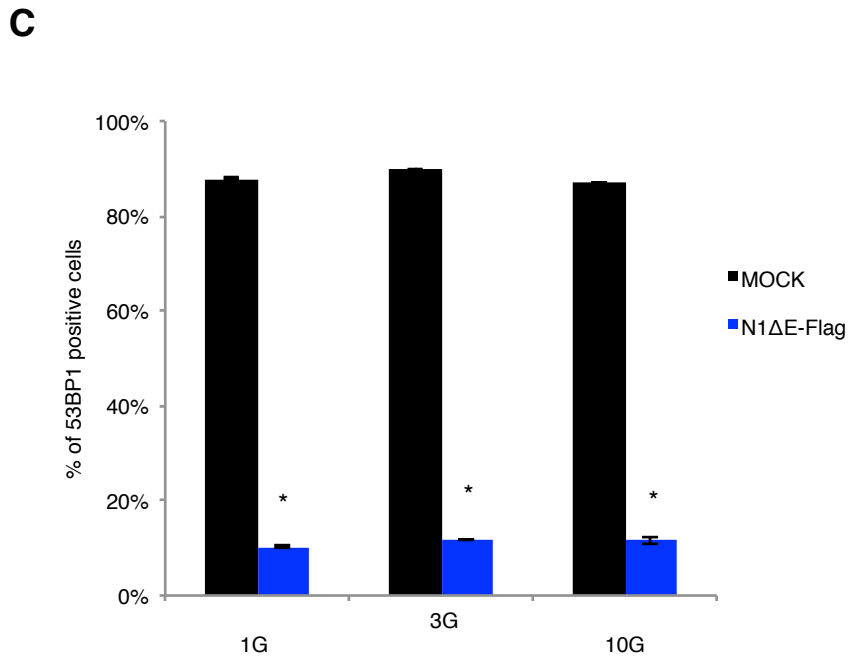

C
100%
80%
60%
40%
20%
0%
% of 53BP1 positive cells
*
*
*
1G
3G
10G
MOCK
N1ΔE-Flag

Figure 7. NOTCH1 inhibits DDR. Immunofluorescence analysis of the DDR activation upon IR treatment (2G; 1h) in HeLa cells transfected with N1ΔE-Flag: pATM (A) and 53BP1 (B). (C) Quantification of the immunofluorescence analysis of the DDR activation (53BP1) in the HeLa cells transfected with N1ΔE-Flag and subjected to different doses of IR (1G, 3G and 10G; 1h).

As shown in the Figure 8A, I observed that HeLa cells expressing N1IC had impaired activation of the DDR as measured by the reduction of the DNA damage dependent phosphorylation of factors like: ATM, SMC1, KAP1 and CHK2 but not H2AX (Figure 8A and B). Although the observed levels of reduction of the DDR activation were moderate, they were consistent with the low levels of N1IC expression that I detected by measuring NOTCH1-induced transcription of its target gene *HES1* (Figure 8C).

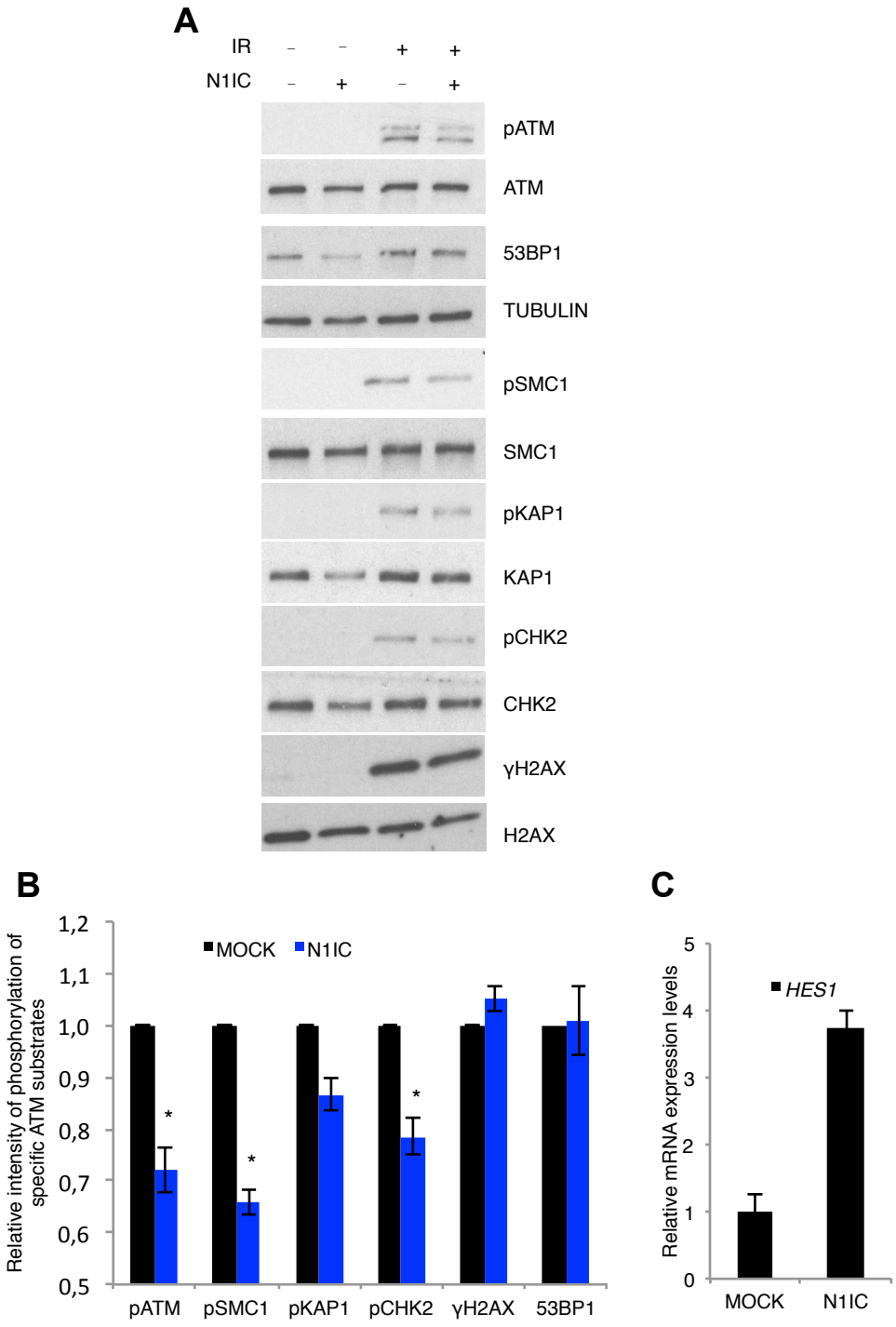


Figure 8. NOTCH1-mediated inactivation of DDR. (A) Immunoblot analysis of the DDR activation upon IR (5G; 1h) in HeLa cells infected with MIGR1-N1IC construct or empty vector. (B) Quantification of the experiment shown in B. (C) qRT-PCR analysis of the mRNA levels of *HES1* in the HeLa cells infected with MIGR1-N1IC construct or empty vector.

To check if the observed phenomenon of NOTCH1-mediated DDR inhibition is not a cell line specific response to NOTCH1 expression in HeLa cells, I performed the same

experiment in normal human mammary epithelial cells (MCF10a). As shown in Figure 9 I observed the same reduction in DNA damage activation as measured by the activation of the ATM kinase (pATM) in MCF10a cells expressing NOTCH1, as in HeLa cells.

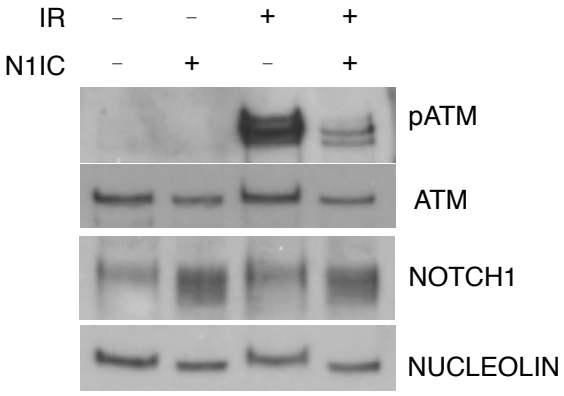


Figure 9. NOTCH1 mediated inactivation of the DDR. Immunoblot analysis of the DDR activation (pATM) upon IR (5G; 1h) in MCF10a cells infected with MIGR1-N1IC construct or empty vector.

NOTCH1-mediated DDR inhibition is persistent in time

The data shown above indicate that NOTCH1 inhibits DDR activation in the cells exposed to IR. It has been reported that inhibition of PARylation, although it does not affect DDR activation, it leads to its delay, stressing the importance of the timing at which DDR activation is assessed (Haince et al., 2007). Therefore I decided to check if NOTCH1-mediated inhibition of the DDR is permanent or just transient. I thus performed immunofluorescence analysis of the DDR activation upon IR (53BP1, pATM) in HeLa cells expressing NOTCH1 (N1ΔE-Flag) at different time points after the DNA damage induction: 1h, 4h, 24h. As shown in Figure 10A and B, NOTCH1 expression was able to inhibit DDR activation throughout all time points analysed, as measured by ATM activation (pATM) and 53BP1 foci formation.

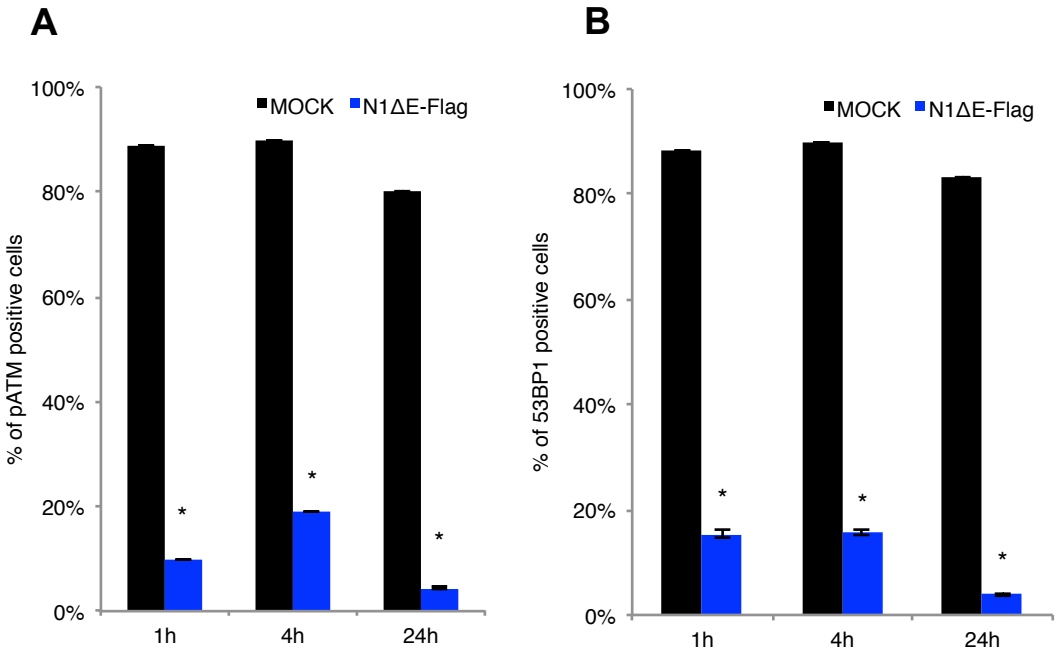


Figure 10. NOTCH1 mediated inactivation of DDR is persistent in time. Quantifications of the immunofluorescence analysis for the DDR activation (pATM, 53BP1) in the HeLa cells expressing NOTCH1 (N1ΔE-Flag) and subjected to 2G of IR.

Nuclear localization of NOTCH1 is necessary for its inhibition of the DDR

Because of the complex functions of NOTCH1 that results in its presence in different cellular compartments at the same time and at the different functional levels, I decided to investigate which form/localization of NOTCH1 is necessary for DDR inhibition (For more details regarding NOTCH1 cellular localization and processing see "NOTCH1 signaling" chapter in the introduction section). To tackle this question, I utilized a set of published constructs encoding for the intracellular form of NOTCH1 (N1IC) bound to GFP with: a nuclear localization signal (GFP-N1IC-NLS) that localizes N1IC to the nucleus, or a nuclear exporting signal (GFP-N1IC-NES) that predominantly localizes N1IC to the cytosol, or a portion of the CD8 receptor that hooks N1IC on the cellular membrane (CD8-N1IC-GFP)(Perumalsamy et al., 2009).

I expressed such constructs in HeLa cells that I subsequently exposed to DSBs-inducing agent neocarzionostatin (NCS). As shown in the immunoblot analysis, I was able to express similar levels of each of the different N1IC constructs (Figure 11). Despite similar

expression levels, I observed a decrease of ATM activation (pATM) preferentially in the cells expressing N1IC construct with a nuclear localization signal (Figure 11). GFP-N1IC-NES also showed a modest impact on ATM activation, which might suggest some leakiness of the system (Figure 11).

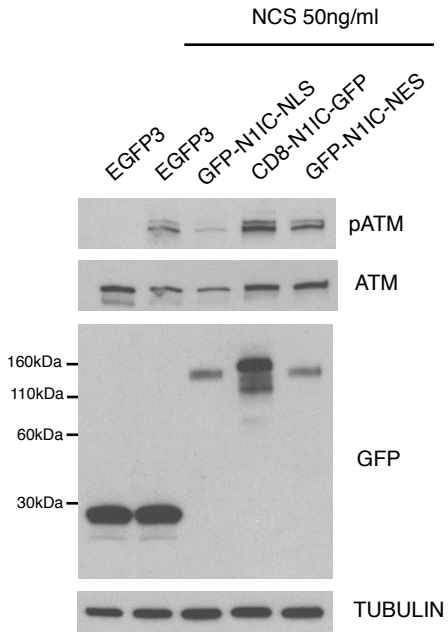


Figure 11. Nuclear localization of NOTCH1 is necessary for DDR inactivation. Immunoblot analysis of the ATM activation in HeLa cells treated with NCS (50mg/ml) and expressing different NOTCH1 constructs: GFP-N1IC-NLS - nuclear form of NOTCH1; CD8-N1IC-GFP - membrane form of NOTCH1; GFP-N1IC-NES - cytosolic form of the NOTCH1.

NOTCH1-mediated inhibition of DDR is not mediated by the transcription activity of NOTCH1

After translocation to the nucleus NOTCH1 controls the transcription of its target genes. To test if NOTCH1-mediated inhibition of the DDR by the nuclear form of NOTCH1 is the result of its transcriptional activity, I decided to check if by impairing NOTCH1 transcriptional functions I was able to prevents its DDR inhibition.

In the nucleus NOTCH1 forms a complex with the CBF1 transcription factor as well as with the MAML1 co-activator to induce the transcription of its target genes. It has been shown that with the use of a short peptide (62aa) encoding for the MAML1 interaction

domain with NOTCH1 it is possible to inhibit NOTCH1-mediated transcription due to the formation of the transcriptionally inert complex between NOTCH1 and the peptide (Weng et al., 2003). Thus, I decided to use a dominant negative form of the MAML1 (DN-MAML1 13-74) in my experiments to inhibit NOTCH1-meditated transcription.

As shown below, the concomitant expression of the DN-MAML1 (13-74) and NOTCH1 (N1ΔE-Flag) in HeLa cells led to the inhibition of NOTCH1-mediated transcription (*HES1*) as validated by the qRT-PCR analysis (Figure 12A). Next, I decided to check the DDR in this setup. Upon treatment of the cells with IR, I was able to see the induction of DDR as measured by the formation of the 53BP1 foci in the MOCK transfected cells, but in the cells expressing either NOTCH1 or NOTCH1 and DN-MAML1 (13-74) the DDR was impaired (Figure 12B and C).

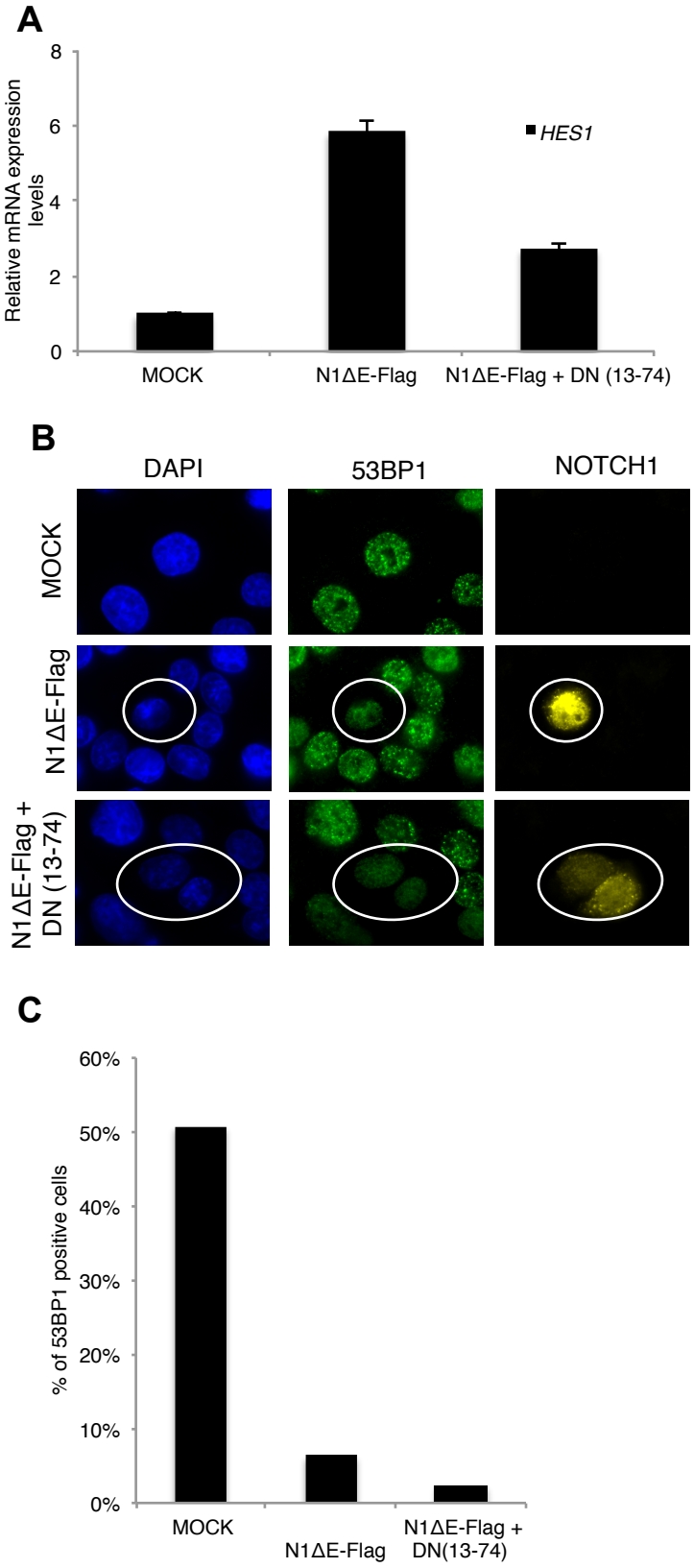


Figure 12. Transcriptional activity of the NOTCH1 is dispensable for NOTCH1-mediated DDR inactivation. (A) Analysis of the mRNA expression levels of the *HES1* by the qRT-PCR in HeLa cells transfected with the N1ΔE-Flag or/and DN-MAML1 (13-74) constructs. (B) Immunofluorescence analysis of the DDR activation (53BP1) in HeLa cells transfected with the N1ΔE-Flag or/and DN-MAML1 (13-74) constructs and subjected to IR (2G; 1h) (C) Quantification analysis of the experiment shown in B.

To strengthen this observation we performed an analysis of the ATM activation (pATM) upon IR in HeLa cells expressing wild type NOTCH1 (NOTCH1 WT-Flag) or NOTCH1 missing transactivation domain (NOTCH1ΔTAD-Flag), which is necessary for NOTCH1-mediated transcription activation (for more details please see the "Structure of the mammalian NOTCH receptors" chapter).

As presented in the immunofluorescence analysis (Figure 13A and B), we were able to observe reduction of ATM activation (pATM) in the HeLa cells expressing full length NOTCH1 as well as NOTCH1 missing its TAD domain (Figure 13A and B). qRT-PCR analysis of the *HES1* transcription confirmed that NOTCH1ΔTAD-Flag mutant was transcriptionally inactive as compared to the wild type NOTCH1 (Figure 13B). Experiments performed with the use of NOTCH1ΔTAD-Flag were carried by Jelena Vermezovic (Vermezovic et al., 2015).

Presented results indicate that the transcriptional activity of NOTCH1 is dispensable for its DDR-inhibiting functions.

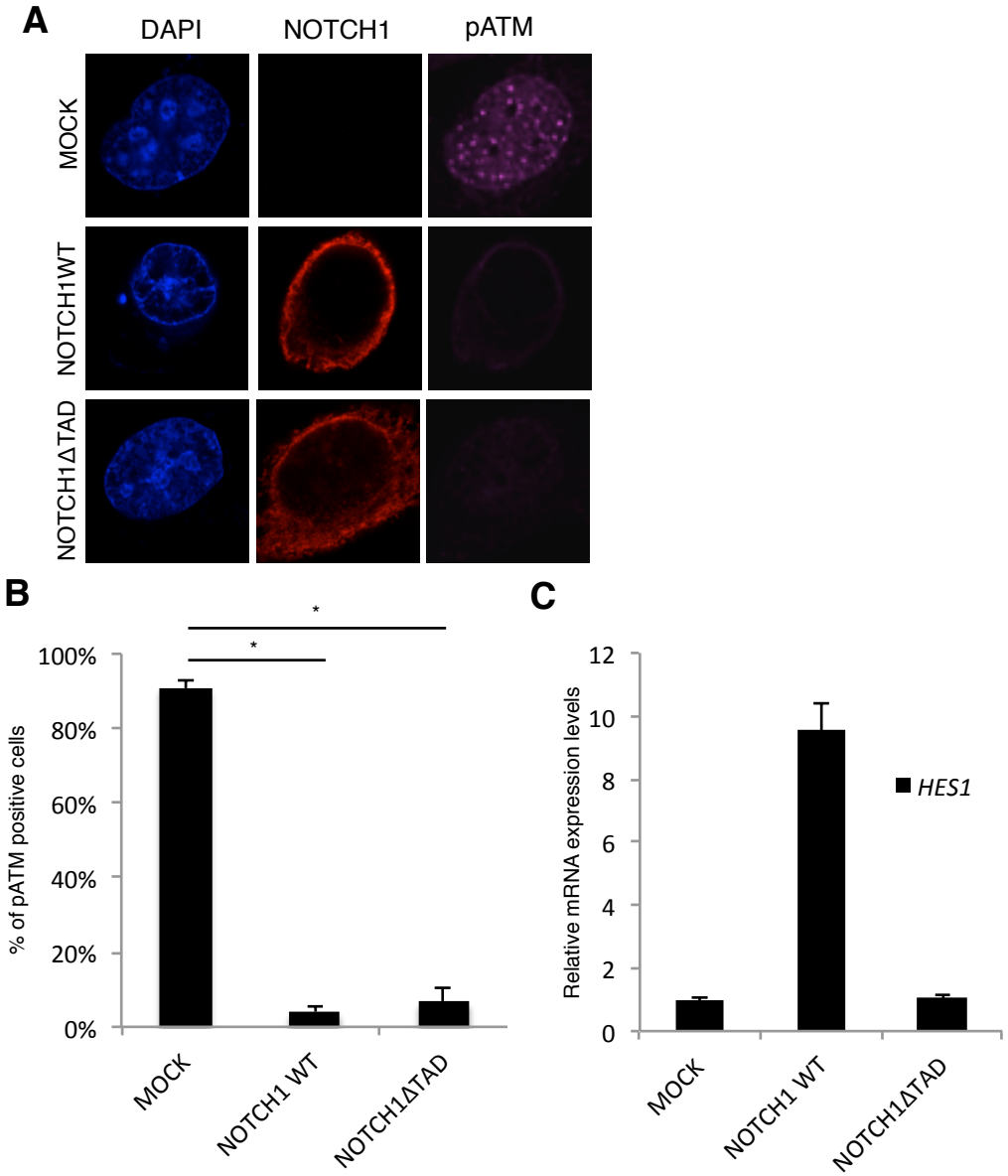


Figure 13. Transcriptional activity of NOTCH1 is dispensable for NOTCH1-mediated DDR inactivation. (A) Immunofluorescence analysis of the DDR activation (pATM) in HeLa cells transfected with the NOTCH1Flag or NOTCH1ΔTAD-Flag constructs and subjected to IR (2G; 1h). (B) Quantification of the immunofluorescence shown in A. (C) Analysis of the mRNA expression levels of the *HES1* by the qRT-PCR in HeLa cells transfected with the NOTCH1-Flag or NOTCH1ΔTAD-Flag constructs. Experiment presented in this figure were performed by Jelena Vermezovic (Vermezovic et al., 2015)

NOTCH1 does not affect ATM kinase nuclear localization

One of the possibilities of the observed NOTCH1-mediated DDR inactivation in the nucleus independently from its transcriptional activity could be to affect the localization of key DDR factors. Therefore I decided to check if NOTCH1 (N1ΔE-Flag) expression affects the nuclear localization of ATM.

I thus performed an immunofluorescence analysis of the ATM protein localization together with its activation (pATM) upon DNA damage induction in the HeLa cells expressing NOTCH1 (N1ΔE-Flag). As presented in the panel below I was not able to observe any differences in the localization of ATM in NOTCH1 expressing despite its inactivation (Figure 14).

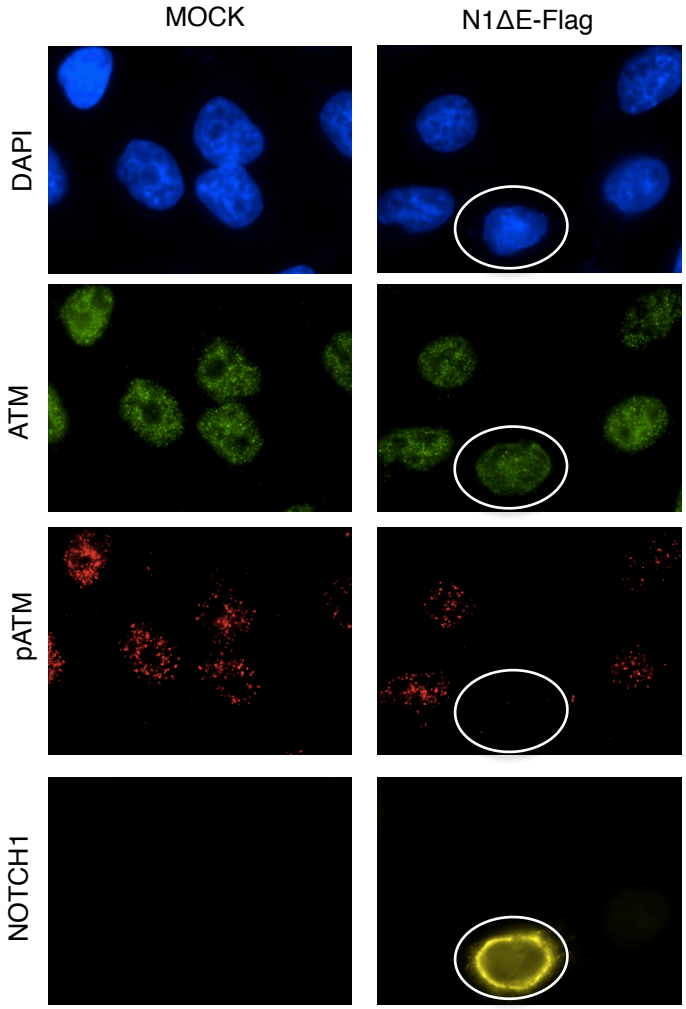


Figure 14. NOTCH1 does not affect ATM kinase nuclear localization. Immunofluorescence analysis of the ATM activation and localization in the HeLa cells expressing NOTCH1 (N1ΔE-Flag) and exposed to IR (2G; 1h).

NOTCH1 forms a protein complex with ATM

Based on the previous results I concluded that the inhibition of the DDR by NOTCH1 is mediated by the nuclear form of NOTCH1 and it is not dependent on its transcriptional activity. Therefore to understand the way in which NOTCH1 is inactivating the DDR, I decided to check if NOTCH1 forms a protein complex with some key DDR components. As ATM is the apical kinase in the DDR and it has been already published that NOTCH1 can interact with the DNA-PKcs, which like ATM is a PI3K like kinase that structurally resembles ATM (Yatim et al., 2012), I tested if NOTCH1 can interact with ATM.

To assess if ATM and NOTCH1 can form a protein complex I first expressed Flag-tagged NOTCH1 (N1ΔE-Flag) in HEK293T cells and immunoprecipitated it with antibodies against the Flag tag. As shown in the Figure 15A, I detected a protein complex between ATM and NOCTH1. ATM as well as NOTCH1 has been shown to have the ability to bind DNA (Aster et al., 1997; Smith et al., 1999). For this reason, to rule out the possibility that such an interaction is mediated by contaminating DNA, I repeated this immunoprecipiation in the presence of high concentrations of Ethidium Bromide, which by intercalation into DNA prevents DNA binding by DNA-binding proteins (Lai and Herr, 1992), or in the presence of DNase that degrades any potential contaminating DNA. Upon treatment of NOTCH1 immunoprecipitations with the Ethidium Bromide or DNase, I could still observe an interaction between ATM and NOTCH1 indicating that the formation of the protein complex between ATM and NOTCH1 is not mediated by the DNA binding abilities of both proteins (Figure 15A).

Next, I decided to check if the observed interaction between ATM and NOTCH1 is affected by DNA damage induction. I performed therefore an immunoprecipitation of either NOTCH1 (N1ΔE-Flag) or ATM from HEK293T cells subjected or not to IR. As shown in the Figures 15B and C, I did not notice any differences in the interaction between ATM and NOTCH1 in the presence or absence of the DNA damage. To control for the specificity of my immunoprecipitations, I used a Flag-tagged protein that does not interact

with ATM (MEIS-Flag). As shown in the Figure 15C, I did not detect any interaction between MEIS and NOTCH1 or ATM.

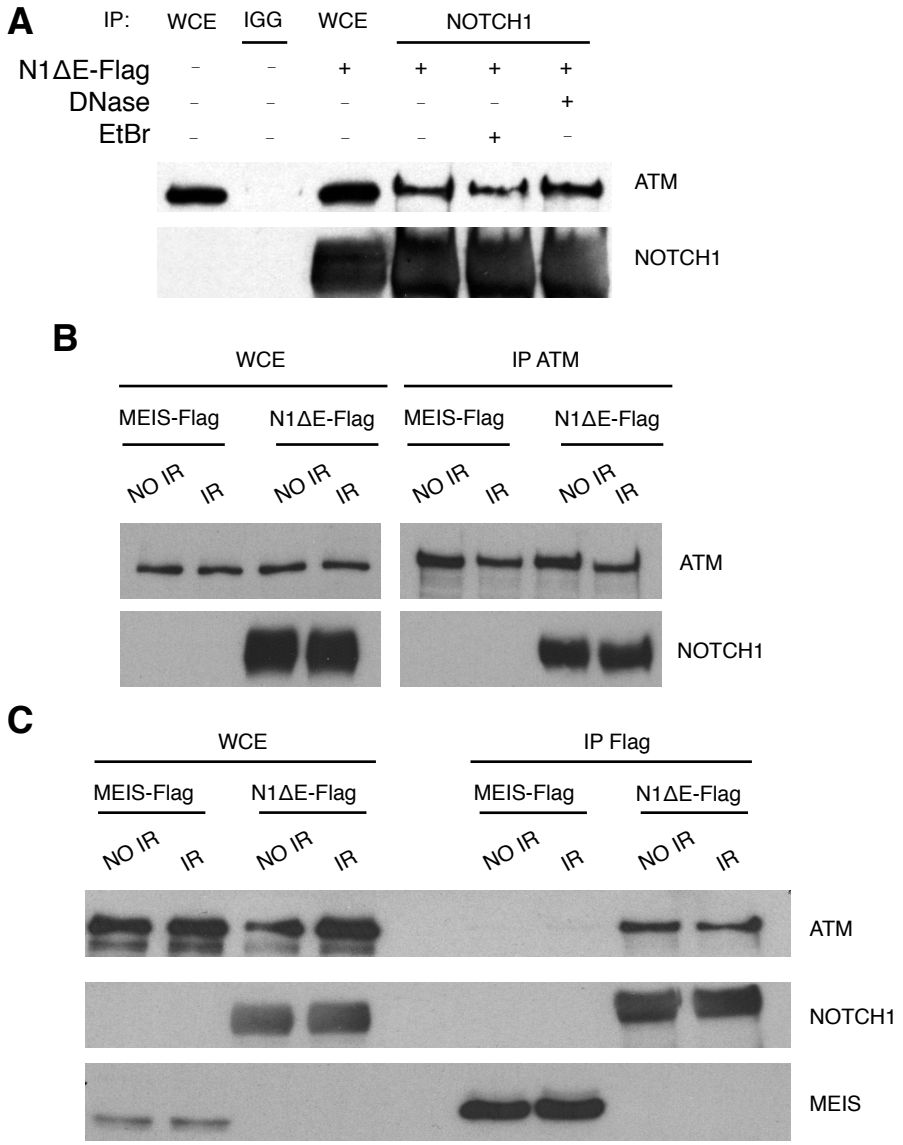


Figure 15. ATM forms a protein complex with NOTCH1. (A) Immunoblot analysis of the immunoprecipitation of NOTCH1 (N1ΔE-Flag) in the presence or absence of Ethidium Bromide (EtBr)(50 μg/ml) or DNase (2U). (B) Immunoblot analysis of the immunoprecipitation of the ATM in the presence or absence of the IR (5G; 1h). (C) Immunoblot analysis of the immunoprecipitation of the Flag tag in the cells transfected with N1ΔE-Flag or MEIS-Flag in the presence or absence of the IR (5G; 1h). WCE - Whole cell extract.

Since these interaction studies were performed in HEK293T cells in which I expressed exogenous NOTCH1, I decided to test if I can recapitulate these observation in cells expressing endogenous NOTCH1. Thus, I used T-cell acute lymphoblastic leukemia cells

called CUTLL1 that express high levels of endogenous NOTCH1 and indeed they are sensitive to the treatment with gamma secretase inhibitors (Palomero et al., 2006).

First, I performed immunoprecipitations of the endogenous intracellular form of NOTCH1 (N1IC) or ATM from CUTLL1 cells. As shown in Figure 16A, consistent with the results obtained in HEK293T cells with the N1ΔE-Flag construct, I was able to observe an interaction between endogenous ATM and endogenous NOTCH1. To further strengthen this result, I decided to perform an *in situ* proximity ligation assay (PLA). PLA assay allows to visualize interactions between proteins (in the 40nm range or less) in the form of fluorescence dots that represent interactions between proteins. I performed PLA between ATM and NOTCH1 in CUTLL1 cells treated or not with the NOTCH1 inhibitor (GSI), which inhibits processing of NOTCH1 on the plasma membrane resulting in the accumulation of NOCTH1 on the cellular membrane decreasing at the same time the nuclear levels of NOTCH1. As shown in Figure 16B, I was able to detect specific dots indicative of the ATM-NOTCH1 complex in CUTLL1 cells, which decreased dramatically upon treatment with GSI, as shown in Figure 16B and its quantification (Figure 16C)

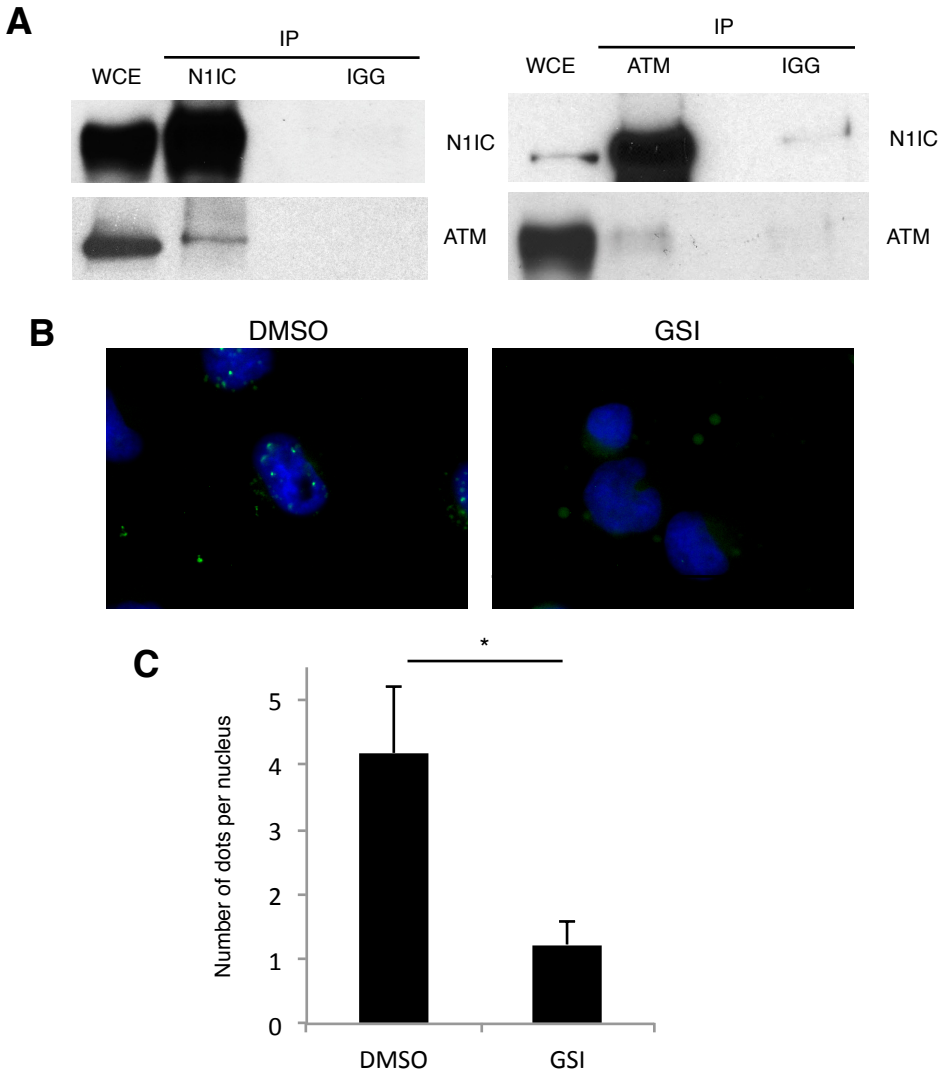


Figure 16. Endogenous ATM and NOTCH1 form a protein complex. (A) Immunoblot analysis of the immunoprecipitation of ATM or intracellular form of NOTCH1 (N1IC) in CUTLL1 cells (B) Immunofluorescence analysis of PLA assays between ATM and the intracellular form of NOTCH1 (N1IC) in CUTLL1 cells treated or not with GSI (Compound E)(1uM; 24h). (C) Quantification of the experiment shown in C. WCE - whole cell extract.

FATC domain of the ATM and Ankyrin repeats of NOTCH1 are necessary for the formation of the ATM-NOTCH1 protein complex

To better understand the details of the ATM-NOTCH1 interaction, I decided to study which domains of these proteins are involved.

First, we used short and sequential fragments of ATM protein fused with the glutathione S-transferase (GST) encompassing the entire protein length (Khanna et al., 1998). I performed the pull down experiment with the recombinant intracellular part of NOTCH1

(recN1IC) and the 12th GST-ATM each encoding for the different part of ATM protein (as indicated in the Figure 17A).

We were able to observe a specific interaction between recombinant NOTCH1 and GST-ATM fragment encoding for the very C-terminal part of ATM (2842-3056aa)(Figure 17A), containing the FATC domain that was previously described to be a critical domain necessary for the ATM activation (Jiang et al., 2006). We could not observe binding with any other fragment, showing at the same time that the observed interaction between ATM (2842-3056aa fragment) and NOTCH1 is specific. As in the pulldown we used just recombinant NOTCH1 and GST-ATM fragments observed interaction between NOTCH1 and ATM is direct and not mediated by the presence of other proteins (Result obtained by Jelena Vermezovic)(Vermezovic et al., 2015).

Next, I decided to investigate which domain of NOTCH1 is necessary for the interaction with ATM. For that I used NOTCH1 (N1ΔE-Flag) and NOTCH1 mutant (N1ΔEΔANK-Flag) missing six Ankyrin repeats (between 1873–2082aa). Ankyrin repeats are the protein-protein interaction domain of NOTCH1 (Bork, 1993). I performed an immunoprecipitation with Flag tag antibodies and as shown in Figure 17B, I was able to observe a reduction of the interaction between ATM and mutant NOTCH1 missing the Ankyrin repeats as compared to the N1ΔE-Flag control. Those result point that Ankyrin repeats are necessary for NOTCH1-ATM binding.

To further understand if the interaction between ATM and NOTCH1 is critical for NOTCH1-mediated ATM inhibition, I performed a set of immunofluorescence experiments to study ATM activation (pATM) upon IR in HeLa cells expressing either full length NOCTH1 (N1ΔE-Flag) or the form missing the ankyrin repeats (N1ΔEΔANK-Flag). As shown in Figure 17C, I observed that the expression of the N1ΔE-Flag resulted in the inhibition of ATM activation upon IR (pATM) and that the expression of the NOTCH1 missing the Ankyrin repeats showed reduced ability to inhibit ATM activation

when compared to full length NOTCH1, suggesting that NOTCH1 binding to ATM is necessary for the NOTCH1-mediated DNA damage activation.

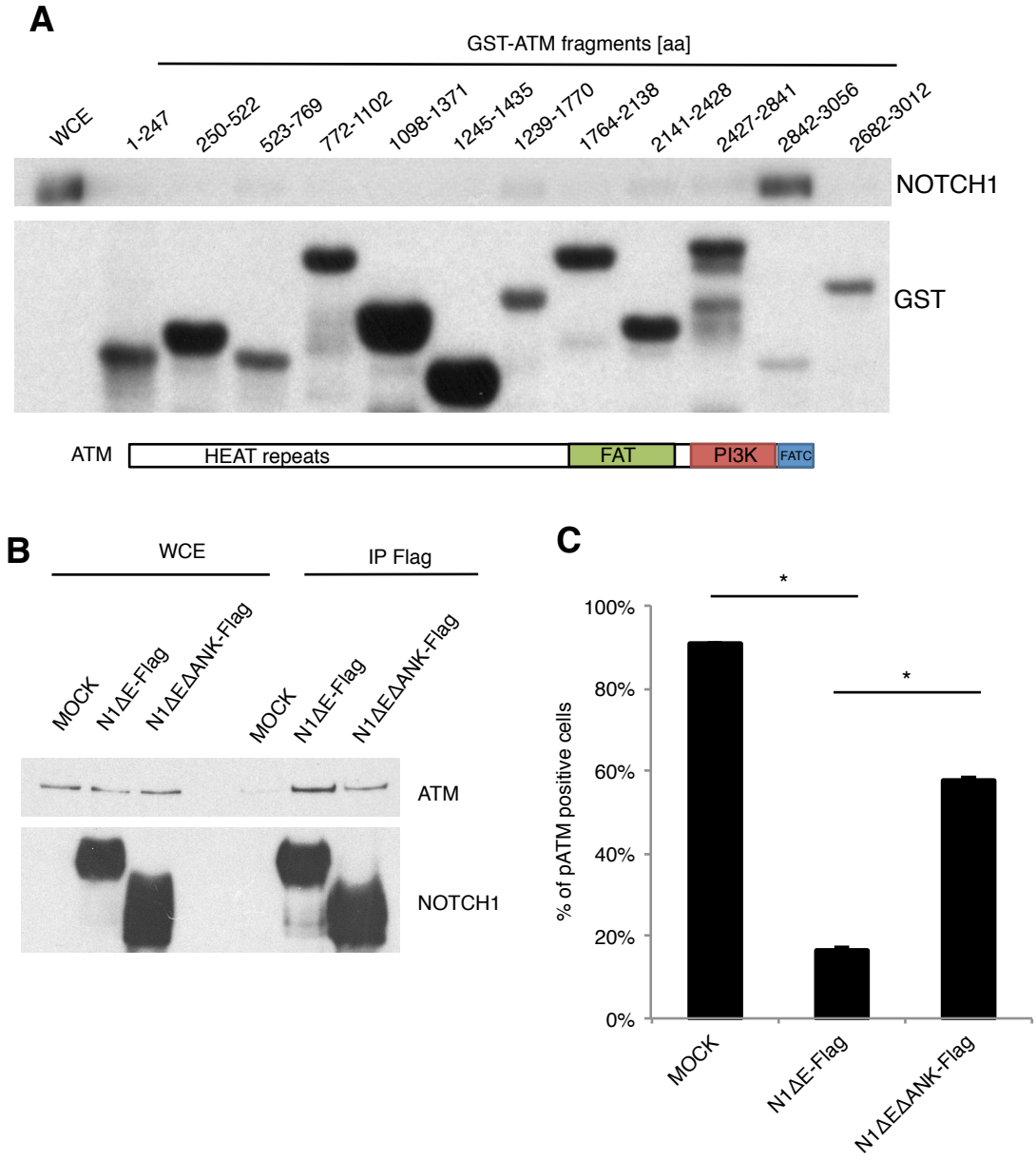


Figure 17. ATM and NOTCH1 interact with each other through the FATC and Ankyrin domains respectively. (A) Immunoblot analysis of the GST pull down of the recombinant NOTCH1 (recN1IC) with the use of GST-ATM fragments (Result generated by Jelena Vermezovic)(Vermezovic et al., 2015). (B) Immunoblot analysis of the immunoprecipitation of the NOCTH1: full length (N1ΔE) or mutant missing Ankyrin domain (N1ΔEΔANK). (C) Quantification of the immunofluorescence analysis of the ATM activation (pATM) in HeLa cells expression either full

length NOTCH1 (N1ΔE) or mutant missing Ankyrin domain (N1ΔEΔANK) upon IR (2G; 1h). WCE - whole cell extract.

NOTCH1 directly inhibits ATM kinase activity

To understand if NOTCH1 can directly inhibit ATM kinase activity, I decided to perform an *in vitro* ATM kinase assay.

A number of *in vitro* ATM kinase assays have been established. Based on the literature it is possible to highlight three most commonly used ways of performing an *in vitro* ATM kinase assays. One of the most and well described methods is the one, in which ATM is purified (by double immunoprecipitation) in a dimer form from HEK293T cells expressing Flag and HA tagged ATM. In this assay purified ATM is incubated with recombinant MRN complex in the presence of linear double-stranded DNA, that mimics DNA DSBs (Lee and Paull, 2004). It is important to mention that this kind of assay is performed in the absence of Mn^{2+} ions that artificially induce ATM activation (regardless of the presence of the damaged DNA) by inducing the formation of a stable catalytically active ATM dimer (Guo et al., 2010). Another well established method to measure ATM kinase activity is to immunopurify endogenous ATM and incubate it with a linear double stranded DNA in the presence of Mn^{2+} ions. This method, although relatively simple to perform, usually generates higher background of the ATM activity, due to the presence of Mn^{2+} ions (Smith et al., 1999). Finally, it has been shown that with the use of *Xenopus leavis* egg extract it is possible to monitor ATM activity by the incubation of the egg extract with linear double stranded DNA (Dupré et al., 2006). Unfortunately despite its simplicity, with the use of this method it is not possible to claim a direct effect of the tested factors on ATM kinase activity because of the presence of other proteins in the egg extract. Therefore I decided to perform an *in vitro* ATM assay with the use of endogenous immunopurified ATM in the presence of linear double-stranded DNA, but in the absence of the Mn^{2+} ions.

For that I performed immunopurification of the endogenous ATM from HeLa cells. As indicated in Figure 18 silver staining of the immunopurifed ATM showed a single pure band of the ATM as compared to the IgG control (MOCK IP).

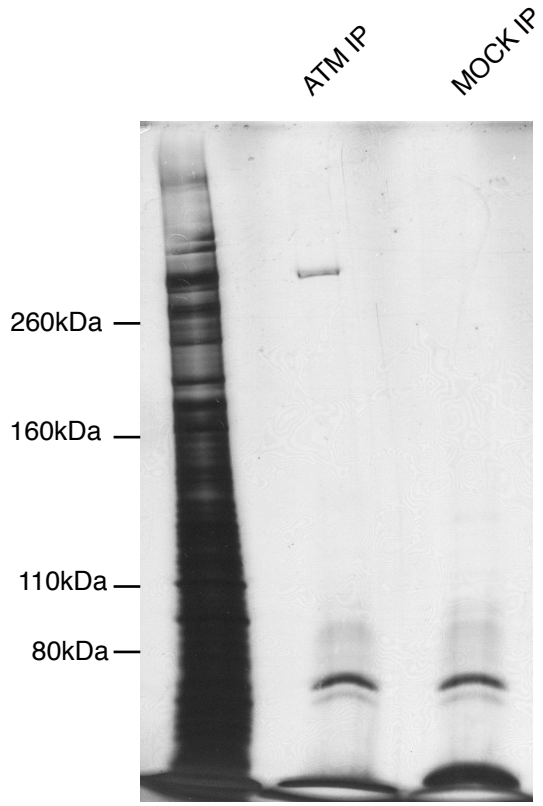


Figure 18. Immunopurification of the ATM. Silver staining of the ATM immunopurification.

Next, I incubated ATM purified this way with recombinant GST-p53 (as a phosphorylation substrate) and recombinant NOTCH1 (N1IC-Flag) in the presence or absence of a linear double stranded DNA (1kb DNA ladder). After the incubation, I analysed GST-p53 phosphorylation at the Serine 15 by immunoblotting. As shown in Figure 19A and B, I was able to observe an induction of GST-p53 phosphorylation by ATM upon addition of linear double stranded DNA. However, when I pre-incubated immunopurified ATM with recombinant NOTCH1 (N1IC-Flag) before addition of DNA and GST-p53, I detected a decrease in GST-p53 phosphorylation upon increasing amounts of NOTCH1. Finally, to determine the specificity of the signal detected, I per-incubated immunopurified ATM with a small molecule ATM kinase inhibitor (ATMi). As shown in Figure 19A, I observed a

specific decrease of GST-p53 phosphorylation in the samples treated with ATMi as compared to the control (DMSO), showing that the observed GST-p53 phosphorylation is specifically mediated by the ATM kinase under the conditions emplyed.

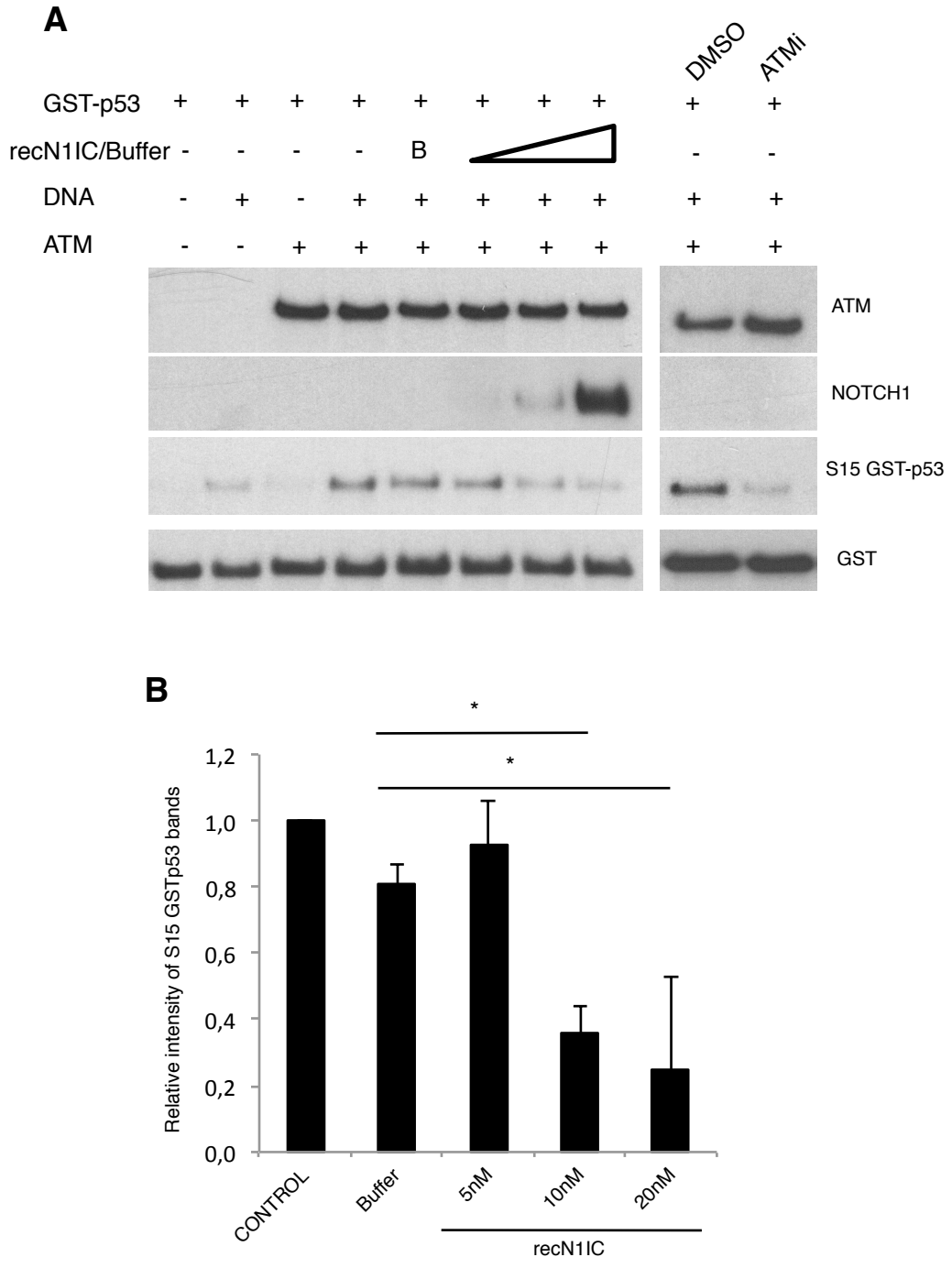


Figure 19. *In vitro* ATM kinase assay. (A) Immunoblot analysis of the *in vitro* ATM kinase assay. Immunopurified ATM was incubated with recombinant GST-p53 in the presence of increasing amounts of recombinant NOTCH1 (N1IC-Flag) or ATMi. (B) Quantification of GST-p53 phosphorylation at Serine 15 shown in A.

Inhibition of NOTCH1 increases ATM activation in NOTCH1-driven cancers

NOTCH1 is mutated and activated in many cancers. One of the most studied are T-acute lymphoblasic leukemias (T-ALL), 50% of which carry a mutation in the NOTCH1 gene (Grabher et al., 2006; Weng et al., 2004). Indeed NOTCH1 mutations are considered driving mutations leading to T-ALL and necessary for T-ALL cells survival (Pear et al., 1996; Weng et al., 2003). Therefore I decided to use T-ALL cells as the physiological cellular context of NOTCH1 expression to study its impact on the DDR and DNA damage-induced cell death.

First, I performed a qRT-PCR analysis of the expression of *HES1* gene, a NOTCH1 target gene, to check if by the use of gammasecretase inhibitor (GSI) I was able to inhibit the translocation, and this way activation of NOTCH1 to the nucleus. As shown in the Figure 20A treatment of TALL-1 cells with GSI resulted in a decrease of NOTCH1-mediated transcription of *HES1*. Next, I performed an analysis of the DDR activation in TALL-1 cells treated or not with GSI. I observed an increase in ATM activation upon IR in GSI-treated cells, compared to controls, as measured by an increase in the autophosphorylation of ATM (pATM) and ATM-dependent phosphorylation of its target proteins such as: Serine 966 SMC1 and Threonine 68 CHK2 (Figure 20B and C).

On the basis of these results I concluded that the inhibition of NOTCH1 in TALL-1 cells leads to an increase in DDR activation.

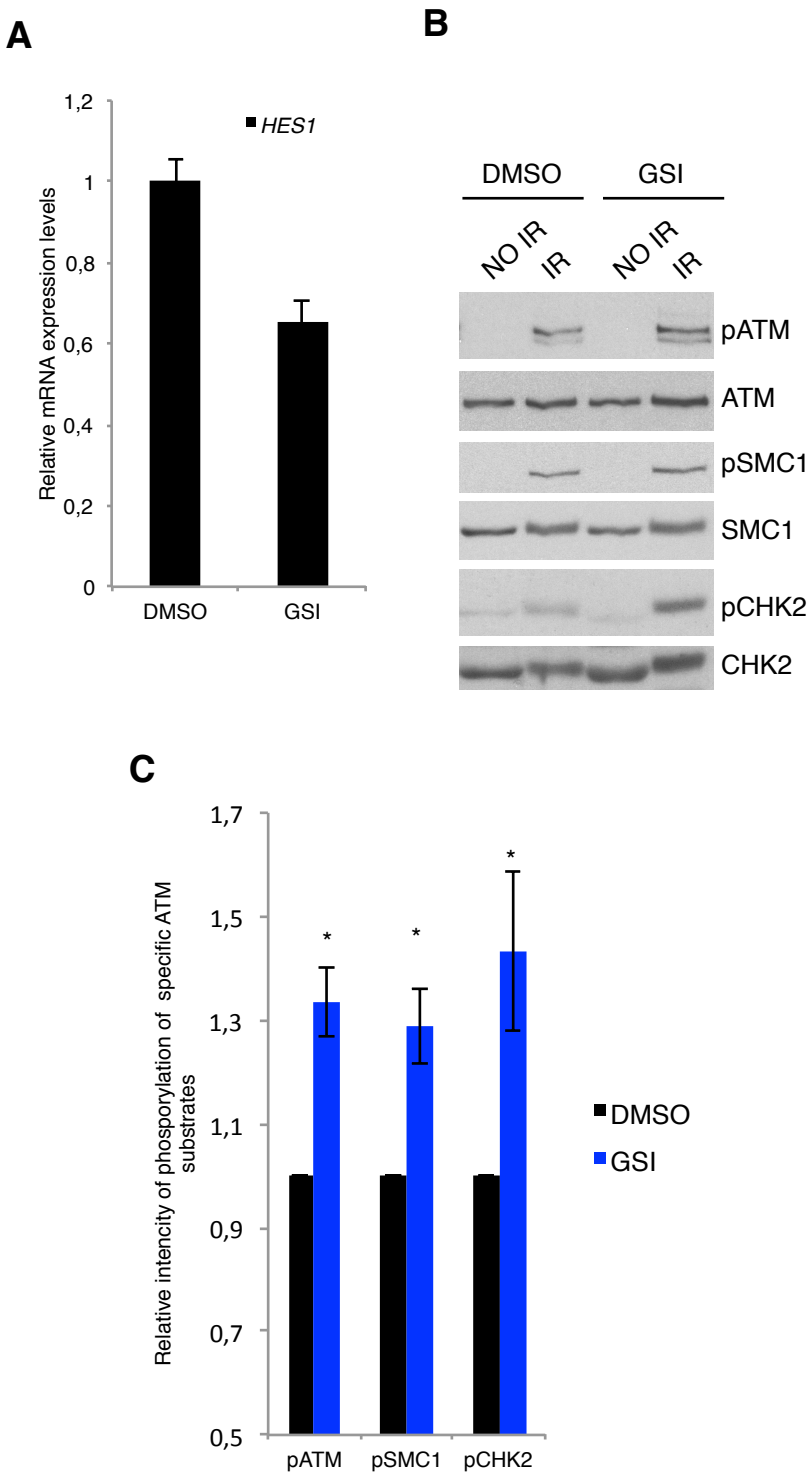


Figure 20. Inhibition of NOTCH1 increases activation of the DDR in TALL-1 cells. (A) qRT-PCR analysis of the expression of *HES1* in TALL-1 cells treated or not with the GSI. (B) Immunoblot analysis of the DDR activation in TALL-1 cells pretreated with the GSI and subjected to IR (5G; 1h). (C) Quantification of the immnoblot analysis shown in B.

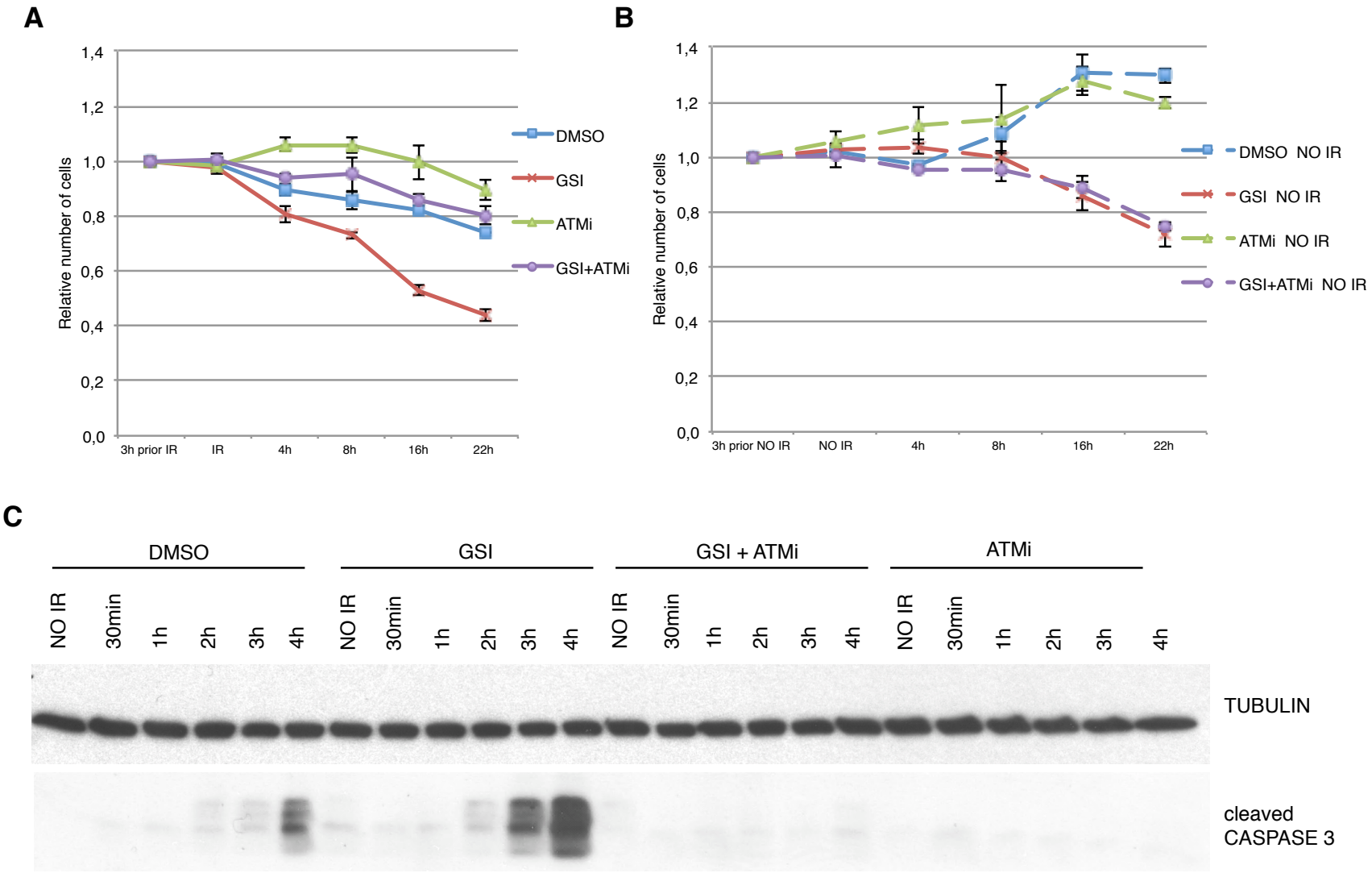
Inhibition of NOTCH1 increases DNA damage-induced cell death in NOTCH1-driven cancers

The results shown before indicate that inhibition of NOTCH1 with the use of gamma secretase inhibitor (GSI) in NOTCH1-driven cancer cells (TALL-1) leads to an increase of the DDR in cells subjected to DNA damage-inducing agents, like IR. Therefore, I tested if, by the boosting the DDR in TALL-1 cells with GSI, I could increase DNA damage-induced cell death in such cells.

For that, I pretreated TALL-1 cells with GSI or an ATM inhibitor (ATMi) before subjecting them to IR. As shown in the chart displaying the survival of TALL-1 cells upon treatment with IR, I observed that cells pre-incubated with GSI had decreased survival as compared to control cells (Figure 21A). Moreover the effect of the GSI on the survival of TALL-1 cells was abolished by the use of the ATMi (Figure 21A). The effect was not a result of GSI cytotoxicity per se since cells treated with GSI, but not subjected to IR, survived better then IR-treated ones (Figure 21B).

To understand if reduced survival of the GSI-treated cells is a result of increased cell death or decreased cell proliferation, I performed an immunoblot analysis of GSI and ATMi pretreated cells exposed to IR. As shown in Figure 21C, I observed that GSI-mediated NOTCH1 inhibition leads to an increase of DNA damage-induced cell death as measured by the cleavage of CASPASE3, a marker of apoptotic cells. This effect was inhibited by the use of ATMi, demonstrating that the induced increase of apoptosis in GSI-treated cells is dependent on ATM activation.

This results together with the previously presented ones demonstrate that NOTCH1 inhibits DDR activation in TALL-1 cells, which results in the inhibition of DNA damage-induce cell death in such cells.


A
B
C
Relative number of cells
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
3h prior IR
IR
4h
8h
16h
22h
DMSO
GSI
ATMi
GSI+ATMi
3h prior NO IR
NO IR
DMSO NO IR
GSI NO IR
ATMi NO IR
GSI+ATMi NO IR
DMSO
GSI
GSI + ATMi
ATMi
NO IR
30min
1h
2h
3h
4h
TUBULIN
cleaved
CASPASE 3

.

Figure 21. Inhibition of NOTCH1 increases DNA damage-induced cell de exposed to IR. (A) and (B) survival analysis of the TALL-1 cells upon tre drugs. TALL-1 cells were pretreated for 3h with indicated drugs (GSI - NOTC ATM inhibitor) followed by the exposure to IR (10G)(A) or not (B). (C) Immur DNA damage-induced cell death (cleaved CASPASE 3) in TALL-1 cells pretre or GSI and ATMi and exposed to IR (10G).

NOTCH1 does not impair the interaction between ATM and NBS1

To study the exact mechanism by which NOTCH1 prevents ATM activ analyze all the known steps that lead to ATM activation upon DNA DSI One of the first steps in DDR is the initial recruitment of ATM to DN Jackson, 2011). This step is mediated by the direct interaction between of ATM kinase and the very C-terminus part of NBS1 protein (Falck e et al., 2003; You et al., 2005), which as a part of MRN complex brings DNA damage.

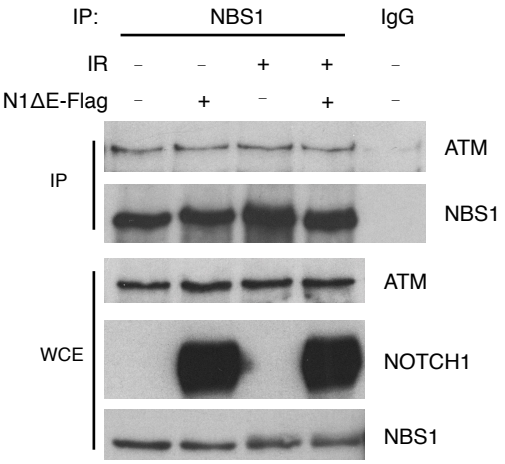


Figure 22. NOTCH1 does not impair the interaction between ATM and analysis of NBS1 immunoprecipitation. HEK293T cell were transfected with vector and subjected or not to IR (5G; 1h) followed by the immunoprecipitat whole cell extract.

Therefore I immunoprecipitated NBS1 in cells expressing or not NOTCH1 (N1ΔE-Flag). As previously reported, I was able to detect NBS1 in a complex with ATM before and after DNA damage induction (Figure 22)(Falck et al., 2005). Despite high levels of NOTCH1 expression, I was not able to see any difference in interaction between ATM and NBS1 in N1ΔE-Flag as compared to MOCK transfected cells (Figure 22).

NOTCH1 does not inhibit ATM recruitment to DSBs

As described above, NOTCH1 does not impact on ATM-NBS1 interaction. I therefore decided to test if NOTCH1 expression can impact on ATM accumulation at the DSBs upon IR. For this purpose I performed a cell lysate fractionation, which allowed me to extract the chromatin bound fraction of proteins. It has been already published that an ATM autophosphorylation mutant (S1981A), although initially recruited to DSBs (up to 10min after DNA damage induction), was not retained at the DNA damage sites (1h after DNA damage induction) (So et al., 2009). Because I already observed that NOTCH1 expression inhibits ATM autophosphorylation at serine 1981, I decided to test ATM recruitment to damaged chromatin by cell lysate fractionation one hour post irradiation.

I transfected HEK293T cells with the EGFP or N1IC-GFP constructs that I subsequently FACS-sorted to enrich for NOTCH1-transfected cells. After sorting, cells were left to recover and exposed to IR.

As expected I observed reduction in the ATM activation (pATM) in NOTCH1 (N1IC-GFP) expressing cells as compared to EGFP transfected cells (Figure 23A). I could also observe in the chromatin bound fraction clear accumulation of ATM upon DNA damage to a similar extent in EGFP and N1IC-GFP transfected cells (Figure 23A and B).

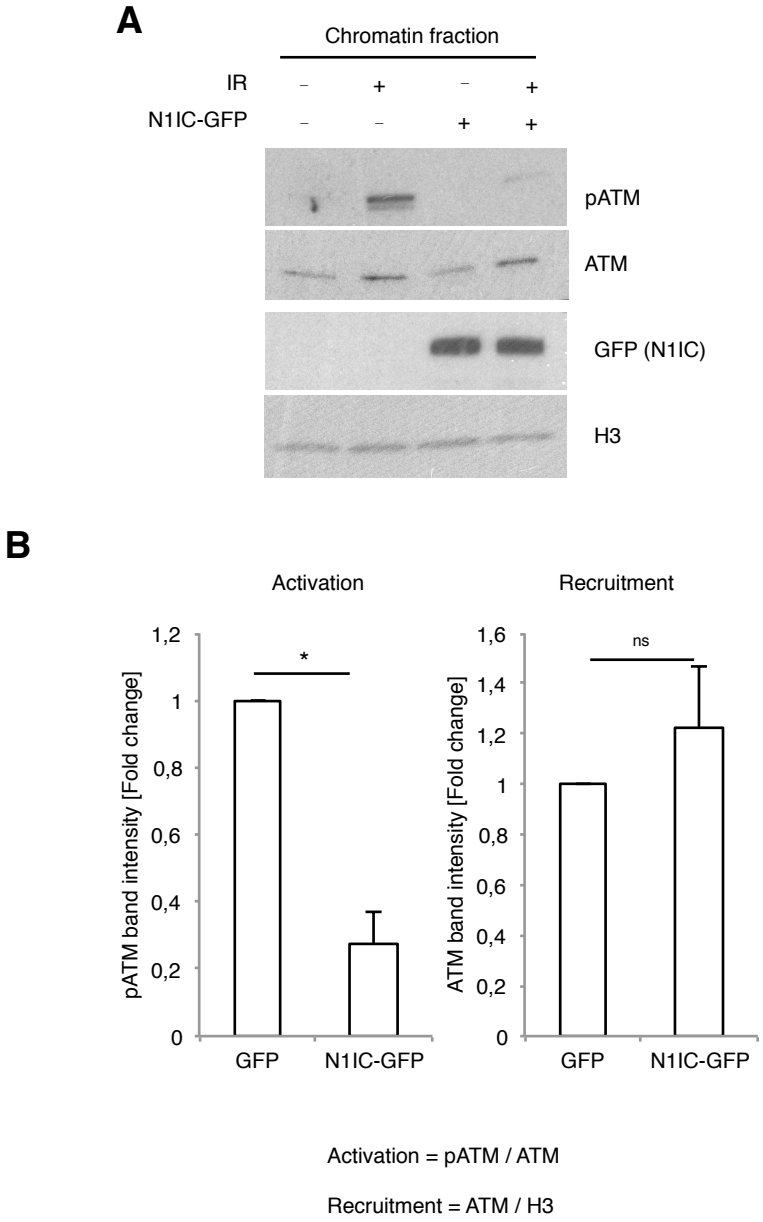


Figure 23. NOTCH1 does not inhibit ATM recruitment to DSBs. (A) Immunoblot analysis of HEK293T cells transfected with EGFP or N1IC-GFP followed by FACS sorting and exposed to IR (10G; 1h). Chromatin fraction of the cell lysate was analyzed. (B) Quantification of the immunoblots are shown.

This result, together with that showing lack of an impact of NOTCH1 on ATM-NBS1 interaction, clearly show that NOTCH1 dose not affect ATM recruitment to DSBs, although confirming ATM inhibition at sites of damage.

ATM forms a protein complex with FOXO3a

As I could not observe any kind of impact of NOTCH1 expression on ATM recruitment to the DNA DSBs, I decided to look into the protein factors that have been reported to play a direct role in ATM activation upon DNA damage. Among the many reported ATM interactors, like PP2A or SMAD7, the FOXO3a transcription factor stood out as a potential good candidate to mediate NOTCH1 functions on ATM (Goodarzi et al., 2007; Park et al., 2015a; Tsai et al., 2008). FOXO3a has been shown to bind directly to the C-terminal part of ATM (between 1764-2841aa) upon DNA damage (Tsai et al., 2008). Knock-down of FOXO3a has been reported to result in impaired DDR activation and, more importantly, ATM kinase activity (Chung et al., 2012b; Tsai et al., 2008). Therefore to understand if FOXO3a-mediated ATM activation process is the likely mediator of ATM inactivation by NOTCH1, I decided to characterize ATM-FOXO3a interaction more in depth.

FOXO3a interacts with ATM despite the presence of the DNA damage

First I preformed an immunoprecipitation of endogenous FOXO3a from cells subjected or not to IR. As shown in the Figure 24A, ATM forms a protein complex with the FOXO3a both in the presence and in the absence of DNA damage. To further understand the nature of the observed interaction, I performed sequential immunodepletion of endogenous ATM or FOXO3a from the nuclear cell lysate. This kind of experiment allows to investigate if one of the two proteins that are in a complex is in excess and not uniquely bound to the other protein of interest.

After performing a set of immunoprecipitations leading to effective immunodepletion of ATM from the nuclear lysate, I could not see any significant change in the levels of FOXO3a (Figure 24B). Instead, when I sequentially immunodepleted FOXO3a from the lysate, I observed a dramatic decrease in the levels of ATM (Figure 24B).

This result indicate that the entire amount of ATM is in a complex with FOXO3a and only a fraction of nuclear FOXO3a is in the same complex with ATM. This is in agreement with

already established role of FOXO3a as a transcription factor, thus likely interacting with several other partners, and it is compatible with other roles of FOXO3a unrelated to ATM interactions.

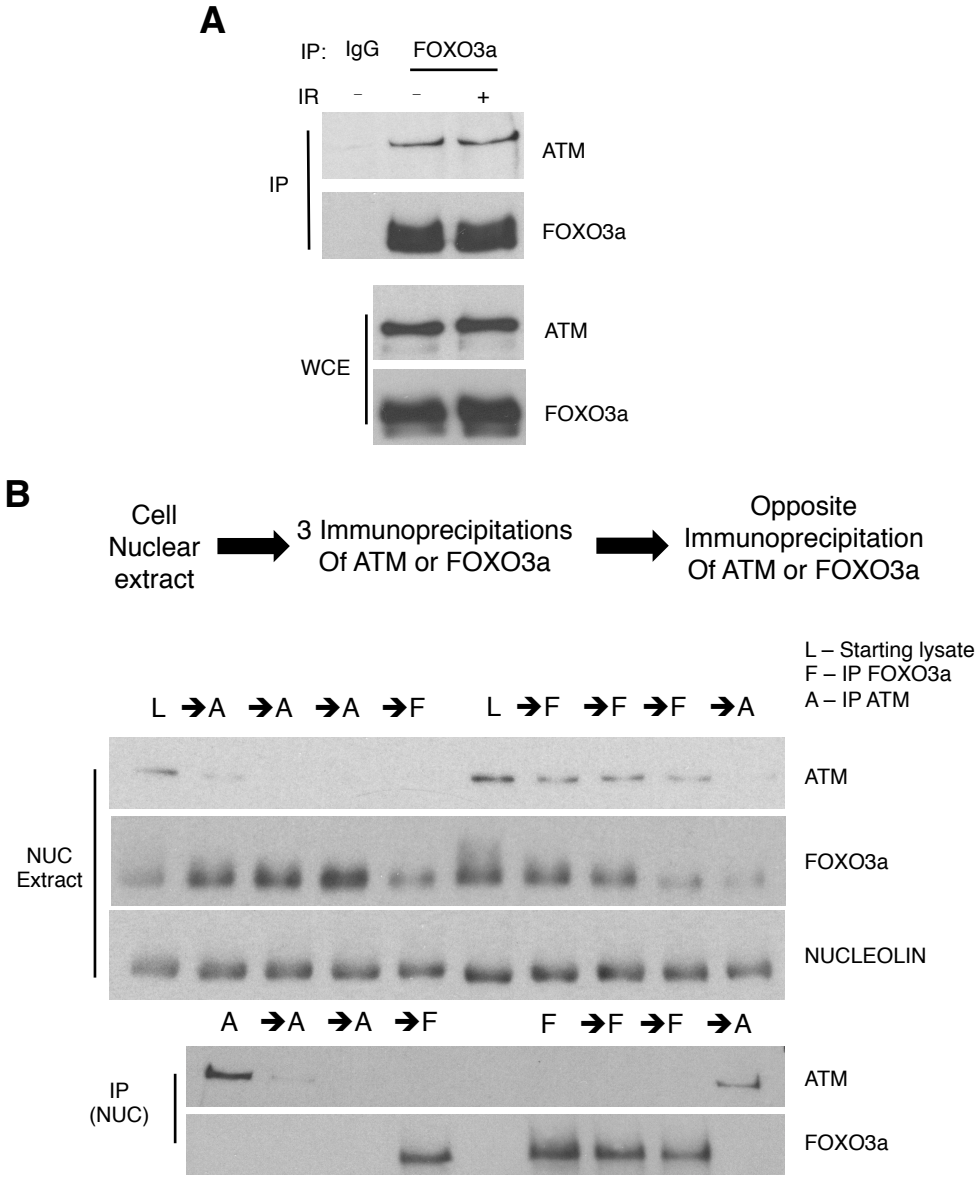


Figure 24. FOXO3a forms a protein complex with ATM in the presence and absence of the DNA damage. (A) Immunoblot analysis of the FOXO3a immunoprecipitation. HEK293T cells were lysed and subjected to IR (5G; 1h) followed by the immunoprecipitation of the FOXO3a. (B) Immunoblot analysis of the ATM or FOXO3a immunoprecipitation. HEK293T cells were lysed and the nuclear fraction was extracted (NUC), followed by the immunoprecipitation of either ATM or FOXO3a

FOXO3a binds directly to the FATC domain of ATM

The domain of ATM involved in the interaction between ATM and FOXO3a has been previously identified (between 1764-2841aa)(Tsai et al., 2008). In this study, authors utilized GST-ATM fragments that cover entire length of ATM (Khanna et al., 1998). Although authors observed specific binding of FOXO3a to the three GST-ATM fragments (between 1764-2841aa) they did not test one of GST-ATM fragment covering the very C-terminal part of ATM, containing FATC domain of ATM (Tsai et al., 2008). Therefore I decided to re-evaluate ATM domain involved in the ATM-FOXO3a interaction.

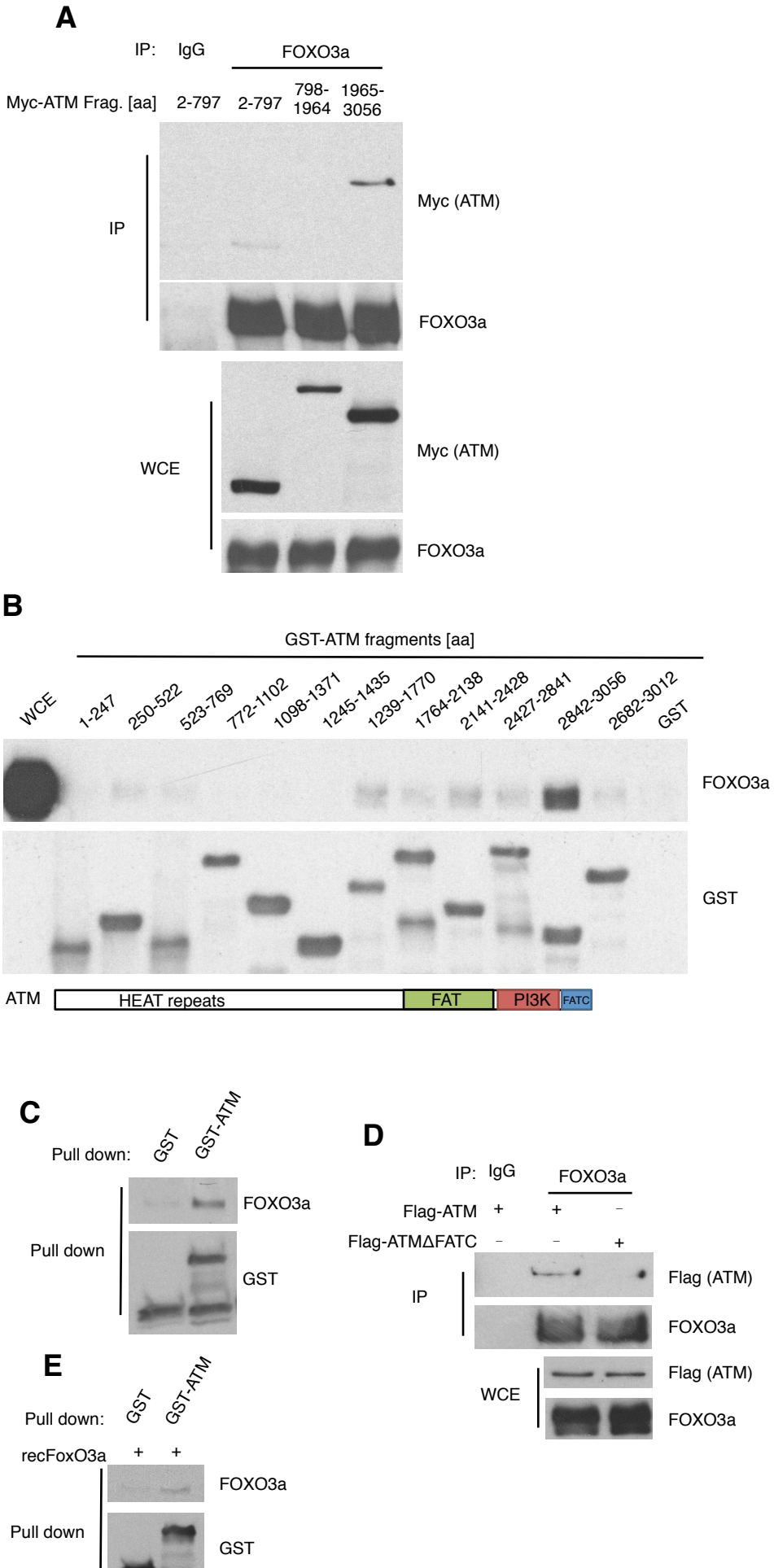

A
IP: IgG
FOXO3a
Myc-ATM Frag. [aa]
2-797
2-797
798-1964
1965-3056
IP
Myc (ATM)
FOXO3a
WCE
Myc (ATM)
FOXO3a
B
GST-ATM fragments [aa]
WCE
1-247
250-522
523-769
772-1102
1098-1371
1245-1435
1239-1770
1764-2138
2141-2428
2427-2841
2842-3056
2682-3012
GST
FOXO3a
GST
ATM
HEAT repeats
FAT
PI3K
FATC
C
Pull down:
GST
GST-ATM
FOXO3a
Pull down
GST
D
IP: IgG
FOXO3a
Flag-ATM + + –
Flag-ATMΔFATC – – +
IP
Flag (ATM)
FOXO3a
WCE
Flag (ATM)
FOXO3a
E
Pull down:
GST
GST-ATM
recFoxO3a + +
FOXO3a
Pull down
GST

Figure 25. FOXO3a interacts directly with the FATC domain of ATM. (A) Immunoblot analysis of immunoprecipitated Myc-ATM fragments. HEK293T cells were transfected with different Myc-ATM constructs followed by the immunoprecipitation of the Myc tag. (B) Immunoblot analysis of the GST pulldowns with different GST-ATM fragments. HEK293T cells were transfected with Myc-FOXO3a, lysed and used in the pull down experiment. (C) Immunoblot analysis of GST pulldowns with GST-ATM fragment (2842-3056aa) and endogenous FOXO3a. HEK293T cells were lysed and used in the pulldown experiment. (D) Immunoblot analysis of immunoprecipitated FOXO3a. HEK293T cells were transfected with different Flag-ATM constructs followed by immunoprecipitation of FOXO3a. (E) Immunoblot analysis of the GST pulldowns with GST-ATM fragment (2842-3056aa) and recombinant FOXO3a. WCE - whole cell extract.

First, I performed an imunnoprecipitation of endogenous FOXO3a from lysate of cells transfected with three different Myc-ATM fragments spanning the entire length of ATM (Myc-ATM: 2-797aa; 798-1964aa and 1965-3056aa). I observed that FOXO3a is predominantly interacting with C-terminal fragment of ATM, although I could notice a weak interaction also with the N-terminal one (Figure 25A). To identify more precisely the ATM interaction domain with FOXO3a, I used GST-ATM fragments including GST-ATM fragment (2842-3056aa) that in the previous study charactering ATM-FOXO3a interaction was not tested (Tsai et al., 2008). As shown in the Figure 25B, 2842-3056aa GST-ATM fragment displayed the strongest binding affinity to ectopically expressed Myc-FOXO3a. Moreover, I noticed additional binding, although with a lower affinity, with a previously reported GST-ATM fragments (between 1239-2841aa). As I could not observe any binding to GST-ATM fragment encoding for 2682-3012aa of ATM I concluded that the domain of ATM responsible for FOXO3a binding is between 3012-3056aa of ATM. As the pull down with all the GST-ATM fragments was performed in cells ectopically expressing Myc-FOXO3a, I performed a pull down with the 2842-3056aa GST-ATM fragment from a lysate of cells expressing just endogenous FOXO3a. As shown in Figure 25C, I confirmed the previous observation that FOXO3a binds specifically to the 2842-3056aa GST-ATM fragment. To further confirm the involvement of very C-terminal part of ATM in FOXO3a binding, I expressed in cells either a Flag-ATM or Flag-ATM mutant, missing the last 64aa

(Flag-ATMΔFATC). When I immunoprecipitated endogenous FOXO3a from the lysate of the cells expressing either of the above-mentioned constructs, I observed that Flag-ATMΔFATC does not stably interact with FOXO3a (Figure 25D), confirming that the last 64aa of ATM containing the FATC domain are essential for the ATM-FOXO3a interaction.

To understand if the observed interaction between the C-terminal domain of ATM and FOXO3a is direct or it is mediated by other proteins, I purified from bacteria human recombinant FOXO3a, which I next used in pulldown experiment together with the 2842-3056aa GST-ATM fragment. As shown in Figure 25E, I was able to pulldown recombinant FOXO3a together with C-terminal fragment of ATM in the absence of any other protein, suggesting that observed interaction between ATM FATC domain and FOXO3a is direct.

NOTCH1 competes with FOXO3a for the binding to the FATC domain of ATM

As I have shown before the interaction between NOTCH1 and ATM is mediated by FATC domain of ATM and ankyrin repeats of NOTCH1, I hypothesized that NOTCH1 might be competing with the FOXO3a for the binding to the FATC domain of ATM. To address this question I performed a number of pulldowns and immunoprecipitations in the presence of an excess of either FOXO3a or NOTCH1.

First, to see if NOTCH1 can affect the binding between ATM and FOXO3a I performed a pulldown of the endogenous FOXO3a with the 2842-3056aa GST-ATM fragment in the cell lysate from the cells transfected with either empty vector or N1ΔE-Flag construct.

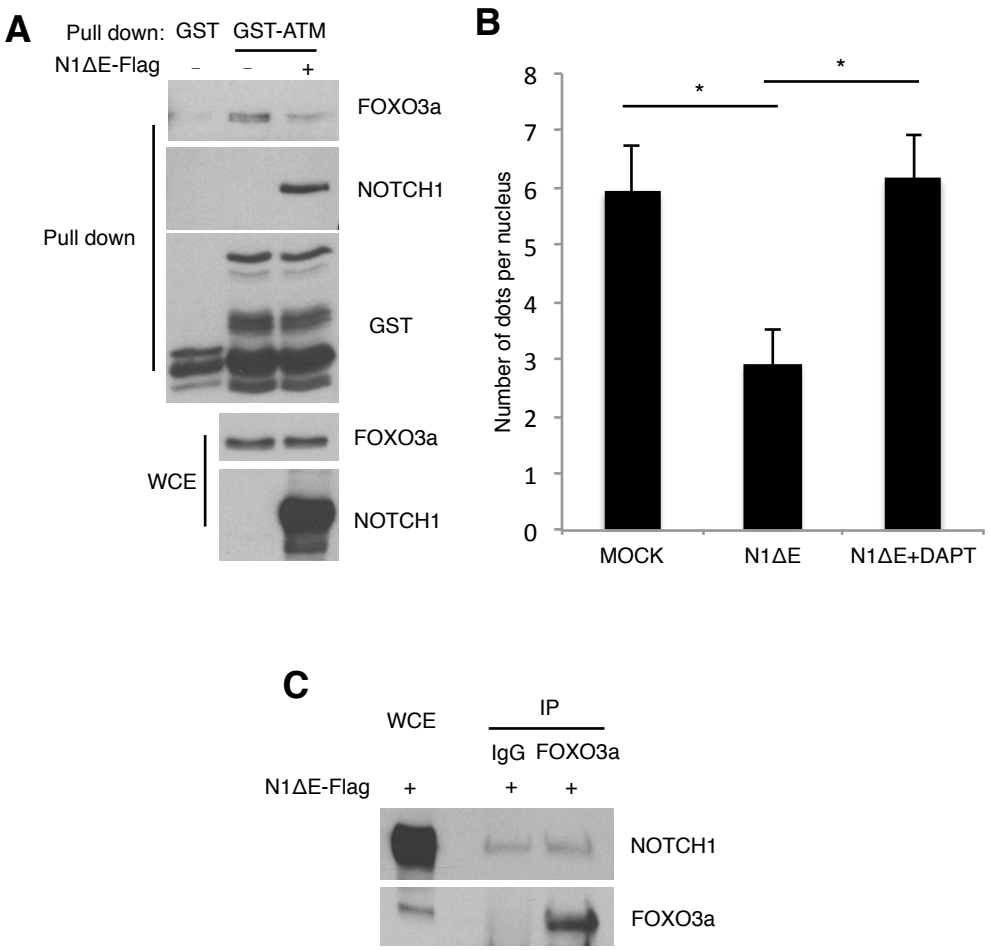


Figure 26. NOTCH1 impairs FOXO3a binding to ATM. (A) Immunoblot analysis of GST pulldowns with GST-ATM fragment (2842-3056aa). HEK293T cells were transfected with the N1ΔE-Flag construct lysed and incubated with the GST-ATM fragment. (B) Quantification of the immunofluorescence analysis of the PLA signal between ATM and FOXO3a. HeLa cells were transfected with the N1ΔE-Flag construct or empty vector. (C) Immunoblot analysis of the FOXO3a immunoprecipitation. HEK293T cells were transfected with N1ΔE-Flag construct followed by the immunoprecipitation of FOXO3a. WCE - whole cell extract.

As shown in the Figure 26A I observed that endogenous FOXO3a forms a protein complex with the 2842-3056aa GST-ATM fragment, but in the presence of NOTCH1 (cells transfected with the N1ΔE-Flag construct) such interaction between ATM and FOXO3a was strongly reduced (Figure 26A).

Next, to validate this conclusion also in living cells, I performed a PLA assay between ATM and FOXO3a in cells expressing or not N1ΔE-Flag construct. The immunofluorescence analysis revealed that the interaction between ATM and FOXO3a (measured as a number of dots per nucleus) in N1ΔE-Flag expressing cells was

significantly decreased as compared to cells transfected with the empty vector (Figure 26B). Moreover, to test if the observed reduction of the PLA signal is specifically mediated by NOTCH1, I treated N1ΔE-Flag expressing cells with a NOTCH1 inhibitor (GSI). Rescue of the PLA signal in the GSI-treated cells to the levels observed in the MOCK transfected cells proved that observed reduction of the PLA signal in the N1ΔE-Flag expressing cells is specific and mediated directly by NOTCH1 (Figure 26B).

NOTCH1 has never been reported to interact with FOXO3a. Therefore to exclude the possibility that observed impairment in the interaction between ATM and FOXO3a in NOTCH1 expressing cells is the result of an interaction between NOTCH1 and FOXO3a I performed an immunoprecipitation of FOXO3a in NOTCH1 expressing cells: I could not detect any interaction between NOTCH1 and FOXO3a (Figure 26C).

Overexpression of FOXO3a impairs NOTCH1 binding to ATM

Next, I decided to test if the observed impairment of ATM-FOXO3a binding in NOTCH1 expressing cells could be reversed.

I therefore transfected cells with the N1ΔE-Flag and Myc-FOXO3a constructs. When I immunopurecipitated NOTCH1 from N1ΔE-Flag transfected cells, I could detect an interaction between ATM and NOTCH1 (Figure 27A). However in cells expressing both N1ΔE-Flag and Myc-FOXO3a, the formation of a protein complex between ATM and NOTCH1 was dramatically perturbed (Figure 27A). I obtained similar results in the reciprocal immunoprecipitations (Figure 27B).

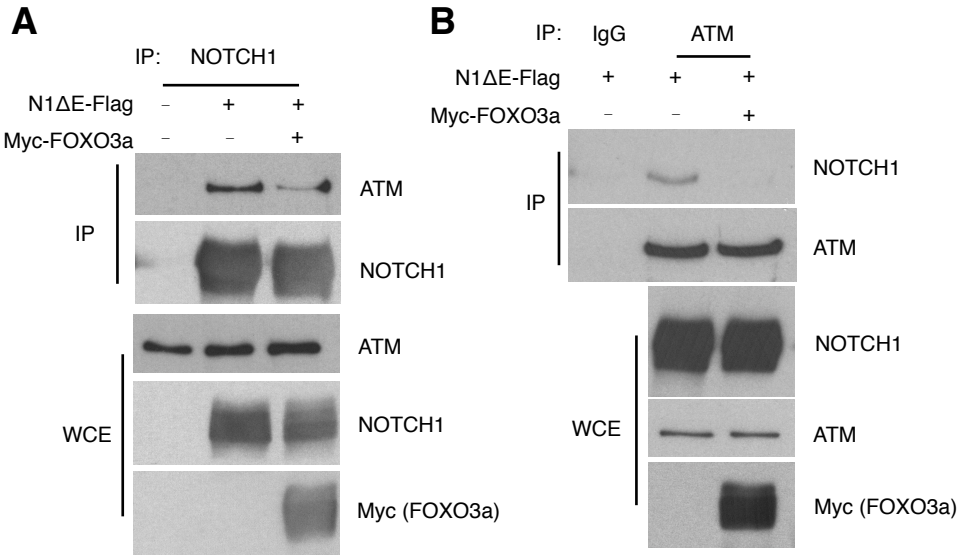


Figure 27. Overexpression of FOXO3a impairs binding of NOTCH1 to ATM. (A) Immunoblot analysis of NOTCH1 immunoprecipitation. HEK293T cells were transfected with N1ΔE-Flag and Myc-FOXO3a constructs followed by immunoprecipitation of NOTCH1. (B) Immunoblot analysis of the ATM immunoprecipitation. HEK293T cells were transfected with N1ΔE-Flag and Myc-FOXO3a constructs followed by the immunoprecipitation of ATM. WCE - whole cell extract.

NOTCH1 binds to the ATM with the higher affinity compared to FOXO3a

To better understand the competition between FOXO3 and NOTCH1 for ATM binding, I decided to evaluate the strength of these interactions.

For this purpose I transfected cells with either N1ΔE-Myc or Myc-FOXO3a constructs. Next, I immunoprecipitated Myc-tagged proteins from cell lysates in parallel. As shown in Figure 28A, both proteins (N1ΔE-Myc and Myc-FOXO3a) were expressed at similar levels, as judged by immunoblotting. Surprisingly, Myc immunoprecipitation reveled that NOTCH1 dispalys higher binding strength to ATM compared to FOXO3a in both irradiated and not irradiated cells (Figure 28A). To rule out that the observed difference in ATM binding is the result of different subcellular localizations of Myc-tagged proteins, I fractionated cell lysate. I observed that Myc-FOXO3a was expressed in the nucleus to a higher extent then N1ΔE-Myc, excluding possibility that observed higher strength of the ATM-NOTCH1 binding as compared to FOXO3a one is a result of the difference of the

proteins cellular localization and emphasizing at the same time previously observed difference in the ATM binding ability of NOTCH1 (Figure 28B).

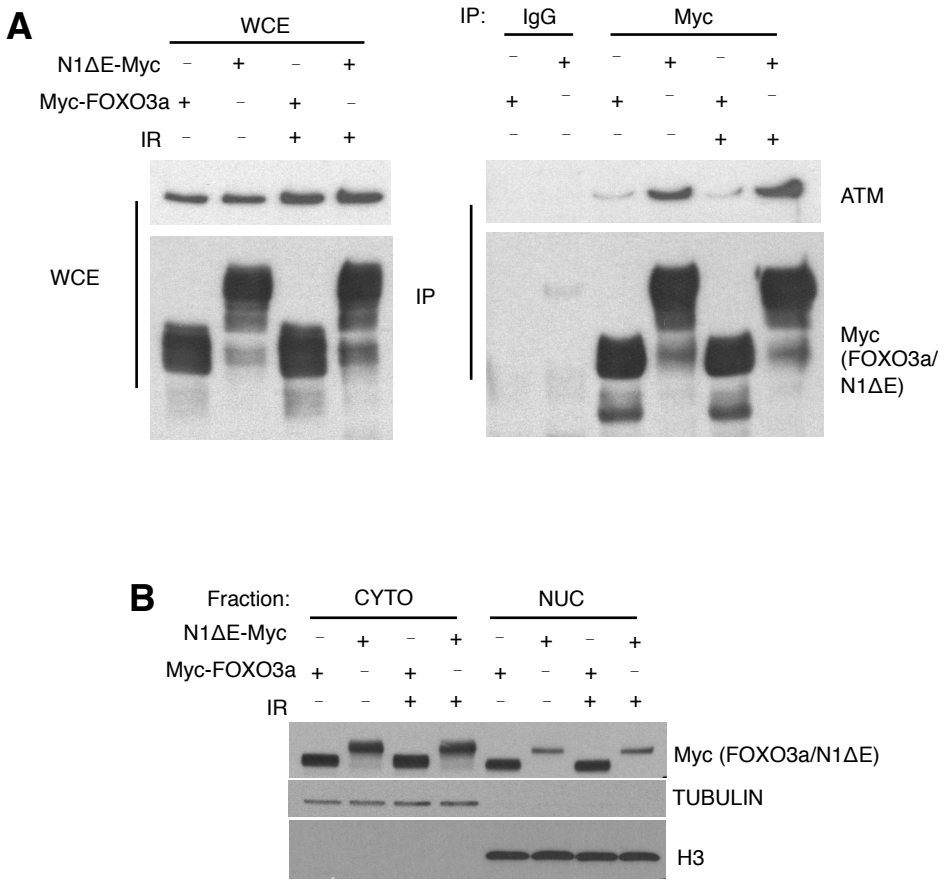


Figure 28. NOTCH1 binds to ATM with higher strength as compared to FOXO3a. (A) Immunoblot analysis of NOTCH1 or FOXO3a immunoprecipitation. HEK293T cells were transfected with N1ΔE-Myc or Myc-FOXO3a construct and subjected to IR (5G; 1h) followed by immunoprecipitation of the Myc tag. (B) Immunoblot analysis of the expression of N1ΔE-Myc and Myc-FOXO3a. HEK293T cells were transfected with N1ΔE-Myc or Myc-FOXO3a construct, subjected to irradiation (5G; 1h) and fractionated (CYTO-cytosolic fraction; NUC-nuclear fraction). WCE - whole cell extract.

These results indicate that NOTCH1 competes with FOXO3a for binding to the FATC domain of ATM thanks to its higher binding affinity toward ATM as compared to FOXO3a.

Overexpression of FOXO3a rescues NOTCH1-mediated ATM inhibition

So far, these results indicate that NOTCH1 and FOXO3a can mutually impair binding of each other to ATM. Therefore, I decided to test if the overexpression of FOXO3a could rescue NOTCH1-mediated ATM inhibition.

I thus performed an immunofluorescence analysis of ATM activation (pATM) in HeLa cells expressing N1ΔE-Flag as well as Myc-FOXO3a constructs. As expected, expression of NOTCH1 blocked ATM activation upon DNA damage, as shown in Figure 29A and B. However in cells expressing both N1ΔE-Flag and Myc-FOXO3a, I was able to observe a rescue of ATM activation. However, such recovery never reached the levels of MOCK transfected cells (Figure 29A and B).

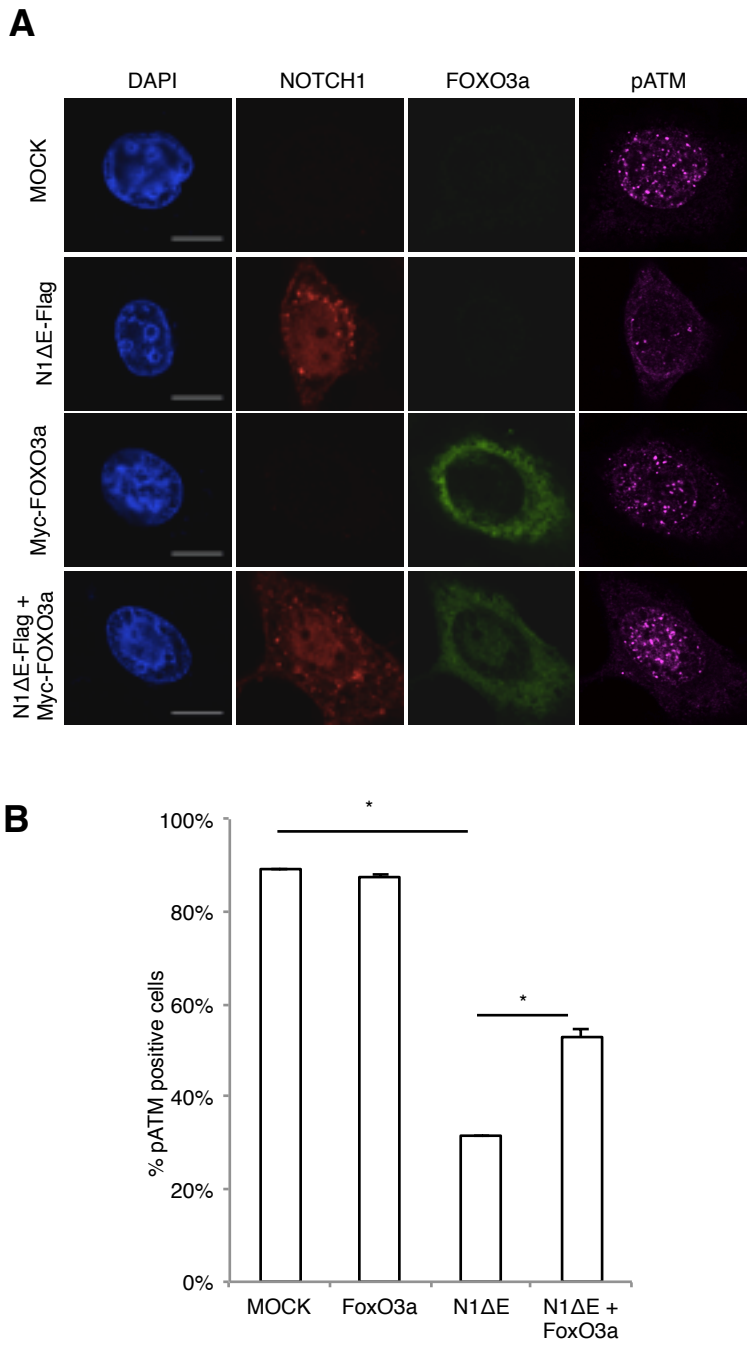


Figure 29. Overexpression of FOXO3a rescues NOTCH1-mediated ATM inhibition. (A) Immunofluorescence analysis of ATM activation upon DNA damage (pATM). HeLa cells were transfected with N1ΔE-Flag and Myc-FOXO3a and subjected to IR (2G; 1h). (B) Quantification of immunofluorescence shown in A.

KAT5 forms a direct protein complex with FOXO3a

The exact role of FOXO3a in ATM activation is unclear. As FOXO3a is a transcription factor which lacks any known enzymatic activity, I hypothesized that FOXO3a can play a role of a bridging protein between ATM and other factor(s) necessary for its activation.

The only factor described in the literature that is necessary for ATM activation and has been reported to bind to the FATC domain of ATM is the lysine acetyltransferase 5 (KAT5). KAT5 has been reported to acetylate ATM upon DNA damage and necessary to induce ATM monomerization and activation (Sun et al., 2005; Sun et al., 2010). Therefore I decided to investigate a possible functional connection between FOXO3a and KAT5.

KAT5 interacts directly with FOXO3a

It has been shown that KAT5 interacts with ATM in the presence and absence of DNA damage (Sun et al., 2005), therefore to verify in my hands this observation I performed an immunoprecipitation of Flag-tagged KAT5 from cells exposed or not to IR. As shown in Figure 30A I observed an interaction between ATM and KAT5 that did not change upon induction of DNA damage. This is reminiscent of the behaviour of the interaction between FOXO3a and ATM (Figure 24).

According to the available database of the protein-protein interactions (Biogrid; July 2016), no interaction between FOXO3a and KAT5 has been described. Therefore I decided to perform an immunoprecipitation of FOXO3a and see if I was able to detect KAT5 with it. As shown in Figure 30B, I observed that FOXO3a forms a protein complex with KAT5 both in the presence and absence of DNA damage. To further prove that the observed interaction is specific and not a result of the DNA binding abilities of both proteins, I performed the immunoprecipitation of FOXO3a in the presence of Benzonase nuclease, which degrades DNA and RNA. Despite the treatment with Benzonase, I could still observe an interaction between FOXO3a and KAT5 (Figure 30C).

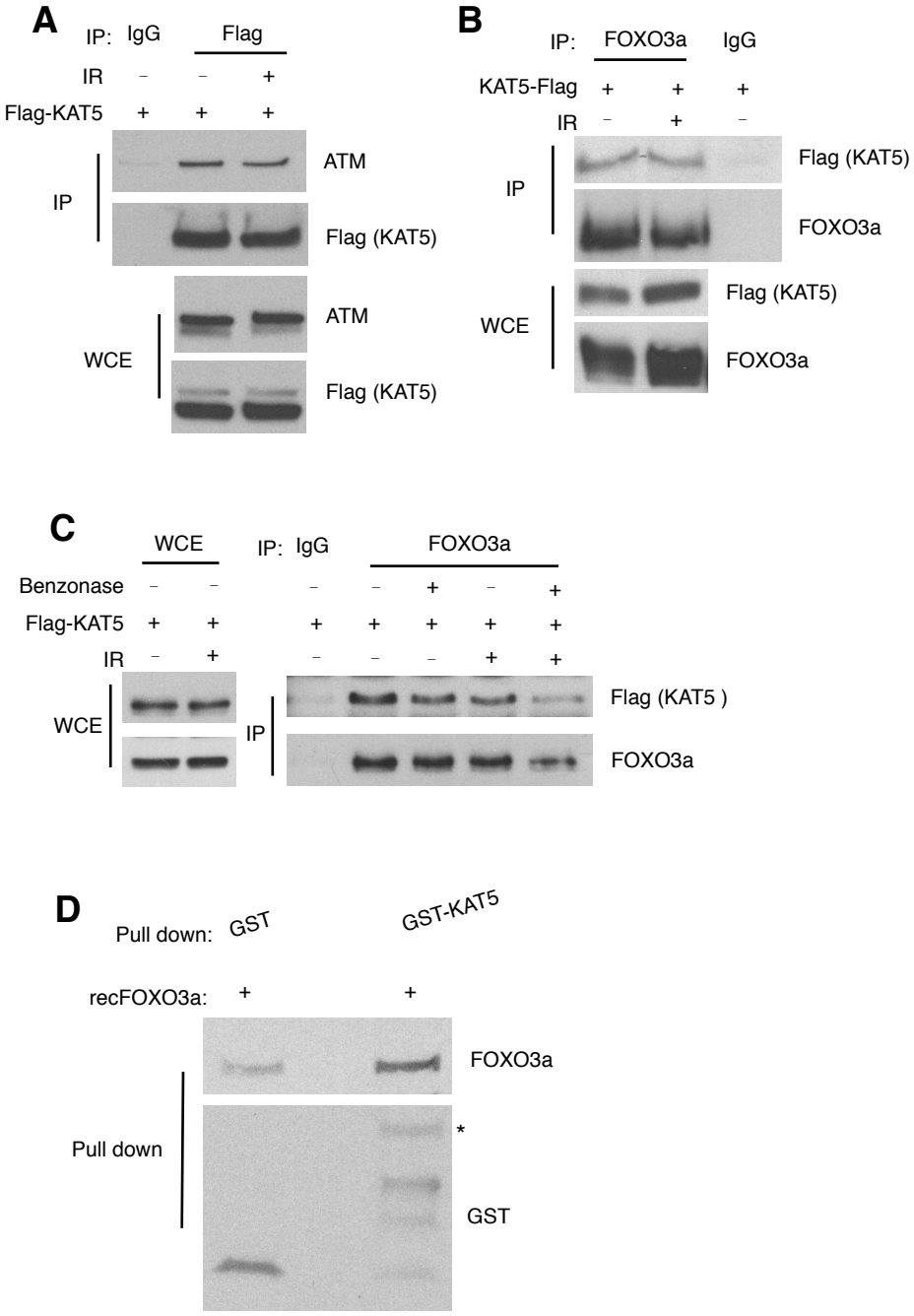
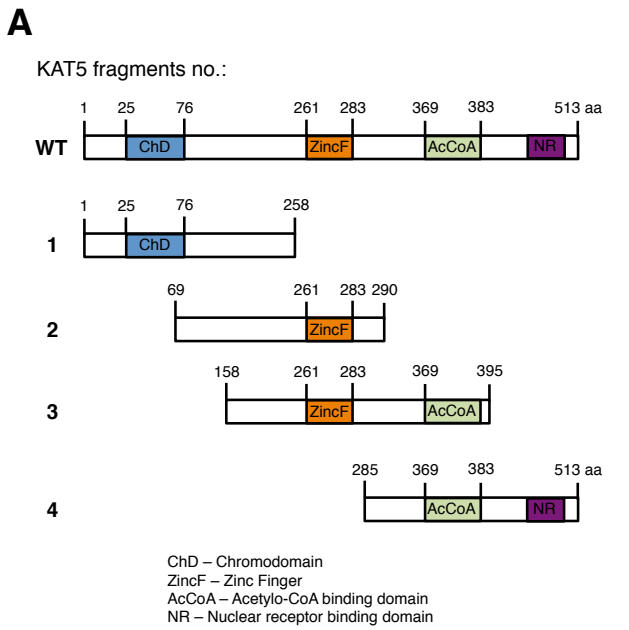


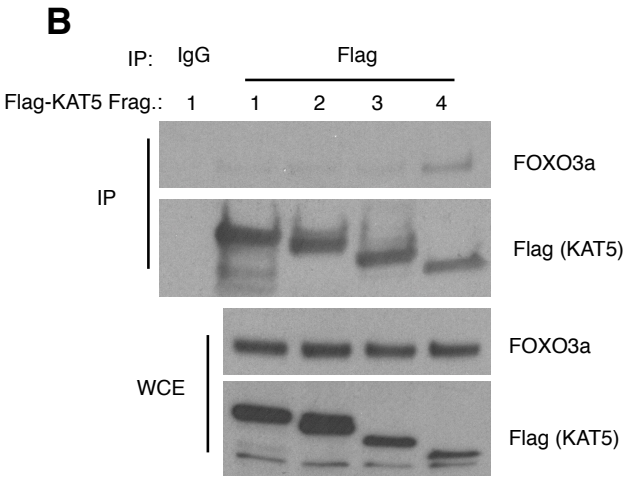
Figure 30. FOXO3a forms a protein complex with KAT5. (A) Immunoblot analysis of the Flag immunoprecipitation. HEK293T cells were transfected with a Flag-KAT5 construct, exposed to IR (5G; 1h), followed by the immunoprecipitation of the Flag tag. (B) Immunoblot analysis of the FOXO3a immunoprecipitation. HEK293T cells were transfected with Flag-KAT5 construct, exposed to IR (5G; 1h) and followed by immunoprecipitation of FOXO3a. (C) Immunoblot analysis of FOXO3a immunoprecipitation. HEK293T cells were transfected with Flag-KAT5 construct exposed to IR (5G; 1h) and followed by immunoprecipitation of FOXO3a in the presence of Benzonase nuclease. (D) Immunoblot analysis of the GST pulldowns. Recombinant FOXO3a was incubated with the GST or GST-KAT5 (asterisk). WCE - whole cell extract.

To understand if the interaction between FOXO3a and KAT5 is direct or mediated by the presence of other protein(s), I performed GST pulldown experiments with either GST or GST-KAT5 incubated with recombinant FOXO3a protein. In the absence of other proteins interaction between FOXO3a and KAT5 was still detected, although weakly (Figure 30D).

The C-terminal domain of KAT5 is necessary for binding to FOXO3a

To map the domain of KAT5 involved in the interaction with FOXO3a, I decided to use a set of Flag-tagged fragments of KAT5 that span the entire length of KAT5 protein (Figure 31A). When I immunoprecipitated those fragments I noticed that FOXO3a was specifically binding to the C-terminal part of KAT5 (fragment number 4) (Figure 31B). Moreover when I preformed the same immunoprecipitation experiment with the KAT5 fragments I could observe that ATM was binding to the same C-terminal fragment just like FOXO3a (Figure 31C). To map precisely the interaction domain of KAT5 with FOXO3a and ATM, I immunoprecipitated the very short C-terminal fragment of KAT5 (450-513aa). As shown in Figure 31D, I observed that both FOXO3a and ATM bind to the very C-terminal fragment of KAT5 (450-513aa), suggesting that KAT5 may bind to ATM through FOXO3a.


A
KAT5 fragments no.:
WT
1
2
3
4
1 25 76 261 283 369 383 513 aa
ChD
ZincF
AcCoA
NR
1 25 76 258
69 261 283 290
158 261 283 369 395
285 369 383 513 aa
ChD – Chromodomain
ZincF – Zinc Finger
AcCoA – Acetylo-CoA binding domain
NR – Nuclear receptor binding domain


B
IP: IgG Flag
Flag-KAT5 Frag.: 1 1 2 3 4
IP
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FOXO3a
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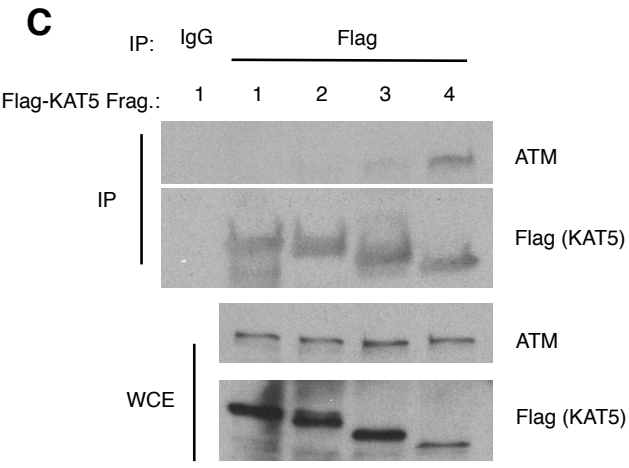
FOXO3a
Flag (KAT5)


C
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IP
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ATM
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ATM
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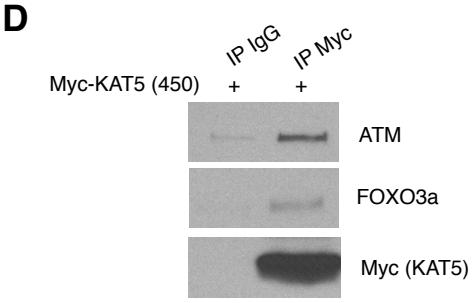
D
IP IgG
IP Myc
Myc-KAT5 (450) + +
ATM
FOXO3a
Myc (KAT5)

Figure 31. The C-terminal domain of KAT5 is necessary for ATM as well as FOXO3a binding. (A) Cartoon representation of the 3xFlag-KAT5 fragments. (B) Immunoblot analysis of the KAT5 immunoprecipitation. HEK293T cells were transfected with the different 3xFlag-KAT5 constructs followed by the immunoprecipitation of the Flag tag. (C) Immunoblot analysis of the KAT5 immunoprecipitation. HEK293T cells were transfected with the different 3xFlag-KAT5 constructs followed by the immunoprecipitation of the Flag tag. (D) Immunoblot analysis of the KAT5 immunoprecipitation. HEK293T cells were transfected with the Myc-KAT5 construct (450-513aa) followed by the immunoprecipitation of the Myc tag. WCE - whole cell extract.

The C-terminal domain of FOXO3a is necessary to bind KAT5

To further investigate nature of the interaction between FOXO3a and KAT5, I created five different Myc-tagged fragments of FOXO3a protein spanning its entire length (Figure 32A). First, I performed an immunoprecipitation of fragments 1-3. I observed that KAT5 was interacting specifically with the third fragment spanning the C-terminal part of FOXO3a (500-673aa)(Figure 32B). To determine the exact domain responsible for binding between KAT5 and FOXO3a, I immunoprecipitated Myc-FOXO3a fragments 3-5 representing short fragments of the C-terminal part of FOXO3a. As shown in Figure 32C, after immunoprecipitation of these fragments, I detected a specific binding between KAT5 and third and fourth Myc-FOXO3a fragments only, suggesting that the domain responsible for KAT5 binding lies between 620-650aa of FOXO3a.

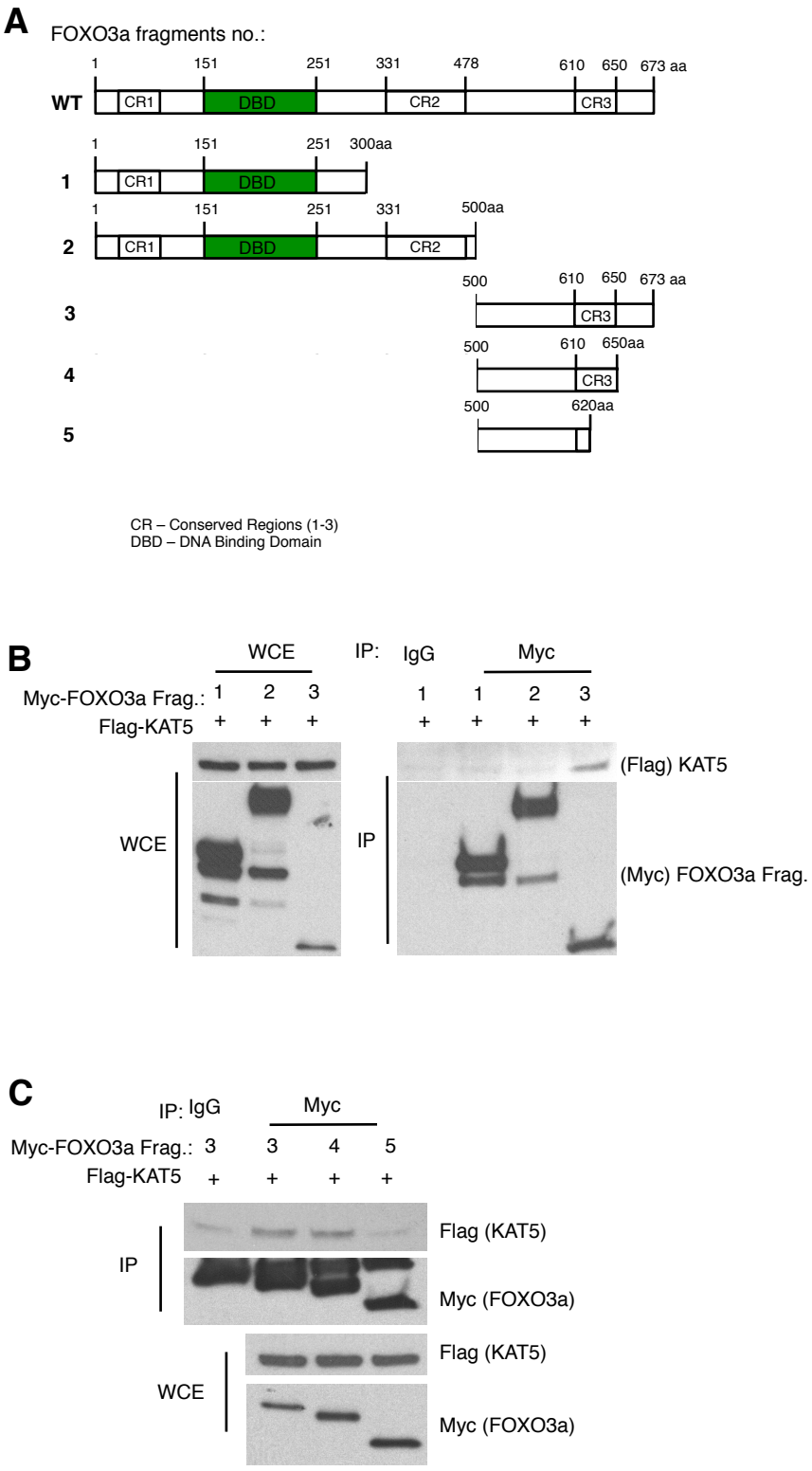

Figure 32. The C-terminal domain of FOXO3a is necessary for KAT5 binding. (A) Cartoon representation of the generated Myc-FOXO3a fragments. (B) Immunoblot analysis of the FOXO3a immunoprecipitation. HEK293T cells were transfected with Flag-KAT5 and different Myc-FOXO3a constructs followed by the immunoprecipitation of the Myc tag. (C) Immunoblot analysis of the FOXO3a immunoprecipitation. HEK293T cells were transfected with the Flag-KAT5 construct and different Myc-FOXO3a constructs followed by the immunoprecipitation of the Myc tag. WCE - whole cell extract.

FOXO3a is necessary for KAT5 binding to ATM

Next, I tested whether FOXO3a is necessary for the binding of KAT5 to ATM. To tackle this question, I used siRNA against *FOXO3a* (siFOXO3a) to knock down the expression of the FOXO3a protein and test its impact on the formation of the KAT5-ATM complex.

First, I tested if I could detect an impairment of the DDR in siFOXO3a treated cells. As shown in the Figure 33A, upon knock down of the *FOXO3a* I could observe a partial but not full impairment of the ATM activation as judged by the decrease in its autophosphorylation (pATM) upon IR. Next, I performed an immunoprecipitation of endogenous KAT5 from cells treated or not with siRNAs against FOXO3a. I observed that, while I could coimmunoprecipitate ATM with KAT5 in cells treated with the control siRNA, the interaction between KAT5 and ATM was somehow decreased in siFOXO3a treated cells (Figure 33B).

To strengthen this result, I preformed the same experiment in cells expressing Flag tagged KAT5. When I immunoprecipitated Flag tagged KAT5, I could observe the same partial impairment of the KAT5-ATM interactions as observed in the previous immuprecipitation with endogenous KAT5 (Figure 33C).

These results show that FOXO3a is necessary for the KAT5 binding to ATM, although knockdown of FOXO3a leads only to a partial impairment of ATM-KAT5 interaction, which results also in a partial impairment of ATM activation in siFOXO3a-treated cells.

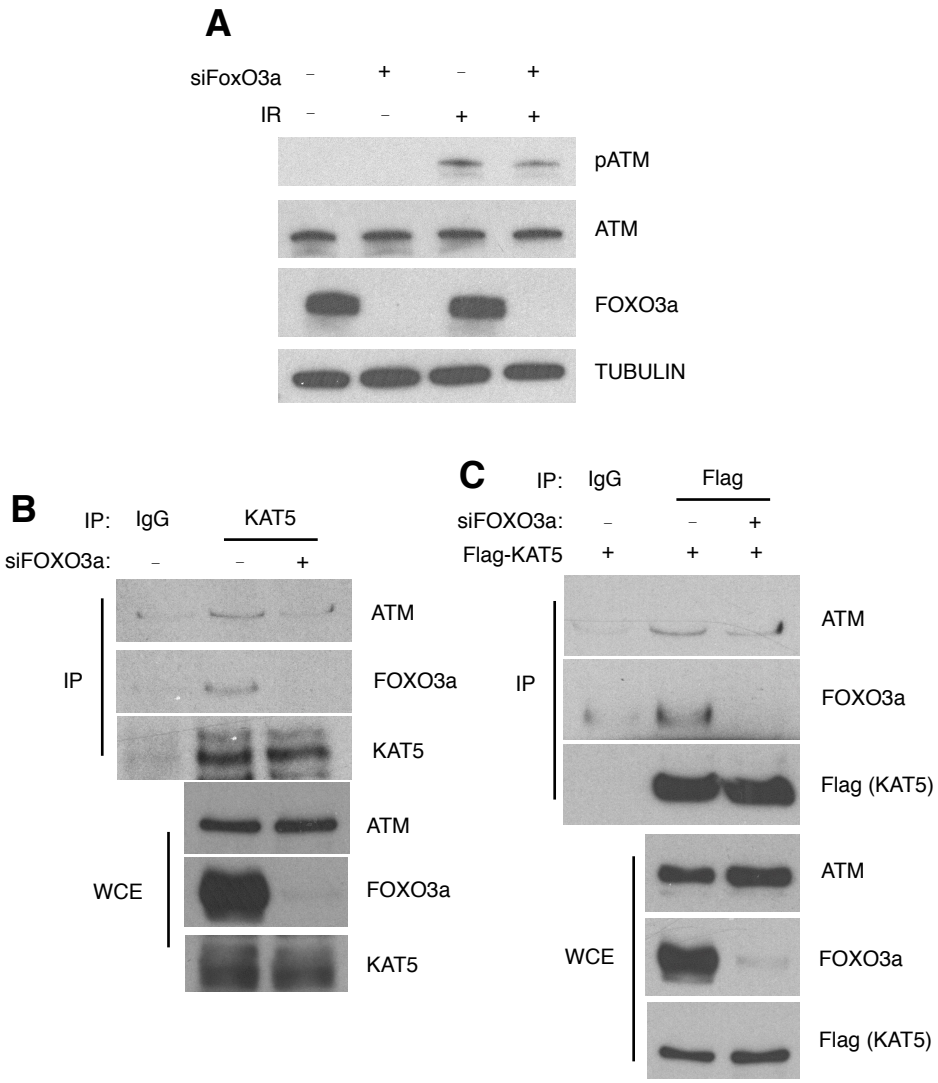

Figure 33. FOXO3a in necessary for KAT5 binding to ATM. (A) Immunoblot analysis of ATM activation. HeLa cells were transfected with siRNA against *FOXO3a* (siFOXO3a), exposed to IR (2G; 1h) and lysed. (B) Immunoblot analysis of KAT5 immunoprecipitation. HEK293T cells were transfected with siRNA against *FOXO3a* (siFOXO3a) and lysed, followed by immunoprecipitation of KAT5. (C) Immunoblot analysis of Flag immunoprecipitation. HEK293T cells were transfected with siRNA against *FOXO3a* (siFOXO3a) and Flag-KAT5 construct followed by immunoprecipitation of the Flag tag. WCE - whole cell extract.

FOXO1 and FOXO4 can bind to ATM as well as to KAT5

To understand why despite a substantial knock down of FOXO3a expression I could detect only a partial effect on ATM activation and ATM-KAT5 complex stability, I decided to investigate the potential redundancy mechanism among different members of the FOX-O family (for more details please see the "FOXO3a structure and regulation" chapter).

First, I compared the sequence of the C-terminal fragment of FOXO3a with other FOX-O family members (FOXO1 and FOXO4) (Figure 34A). As shown, I noticed some regions of the strong conservation between FOXO3a, FOXO1 and FOXO4. Therefore I decided to test if, like FOXO3a, FOXO1 and FOXO4 could bind to ATM and KAT5.

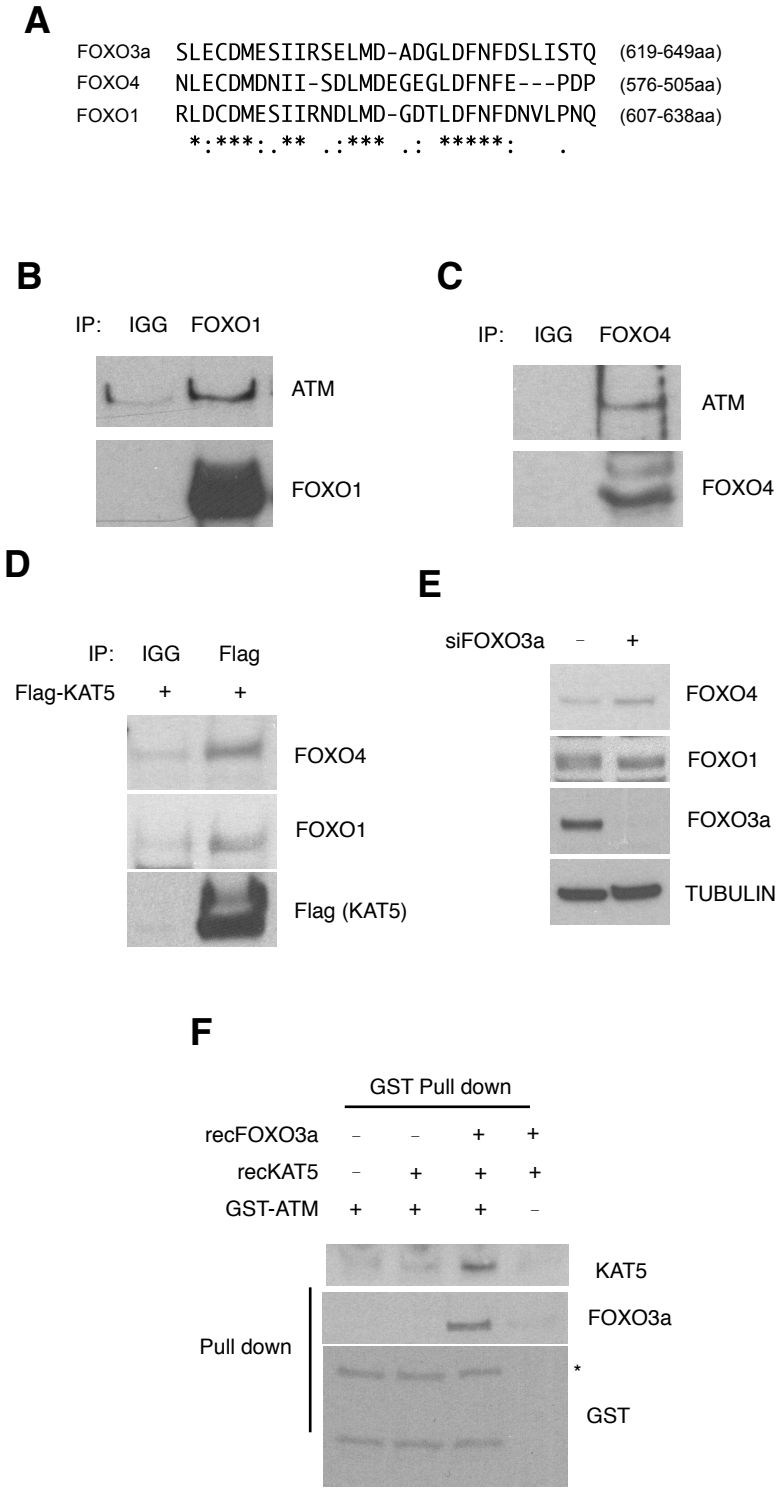

Figure 34. FOXO3a is necessary for KAT5 binding to ATM. (A) Protein sequence alignment of

human FOXO3a, human FOXO4 and human FOXO1 as generated by Clustal X software. "*" - fully conserved residue. ":" - strongly similar properties of the residues. "." - weakly similar properties of the residues (B) Immunoblot analysis of the FOXO1 immunoprecipitation. HEK293T cells were lysed, followed by the immunoprecipitation of FOXO1. (C) Immunoblot analysis of FOXO4 immunoprecipitation. HEK293T cells were lysed, followed by immunoprecipitation of FOXO4. (D) Immunoblot analysis of the Flag immunoprecipitation. HEK293T cells were transfected with Flag-KAT5 construct and lysed, followed by the immunoprecipitation of Flag tag. (E) Immunoblot analysis of the HEK293T cells treated with siFOXO3a. (F) GST pulldown experiment. 2842-3056aa GST-ATM fragment was incubated with recombinant KAT5 (recKAT5) and/or with recombinant FOXO3a (recFOXO3a).

As shown in Figure 34B and C, immunoprecipitation experiments revealed that both FOXO1 as well as FOXO4 can form a protein complex with ATM. Next, I performed an immunoprecipitation of KAT5 (Flag-KAT5) and I observed that as in the case of ATM, FOXO1 and FOXO4 can interact with KAT5 too (Figure 34D).

In addition, I performed analysis of the expression levels of each member of the FOX-O family in cells treated or not with siFOXO3a. As shown in Figure 34E, I observed a moderate increase in the expression of FOXO4 in siFOXO3a treated cells.

Finally, following the realization that other FOX-O family proteins can bind to ATM and KAT5 and that FOXO4 is up-regulated in siFOXO3a treated cells, I decided to perform an *in vitro* binding assay in the absence of any redundant mechanism to test whether FOXO3a can mediate the interaction between ATM and KAT5. I thus performed GST pulldown experiments with the use of a 2842-3056aa GST-ATM fragment, recombinant KAT5 and recombinant FOXO3a. As shown in the immunoblot analysis of the material pulled down (Figure 34F), I could not detect any direct interaction between GST-ATM fragment and recombinant KAT5, which is in the agreement with the already published data (Sun et al., 2010). However, in the presence of recombinant FOXO3a, binding between recombinant

KAT5 and GST-ATM fragment became robustly detectable, convincingly proving that FOXO3a mediates ATM-KAT5 interaction (Figure 34F).

NOTCH1 impairs KAT5 binding to ATM

In the previous set of results, in which I characterized the competition between NOTCH1 and FOXO3a for the binding to the FATC domain of ATM, I showed that overexpression of NOTCH1 impairs the binding between FOXO3a and ATM (Figure 26). Therefore in the light of the results showing that FOXO3a forms a direct protein complex with KAT5 and it is necessary for KAT5-ATM association, I decided to test if the expression of NOTCH1 can impact on the binding between ATM and KAT5.

It has been reported that NOTCH1 can interact with KAT5 to repress NOTCH1 driven transcription of its target genes through KAT5 mediated acetylation (Kim et al., 2007a). Binding between NOTCH1 and KAT5 is mediated by Zinc finger and Aceltylo-CoA binding domains of KAT5 (between 158-395aa). Therefore to avoid the binding between NOTCH1 and KAT5, I decided to use a Myc-KAT5 construct expressing just the last 65aa of the KAT5 protein (Myc-KAT5 450-513aa).

As shown in Figure 35, when I immunoprecipitated Myc-tagged KAT5 (450-513aa) I observed that NOTCH1 expression impairs the interaction between ATM and KAT5. Unexpectedly, I noticed that in addition to disrupting such interaction, NOTCH1 expression also impaired the binding between KAT5 and FOXO3a (Figure 35).

This result could arise from NOTCH1-mediated sequestration of either FOXO3a or KAT5. However, I had already showed that NOTCH1 does not interact with FOXO3a (Figure 26). I have also ruled out the possibility that NOTCH1 could sequester KAT5 since I used a C-terminal fragment of KAT5, which should not form a binding with NOTCH1 according to the previously published study (Kim et al., 2007a).

Therefore I concluded that observed reduction in the interaction between FOXO3a and KAT5 in cells expressing NOTCH1 is specific.

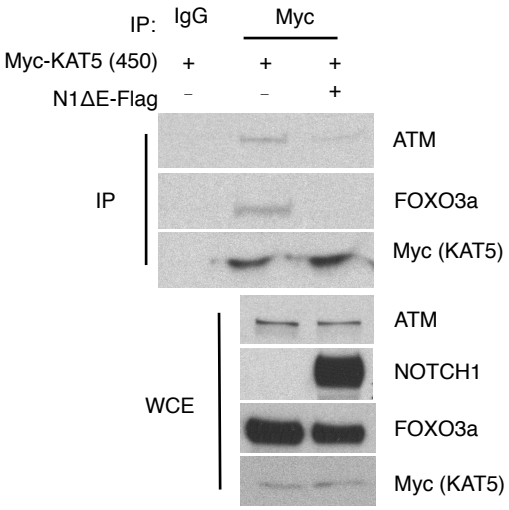

Figure 35. Expression of NOTCH1 impairs binding of KAT5 to ATM. Immunoblot analysis of the Myc tag immunoprecipitation. HEK293T cells were transfected with Myc-KAT5 (450-513aa) and N1 Δ E-Flag constructs followed by the immunoprecipitation of Myc tag. WCE - whole cell extract.

FOXO3a binding to ATM is necessary for the formation of FOXO3a-KAT5 protein complex

I decided to further pursue the observation of the loss of interaction between KAT5 and FOXO3a in the presence of NOTCH1.

Till now I have shown that KAT5, FOXO3a and ATM form a three proteins complex in which FOXO3a plays a role of a bridging protein between KAT5 and ATM. Furthermore, I showed that in the presence of NOTCH1, the interactions between ATM and FOXO3 as well as FOXO3a and KAT5 are impaired. Therefore I decided to investigate whether the interaction between FOXO3a and KAT5 is preserved or not in the absence of ATM (Figure 36A).

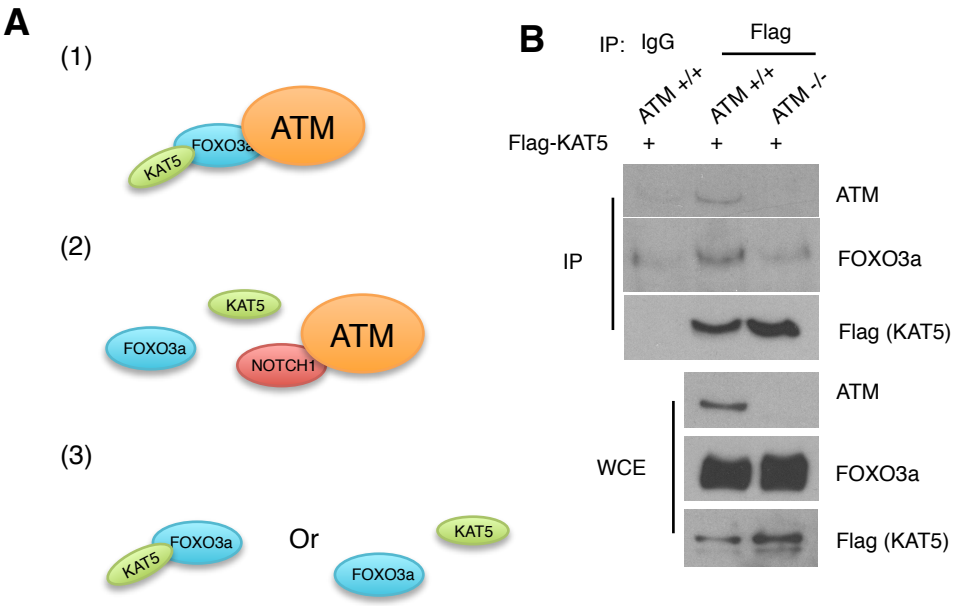

Figure 36. ATM is necessary for the formation of FOXO3a-KAT5 protein complex. (A) Cartoon model of the interactions between ATM/FOXO3a/KAT5 proteins. (1) Interactions between ATM, FOXO3a and KAT5 in the absence of NOTCH1; (2) Interactions between ATM, FOXO3a and KAT5 in the presence of NOTCH1; (3) Possible interactions between FOXO3a and KAT5 in the absence of ATM. (B) Immunoblot analysis of the Flag immunoprecipitation in WT or *Atm* knockout mESC cells. mESC cells were transfected with the Flag-KAT5 construct followed by the immunoprecipitation of the Flag tag. WCE - whole cell extract.

I immunoprecipitated Flag-tagged KAT5 in wild type or *Atm* knockout mouse embryonic stem cells (mESCs). As shown in the Figure 36B, I was able to coimmunoprecipitate FOXO3a with KAT5 in the wild type mESCs, but the interaction between KAT5 and FOXO3a was lost in *Atm* knockout cells.

These results, together with the previous immunoprecipitations experiments showing the disruption of FOXO3a-KAT5 interaction in the presence of NOTCH1, strongly suggest that the binding between KAT5 and FOXO3a is dependent on the formation of the three proteins complex with ATM.

Induction of FOXO3a nuclear localization sensitizes T-ALL cells to DNA damage-induced cell death

I have previously suggested a potential use of NOTCH1 inhibitors in combination with DNA damage inducing agents (IR) to sensitize NOTCH1-driven cancers to DNA damage-induced cell death (Figure 21).

Following the discovery of an ATM-FOXO3a-KAT5 three proteins complex, I decided to investigate the potential translational impact of these findings in the treatment of NOTCH1-driven cancers. I therefore decided to test the use of different drugs to induce FOXO3a nuclear localization, thus allowing it to increase the competition between FOXO3a and NOTCH1 for ATM binding leading to a more robust DDR activation in NOTCH1-driven cancers.

As there are no drugs available to target specifically FOXO3a in the nucleus, I decided to act on the upstream signalling pathways that are involved in its nuclear localization. It has been shown that activation of AMPK kinase induces FOXO3a nuclear localization and activation of the DDR in a FOXO3a dependent manner (Hu et al., 2014). Additionally it has been reported that inhibition of the p38 kinase in cells not exposed to any stimuli increases nuclear FOXO3a accumulation (Chiacchiera et al., 2009). Therefore I decided to use Metformin, a widely used AMPK kinase activator, and SB203580, which inhibits p38 kinase activity, in T-ALL cells to increase levels of FOXO3a in the nucleus.

First, I tested if the treatment of TALL-1 cells with Metformin results in FOXO3a nuclear accumulation. This was studied by monitoring the changes in the expression of the FOXO3a target gene *GADD45α*. As shown in Figure 37A, I observed increased *GADD45α* expression in TALL-1 cells treated for 24h with 500μM dose of Metformin.

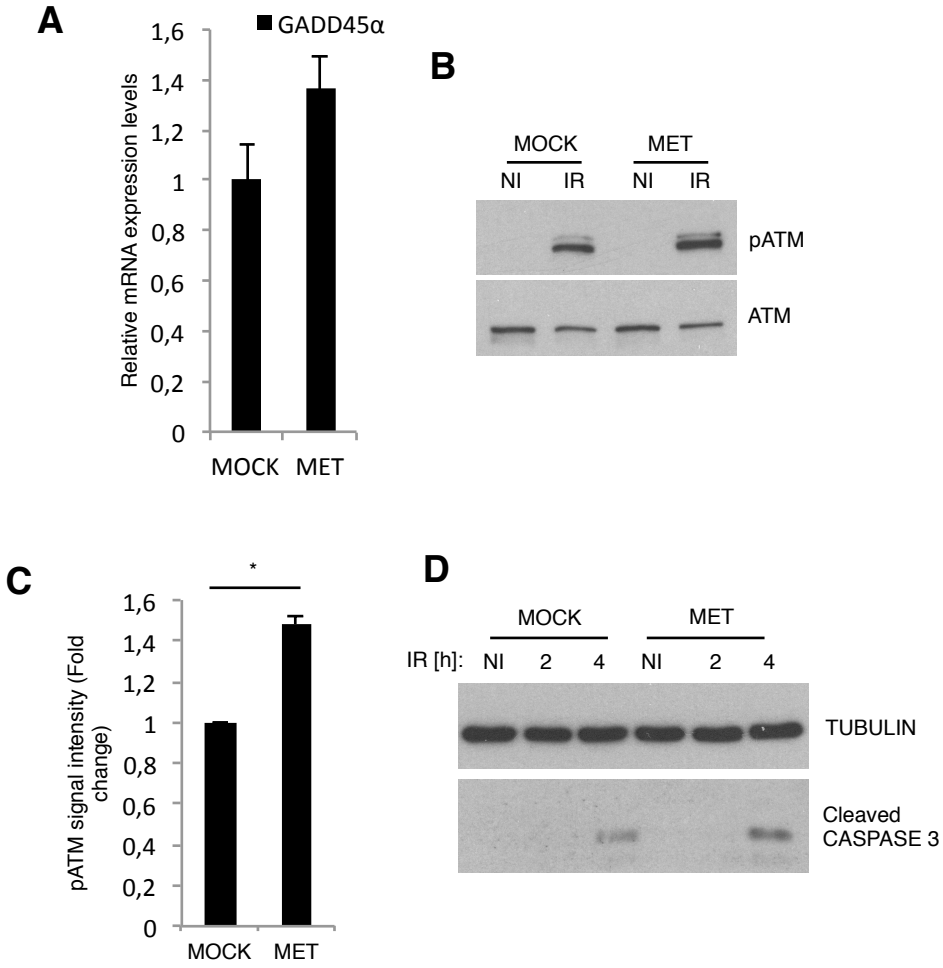

Figure 37. Metformin sensitize TALL-1 cells to DNA damage-induced cell death. (A) qRT-PCR analysis of the *GADD45α* in TALL-1 cells treated with Metformin (MET). (B) Immunoblot analysis of the ATM activation (pATM). TALL-1 cells were pretreated with Metformin (MET) and exposed to IR (5G; 1h). (C) Quantification of the immunoblot shown in B. (D) Immunoblot analysis of DNA damage-induced cell death (cleaved CASPSE 3). TALL-1 cells were pretreated with Metformin (MET) and subjected to IR (10G).

Next, I exposed to IR cells treated as above and I performed an immunoblot analysis of ATM activation (pATM). As shown in the Figure 37B and C, I observed an increase in ATM activation upon DNA damage in Metformin-treated cells as compared to MOCK-treated ones. To investigate if the observed increase in DDR activation resulted in an increase of DNA damage-induced cell death, I measured the cleavage of the CASPASE3, a marker of apoptosis, in cells treated or not with Metformin followed by IR. As shown in the Figure 37D, Metformin-treated cells displayed a significant increase in CASPASE3 cleavage as compared to control cells.

In another set of experiments I pretreated TALL-1 cells with the 10μM of SB203580 for 24h. As in the case of Metformin I checked if the treatment with the SB203580 resulted in FOXO3a nuclear accumulation. As shown in the Figure 38A SB203580, pretreatment resulted in the increase in *GADD45α* transcription. Moreover I could detect an increase of ATM activation (pATM) in SB203580 pretreated cells upon exposure of those cells to IR Figure 38B and C.

Next, I performed an analysis of the DNA damage-induced cell death (CASPASE3 cleavage) in SB203580 pretreated cells. As shown in Figure 38D, cells treated with the SB203580 showed a striking increase in DNA damage-induced cell death as compared to untreated cells. To check if the observed cell death was restricted to ATM activation, I pretreated cells with an ATM inhibitor (ATMi). As shown by the immunoblot analysis (Figure 38E), treatment with the ATM inhibitor resulted in the complete loss of DNA damage-induced cell death in SB203580-treated cells, clearly demonstrating that the observed apoptosis was strictly ATM-dependent.

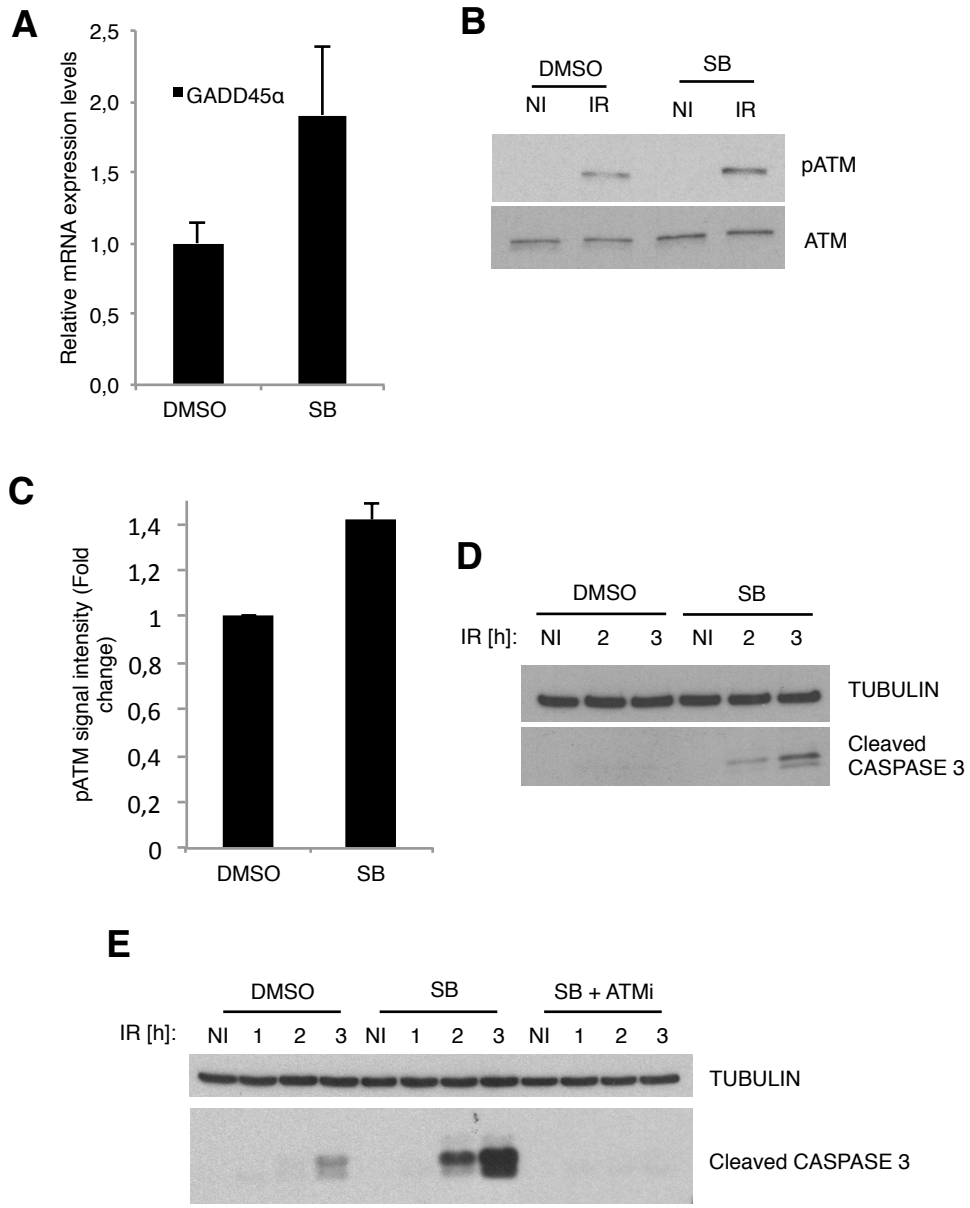

Figure 38. SB58023 sensitize TALL-1 cells to DNA damage. (A) qRT-PCR analysis of GADD45α in TALL-1 cells treated with SB203580 (SB). (B) Immunoblot analysis of ATM activation. TALL-1 cells were pretreated with SB203580 (SB) and exposed to irradiation (5G; 1h). (C) Quantification of the blot shown in B. (D) Immunoblot analysis of DNA damage-induced cell death. TALL-1 cells were pretreated with SB58023 (SB) and subjected to irradiation (10G). (E) Immunoblot analysis of DNA damage-induced cell death. TALL-1 cells were pretreated with SB203580 (SB) and ATM inhibitor (ATMi) and subjected to irradiation (10G).

NOTCH1 interacts with other PI3-like kinases

I have previously shown that NOTCH1 interacts with ATM through the FATC domain of ATM. Other PI3K-like kinases involved in the DDR like ATR and DNA-PKcs posses similar domain structure (Figure 39A). It has been shown that FATC domains of PI3K-like kinases of ATM, ATR and DNA-PKcs are functionally equivalent and that the FATC domains of ATR and DNA-PKcs can functionally substitute for the FATC domain of ATM (Jiang et al., 2006). Therefore I decided to test if NOTCH1 can bind to ATR and DNA-PKcs.

I performed immunoprecipitation of NOTCH1 or DNA-PKcs in HEK293T cells expressing N1ΔE-Flag construct. As shown in (Figure 39B), I observed that NOTCH1 can form a protein complex with ATR and DNA-PKcs

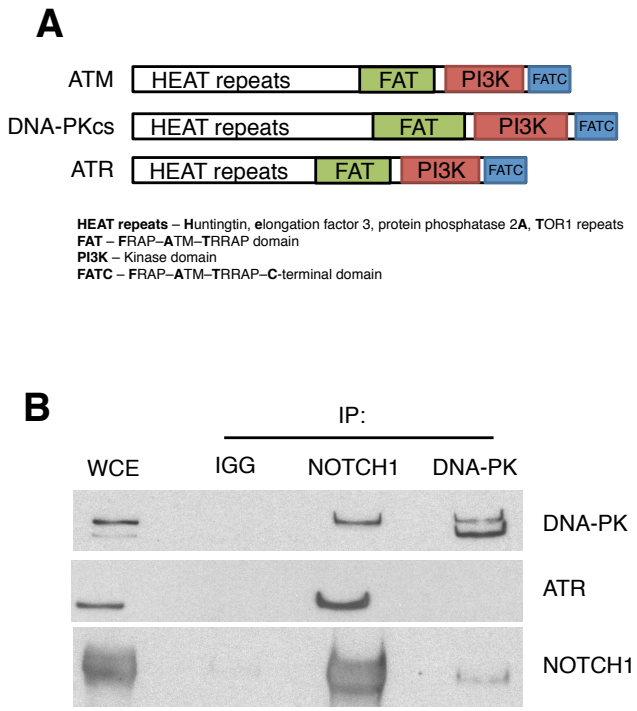

Figure 39. NOTCH1 forms a protein complex with DNA-PKcs and ATR kinases. (A) Cartoon depicting domain structure of the PI3K-like kinases (ATM, ATR and DNA-PKcs). (B) Immunoblot analysis of the DNA-PKcs or NOTCH1 immunoprecipitations. HEK293T cells were transfected with the N1ΔE-Flag construct followed by the immunoprecipitation of NOTCH1 or DNA-PKcs. WCE - whole cell extract.

NOTCH1 inhibits DNA-PKcs but not ATR kinase

Due to the observed binding of NOTCH1 to ATR and DNA-PKcs kinases and the data showed above indicating that NOTCH1 inhibits ATM kinase activity, I tested whether NOTCH1 could inhibit ATR and DNA-PKcs kinases.

For this purpose, I transfected HEK293T cells with a N1IC-GFP construct, FACS sorted GFP-positive cells and subjected them to IR, to test for DNA-PKcs and ATR activation, or UV light to test for ATR activation in the presence of NOTCH1. My immunoblot analyses revealed that in IR-treated cells NOTCH1 could dramatically inhibit the activation of ATR kinase functions as judged by the reduction of ATR-mediated phosphorylation of CHK1 kinase at Serine 345 and Serine 317 (Figure 40). Similarly DNA-PKcs kinase activity was strongly reduced in NOTCH1 expressing cells exposed to IR as evaluated by the reduction in DNA-PKcs autophosphorylation at Serine 2056 (Figure 40), which is an exclusively DNA-PKcs target phosphorylation site (Uematsu et al., 2007). The reduction of phosphorylation of DNA-PKcs at the T2609 and T2647 sites observed in NOTCH1 expressing cells (Figure 40), can be attributed to the reduction in ATM kinase activity, since it has been shown that these sites are phosphorylated by ATM (Uematsu et al., 2007). Surprisingly, I did not observe any significant difference in ATR-dependent phosphorylation of CHK1 kinase at Serine 345 and Serine 317 upon UV light treatment of NOTCH-expressing cells (Figure 40). These results are consistent with those showing that ATR activation upon treatment with IR is dependent on ATM activation and resection at DNA damage sites (Jazayeri et al., 2006). Therefore my results indicate that ATR kinase activity is not affected by NOTCH1 expression and lack of ATR activation upon treatment with IR is the result of NOTCH1-meditaed ATM inhibition. Differently, the kinase activity of DNA-PKcs seams to be affected by NOTCH1 expression.

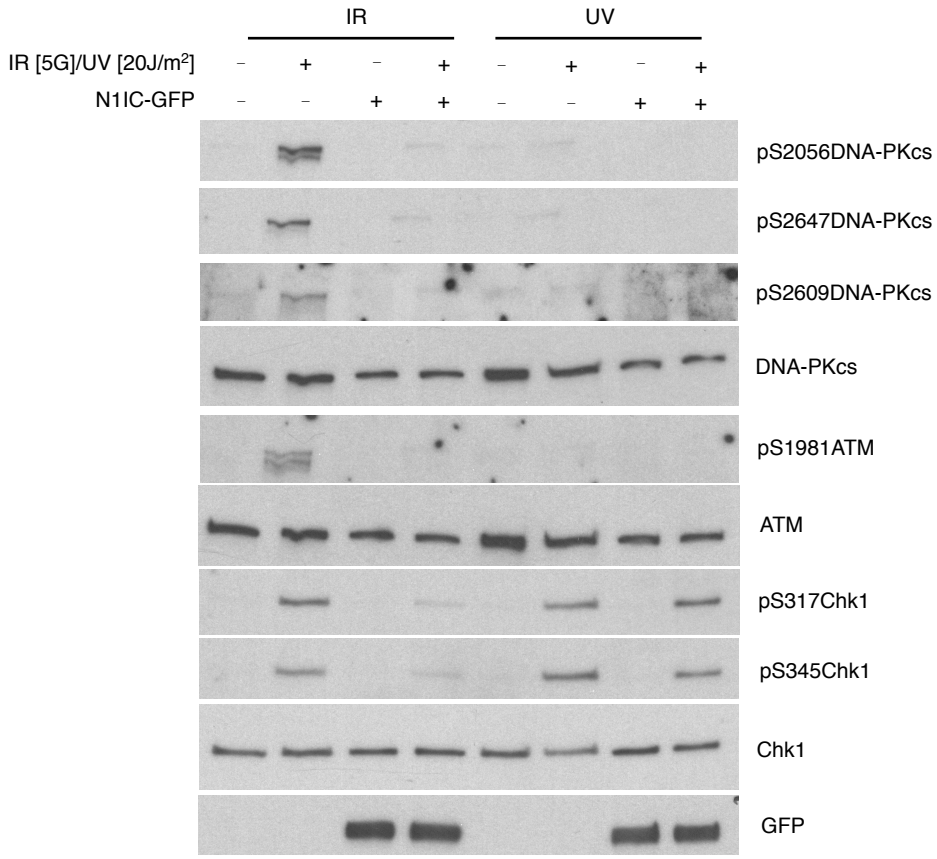

Figure 40. NOTCH1 inhibits kinase activity of the DNA-PKcs but not ATR kinase. Immunoblot analysis of the DNA-PKcs and ATR kinases activation upon treatment with IR or UV light. HEK293T cells were transfected with N1IC-GFP, FACS sorted for GFP positive cells and exposed to IR or UV light and collected 1h afterward.

NOTCH1 dose not inhibit repair of DSBs

DNA-PKcs has been shown to be a key kinase involved in DNA damage repair by non homologus end joining.

The data shown above indicate that NOTCH1 impacts on DNA-PKcs transphosphorylation and autophosphorylation upon DSBs induction. To test if NOTCH1 can influence the function of DNA-PKcs in DNA damage repair, I decided to perform a repair assay in cells expressing NOTCH1 in the presence or absence of the DNA-PKcs inhibitor (DNA-PKcsi). I thus studied over time the kinetics of phosphorylation of the histone H2AX upon DNA damage that has been shown to be a specific marker of DSBs accumulation.

As shown in Figure 41, consistently with previous results, I observed an induction of the phosphorylation of histone H2AX upon IR, both in the MOCK as well as NOTCH1 (N1 Δ

E) transfected cells (1h time point). With time, DNA damage was detectably being repaired as judged by the decrease of the phosphorylation of histone H2AX (8h time point)(Figure 41). Such decrease was the result of DNA repair as cells treatment with DNA-PKcsi prevented such a decrease (Figure 41). Unexpectedly I could not observe any strong differences in the decrease of histone H2AX phosphorylation between NOTCH1 and MOCK transfected cells, showing that NOTCH1 does not significantly impact on the repair of DNA DSBs, at least as judge by the assay so far employed (Figure 41).

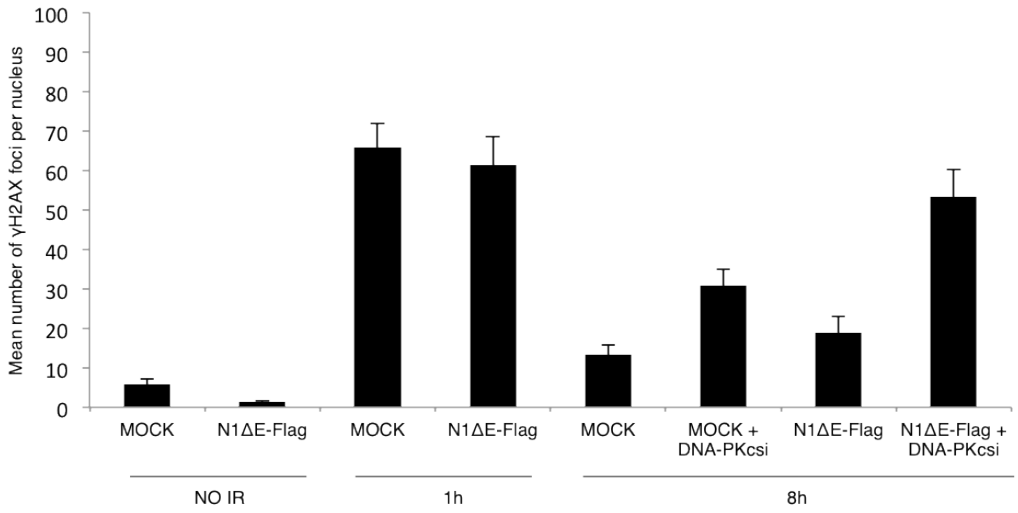

Figure 41. NOTCH1 does not inhibit DNA damage repair. Immunofluorescence analysis of histone H2AX phosphorylation on Serine 139 upon IR (2G) at the indicated time points in U2OS cells transfected with NOTCH1 (N1ΔE-Flag) and treated with DNA-PKcs inhibitor (DNA-PKcsi). MOCK - mock transfected cells.

Discussion

Impact of NOTCH1 on ATM activation and its substrate specificity

Despite intense efforts starting from the time of ATM discovery, over twenty years ago, the full mechanism behind ATM activation remains unknown (Savitsky et al., 1995). This lack of a clear vision over the processes governing ATM activation does not allow to pinpoint the individual contributions of different factors in the regulation of DDR activation.

In this part of my thesis, I will discus how my results fit in the context of published literature in regard to the mechanisms regulating ATM activation.

NOTCH1 and recruitment of ATM to the DSBs

FATC domain is positioned at the very C-terminal end of ATM (between 3023-3056aa). It has also been shown that NBS1 binds to the N-terminal part of ATM, between 250-522aa (Pessina and Lowndes, 2014; You et al., 2005). Although ATM is a large protein, the recently published structures of ATM monomer and dimer indicate that FATC domain is in physical proximity to the N-terminal part of ATM (Lau et al., 2016). Moreover, it has been shown that mutations in the FATC domain of Tel1 (yeast ortholog of ATM) results in the perturbation of the binding of Xrs2 (yeast ortholog of NBS1) to ATM, which ultimately results in the impaired recruitment of Tel1 to DSB (Ogi et al., 2015). My results indicate that NOTCH1 directly binds to the FATC domain of ATM. I additionally observed that formation of the protein complex between ATM and NOTCH1 does not affect the interaction between ATM and NBS1. Moreover, NOTCH1 binding to the FATC domain of ATM does not perturb the accumulation of ATM at DSBs. This suggests that NOTCH1, despite binding to the FATC domain of ATM, does not impair the interaction of ATM with other proteins like NBS1 through its N-terminal part. It is worth noting that not all of Tel1 FATC mutants in the study quoted above (Ogi et al., 2015) impaired Tel1 interaction with Xrs2, or Tel1 recruitment to the DSBs.

ATM autophosphorylation. What is its role?

ATM kinase has been shown to phosphorylate itself more then a decade ago (Kim et al., 1999). So far many autophosphorylation sites of ATM have been discovered and shown to be the result of ATM activation upon DNA damage (Kozlov et al., 2006; Matsuoka et al., 2007). ATM autophosphorylation at Serine 1981, has been shown to be necessary for the first step of ATM activation, which is dissociation of an inactive ATM dimer into an active monomer in a process of transphosphorylation (Bakkenist and Kastan, 2003). Moreover, it was shown that expression of S1981A ATM mutant of in A-T cells did not restore the full ATM activity as compared to wild type ATM (Bakkenist and Kastan, 2003). I observed that NOTCH1 blocks ATM autophosphorylation at Serine 1981 (from now on referred as ATM autophosporylation), which results in the inhibition of ATM kinase activity, as judged by the decrease in phosphorylation of many ATM substrates. Thus, my observation of NOTCH1-mediated inhibition of ATM autophosphorylation and ATM kinase activity is consistent with the literature cited above.

Unexpectedly, a seemingly contradictory set of results showed that mice and cells carrying an S1987A mutation in Atm (equivalent to human S1981A mutation) display normal unaffected DNA damage response and ATM kinase function both *in vitro* as well as *in vivo* (Pellegrini et al., 2006). Apart from Serine 1987, also other autophosphorylation sites (Serine 367 and 1893) have been shown to be dispensable for ATM activation upon DNA damage (Daniel et al., 2008). Therefore, to determine whether NOTCH1 mediated inhibition of ATM autophosphorylation results from the inhibition of ATM kinase activity or *vice versa,* I performed an ATM *in vitro* kinase assay, which demonstrated a NOTCH1-mediated inhibition of the ATM kinase. It has been published that ATM mutant (S1981A) was able to phosphorylate p53 as well as CHK2 *in vitro* upon stimulation with linear double-stranded DNA (Lee and Paull, 2005). As I performed an ATM *in vitro* kinase assay with the use of immunopurified ATM, I could exclude any involvement of ATM autophosphorylation in the process of ATM kinase activation, since the anti ATM antibody

that I used for ATM purification was masking ATM autophosphorylation site, suggesting that NOTCH1-mediated inhibition of ATM kinase is direct and it is not connected with ATM autophosphorylation.

It has been reported that lack of ATM autophosphorylation results in a decrease of ATM retention at DSBs (1h after DNA damage)(So et al., 2009), without affecting ATM primary recruitment (up to 10 minutes after DNA damage)(So et al., 2009). I have observed that NOTCH1 despite blocking ATM autophosphorylation, does not block ATM recruitment to DSB and its retention (up to 1h after DNA damage induction). The differences between my observations and the report mentioned above may arise from different experimental setups. I studied recruitment of ATM in a complex with NOTCH1 that inhibits ATM kinase activity. Differently, in this report (So et al., 2009) the authors used ATM knockout cells that were stably expressing YFP tagged ATM or YFP-ATM carrying a point mutation (S1981A) (So et al., 2009). Therefore it would be best to compare recruitment of ATM-NOTCH1 complex with the recruitment of catalytically inactive ATM. Few years ago, two studies investigating DNA damage response in cells expressing kinase dead ATM mutant have been published (Daniel et al., 2012; Yamamoto et al., 2012). Here the authors showed that catalytically inactive ATM, although not able to autophosphorylate itself, was efficiently recruited to the DSBs (Daniel et al., 2012; Yamamoto et al., 2012). Additionally it has been reported that in the absence of KU70 and MRE11 activity, although ATM autophosphorylation upon DNA damage was perturbed, ATM protein was still efficiently recruited to DSBs (Hartlerode et al., 2015).

Hence, it is clear that ATM autophosphorylation and its recruitment to DSBs is differentially regulated as shown by my results and others'. It is an exciting area of research that needs further investigation.

Impact of NOTCH1 binding to the FATC domain of ATM on its substrate recognition

One of the recently suggested roles of ATM FATC domain is its participation in ATM substrate recognition. Indeed, when mutations were introduced in the FATC domain of Tel1 kinase, its ability to phosphorylate its main substrate Rad53 (yeast ortholog of the CHK2 kinase) was significantly reduced without any impact on Tel1 autophosphorylation (Ogi et al., 2015). Is it possible that the observed reduction in ATM autophosphorylation and phosphorylation of its substrates in NOTCH1 expressing cells is the result of impaired ATM substrate recognition rather than impaired ATM kinase activity? In my work I showed that NOTCH1-mediated inhibition of ATM kinase activity is specific and directly mediated by NOTCH1 binding to the FATC domain of ATM. I have shown that phosphorylation of a broad spectrum of ATM substrates like CHK2, SMC1, KAP1, p53, DNA-PKcs and ATM itself are affected by NOTCH1 binding to ATM. Moreover, in our laboratory we have performed an *in vitro* ATM kinase assay in *Xenopus laevis* egg extract, in which we detected by immunoblot analysis a striking reduction in ATM-mediated phosphorylation of tens of ATM substrates (detected by pS/TQ antibody that detects ATM-mediated phosphorylations)(Vermezovic et al., 2015). In addition, we showed that NOTCH1-mediated ATM inactivation results in checkpoint inactivation, which is a functional readout of ATM activation (Vermezovic et al., 2015). Despite all of these results indicating that inhibition of phosphorylation of ATM substrates in NOTCH1 expressing cells is the result of inhibition of ATM kinase activity, one observation stood out: phosphorylation of histone H2AX at Serine 139 (γH2AX), which was not affected by NOTCH1 expression. A separate chapter in this Discussion has been devoted to this observation (see chapter below).

In regard to the possible involvement of ATM FATC domain in ATM substrate recognition it has been reported that an ATM mutant, missing the last 10aa of FATC domain, shows reduced ability to phosphorylate its substrates (Banin et al., 1998). It was

reported that the interaction of ATM with MRN complex enhances ATM substrate recognition and thus their phosphorylation (Lee and Paull, 2004), which together with previously mentioned report showing the importance of FATC domain in Tel1-Xrs2 interaction (Ogi et al., 2015) suggests that the FATC domain through its ability to regulate ATM-MRN interaction can impact on ATM substrate recognition. Additionally, it has been shown that lack of FATC domain impairs ATM activation upon oxidative stress in an MRN independent manner, indicating that the FATC domain under these conditions is necessary for ATM substrate recognition (Guo et al., 2010). Despite all of the data implicating FATC domain in ATM substrate recognition, in my studies I did not observe any impact of NOTCH1 on the ATM-MRN interaction and MRN mediated ATM recruitment. This suggests that if NOTCH1 impacts on ATM substrate recognition it is not through its impact on the interaction between MRN complex and ATM, but rather through the MRN-independent FATC domain substrate recognition function, like the one reported by Guo et al. (Guo et al., 2010).

NOTCH1 does not affect phosphorylation of histone H2AX

As shown in this thesis, I have observed that NOTCH1 blocks ATM kinase activation. This conclusion is based on the observed reduction of ATM autophosphorylation. Upon autophosphorylation, ATM mediates phosphorylation of its substrates on Serine (Ser) or Threonine (T) residues (Kim et al., 1999). Many of ATM phosphorylation sites have been identified: T68CHK2, Ser139H2AX, Ser824KAP1, T2647DNA-PKcs, T2609DNA-PKcs, Ser15p53 or Ser966SMC1. I observed that NOTCH1 was able to inhibit ATM-mediated phosphorylation of CHK2, KAP1, DNA-PKcs, p53 and SMC1 but not phosphorylation of H2AX. This result may seem unexpected considering that phosphorylation of H2AX by ATM has been described as one of the most important steps of DDR activation necessary for further accumulation and recruitment of secondary response proteins like 53BP1 and MDC1 (Kleiner et al., 2015; Stucki et al., 2005). Lack of the impact of NOTCH1 on

H2AX phosphorylation might result from the redundancy among PI3K-like kinases, since DNA-PKcs, like ATM, has been described to phosphorylate H2AX in response to DNA damage (Stiff et al., 2004). Additionally, it has been shown that in ATM knockout cells H2AX can be phosphorylated to an extent observed in ATM proficient cells (Riballo et al., 2004). NOTCH1 expressing cells, which were additionally treated with a DNA-PKcs inhibitor and exposed to IR, did not show reduced levels of H2AX phosphorylation (data not shown). This result differs from the one described by Stiff and colleagues (Stiff et al., 2004), who reported that in the absence of ATM and DNA-PKcs, phosphorylation of H2AX is perturbed.

It is possible that phosphorylation of H2AX upon DNA damage in NOTCH1 expressing cells might be mediated by other kinases that do not belong to the PI3K-like kinase family. Indeed, it has been reported that other protein kinases, like JNK1 and p38 can phosphorylate H2AX under stress conditions like UV light irradiation or serum starvation (Lu et al., 2008; Lu et al., 2006), Additionally it has been recently reported that VRK1 kinase (Vaccinia related kinase 1) can phosphorylate H2AX upon DNA damage induction (Salzano et al., 2015). VRK1 has been previously shown to be necessary for accumulation and activation of DDR factors at DSBs (Sanz-García et al., 2012). Now, it has been reported that it can phosphorylate H2AX upon DSB formation parallel to ATM kinase. Moreover, it has been shown that inhibition of both kinases (ATM and VRK1) has an additive effect on H2AX phosphorylation (Salzano et al., 2015).

NOTCH1, KAT5, ATM acetylation and its monomerization

Monomerization of ATM kinase is a key step in its activation. This process is preceded by KAT5-mediated acetylation that is followed by ATM autophosphorylation (Bakkenist and Kastan, 2003; Sun et al., 2005). It has been shown that ATM mutants that are unable to undergo KAT5-mediated acetylation can neither undergo autophosphorylation nor dissociate from a dimer state upon DNA damage (Sun et al., 2005). Moreover authors of

this report observed that catalytically inactive ATM despite KAT5-mediated acetylation remains in its dimer state (Sun et al., 2005).

In my work, I suggest that NOTCH1 blocks KAT5-mediated ATM acetylation, as I observed that NOTCH1 binding to ATM results in displacement of KAT5 from ATM. Although I have never performed an experiment to test whether NOTCH1 blocks ATM acetylation, it has been reported that impairment of the binding between KAT5 and ATM results in a lack of KAT5-medited ATM acetylation (Sun et al., 2005). This rises a question, why would NOTCH1 impair ATM acetylation when it is already blocking its kinase activity? It is possible that KAT5-mediated ATM acetylation may impact on the ability of ATM to recognize itself as a substrate and undergo autophosphorylation. Although NOTCH1 displaces KAT5 from its complex with ATM, it is necessary to mention that KAT5 has been shown to interact directly with NOTCH1 by binding (like ATM) to its Ankyrin repeats (Kim et al., 2007a). As a result, it is possible that ATM that is in a complex with NOTCH1 is still in a complex with KAT5, which instead of FOXO3a binds to ATM through NOTCH1. Therefore, performing additional experiments aimed at understanding the impact of NOTCH1 on ATM acetylation and monomerizarion upon DNA damage would clarify the mechanisms of NOTCH1-mediated ATM inactivation.

FOXO3a binding domain of ATM

It has been shown that FOXO3a interacts with ATM upon DNA damage induction (Tsai et al., 2008). I my experiments I showed that the formation of the protein complex between FOXO3a and ATM is independent from the presence of DNA damage. Although this observation differs from previously reported ones (Chung et al., 2012a; Tsai et al., 2008), it has to be stressed that in those studies authors in their immunoprecipitation experiments have always used an antibody against the phosphorylated form of ATM (Serine 1981), which did not bring down ATM in cells not exposed to DNA damage, as expected.

In the same study, it has been shown that FOXO3a binds to ATM within a domain spanning 1764-2841aa (Tsai et al., 2008). Though I observed some binding of FOXO3a in this region, I have detected a stronger binding with the use of the GST-ATM 2842-3056aa fragment. It is necessary to mention that for unknown reasons this particular GST-ATM fragment has not been tested in the report of Tsai et al. (Tsai et al., 2008). It is possible that such binding of FOXO3a to the 1764-2841aa region of ATM is the result of some non-specific binding of GST-ATM fragments, as I have observed some binding to this region also with FOXO3a or NOTCH1 (Vermezovic et al., 2015). This observation (interaction between FOXO3a and 1764-2841aa ATM region) can also be explained by the ability of ATM to form a homodimer (Bakkenist and Kastan, 2003). It has been shown that an ATM fragment containing kinase domain, can interact directly with the unphosphorylated ATM fragment containing ATM autophosphorylation site at Serine 1981, exactly like GST-ATM 1764-2138aa fragment with which the biggest FOXO3a-ATM interaction was observed (Tsai et al., 2008). Therefore it is possible that binding between 1764-2138aa GST-ATM fragment and FOXO3a reported by Tsai et al., is the result of the formation of a protein complex between GST-ATM fragment and endogenous ATM-FOXO3a complex. In addition to the results obtained with the use of GST-ATM fragments while determining ATMs FOXO3a binding domain, to prove the involvement of FATC domain in FOXO3a binding, I showed that ATM lacking FATC domain loses its ability to bind FOXO3a. In my opinion this set of results clarifies the domains of ATM involved in its interaction with FOXO3a.

Additionally, it is necessary to stress that it is not possible to exclude that the ATM region between 1764-2138aa contributes or stabilizes ATM-FOXO3a binding once created. For this purpose further experiments should be performed with the use of ATM mutants missing both FATC domain as well as 1764-2138aa region.

FOXO3a and the ATM-KAT5 interaction

In the initial report characterizing FOXO3a as a factor necessary for ATM activation upon DNA damage, an exact mechanism of FOXO3a-mediated ATM activation has not been elucidated (Tsai et al., 2008). I observed that FOXO3a impacts on ATM activation by bridging KAT5 binding to ATM. An interaction between KAT5 and ATM had been previously characterized (Sun et al., 2005). KAT5 has been reported to bind to the FATC domain of ATM, which is necessary for KAT5-mediated ATM acetylation (Jiang et al., 2006). Authors admitted that it was impossible to observe a direct interaction between ATM and KAT5 (Sun et al., 2010). Additionally, KAT5 has been shown to interact with ATM both in the presence and absence of DNA damage and that entire nuclear amount of ATM was in the complex with KAT5 (Sun et al., 2005).

Consistently with those studies, I have observed that FOXO3a as KAT5 binds specifically to the FATC domain of ATM but unlike KAT5, such binding is direct. Furthermore, the interaction between ATM and FOXO3a displayed features similar to ATM-KAT5 binding: the interaction was independent from DNA damage and the entire nuclear ATM was in a complex with FOXO3a but not all of FOXO3a was in a complex with ATM. This led me to the discovery that KAT5 interacts directly with FOXO3a. Although it was shown that some members of FOX family like FOXP3 and FOXR1 are able to interact with KAT5 (Li et al., 2007; Li et al., 2015), this ability has never been described for FOXO3a. Additionally, I have shown that the interaction between KAT5 and FOXO3a, like ATM-KAT5 and ATM-FOXO3a interactions, takes place independently from DNA damage.

To understand whether FOXO3a can mediate the interaction between ATM and KAT5, I studied the domains involved in binding between ATM, FOXO3a and KAT5. KAT5 was binding to both FOXO3a and ATM through the domain located at its C-terminal part (450-513aa), which suggested that KAT5 could not interact directly at the same time with ATM and FOXO3a. Furthermore, I observed that FOXO3a interacted with KAT5 through a domain located in its C-terminal fragment, between 620-650aa. At the same time I could

not detect any interaction between this FOXO3a fragment and ATM (data not shown). Consistently, FOXO3a domain necessary for interaction with ATM was characterized to be located between 618-625aa of FOXO3a (Tsai et al., 2008). Hence, those results suggest that FOXO3a can at the same time interact with ATM and KAT5, supporting a role of FOXO3a as protein bridging ATM-KAT5 interaction.

I have performed an immunoprecipitation of KAT5 in cells knockdown for FOXO3a. However, I have repeatedly observed only a partial reduction of the interaction between ATM and KAT5, despite a significant reduction of FOXO3a protein levels. This observation led me to the discovery that also other FOX-O family members, like FOXO1 and FOXO4, which share the conserved C-terminal structure with FOXO3a, display the ability to bind to KAT5. FOXO1 has been already reported to form a protein complex with ATM (Tsai et al., 2008). Additionally, it is possible that other FOX family members that were already characterized to interact with KAT5 (FOXP3; FOXR1) could bridge binding between ATM and KAT5. Finally, it is probable that even small levels of FOXO3a protein are sufficient for ATM activation, as I have observed that only a fraction of FOXO3a is in a complex with ATM.

RAM and Ankyrin repeats. NOTCH1 or something more?

I have observed that NOTCH1 interacts with ATM through its FATC domain. On the other hand, the domain of NOTCH1 I identified as necessary for ATM binding is the Ankyrin repeats domain. Unexpectedly, upon deletion of Ankyrin repeats, although I observed a decrease in the binding between ATM and NOTCH1, such reduction was partial. This result was consistent with a partial rescue of ATM activation by NOTCH1 Ankyrin repeats mutant as compared to wild type NOTCH1.

NOTCH1 has a complex structure with many domains that participate in the formation of transcriptionally active complex (for more details please see “Structure of the mammalian NOTCH receptors” chapter of introduction). It has been reported that both RAM and

Ankyrin repeats domains of NOTCH1 participate in formation of NOTCH1 transcription initiation complex through their interaction with CBF1 (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995). Thus, partial reduction of binding between ATM and Ankyrin repeats mutant of NOTCH1 might result from the presence of the RAM domain. It would be interesting to test whether the RAM domain contributes to the binding of NOTCH1 to ATM, as this information would be important for designing drugs targeting ATM-NOTCH1 interaction.

My results indicate that Ankyrin repeats probably together with the RAM domain are mediating NOTCH1 binding to ATM. Is it therefore possible that other proteins, which contain Ankyrin repeats or RAM domains interact with ATM? Indeed other Notch family members like NOTCH2, NOTCH3 and NOTCH4 possess Ankyrin repeats as well as RAM domain. I have never assessed if other Notch receptors can impact on DDR or form a protein complex with ATM. Thus, it is possible to conclude that NOTCH2, NOTCH3 as well as NOTCH4 receptors might indeed interact with ATM. Additionally, it would be interesting to investigate whether each of Notch receptors affect ATM activity in exactly the same way as NOTCH1, or differently.

It is entirely possible that in case of NOTCH4 receptor, which much smaller in size as compared to NOTCH1, the effect on ATM kinase activity would be different. As I have discussed before, it is possible that if NOTCH1 binding to ATM affects ATM substrate recognition, binding of protein/s of a different structure/size could affect ATM substrate recognition in a different way/manner as compared to NOTCH1. Indeed in case of Notch receptors this kind of DDR regulation could be additionally connected to their primary role as transmembrane receptors, transmitting signals from environment to the nucleus.

Is there more then one ATM complex?

It has been recently reported that ATM kinase is involved in the process of peroxisome autophagy (pexophagy)(Zhang et al., 2015). In this study authors have reported that ATM

can localize at peroxisomes. Recruitment of ATM to the outer membrane of peroxisomes is mediated by a newly discovered ATM interactor, peroxisomal biogenesis factor 5 (PEX5)(Zhang et al., 2015). It is important to mention that although PEX5 has been initially characterized to bind FATC domain due to the lack of ATM-PEX5 interaction in the FATC domain point mutant, it is still possible that PEX5 might be binding to N-terminal part of ATM (for more details please see "NOTCH1 and the ATM recruitment to the DSBs" chapter of discussion). Described above by Zhang et al. study provides new insight in ATM kinase biology, which till now was mainly focused around ATM involvement in the DDR.

PI3K-like kinases show many similarities, which are connected with their conserved structure, but also with their coactivators. ATM, ATR, DNA-PKcs and mTOR have been characterized to interact with their main coativators: MRN complex, PEX5, ATRIP, KU70/80, RICTOR and RAPTOR, through their N-terminal part (Ball et al., 2005; Davis et al., 2013; Watanabe et al., 2011; You et al., 2005). It has been recently described that binding of RICTOR, but not RAPTOR to mTOR kinase stimulates its newly discovered Tyrosine kinase activity (Wang and Proud, 2016; Yin et al., 2016). Thus, it is possible that due to structural proximity between FATC domain and N-terminal part of PI3K-like kinases, proteins binding to the N-terminal end of PI3K-like kinases may impact not only on the substrate recognition of PI3K-like kinases (like MRN in case of ATM) but also on their kinase activity (like RICTOR in case of mTOR).

Consequently, as explained above, ATM can form different protein complexes: with MRN complex as well as with PEX5 (ATM complex 1 and 2 respectively (ATMC1 and ATMC2))(Figure 42). Thus, it is possible that due to an involvement of different proteins in the formation of ATM complexes, each ATM complex might be characterized by a different substrate recognition as well as kinase activity, like the one described for mTORC2 (Wang and Proud, 2016; Yin et al., 2016)

It is necessary to mention that ATM has been already characterized to exist in two different complexes in the nucleus. It has been reported that there is an active competition between MRN complex and ATM interacting protein (ATMIN) for the binding to ATM (Zhang et al., 2014; Zhang et al., 2012). Competition between those two factors regulates ATM activity in response to different stimuli: MRN - double strand breaks, ATMIN - oxidative and hypotonic stresses (Kanu and Behrens, 2007; Kanu et al., 2010). This suggests that even within one possible ATM complex, competition between ATM coactivators may control its function and activity.

It would be interesting to investigate whether NOTCH1, which can affect the activation of ATM in a complex with MRN, can also perturb the activation of a different ATM complexes, like the one with ATMIN or PEX5. It is thought-provoking to describe ATM kinase as part of functionally different complexes, knowing that each of complexes might regulate ATM activity in a different way. Hence, I reckon that we should reconsider our knowledge in regard to ATM kinase activity. ATM may not be only phosphorylating its substrates on Serine and Threonine resides as previously established (Kim et al., 1999), but maybe also on Tyrosine ones. Based on above mentioned reports and discussion about ATM protein complexes, I have depicted below three possible ATM complexes, although it cannot be excluded that they may be even more (Figure 42).

In my work I have described that NOTCH1 blocks ATM kinase activity by inhibiting the formation of a three-protein complex with FOXO3a and KAT5. Moreover, I have showed that NOTCH1 possesses higher affinity for ATM binding then FOXO3a. It would be tempting to investigate whether NOTCH1 could impact on other ATM complexes, allowing for a distinction between different ATM complexes, their substrate recognition and functions. Thus, NOTCH1 might appear as an attractive tool to study possible differences in the activities of ATM complexes.

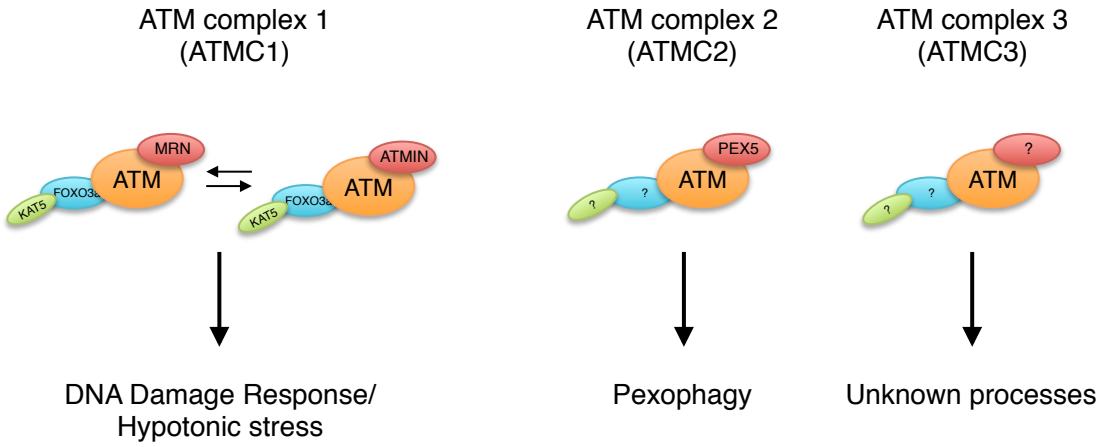

Figure 42. Scheme of the potential ATM complexes. ATMC1 - the most well described ATM complex involved in DNA damage response/hypotonic stress. ATMC2 - recently described ATM complex involved in the pexophagy. ATMC3 - ATM complex yet to be described.

How does NOTCH1 impact on DNA damage repair?

I have observed that besides ATM, NOTCH1 can bind to other PI3K-like kinases: ATR and DNA-PKcs. My analysis of ATR and DNA-PKcs kinase activities has showed that NOTCH1 was not able to inhibit ATR activation upon UV light irradiation. On the other hand, I have observed a dramatic decrease of ATM-mediated transphosphorylation of DNA-PKcs as well as DNA-PKcs autophosphorylation in NOTCH1 expressing cells. This observation was in contrast with the lack of an impact of NOTCH1 on DNA damage repair, which unexpectedly was virtually unaffected.

It has been reported that ATM-mediated transphosphorylation of DNA-PKcs is necessary for DNA-PKcs-mediated repair (Cui et al., 2005), as its inhibition may result in problems with DNA ends processing as well as dissociation of DNA-PKcs from DSBs, blocking access to XRCC4-LIGASE4 complex to DNA ends (Cui et al., 2005; Jiang et al., 2015; Uematsu et al., 2007). Therefore my results are in line with reports quoted above. More strikingly, lack of DNA-PKcs autophosphorylation (Serine 2056), which indicates perturbation in DNA-PKcs kinase activity, contradicts studies showing that cells expressing catalytically inactive DNA-PKcs kinase exhibit impairment in DSBs repair (Jiang et al., 2015; Kurimasa et al., 1999).

To understand this conundrum, it is necessary to stress that in the presence of NOTCH1 and a specific small molecule DNA-PKcs inhibitor, I have observed an inhibition of DNA damage repair. This directly shows that NOTCH1 is not inhibiting DNA-PKcs kinase activity, but rather just its autophosphorylation. Additionally levels of DNA damage repair inhibition by DNA-PKcs inhibitor were moderately increased in NOTCH1 expressing cells as compared to MOCK transfected cells. Thus, it seems that NOTCH1 does not inhibit DNA-PKcs kinase activity and therefore DNA damage repair, in agreement with published studies.

An explanation for these opposing results of DNA damage repair assay and inhibition of DNA-PKcs autophosphorylation may be related to the impact of NOTCH1 on ATM substrate recognition. We can speculate that NOTCH1 does not inhibit DNA-PKcs kinase activity but, rather, its substrate recognition that would result in an inability of DNA-PKcs to autophosphorylate itself, while at the same time being able to recognize and phosphorylate other substrates necessary for DNA damage repair. If correct, this is an exciting result showing that not all of DNA-PKcs dependent phosphorylations are necessary for DNA damage repair, something which has been already proven for some of DNA-PKcs substrates like: XRCC4, LIGASE IV, ARTEMIS (Goodarzi et al., 2006; Sharma et al., 2016; Wang et al., 2004) or KU70/80 heterodimer (Douglas et al., 2005). More importantly, NOTCH1-mediated impairment of DNA-PKcs substrate recognition, would allow researchers to investigate the difference between DNA-PKcs substrates phosphorylation in the presence or absence of NOTCH1 to identify the most critical targets of DNA-PKcs, necessary for DNA damage repair.

What is the difference between ATM and DNA-PKcs that allows DNA-PKcs to preserve its critical activity and facilitate DNA damage repair as compared to ATM? Although it has been shown that DNA-PKcs and ATM share the same domain structure, both proteins differ dramatically in size (ATM - 3056aa; DNA-PKcs - 4128aa)(Paull, 2015). This results in much bigger length of DNA-PKcs domains as compered to ATM (52aa difference for

FAT domain and 102aa difference for kinase domain) with the exception of the FATC domain (33aa each protein). This could explain a similar NOTCH1 binding ability toward both kinases. It is possible that the bigger size of DNA-PKcs FAT and kinase domains may result in a milder effect of NOTCH1 on its substrate recognition abilities as compered to ATM.

Another possible explanation for the differential effect of NOTCH1 on ATM and DNA-PKcs activities might be connected with the processes that lead to ATM and DNA-PKcs activation. The observed differences in NOTCH1-mediated inhibition of ATM and DNA-PKcs may arise from their different mechanisms of activation upon DNA damage. ATM in a process of activation undergoes acetylation followed by its autophosphorylation and monomerization (Bakkenist and Kastan, 2003; Sun et al., 2005). Differently, DNA-PKcs in response to DNA damage first forms a heterodimer with KU70/80 subunits and later a synapsis at the DNA ends with another DNA-PKcs- KU70/80 heterodimer (Spagnolo et al., 2006). Therefore if NOTCH1 inhibits ATM activation by blocking its monomerization mediated by KAT5 acetylation (Sun et al., 2005), maybe in the case of DNA-PKcs this form of inhibition does not occur.

I have observed that NOTCH1 displaces KAT5 from FATC domain of ATM. It has been reported that FATC domains of ATM, ATR and DNA-PKcs are functionally equivalent (Jiang et al., 2006). Therefore it is possible that NOTCH1 binds to DNA-PKcs through its FATC domain and impairs binding of KAT5. As the absence of KAT5 was shown to affect DNA-PKcs autophosphorylation (Jiang et al., 2006) it cannot be excluded that NOTCH1-mediated inhibition of DNA-PK autophosphorylation results from impaired interactions between KAT5 and DNA-PKcs. However it has been reported that KAT5-mediated acetylation on DNA-PKcs is of much lower level when compared with ATM (Jiang et al., 2006). Moreover, a recent study has shown that mutation of DNA-PKcs acetylation sites, has no impact on the efficiency of DNA damage repair or on the kinetics of DNA-PKcs recruitment to DSBs (Mori et al., 2016). It is necessary to mention that it has not been

assessed whether the DNA-PKcs acetylation sites analyzed were actual KAT5 target sites (Mori et al., 2016).

NOTCH1 has been shown to be expressed in neural stem cells (NSCs) (Hitoshi et al., 2002). Our group has reported that NSCs upon treatment with DNA damaging agents undergo differentiation (Schneider et al., 2013) It is possible to speculate that one of the roles of NOTCH1 in NSCs could be preventing DNA damage-induced differentiation, by inhibiting DDR signaling at its initiation point, that is ATM activation. This would be an effective mechanism to preserve NSCs identity. Indeed it has been shown that DNA damage-induced differentiation of NSCs into astrocytes is an ATM dependent process (Schneider et al., 2013). Every day cells of our body have to deal with substantial amounts of DNA damage (Ciccia and Elledge, 2010), thus it would be useful for an organism to have an mechanism to modulate DDR in the NSCs, allowing for the repair mechanism to deal with DNA damage, preventing at the same time from spontaneous differentiation and eradication of NSCs from organism.

NOTCH1 could tune down DDR and consequently with it apoptosis and stem cell differentiation, to favor DNA damage repair instead. Thus, one could ask whether NOTCH1 would favor an error-prone DNA damage repair by NHEJ, as HDR due to NOTCH1-mediated ATM inhibition should be perturbed (Bakr et al., 2015; Beucher et al., 2009). I have never tested HDR in NOTCH1 expressing cells, I cannot exclude that NOTCH1 could impact on HDR. It has been recently reported that inhibition of HDR factors in NOTCH1-driven tumor in *C. elegans* leads to its sensitization to DNA damage inducing agents (Deng et al., 2015). This result suggests that HDR is not affected in the presence of NOTCH1, despite the high radioresistance of those tumor cells, probably due to NOTCH1-mediated ATM inhibition (Deng et al., 2015).

Recently a report has been published showing that the stem cell specific transcription factor SALL4 favors ATM activation and DSBs repair in stem cells through its ability to bind to RAD50 and stabilize the MRN complex (Xiong et al., 2015). This study shows that

there are yet additional mechanisms, which can impact on DDR as well as DNA damage repair apart from NOTCH1. It is necessary to stress that while performing my experiments, I used systems in which I ectopically expressed large quantities of NOTCH1 (N1ΔE or N1IC constructs), which resulted in dramatic decrease of ATM kinase activity. It is thus unlikely that this kind of situation would ever take pace in biological/endogenous systems where the NOTCH1 expression levels are much lower. Indeed when we have performed assessment of DDR activation in endogenous NOTCH1 system, by coculturing together cells expressing NOTCH1 receptor with cells expressing NOTCH1 ligand, we observed that downregulation of DDR was only moderate although reproducible (Vermezovic et al., 2015).

I would like to propose that role of NOTCH1 in DDR is not a full ATM inhibition, rather moderate impairment of its activity, to modulate the balance between amount of DNA damage that cell has to deal with and signaling it induces. That kind of DDR modulation would result in suppression of DNA damage-induced cell apoptosis or in case of NSCs, differentiation, giving cells time for repair to occur.

Summary - working model of the ATM activation

Here I would like to summarize all of the results described in this thesis regarding the mechanism of NOTCH1-mediated modulation of ATM activation. Based on my observations that NOTCH1 mediates ATM inactivation by perturbing the formation of a three-protein complex (ATM, FOXO3a and KAT5) and published reports characterizing involvement of: NOTCH1 (Vermezovic et al., 2015), FOXO3a (Chung et al., 2012a; Tsai et al., 2008) and KAT5 (Ayrapetov et al., 2014; Jiang et al., 2006; Sun et al., 2005; Sun et al., 2010; Sun et al., 2009) in ATM activation, I have elaborated a model, which includes all of the results presented in this thesis and places them in the context of published studies (Figure 43).

In this model, I propose that upon DNA damage induction ATM is recruited to the DNA DSBs in a form of three-protein complex together with KAT5 and FOXO3a. KAT5 at the site of the damage interacts with tri-methylated histone H3K9 that is generated by SUV39h1 methyl trasferase immediately after DNA damage. This leads to the activation of KAT5 acetlytrasferase activity that results in acetylation. Upon acetylation ATM undergoes autophosphorylation, monomerization and starts phosphorylation of its substrates priming DDR cascade induction (Figure 43).

In the presence of NOTCH1, ATM is still recruited to DSB, but due to NOTCH1 binding to ATM, the formation of the three-protein complex between FOXO3a, KAT5 and ATM is perturbed. This results in the displacement of FOXO3a that mediates ATM-KAT5 interaction, from ATM. At the site of DNA damage, ATM in complex with NOTCH1 is unable to undergo activation due to the lack of KAT5-mediated acetylation. Therefore, ATM in complex with NOTCH1 despite being recruited to the DSBs persists in its inactive dimeric state (Figure 43)

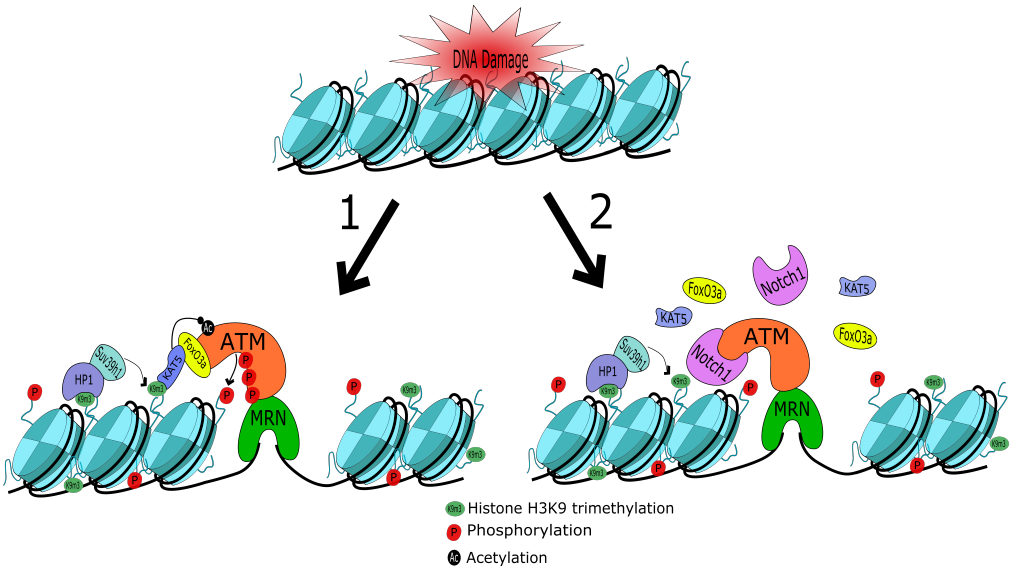

Figure 43. Working model of ATM activation in the absence (1) and presence (2) of NOTCH1.

On a hunt for an application

Based on the molecular mechanism of NOTCH1-mediated ATM inactivation, I have performed a set of experiments to evaluate if by increasing levels of FOXO3a in the

nucleus and by that competition between FOXO3a and NOTCH1 in NOTCH1-driven cancer cells, I could boost DDR activation. I have observed that with the use of drugs like Metformin and SB203580, I was able to increase DDR activation as well DDR-induced cell death in NOTCH1-driven cancers. Moreover I have observed similar effects with the use of NOTCH1 inhibitors (GSIs). As I have mentioned in the introduction, NOTCH1 has been proposed to be a prototypical oncogene (Pear et al., 1996). It has been reported that NOTCH1 is a target of many mutations and translocations that results in an increase in its expression and/or stabilization. Mutations affecting NOTCH1 have been shown to be very frequently present in T-ALL (almost 50% of cases) or breast tumors (Pece et al., 2004; Weng et al., 2004). Additionally it has been recently reported that NOTCH1 expression leads to the radioresistance of tumor cells in mice xenografts (Theys et al., 2013). Therefore increasing the competition between NOTCH1 and FOXO3a for ATM binding could occur beneficial in combination with genotoxic agents. Due to a high toxicity of GSIs and their side effects, it may not be feasible to use GSIs in the near future in a combinational therapy with DNA damage inducing agents to treat NOTCH1-driven cancers (Andersson and Lendahl, 2014). Indeed, no GSI have been approved by the Food and Drug Administration (U.S.A.) for the treatment of NOTCH-driven cancers (Andersson and Lendahl, 2014).

Unlike GSIs, Metformin is a non-toxic drug widely used in treatment of the type II diabetics (Witters, 2001). Although more in vivo experiments are necessary, my results fit with published reports indicating that Meformin can induce ATM activation in FOXO3a dependent manner (Hu et al., 2014; Vazquez-Martin et al., 2011). Additional studies have showed Metformin-mediated increase in DDR activation (Fasih et al., 2014; Menendez et al., 2011). It is necessary to mention that I have observed a striking effect of SB203580 on DNA damage-induced cell death. Such effect can be attributed to a complex role of p38 kinase in different cellular processes, indeed it has been shown that p38 can play a pro-survival role in the response to the DNA damage (Thornton and Rincon, 2009).

Apart from using drugs already available, it could be useful to search for a new inhibitor targeting specifically ATM-NOTCH1 interaction. As NOTCH1 and FOXO3a compete with each other for the ATM binding, a small molecule inhibitor that would target and inhibit binding between ATM and NOTCH1 at the same time should not be able to impair ATM-FOXO3a interaction. It should therefore target directly the NOTCH1 domain involved in the interaction with ATM. A compound with these features might be useful not only in the previously mentioned T-ALL cancers, but also in other types of tumors, in which DDR is inhibited by NOTCH1.

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Acknowledgments

Na samym początku chciałbym podziękować swoim Rodzicom, którzy inspirowali mnie przez cały czas.

Chcialbym też przed wszystkim podziękować Jelenie Vermezovic, która wzięła mnie pod swoje skrzydła i była moim naukowym opiekunem w przeciągu trwania mojej pracy w IFOM.

Serdeczne podziękowania dla Fabrizio d'Adda di Fagagna za stworzenie możliwości i warunków do przeprowadzenia powyższej pracy.

Kończąc chciałbym podziękować wszystkim których udało mi się poznać w Mediolanie.

Dziękuję za życzliwość, cierpliwość i wspomnienia.