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**A Novel Function of Tumor Suppressor Menin in the  
Cellular Response to DNA Damage**

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## **1. INTRODUCTION**

### **1.1 Multiple endocrine neoplasia type 1**

Multiple endocrine neoplasia type-1 (MEN1) is an autosomal dominantly inherited disease caused by mutations in the Men1 tumor suppressor gene. The main endocrine expressions of MEN1 are hormone hypersecretion due to clonal tumors of the parathyroids, enteropancreatic neuroendocrine tissue and anterior pituitary (Marx SJ, 2002). The main non hormonal manifestations of MEN1 are facial angiofibroma, lipoma, collagenoma, meningioma, and smooth muscle tumor. Men1 mutations can contribute to hereditary and non hereditary tumors. Germline and somatic mutations are distributed over the entire Men1 encoding region, without mutations hot spots and no suggestion of a genotype-phenotype correlation (Wautot et al, 2002).

Men1 gene was identified by positional cloning in 1997 and mapped to chromosome 11 (Chandrasekharappa S.C. et al, 1997). The product of Men1, Menin, encodes a protein of 610 amino acids residues (Chandrasekharappa S.C. et al, 1997). Menin harbors two nuclear localization signals at the C terminus and it has been detected in the nucleus in different cell types (Guru S.C. et al., 1998) (Ikeo Y. et al., 1999) (Stewart C. et al., 1998) but its subnuclear distribution is not yet clear.

## **1.2 *Men1* is a tumor suppressor gene**

Kinzler and Vogelstein introduced the term 'gatekeeper' to describe a class of genes that directly control cellular proliferation and whose dysregulation is necessary to cause neoplastic growth in specific tissues (Kinzler, K.W et al., 1997). Gatekeepers include many classical tumor suppressors, such as RB1 and APC. Inactivation of both homologs of these genes, often through mutation of one copy and deletion of the second copy, is found both in hereditary forms of cancer and in the great majority of sporadic tumors of the types seen in the hereditary disease.

Another class of tumor predisposition genes, the 'caretakers' does not directly affect growth control. The loss of caretakers causes genomic instability and promotes mutation of gatekeepers and other genes directly involved in growth control. Mutations in caretakers, for example BRCA1-2 and HNPCC, are not often found in sporadic cancers.

Tumors in *Men1* arise through a two hit mechanism (Larsson et al., 1988) (Friedman E. et al., 1989) (Thakker R.V., et al., 1989). Typically, loss of function of *MEN1* occurs as a single-base DNA change within *MEN1* for a first hit and a large rearrangement of chromosome 11 for the second hit (Pannett A.A. et al, 2001)

As mentioned above, this genetic mechanism of pathogenesis is typical of classic tumor suppressor genes, as Rb or hereditary cancer genes involved in the maintenance of genomic integrity as BRCA1 and HNPCC.

MEN1 is a tumor suppressor gene by several criteria:

- First of all it has shown that Menin overexpression in tumor cells partially suppresses the tumor phenotype. Infact initial studies have revealed that menin overexpression inhibits the proliferation of Ras transformed fibroblasts (NIH3T3) in soft agar as well as the Ras induced tumor growth in nude mice (Kim Y.S. et al, 1999).
- Most of the germline and somatic MEN1 mutations lead to protein truncation or absence. In this way the mutations cause “loss of function” of the gene, similar to the mutations of the most human tumor suppressor genes.
- Loss of heterozigosity at 11q13 is evident in most of the tumors of MEN1 syndrome as well as in many sporadic tumors with a Men1 mutation in the other allele.
- Complete loss of Menin has been identified by immunohistology in tumors from patients with Men1 or from mouse models of Men1.
- Similar to knockout of the most human tumor suppressor genes tested in mice, homozygous loss of Menin causes death in mice in utero.
- Furthermore mice with heterozygous Men1 loss gestate normally but after 9-16 months, they develop endocrine tumors similar to those observed in the human Men1 disease.

### ***1.3 Menin interacts with several nuclear factors involved in transcription's regulation***

The biological role of Menin in tumorigenesis remains unknown at this time. Protein database searches fail to demonstrate any protein sequences similar to Menin and therefore do not provide clues to its function. Unlike other tumor suppressor proteins such as p53, MEN1 disease-associated missense mutations are distributed along the length of the protein and therefore fail to reveal a specific site to focus study (Guo SS et al, 2001) Investigative efforts aimed to identify Menin's function by studying interacting proteins have not resulted in a clear canonical biological pathway involving endocrine tumor development.

To date, Menin has been found to interact with a variety of transcription factors, suggesting menin may regulate transcription (Figure 1). Among these proteins there are a series transcription factors involved in cell survival and apoptosis regulation such as JunD. JunD, which belongs to the AP1 family, and was the first Menin partner to be identified. Menin binds to the amino terminus of JunD (Agarwal S.K. et al., 1999) and when overexpressed it inhibits JunD-dependent transcription. Although JunD does not promote cell proliferation and has been proposed to act as suppressor of cell growth (Pfard et al., 1994), in absence of menin expression it switches from a growth suppressor to a growth promoter activity (Agarwal S.K. et.al., 2003), suggesting a possible of Menin induced tumorigenesis.

In addition to these studies, we have shown that Menin functions as a suppressor of MAPK induced phosphorylation of JunD, c-Jun without directly altering the activity of ERK and JNK kinases and the activity (Gallo A. et al., 2002). Besides, we also found that Menin overexpression represses serum-induced activity of the c-fos promoter, by down-regulating ERK-dependent transcriptional activity of Elk1 (Gallo A. et al., 2002). These findings suggest that Menin uncouples ERK and JNK activation from phosphorylation of their nuclear targets and inhibits the accumulation of Fos/Jun heterodimers.

Menin is also necessary for the transcription of transforming growth factor  $\beta$ -responsive genes, an activity mediated through a direct interaction of Menin and Smad3 (Kajii H. et al., 2001).

While Menin does not influence Smad3-Smad4 dimerization or nuclear localization, it enhances Smad3 DNA binding and Smad3-mediated transcriptional activation. The finding that Menin is a key regulator of growth inhibitory signals arising from the activation of the transforming growth factor  $\beta$  pathway strongly supports its role as a tumor suppressor.

Menin also interacts with NF $\kappa$ B proteins involved in immune response, in cellular transformation and oncogenesis and it represses p65 mediated transcriptional activity (Heppner C. et al., 2001).

Menin interacts with ASK (activator of S phase kinase) a component of cdc7/ASK kinase complex that is crucial in DNA replication and repair (Schnepp R.W. et al., 2004). Menin completely represses ASK induced cell proliferation.

Besides, Menin is also involved in epigenetic regulation of transcription by associating with nuclear proteins involved in



chromatin modification. In fact Menin interacts with mSin3A, a subunit of histone deacetylase complex (Hyungsoo et al.,2003). Moreover a recent report has shown that Menin is associated with a Histone methyl transferase complex (HMTase complex) containing two trithorax family members: MLL2 and Ash2L. This complex can methylate H3 on Lys 4, an epigenetic mark associated with transcriptionally active chromatin (Hughes et al., 2004).

In conclusion, a large number of studies suggest that Menin may control gene expression by interacting either with transcription factors or with histone modifying enzymes. In agreement with this model, Elledge and coworkers have shown Menin-dependent repression of human telomerase promoter, suggesting a potential mechanism through which Menin may inhibit the proliferation of tumor cells ( Lin S.Y. et al., 2003).

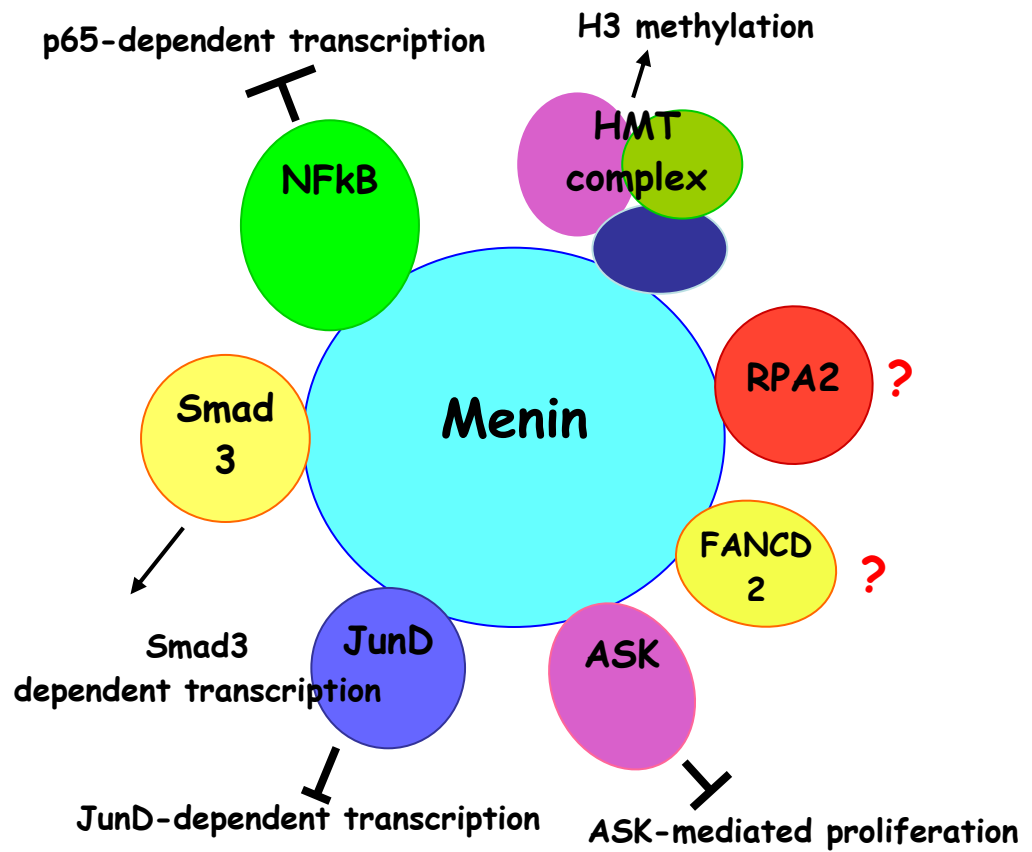


Figure 1 Menin interacts with several nuclear factors

#### ***1.4 Menin and the DNA damage response***

The identification of two further Menin-interactors, as Replication protein A (RPA) and the Fanconi Anemia protein FANCD2, both involved in multiple pathways of the DNA damage response, has brought up a potential role of the MEN-1 gene in the maintenance of DNA integrity.

FANCD2 is a protein encoded by a gene mutated in patients with an inherited cancer prone syndrome, Fanconi Anemia. This syndrome is an autosomal recessive disorder characterized by genomic instability and hematological cancers (D'Andrea et al., 2003). Besides FA cells are hypersensitive to the cross-linker agent mytomyacin C. However biallelic disruption of FANCD2 results both in mytomyacin and IR hypersensitivity (D'Andrea et al., 2003). In response to IR, ATM phosphorylates FANCD2, an event that is required for S-phase checkpoint (Taniguchi et al., 2002). Interestingly, FANCD2-Menin interaction is increased by  $\gamma$  -irradiation suggesting that these proteins cooperate in repairing DNA damage (Jin et al., 2003).

The 32KDa subunit of RPA (RPA2), with which Menin interacts (Sukhodolets KE et al., 2003), is the homolog of SSB protein in *Escherichia Coli*. It is an heterotrimeric protein that binds ssDNA and it is required for DNA replication, recombination and repair.

A further Menin-interacting protein involved in DNA replication and repair is ASK (activator of S phase kinase), a component of cdc7/ASK kinase complex (Schnepp R.W. et al., 2004). Menin interaction completely represses ASK induced cell proliferation.

To corroborate a potential role of Menin in maintaining genome integrity, it has been shown that Menin depletion leads to increased sensitivity to DNA damage, either in embryonic mouse fibroblasts or *Drosophila* larvae (Schnepp R.W. et al., 2004) (Busygina V. et al., 2004).

More recently, it has been shown that either *Drosophila* larvae or mouse embryonic fibroblasts lack of Menin expression is linked to a deficiency for a DNA damage-activated S-phase checkpoint, through interaction suggesting with the forkhead transcription factor CHES1 (FOXN3), a component of a transcriptional repressor complex, that includes mSin3a, histone deacetylase 1 and 2 (Busygina V et al., 2006).

Furthermore, Menin localizes to chromatin after UV irradiation in an ATR-CHK-dependent fashion (Farley S.M. et al., 2006).

In addition to these recent findings, previous studies have associated MEN-1 syndrome with chromosomal instability (Tomassetti et al., 1995) and loss of DNA integrity (Hessman et al., 2001).

In view of these observations I have decided to study the role of Menin in the DNA damage response and in particular in the DNA damage activated checkpoints

#### **1.4 The cell cycle checkpoints**

To ensure survival and propagation of accurate copies of the genome on to subsequent generations, eukaryotic cells respond to damaged or abnormally structured DNA by a multifaceted response that coordinates cell cycle progression with DNA repair, chromatin remodeling, transcriptional programs and other metabolic adjustments or cell death.

The cell cycle checkpoints are defined as biochemical signalling pathways that sense various types of structural defects in DNA, or in chromosome function, and induce a multifaceted cellular response that activates DNA repair and delays cell-cycle progression (Zhou B.B. et al., 2000) (Nyberg, K. A. et al. 2002). These biochemical cascades include sensor proteins that monitor the genome for any abnormalities and help generate the signals that are amplified and propagated by adaptors/mediators and signal transducers to downstream checkpoint effectors that connect the checkpoints with the core cell cycle machinery (Zhou B.B. et al., 2000). To ensure faithful replication and transmission of the genome and to promote survival, checkpoints fulfil at least four tasks: they rapidly induce cell-cycle delay, help activate DNA repair, maintain the cell-cycle arrest until repair is complete, and then actively re.initiate cell-cycle progression. Checkpoint malfunction leads to accumulation of mutations and chromosomal aberrations, which in turn increase the probability of developmental malformations or genetic syndromes and diseases including cancer.

Most checkpoint pathways operate only in cycling cells, which are at higher risk of fixing and propagating deleterious

mutations. But even among proliferating cells, the choice of checkpoint cascade to be alarmed, and the outcome of such response, depends on many variables. These factors include the type, extent and duration of the DNA damage stimulus, the type of cell cycle, the cell type and differentiation stage, and the position of the cell within the cell cycle.

In regard to cell cycle, we can distinguish three different cell cycle checkpoints: G1 DNA damage checkpoint that arrests the cell cycle in G1 before DNA replication; S-phase DNA damage checkpoint that arrests the cell cycle in S-phase and G2 DNA damage checkpoint that blocks the cell cycle in G2 phase before mitosis (Lukas J. et al., 2004) (Shiloh Y. et al., 2003).

During my PhD I have focalized my study on the function of Menin in the S phase checkpoint. This checkpoint is activated by genotoxic insults and causes only a transient and reversible delay in cell cycle progression, mainly by inhibition of new replica initiation and so slowing down DNA replication.

The main sensors for S phase checkpoint are two proteins that belong to PIKK (phosphatidil-inositol-3 kinase-like kinase) family: ATR (ATM and Rad3 related protein kinase) and ATM (ataxia teleangectasia mutated) (Abraham R.T. et al., 2001).

In response to DSBs induced by ionizing radiations or chemical agents, there is the activation of ATM (Pandita T.K. et al., 2000). ATM activated triggers two cooperating parallel cascades to inhibit the replicative DNA synthesis. ATM phosphorylates chk2 on thr 68. Activated chk2 induces, by phosphorylation, the ubiquitin mediated degradation of cdc25A phosphatase. ATM also initiates a second pathway by phosphorylating Nbs1: this protein was found in a complex with

two other genome maintenance proteins, Mre11 and Rad50 (MRN complex) which plays an important role in the recombinational repair of DSBs (D'Amours D. et al., 2002) (Petrini, J. H. et al., 2003). This complex processes the termini of DSBs before initiation of strand invasion by Rad51.

In contrast of ATM as a sensor of DSBs, when DNA is damaged by UV or chemicals that induce SSBs, the main DNA damage sensor is ATR. ATR binds to chromatin and it can bind directly to UV induced lesions or to RPA coated single stranded DNA and it becomes activated. Active ATR phosphorylates chk1 which delays cell cycle progression through S or G2 phases by inhibiting the Cdc25A and Cdc25C phosphatases (Bartek J. et al., 2004). In this way it inhibits firing of replication origins.

As for the ATM mediated checkpoint, ATR mediated pathway results in the phosphorylation of BRCA1 and Nbs1 that are implicated in homologous recombination mediated DNA repair.

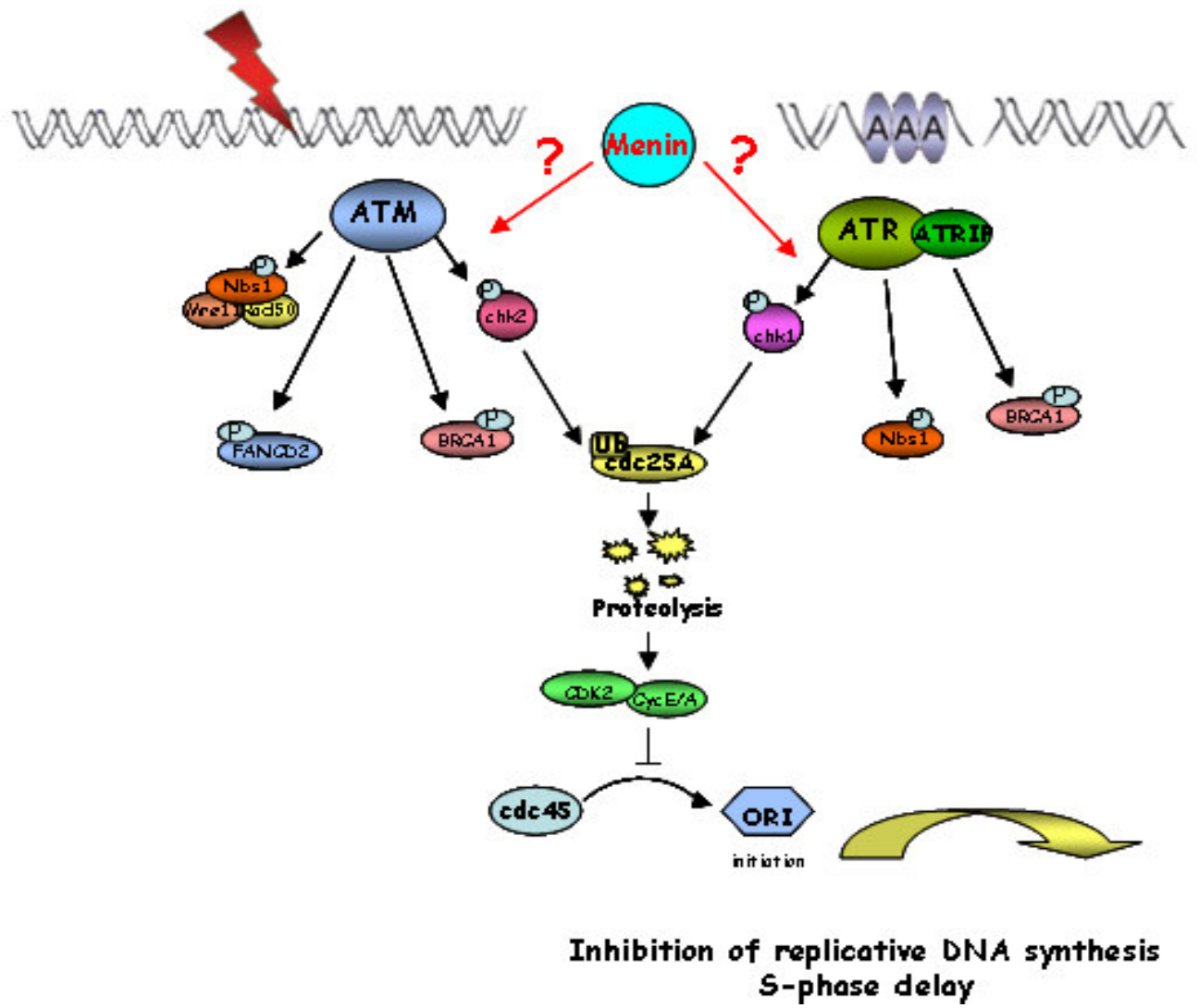


Figure 2 *ATM and ATR mediated S-phase checkpoint*



### **1.5 Double strand breaks and recombination-directed repair**

Maintenance of genome stability relies on the accurate repair of double strand breaks (DSBs) that arise during DNA replication or from DNA damaging agents. Failure to repair such breaks can lead to the introduction of mutations, chromosomal translocations, apoptosis and cancer. Hence, in order to preserve genome integrity, cells have evolved processes to respond and repair DSBs.

In eukaryotes, DNA double strand breaks are repaired mainly by two distinct but interconnected pathways: non homologous end joining (NHEJ) and homologous recombination (HR) (Van Gent D.C. et al., 2001) (Khanna K.K. et al., 2001) (Valerie K. et al., 2003).

Different studies have demonstrated that NHEJ is the predominant form of DSBs repair in G<sub>0</sub>/G<sub>1</sub> cells while HR operates mainly during S and G<sub>2</sub> phases (Essers J. et al., 2000). The NHEJ pathway occurs between sequences with little or no sequence homology.

During NHEJ, the two ends of a DSB are rejoined by simple ligation after little or no nucleolytic processing of the ends. In human this pathway involves the Ku70/80 heterodimer, DNA PK and the Lig4/Xrcc4 ligase.

Since NHEJ is often accompanied by mutations and small deletions at the break-site, this pathway is generally considered error prone.

In mammalian cells, homologous recombination has emerged as the major mechanism for the error-free homology-directed repair of DSBs. In contrast to NHEJ, HR requires extensive 5' resection to generate regions of single-stranded DNA at the break-site. The single stranded ends can subsequently invade a homologous template to prime DNA synthesis and copy and ultimately restore genetic information disrupted by the DSB (Figure 3). Since genetic information is preferentially copied from an intact sister chromatid, this pathway is error-free (Symington L.S. et al., 2002).

Homologous recombination involves the exchange of DNA between sequences of perfect or near perfect homology over several hundreds of base pairs. The process of homologous recombination plays essential roles in the mitotic and meiotic cell cycles of most eukaryotic organisms. It is now widely recognized that the primary function of homologous recombination in mitotic cells is to repair doublestrand breaks (DSBs) that form as a result of replication fork collapse, from processing of spontaneous damage, and from exposure to DNA-damaging agents. In mammalian cells faulty repair of DSB compromises tissue and organ function, and favours the development of cancer and degenerative diseases.

The double strand break (DSB) repair model provides a prototypic molecular mechanism for homologous recombination. It consists of (Figure 3):

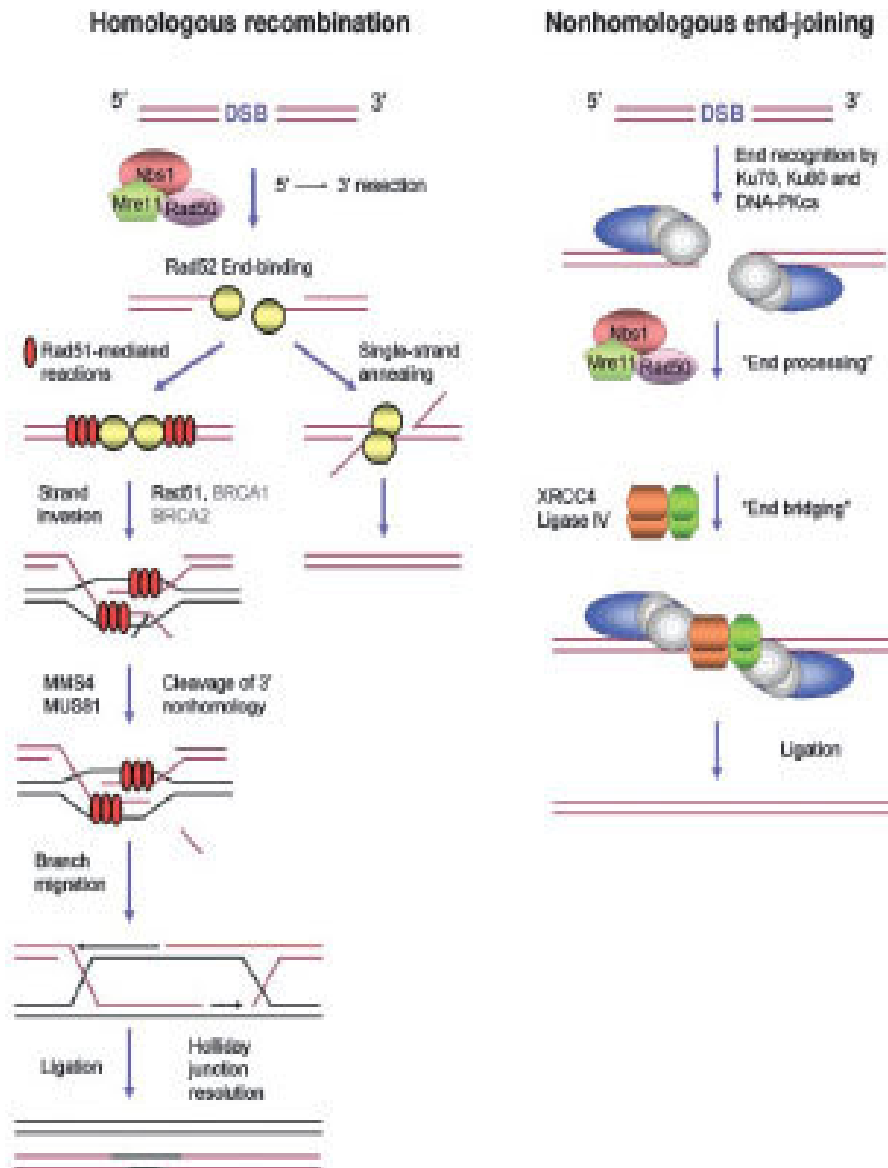
- 1) introduction of the DSB
- 2) processing of the DSB to produce tailed DNA with 3'-ssDNA overhang

- 3) invasion of one ssDNA end into homologous dsDNA
- 4) subsequent invasion or annealing of the other processed end
- 5) DNA synthesis and ligation to form double Holliday junctions
- 6) branch migration and resolution of the double Holliday junctions.

The proteins participating in the DSB repair pathways are highly conserved from yeast to humans, as in the nuclear organization of the repair processes.

In humans, efficient HR requires replication protein A (RPA) and the proteins of the Rad52 group including the Mre11/Rad50/Nbs1 (MRN) complex. Proper DNA damage checkpoint activation requires the MRN complex, Rad17, Rad9/Hus1/Rad1 and ATR/ATRIP complexes and ATM, as well as a number of adaptor proteins including BRCA1 and claspin, and effector kinases Chk1 and Chk2.

The central activity of HR is conferred by the Rad51 protein, a eukaryotic homolog of the *Escherichia coli* RecA recombinase, which catalyses the invasion of the broken ends of the DSB into the intact sister chromatid. Human Rad51 is a relatively small protein (38 Kda). It is functional as a long helical polymer, made of hundreds of monomers, that wraps around the DNA to form a nucleoprotein filament. The relevance of Rad51 in the HR is underlied by the observation that cells deficient in Rad51 accumulate DSBs after replication or at stalled replication forks (Sonoda E et al., 1998).



**Figure 3 Double-strand break/recombinational repair.**

Double-strand breaks are repaired by either homologous recombination, which in eukaryotes depends on the Rad51-family proteins, or by nonhomologous end-joining mediated by the DNA-PK complex. A key intermediate in homologous recombination is the Holliday intermediate, in which the two recombining duplexes are joined covalently by single-strand crossovers.

## **2. RESULTS**

### ***2.1 Menin overexpression promotes the phosphorylation of Chk1 and Chk2 in response to etoposide***

ATM and ATR kinases are central component of the DNA damage response. Upon DNA damage, they activate a network of damage-response pathways by phosphorylating cellular target proteins that activate DNA repair pathways and cell cycle checkpoints. Among these downstream effectors, activated checkpoint kinases Chk1 and Chk2 lead to cell cycle block by inhibiting the Cdk2/Cdc7-Cdc45 cascade that is required for assembling of pre-replication complexes (Sancar A. et al., 2004).

I have investigated the effect of Menin over-expression on the ATM/ATR dependent phosphorylation of Chk1, Chk2 and other proteins involved in the activation of checkpoints, in response to etoposide. Etoposide is an inhibitor of topoisomerase II, causing first the accumulation of DNA double strand breaks (DSB), and later the generation of DNA single breaks (SSB). Chk1 is mainly activated by ATR in a SSB-dependent fashion, while Chk2 is mainly activated by ATM in a DSB-dependent fashion .

To this aim I have transiently transfected Hela or H-293 cells with either an empty vector or a Menin expressing vector.

After transfection, cells were treated with etoposide at 5micromol concentration for different time points, as indicated in the figure 4.

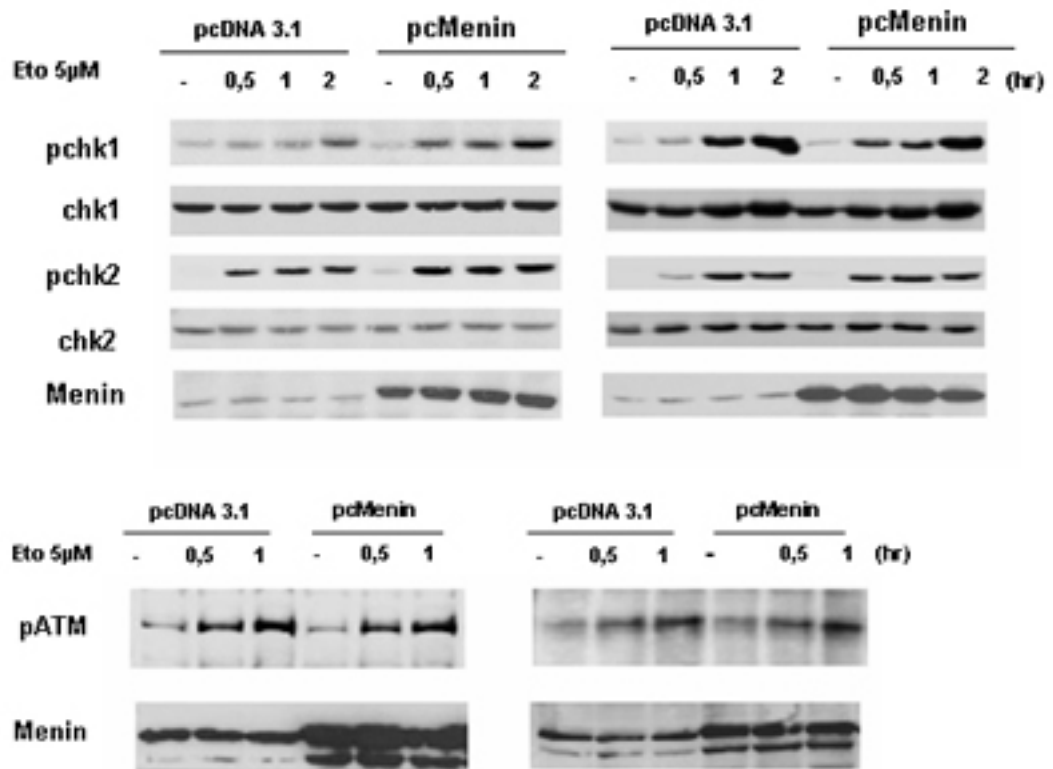
As shown in figure 4, Menin overexpression promoted Chk1 phosphorylation in response to etoposide already after 0,5 hr of treatment as compared with the control and the activation was kept on for two hours.

Similarly, in HEK 293 cells Menin overexpression anticipated the induction of Chk1 phosphorylation by etoposide without affecting its duration time. To at least extent, Menin overexpression also increased Chk2 phosphorylation. Furthermore, Menin overexpression doesn't influence the etoposide-induced autophosphorylation of ATM on serine 1981 or of H2AX (data not shown), ruling out the possibility that it may increase the rate of DNA damage

ATR-mediated activation of Chk1 requires RPA-dependent recruitment of Rad17 and Rad9 complexes at the DNA-damaged sites (Zou L. et al., 2005). Since Menin has been shown to interact with RPA (Sukhodolets K. E. et al., 2003) I investigated whether Menin regulates the recruitment of Rad17 and Rad9 complexes at the site of DNA damage.

As shown in the Figure 5 (panel B), Menin over-expression had no effect on either Rad17 or Rad9 retention to chromatin. Similarly, Menin had no effect on etoposide induced phosphorylation of RPA or p53 (Figure 5, panel A).

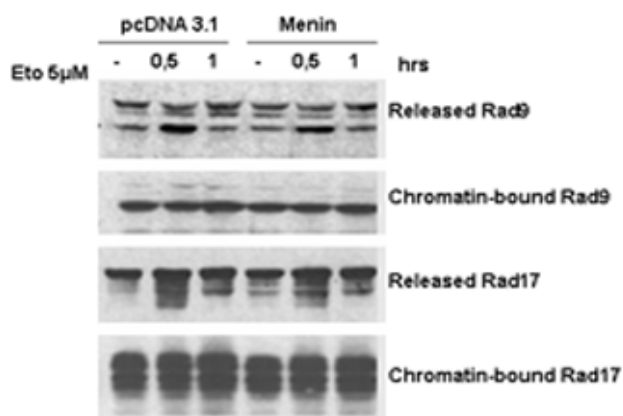
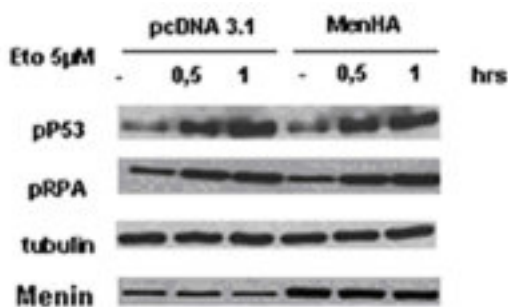
These findings suggest that increased levels of Menin expression specifically promotes Chk1 phosphorylation in response to DNA damage, rather than enhancing overall activation of ATM or ATR dependent pathways,



**Figure 4** *Menin overexpression promotes the phosphorylation of Chk1 and Chk2 in response to etoposide but doesn't influence ATM activation.*

To analyse the effect of Menin overexpression on the ATM/ATR mediated pathways, H-293 and Hela cells were transfected either with an empty vector or with a Menin-encoding vector. 24 hrs after the transfection cells were treated with 5 micromol etoposide and western blot analysis were performed with the indicated antibodies.

**A**



**B**

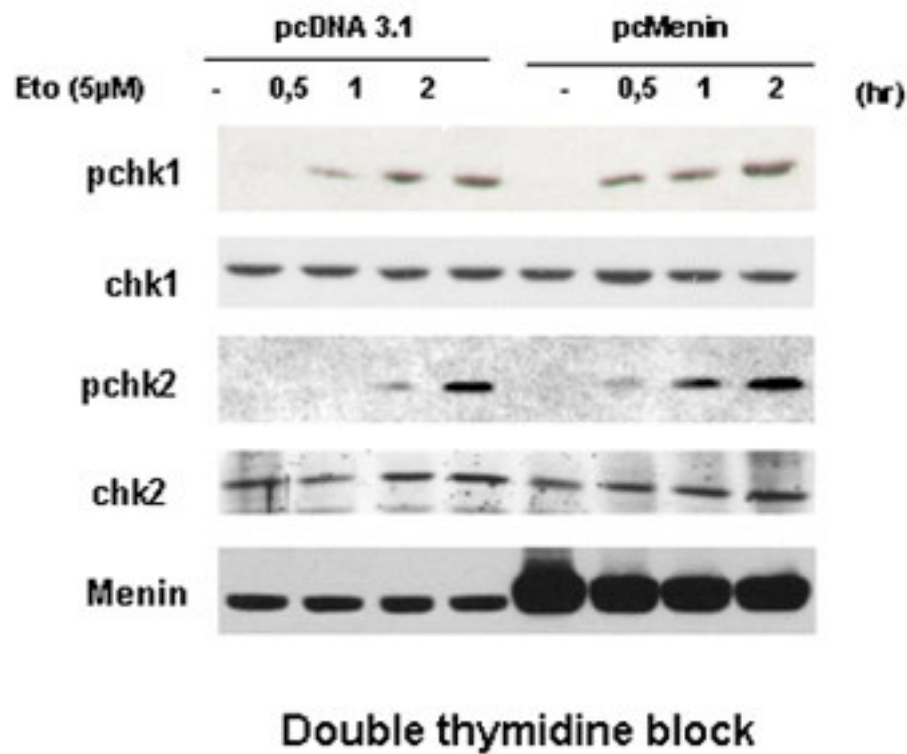
***Figure 5 Menin overexpression has no effect on the activation of RPA, p53, Rad9 and Rad17 proteins***

HeLa cells were treated with etoposide and A) whole extracts and B) fractionated extracts were analyzed by western blot performed with the indicated antibodies. Has shown in the figure Menin doesn't influence the activation of other proteins involved in the DNA damage checkpoints.



## ***2.2 Menin overexpression promotes the phosphorylation of Chk1 and Chk2 in S-phase arrested cells***

To distinguish between G1 and S-phase checkpoint we assessed the effect of Menin overexpression in HeLa cells that were arrested in S-phase by double thymidine block, and then treated with etoposide for different time points after release of thymidine. As shown in the figure, Menin significantly anticipate Chk2 phosphorylation suggesting that it affects S-phase checkpoint. At a least extent Menin also affected Nbs1 and Chk1 phosphorylation.

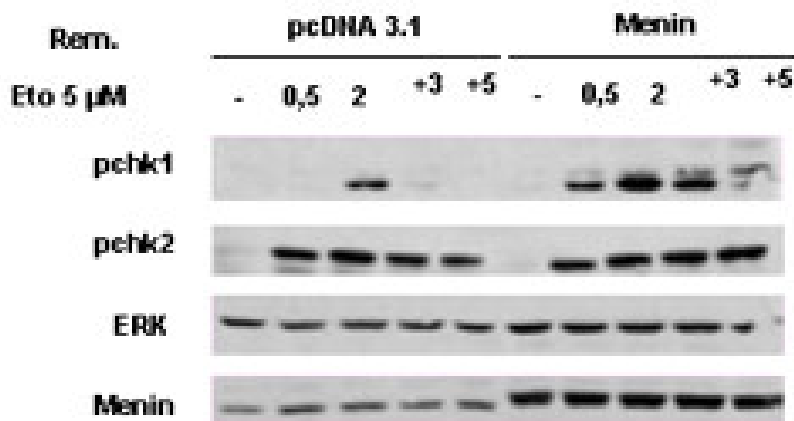
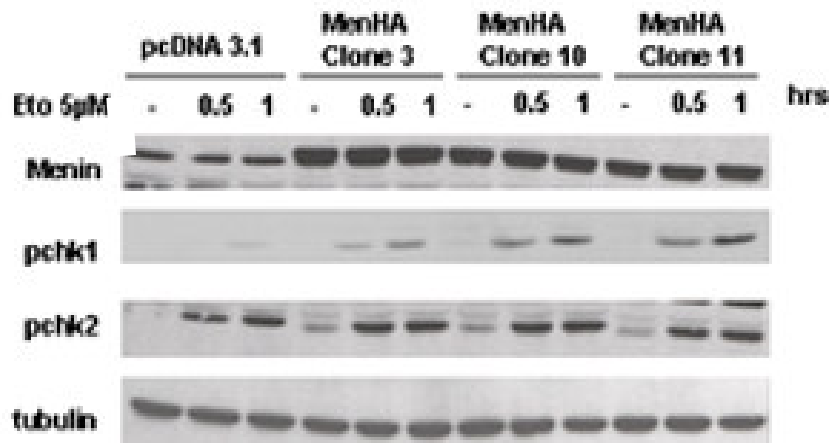


**Figure 6** *Effect of Menin in S phase arrested cells*

Hela cells were synchronized at the beginning of S phase by double thymidine block: 24 h incubation with 1 mM thymidine, an interval of thymidine-free incubation for 8 h, and second thymidine incubation for 14 h. The cells were treated with etoposide and western blot analysis were performed with the indicated antibodies.

### ***2.3 Stable Menin overexpression promotes and protracts the activation of S-phase checkpoint***

To further study the effect of Menin on Chk1 and Chk2 phosphorylation we generated HEK-293 cell lines stably overexpressing Menin. As shown in the Figure 7 three different HEK-293 clones overexpressing Menin showed enhanced phosphorylation of Chk1, but not Chk2, when treated with etoposide. HEK-293/MenHa clones 3 and 10, were further analysed to investigate the effect of Menin over-expression on the extension time of Chk1 and Chk2 phosphorylation after removal of etoposide. As shown in figure, in the control cell line etoposide-induced phosphorylation of Chk1 was turned on 1 hour after the treatment and disappeared after removal of the drug, while Chk2 phosphorylation was detectable already at 30 minutes and remained on after removal of the drug. Presumably, the different kinetic of Chk2 and Chk1 phosphorylation reflects the different kinetic of DSB and SSB accumulation and consequently the different kinetic of DSB mediated activation of ATM and the SSB-mediated activation of ATR. Interestingly in MenHa-clone 3 the kinetic of Chk1 phosphorylation was similar to that of Chk2. In contrast, neither H2AX or ATM phosphorylation changed (data not shown), indicating that Menin over-expression did not cause DNA damage. All the data discussed above, were obtained also with MenHa clone 10 (data not shown). These results suggest that Menin might induce ATM-mediated phosphorylation of Chk1.

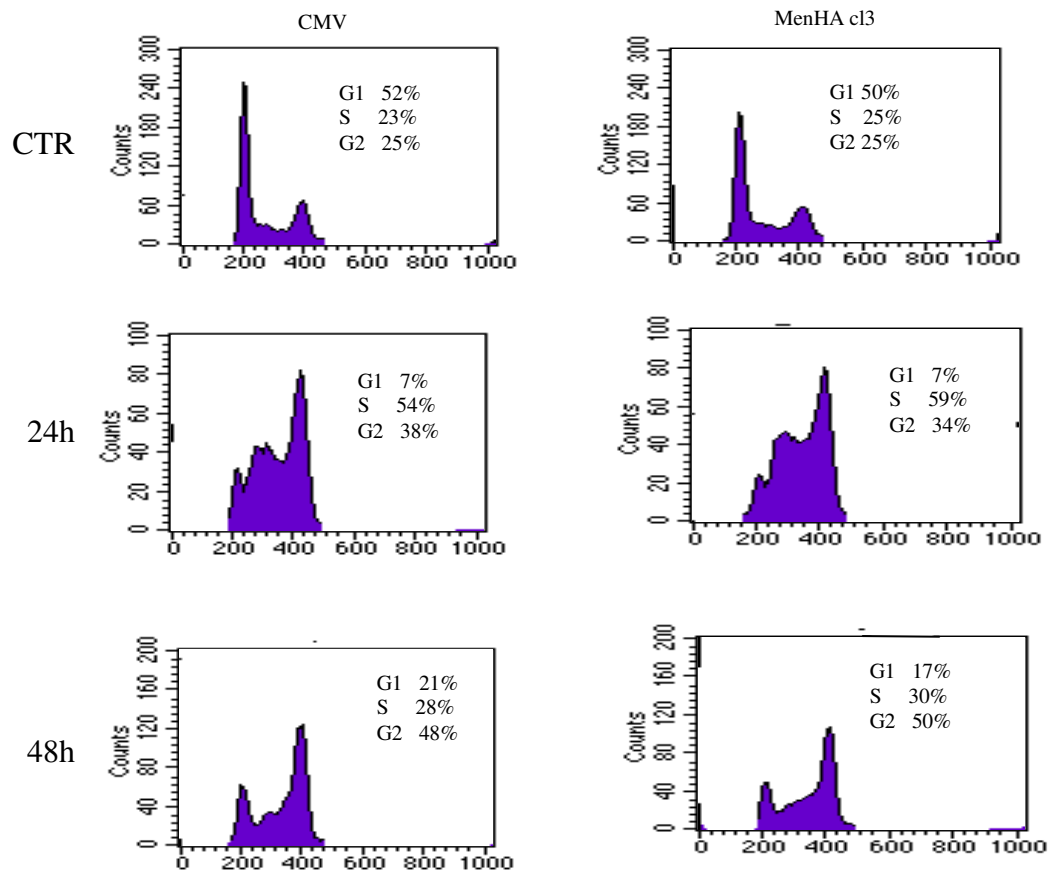
**A**

**Figure 7 *Menin protracts Chk1 phosphorylation in response to etoposide treatment***

H-293 clones stably expressing HA-tagged Menin, or the control pcDNA3.1 empty vector, were treated either with etoposide for 0.5 or 1 hour, as indicated. Western blot analysis were performed with the indicated antibodies. As shown Menin overexpression promotes the phosphorylation of both Chk1 and Chk2 in response to etoposide (panel A). HEK 293/MenHA clones were further analysed to investigate the effect of Menin overexpression on the extension time of Chk1 and Chk2 phosphorylation after removal of etoposide. As shown (panel B) in the control cell line etoposide-induced phosphorylation of Chk1 was turned on 2 hrs after the treatment and disappeared after removal of etoposide, while Chk2 phosphorylation was detectable already at 30 minutes and remained on after removal of the drug.

## ***2.4 Menin overexpression doesn't influence the cell cycle***

Subsequently I have investigated the functional significance of protracted Chk1 phosphorylation. To this aim I have examined the cell cycle distribution of control and MenHA overexpressing clones in presence or absence of etoposide by FACS analysis. Control cells and MenHA clone3 were treated with etoposide for 4 hours and collected after 24 and 48 hours from removal of the drug. As shown in these FACS panels (Figure 8), Menin overexpression doesn't influence the cell cycle distribution, suggesting that the overexpression of Menin may affect a chk1 function different that S-phase arrest.



**Figure 8 Cell cycle analysis of Menin overexpressing clones**

The cell cycle distribution of control and MenHA clones was examined in presence or absence of etoposide with FACS analysis. To this aim control cells and MenHA clone3 were treated with etoposide for 4 hours and collected after 24 and 48 hours from removal of the drug. As shown in these FACS panels, Menin overexpression doesn't influence the cell cycle distribution, suggesting that the overexpression of Menin may affect a Chk1 function different that S-phase arrest.

## ***2.5 Menin overexpression increases the rate of homologous-directed DNA repair***

Homologous recombination, the exchange of DNA sequence between homologous DNA molecules, is essential for accurate genome duplication and preservation of genome integrity. DNA double-strand breaks (DSBs) and single-stranded gaps are efficient initiators of homologous recombination, which results in their accurate repair using an intact homologous template DNA in the same cell (Symington L.S. et al., 2002). Recent studies indicate that HR is a prominent DSBs repair pathway in mammalian cells.

In mammals, at least, the recombination machinery is subject to regulation by tumor suppressors. In somatic cells this provides a correlation between homologous recombination and the maintenance of genome stability to safeguard against genome rearrangements and ultimately for cancer prevention.

As mentioned above Menin interacts with FANCD2, that is involved in HR through its interaction with the BRCA1 pathway. Furthermore a recent report has established that Chk1 is a key regulator of genome maintenance by the HR system (Sorensen CS et al., 2005). As showed above, in spite of the protracted phosphorylation of Chk1 I have found that neither the rate of etoposide-induced cell cycle arrest nor apoptosis were changed in Menin overexpressing clones. This observation suggests that the overexpression of Menin may affect a Chk1 function different than S-phase arrest.

To analyze the functional significance of the effect of Menin overexpression on Chk1 phosphorylation, I have investigated

the involvement of Menin on HR system performing a fluorescence based assay. To this aim I have used a recombination reporter system performed by M. Jasin (Memorial Sloan Kettering Cancer Center, New York) which contains two differentially mutated GFP genes oriented as direct repeats and separated by a drug selection marker, the puromycin N-acetyltransferase gene (Figure 9). The first GFP gene, at the 5' end, contains the recognition site for Sce-I, a rare cutting endonuclease (Colleaux L., 1998 ), which doesn't cleave several eukaryotic genomes tested (Jasin M., 1996).

The Sce-I site was incorporated into a Bcg-I restriction site, present in the functional GFP gene, by substituting 11 bp of wild type gene. These substituted base pairs also supply two in frame stop codons, which terminate translation and inactivate the expression of GFP.

The second gene contains a Bcg site (canonic site in GFP sequence), a small deletion at amino terminal domain and one at carboxy terminal domain, it doesn't contain a promoter and it is no functional. Furthermore this gene will undergo DSBs when Sce-I is expressed in vivo by transfection.

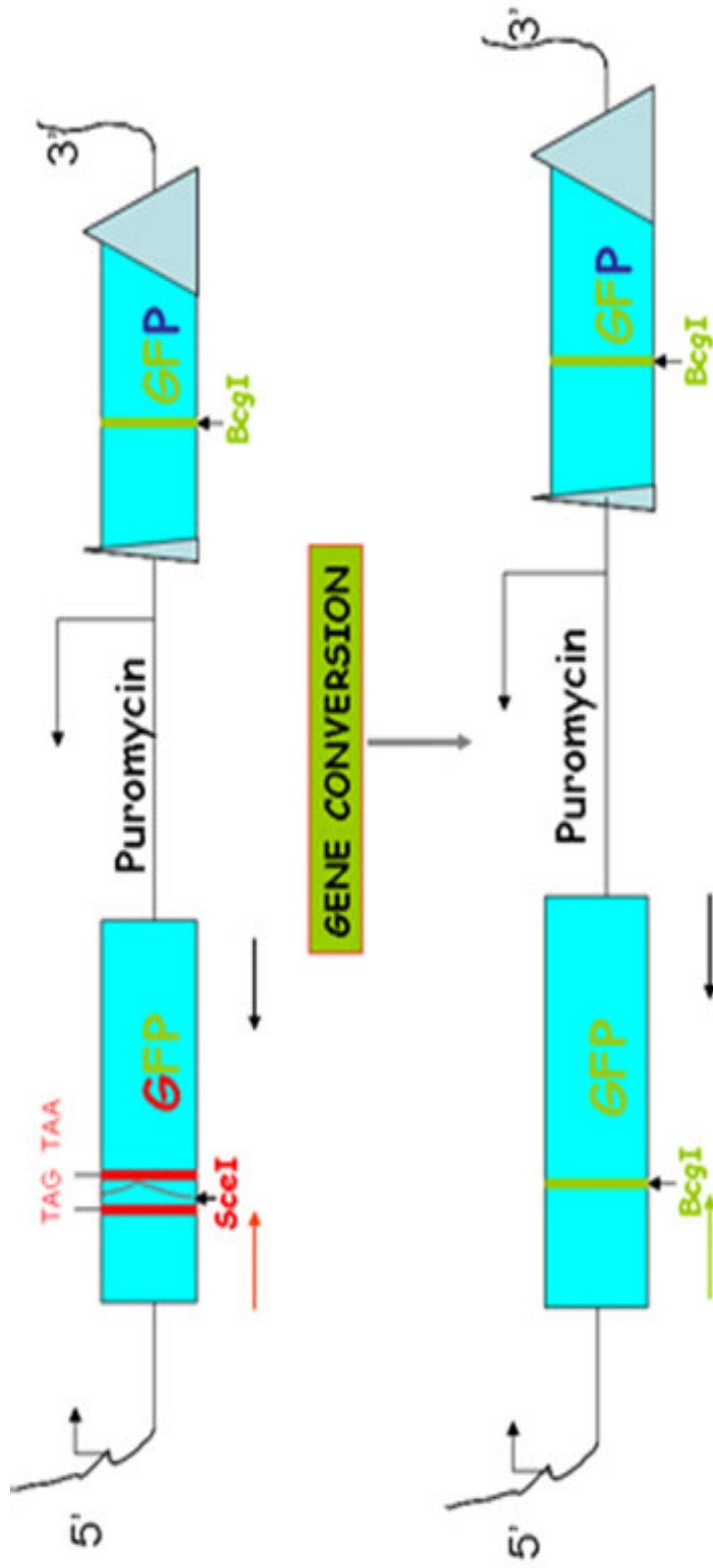
This system was stably integrated in Hela cell line (kindly provided by Prof. V.E. Avvedimento) and DSB is then induced by transient transfection of a plasmid expressing Sce-I endonuclease.

Repair of DSBs by HR leads to a functional GFP gene, allowing to score the gene conversion events by flow cytometry.

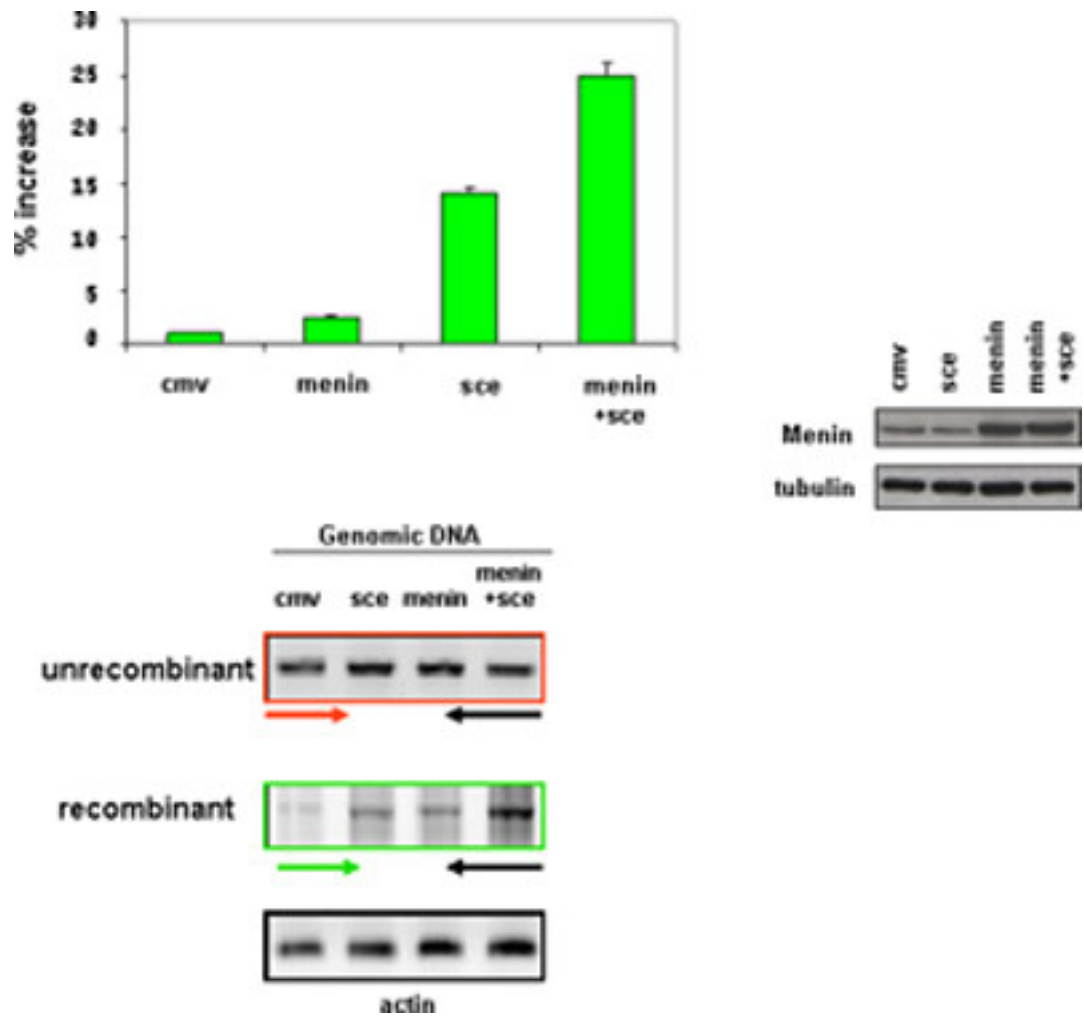
To analyze the effect of Menin on homologous recombination directed repair I have transfected different Hela /DR-GFP clones with Sce-I expressing plasmid in presence or in absence



of Menin and after 7 days the cells were analyzed for HR efficiency by flow cytometry. As shown in the Figure 10 coexpression of Menin and Sce significantly increases the gene conversion events expressed as GFP positive cells compared with the control. Furthermore I have analyzed genomic DNA extracted by the same cells, by performing PCR reactions using a fixed 3' primer together with 5'Sce oligo or with a 5'Bcg oligo. As shown in the figure, Menin overexpression increases also the genomic DNA levels.



**Formation of a functional GFP gene by recombination initiated at a specific double strand break.** The figure shows the events, initiated at the Sce-I site in the DR-GFP reporter plasmid, leading to a functional GFP gene. Translation termination codons at the Sce-I site in the 5' end GFP cassette are indicated in red. Bcg-I site in the homologous wild type GFP sequence in the 3' end cassette is indicated in green. The triangle at the 5' and 3' ends of the GFP cassette indicate the deletions in the amino and carboxy termini of the protein. Short tract gene conversion of the Sce-I site to Bcg-I, initiated by the DSB, leads to a functional GFP gene.



**Figure 10 *Menin increases the rate of homologous-directed DNA***

Different HeLa /DR-GFP clones were transiently transfected with a I-SceI expressing plasmid in presence or in absence of Menin and after 7 days were analysed for HR efficiency by flow cytometry. As shown coexpression of Menin significantly increased the number of GFP positive cells so Menin increases the homologous recombinational events. Below are reported also the PCR analysis of genomic DNA extracted by the same cells.

### **3. DISCUSSION**

Patients affected by Men1 develop multiple primary tumors in the parathyroids, the endocrine pancreas, and the anterior pituitary (Marx SJ, 2002). Also, the Men-1 syndrome has been associated with chromosomal instability (Tomassetti et al., 1995). Besides, many patients also develop tumors in a range of non-endocrine tissues, suggesting that MEN1 may control tumor development in different tissues, depending on genetic interactions with other cancer-associated genes. Menin, the nuclear protein encoded by the MEN1 gene, interacts with a large number of proteins, however the precise biochemical function of Menin mediating its tumor-suppressor activity has yet not been established. The identification of two novel Menin-interactors, Replication protein A (RPA) (Sukhodolets KE et al., 2003) and the Fanconi Anemia protein FANCD2 (D'Andrea et al., 2003) both involved in multiple pathways of the DNA damage response (Zou L. et al., 2003), has brought up a potential role of the MEN-1 gene in the maintenance of DNA integrity. In the same direction, previous studies have associated the MEN-1 syndrome with chromosomal instability and loss of DNA integrity (Ikeo Y. et al., 2000) (Tomassetti P. et al., 2005). Furthermore, extensive chromosomal breakage has been observed in lymphocytes from MEN1 patients but not in lymphocytes from normal individuals, after treatment with an agent cross-linking doublestrand DNA (Itakura Y. et al., 2000). A genome-wide LOH screening of 13 MEN1 patients indicates

that MEN1 pancreatic tumors fail to maintain DNA integrity and chromosomal stability (Hessman O. et al., 2001).

Also, Menin depletion leads to increased sensitivity to DNA damage, either in embryonic mouse fibroblasts or *Drosophila* larvae (Jin S. et al., 2003) (Busygina V. et al., 2004).

In my thesis work I have accumulated evidence suggesting that Menin functions in the DNA damage pathway by regulating the activation of the essential checkpoint kinase Chk1 and the frequency of homologous-directed DNA repair.

DNA damage induced by radiations, chemical agents, or oxidative stress elicits a complex array of checkpoint pathways that result in the onset of cell cycle delay, DNA repair, chromatin remodelling, modulation of transcriptional programs, and the optional triggering of cell death (Zhou B.B. et al., 2000) (Nyberg, K. A. et al. 2002). Central to these processes are ATM (ataxia teleangectasia mutated), ATR (ATM related) and DNA-PK kinases. Upon DNA damage ATM and ATR activate a network of damage-response leading to activation of the checkpoint kinases Chk1 and Chk2 and DNA repair proteins, while DNA-PK is involved in Non-Homologous-End-Joining (NHEJ) repair. The inability to respond properly to DNA damage leads to genetic instability and in turn cancer. Furthermore, early in the progression of major human cancer types (before genomic instability and malignant conversion) tumorigenic events activate the ATR/ATM-regulated checkpoint through deregulated DNA replication and DNA damage. Chk1 and Chk2 are essential for S-phase delays after DNA damage.

Besides, Chk1 is required for homologous recombination repair (Sorensen CS et al., 2005). Several studies have indicated IR

and double strand breaks activate the ATM/Chk2 damage pathway, while UV light, single strand breaks (SSB) and blocked DNA replication activate the ATR/Chk1 pathway. However, accumulating evidence indicates that ATM and ATR can be activated by overlapping damaging agents, but yet they maintain the specificity of their substrates. Accordingly, UV-activated ATM specifically phosphorylates Chk2, while IR-activated ATR specifically activates Chk1. Presumably, the substrate specificity is coordinated by the unique binding and activation of ATM and/or ATR with auxiliary proteins at sites of DNA.

I have investigated the DNA damage response of embryonic kidney cell H-293, stably over-expressing menin. Normally, H-293 cells respond to etoposide, an inhibitor of topoisomerase II causing accumulation of both DSBs and SSBs, by activating first the ATM-Chk2 pathway and subsequently the ATR-Chk1 pathway. Inversely, UV leads first to activation of ATR-Chk1 and later ATM-Chk2. In both cases Chk1 activation is transient, disappearing shortly after removal of the DNA-damaging agent. Conversely, Chk2 activation persists after removal of either agents. Presumably, the fast decay of Chk1 activation is the result of ubiquitin-dependent degradation of active Chk1, once it translocates from chromatin to nucleoplasm (Zhang YW et al., 2005).

I found that stable expression of Menin prolongs the time-course of etoposide or UV-induced phosphorylation of Chk1, without affecting either the ATR pathway or the rate of DNA damage.

My results suggest that Menin promotes and prolongs the time-course of Chk1 phosphorylation induced by etoposide. In spite of the protracted phosphorylation of Chk1, I found that the rate of etoposide-induced cell cycle arrest nor apoptosis were changed in MenHa overexpressing clones. This observation suggests that the overexpression of Menin may affect a Chk1 function different that S-phase arrest.

Recent studies have shown that Chk1 is required for homologous recombination repair through the activation of the essential recombination repair protein rad51 (Sorensen CS et al., 2005). Interestingly, I have observed that Menin overexpression increases the efficiency of homologous-directed DNA repair (HDR) of DSBs, suggesting that that this effect might be a direct consequence of the sustained activation of Chk1, itself involved in this mechanism of DNA repair.

Several studies indicate that HR is a prominent DSBs repair pathway in mammalian cells. In mammals, at least, the recombination machinery is subject to regulation by tumor suppressors. Homologous recombination, the exchange of DNA sequence between homologous DNA molecules, is essential for accurate genome duplication and preservation of genome integrity. DNA double-strand breaks (DSBs) and single-stranded gaps are efficient initiators of homologous recombination, which results in their accurate repair using an intact homologous template DNA in the same cell (Symington L.S. et a., 2002). In somatic cells this mechanism provides a correlation between homologous recombination and the maintenance of genome stability to safeguard against genome rearrangements and ultimately for cancer prevention.

Besides, although Menin is ubiquitously expressed, its expression level is particularly high in the testis, consistent with a potential role in recombination (Stewart C. et al, 1998). Hence, my current findings are consistent with the idea that processes involved in the maintenance of protein integrity may be important for ensuring the integrity of the genome and also they are consistent with the notion that DNA repair might be one of the functions of Menin and suggest an additional mechanism by which Menin functions as a tumor suppressor by promoting and protracting the phosphorylation of Chk1 during DNA damage response.

The inability to respond properly to DNA damage leads to genetic instability, which in turn may enhance the rate of cancer development. Indeed, tumor suppressor genes as p53, ATM and BRCA1-2 have been linked to DNA-damage response, and more accumulating evidence are connecting this cellular response with tumorigenesis. However, mutations in some tumor suppressor genes, as BRCA1 and BRCA2, only predispose to breast and ovarian cancers. Therefore a key issue is to assess the cell-type and tissue-specific differences of DNA damage response pathways. Understanding how Menin influence Chk1 activation and DNA repair will contribute to expand the knowledge of these pathways and facilitate the development of more specific and effective anticancer therapies.



## **4. MATERIALS AND METHODS**

### **4.1 Cell culture**

HEK 293, Hela and Hela Dr-GFP (stable cell line) were grown respectively in complete Dulbecco's modified Eagle's medium (DMEM) or RPMI supplemented with 10% Fetal Bovine Serum (Invitrogen). The cells also received 1% L-glutamine, and 1% penicillin–streptomycin and were housed in an incubator maintaining an atmosphere of 90% of humidity and 5.0 CO<sub>2</sub> at 37°C. All cell samplings for preparation of DNA, RNA and proteins were performed on cells cultured to a confluency of 80%.

### **4.2 Stable and transient transfection**

The plasmids used for the transfections were: pcDNA 3.1 Men-HA (obtained using standard cloning techniques), pC $\beta$ ASce and pcDNA 3.1 HA as a control vector. These constructs were transfected to HEK 293, Hela and Hela DR-GFP cells according to Lipofectamine and Lipofectamine 2000 reagent protocols (Invitrogen). Stable transfectants were selected in gentamycin (Calbiochem) at a concentration of 800 microgr/ml. I clonally expanded three different clones named M3, M10 and M11. Furthermore HEK 293 cells were transfected with pCDNA 3.1 HA without insert (control vector). After clonal expansion, transfectants were cultured in the presence of 400 microgr/ml of gentamycin and used at passages 4 and 5.

### ***4.3 Cell synchronization***

Hela cells were synchronized at the beginning of S phase by double thymidine block and release protocol: first 24 h incubation with 1 mM thymidine, an interval of thymidine-free incubation for 8 h, and second thymidine incubation for 14 h.

### ***4.4 Drug treatment***

Etoposide (Calbiochem) was dissolved in dimethyl sulphoxide (DMSO) and it was added at 5 micromol concentration to the cells after 24 hours of transfection. Aliquots were prepared in DMSO and stored at -20°C.

### ***4.5 Cell extracts preparation and Western blot analysis***

To detect protein expression, I have prepared whole and fractionated cell lysates. The cells were collected by scraping and washed twice in PBS 1X. The whole cell lysates were prepared from freshly isolated cells by using lysis buffer (250 mM sucrose, 20mM TrisHCl, 1,1 mM MgCl<sub>2</sub>, 0,2% Triton, 250 mM NaCl) supplemented with protease inhibitor mixture (Calbiochem), PMSF and sodium orthovanadate as phosphatase inhibitor. The cells were incubated in ice for 15 minutes and centrifuged at 13000 rpm for additional 15 minutes. Chromatin fractionations were performed essentially as described by Zou and Elledge (Zou L. et al., 2002). Briefly, the cells are resuspended in 200 µL of solution A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.34 M sucrose, 10% glycerol, 1mMDTT, 10mMNaF, 1mMNa<sub>2</sub>VO<sub>3</sub>, protease inhibitors). Triton X-100 was added to a final concentration of 0.1%, and the cells were left on ice for 5 min. Cytoplasmic proteins (S1) were

separated from nuclei (P2) by lowspeed centrifugation (1300g for 4 min). Isolated nuclei were washed once with solution A and lysed in 200  $\mu$ L of solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT). After a 10-min incubation on ice, soluble nuclear proteins (S2) were separated from chromatin (P2) by centrifugation (1700g for 4 min). Isolated chromatin was washed once with solution B and spun down at high speed (10,000g for 1 min). Finally, chromatin was resuspended in 200  $\mu$ L of SDS sample buffer. The protein concentration from supernatants was determined by Biorad assay. Protein samples were separated in 10% Tris-glycine SDS/PAGE gels, transferred to nitrocellulose membranes, and hybridized with appropriate antibodies. Blots were developed by enhanced chemiluminescence (Roche).

#### ***4.6 Antibodies***

Menin C-19 (#sc-8200, santa cruz), pChk1-Ser 345 (#2341, cell signaling), Chk1 tot (#sc-8408, santa cruz), pChk2 (#2668, cell signaling), Chk2 tot (#sc-8813, santa cruz), Rad17 (#12416, santa cruz), pP53 Ser 15 (#9284, cell signaling), pRPA Ser 4/8 (#A300-245A, bethyl), Erk 1-2 (#sc 153, santa cruz), pNbs1 Ser 343 (#3001, Cell Signaling), pATM Ser 1981 (#4526, cell signaling), Tubulin (#sc 8035, santa cruz).

#### ***4.7 RNA and DNA extraction***

Total RNA was extracted using Triazol (Gibco BRL) method. Genomic DNA extraction was performed with following protocol: cellular pellet was resuspended in 10mM Tris pH 7.8 and 50mM NaCl solution. After addition of 1% SDS the sample was gently mixed. Proteinase K, at the final concentration of 20 microgr/ml,

was added and the sample incubated at 55°C over night. The day after, hot NaCl at the final concentration of 1.5 M was added and DNA was extracted by phenol/chloroform. The DNA was precipitated by adding ethanol, the pellet was dried and resuspended in TE buffer.

#### **4.8 PCR analysis**

cDNA was synthesized in a 20 microl reaction volume containing 2 micrograms of total RNA, 4 units of Omniscript Reverse Transcriptase (Quiagen), 1 microl random examer [20 ng/microl] (Invitrogen).

mRNA was reverse transcribed for 1 hr at 37°C and the reaction was heat-inactivated for 10 minutes at 70°C. The products were stored at -20°C until use. PCR analysis was performed in a 50 microl reaction mixture containing 2 microl of synthesized cDNA product or 1 microgr of genomic DNA, 5 microl of 10X PCR buffer, 1,5 mM MgCl<sub>2</sub>, 0,3 mM dNTP, 1,25 unit of Taq polymerase (Roche) and 6 micromol of each primer. The primer sequences that were used for the different mRNAs or DNAs were:

5' SclI GCTAGGGATAACAGGGTAAAT

5' Bcg GAGGGCGAGGGCGATGCC

3' Common TGCACGCTGCCGTCCTCG that amplify a fragment of 443 base pairs

Beta-actin/R AAAGCCATGCCAATCTCATC

Beta-actin/L GATCATTGCTCCTCCTGAGC that amplify a fragment of 250 bp

Amplifications were performed in a Primus termocycler (MWG-Biotech) using the following program:

95°C 5 min x 1 cycle

95°C 45 sec

57°C 30 sec

72°C 2 min x 30 cycles

72°C 10 min x 1 cycle

The number of cycles were selected and validated by running several control reactions and determining the linear range of the reaction. 15 microl of the PCR products were applied on a 1.2% agarose gel and visualized by ethidium bromide staining. Densitometric analysis was performed using phosphor-imager. The determination for each point was carried out on at least 3 independent reactions.

#### ***4.9 Fluorescence activated cell sorter (FACS) analysis***

Hela DR-GFP cells were transfected with pcDNA 3.1/MenHA or control plasmid in presence or absence of pCβASce and after six days were treated with tripsyn and collected. The cells were washed twice in cold PBS 1x and after they were resuspended in 500 microl of PBS 1X . 6X10<sup>4</sup> cells were analyzed.

#### ***4.10 Cell cycle analysis***

To perform a cell cycle analysis I have used a propidium iodide staining. The cells were harvested and washed, after they were fixed in ice-cold 70% ethanol while vortexing. Subsequently the cells were centrifuged at 2000rpm for 5 minutes and washed twice in PBS. After a treatment with 1mg/ml ribonuclease for 5 minutes at room temperature, 40µl propidium iodide (50µg/ml) was added to the cells and they were analyzed by flow cytometry

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