Doctorate Program in Molecular Oncology and Endocrinology

XVIII cycle - 2002–2006
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INVOLVEMENT OF H4(D10S170), A GENE FREQUENTLY REARRANGED WITH RET IN PAPILLARY THYROID CARCINOMAS, IN ATM-DEPENDENT RESPONSE TO DNA DAMAGE

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A. **Celetti A, Cerrato A, Merolla F, Vitagliano D, Vecchio G, Grieco M.** H4(D10S170), a gene frequently rearranged with RET in papillary thyroid carcinomas: functional characterization. Oncogene 2004; 23(1):109-21.

B. **Celetti A, Testa D, Staibano S, Merolla F, Guarino V, Castellone MD, Iovine R, Mansueto G, Somma P, De Rosa G, Galli V, Melillo RM, Santoro M.** Overexpression of the cytokine osteopontin identifies aggressive laryngeal squamous cell carcinomas and enhances carcinoma cell proliferation and invasiveness; Clin Cancer Res 2005; 11(22):8019-27.

C. **Motti ML, Califano D, Baldassarre G, Celetti A, Merolla F, Forzati F, Napolitano M, Tavernise B, Fusco A, Viglietto G.** Reduced E-cadherin expression contributes to the loss of p27kip1-mediated mechanism of contact inhibition in thyroid anaplastic carcinomas. Carcinogenesis 2005; 26(6):1021-34.

D. **Merolla F, Pentimalli F, Pacelli R, Vecchio G, Fusco A, Grieco M, Celetti A.** Involvement of H4(D10S170) protein in ATM-dependent response in DNA damage. Oncogene 2006; accepted pending revision.

E. **Celetti A, Staibano S, Merolla F, Testa D, Guarino G, Mascolo M, Di Benedetto M, Iovine R, De Rosa G, Galli V, Melillo RM, Santoro M.** Osteopontin expression predicts the clinical outcome of laryngeal intraepithelial neoplasia. Am J Pathol, submitted

ABSTRACT

This dissertation is focused on the functional characterization of H4(D10S170), a gene frequently rearranged with RET in Papillary Thyroid Carcinomas, and in particular on its involvement in ATM-dependent response to DNA damage.

Exposure to ionizing radiation is a well-known risk factor for thyroid cancer carrying the RET/PTC oncogenes. In fact, radiation exposure results in extensive DNA damage, including double strand breaks and chromosomal rearrangements. In human papillary thyroid carcinomas the rearrangement of the RET protooncogene with a number of heterologous genes generates the RET/papillary thyroid carcinoma (PTC) oncogenes. One of the most frequent variants of these recombination events is the fusion of the intracellular kinase-encoding domain of RET to the first 101 amino acids of a gene named H4(D10S170), where the spatial contiguity of RET and H4 chromosomal loci might be responsible for the high frequency of RET/PTC1 radiation-induced rearrangements in thyroid human cells.

The H4(D10S170) gene product is an ubiquitously expressed 55 KDa nuclear and cytosolic protein that is phosphorylated following serum stimulation. This phosphorylation depends on mitogen-activated protein kinase (MAPK) Erk1/2 activity and is associated to the relocation of H4(D10S170) from the nucleus to the cytosol. Overexpression of the H4(D10S170) gene was able to induce apoptosis of thyroid follicular epithelial cells; conversely a carboxy-terminal truncated mutant, H4(1-101), corresponding to the portion of H4(D10S170) included in the RET/PTC1 oncoprotein, behaved as dominant negative on the proapoptotic function and nuclear localization. More interestingly, the conditional expression of H4(1-101) and of H4(S244)A mutants protect cells from ionizing radiation, too. These latter findings may imply that the transforming potential of RET/PTC1 is not only confined to the RET tyrosine kinase activation, but also to the disruption of the H4 function.

The DNA damage signalling pathway is a highly conserved response to genotoxic stress. In mammalian cells the pathway functions to protect cells from agents that induce cellular death or transformation, where it participates in DNA repair and checkpoint control leading to survival or apoptosis. The ATM kinase, a central mediator of responses to DNA double-strand breaks, in cells phosphorylates a limited number of downstream protein targets in response to DNA damage.

In this dissertation we investigated the potential role of H4(D10S170) in DNA damage signaling pathways.

Several consensus phosphorylation sites for ATM might be present in H4(D10S170) protein, as predicted by profile scan primary sequence analysis, and also conserved in the various species in which H4(D10S170) is present, thus suggesting that H4(D10S170) might be a direct target of ATM.

We found that in cells treated with the DNA damaging agents, i.e. ionizing radiation or etoposide, H4(D10S170) underwent ATM-mediated phosphorylation at Thr 434, stabilizing nuclear H4.

In ataxia teleangectasia lymphoblast cell lines endogenous H4(D10S170) was localized to cytoplasm and was excluded from the nucleus. Moreover, H4(D10S170) was not phosphorylated in ATM-deficient lymphoblasts after ionizing irradiation. Inhibition of ATM kinase interfered with H4(D10S170) apoptotic activity and expression of a dominant negative H4-mutant, H4T434A, protected the cells from genotoxic stress-induced apoptosis. Most importantly, silencing of H4(D10S170) in mammalian cells increased cell survival, DNA synthesis and cell doublings after exposure to ionizing radiation.

Our results suggest that H4(D10S170) is involved in cellular response to DNA damage ATM-mediated and that the loss of H4(D10S170) gene function might have a role in thyroid carcinogenesis by impairing apoptosis and by promoting DNA synthesis resistant to genotoxic stress.

1. INTRODUCTION

1.1 Thyroid Cancer

Carcinoma of the thyroid gland is the most common malignancy of the endocrine system.

The thyroid gland is composed by two distinct hormone-producing cell types: follicular cells and parafollicular *C* cells. Follicular cells comprise most of the epithelium and are responsible for iodine uptake and thyroid hormone synthesis. *C* cells are scattered intrafollicular or parafollicular cells and are dedicated to the production of the calcium-regulating hormone calcitonin. More than 95% of thyroid carcinomas are derived from follicular cells while a minority of tumours (3%), referred to as medullary thyroid carcinoma, are of *C*-cell origin.

Thyroid cancer affects women more often than men and usually occurs in people between the ages of 25 and 65 years. The incidence of this malignancy has been increasing over the last decade.

Patients with a history of radiation administered in infancy and childhood for benign conditions of the head and neck, such as enlarged thymus, acne, or tonsillar or adenoidal enlargement, have an increased risk of cancer as well as other abnormalities of the thyroid gland. In this group of patients, malignancies of the thyroid gland first appear beginning as early as 5 years following radiation and may appear 20 or more years later. Other risk factors for the development of thyroid cancer include a history of goiter, family history of thyroid disease, female gender, and Asian race.

Thyroid carcinomas are broadly divided into well-differentiated, poorly differentiated and undifferentiated types on the basis of histological and clinical parameters (Table 1). Differentiated tumors (papillary or follicular) are highly treatable and usually curable. Poorly differentiated tumors (medullary and anaplastic) are much less common, are aggressive, metastasize early, and have a much poorer prognosis. The 10-year overall relative survival rates for patients in the United States are 93% for papillary cancer, 85% for follicular cancer, 75% for medullary cancer, and 14% for undifferentiated/anaplastic cancer.

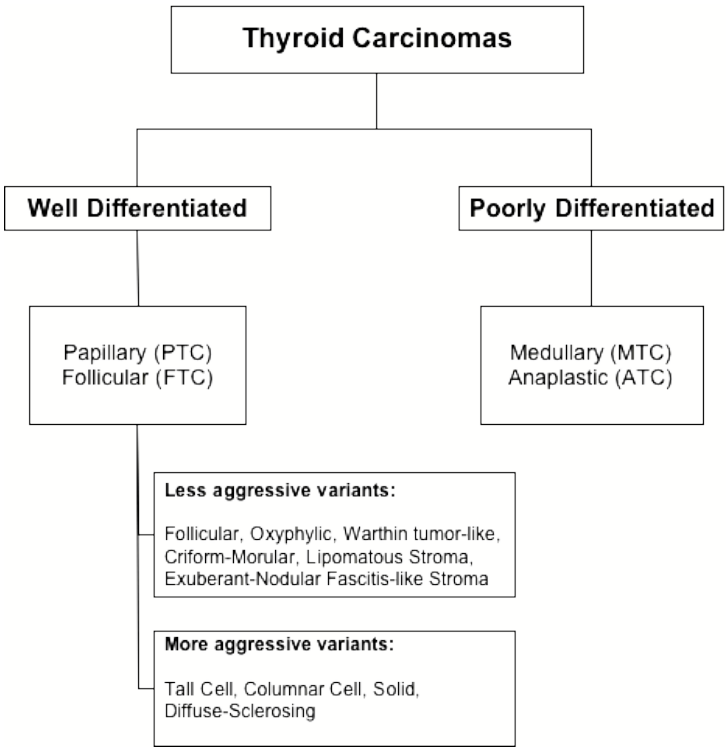

Figure 1. Classification of thyroid carcinomas. *For clinical management of the patient, thyroid cancer is generally divided into 2 categories: well differentiated or poorly differentiated.*

Papillary thyroid carcinomas (PTCs) account for around 60 to 80% of all thyroid cancers and are closely linked to ionizing irradiation (Ron et al. 1995). Young children are particularly susceptible since thyroid growth occurs primarily in childhood. A striking increase in PTC has been reported in Belarus, Ukraine and Western regions of Russia, following the Chernobyl disaster of 1986. The worldwide incidence of thyroid cancer in children has been estimated in the order of 1 per million children per year, while it increased by more than 30 fold after the Chernobyl disaster in the exposed areas (Williams 2002). PTC shows typically multicentricity and a tendency to spread into lymphatic vessels; regional node metastases at presentation are found in a significant proportion of cases. There are several PTC variants including solid-follicular, follicular, tall-cell, hurthle cell variants (Ostrowski et al. 1996).

About 10 to 30% of thyroid cancers are Follicular thyroid carcinoma (FTC). FTC is linked to dietary iodine deficiency (Williams et al. 1977) and shows variable morphology ranging from well-formed colloid-containing follicles, to solid or trabecular growth pattern. Therapy for both PTC and FTC consists in surgery followed by metabolic treatment with ^{131}I. Prognosis is very good with a survival rate at 10 years ranging from 90 to 98%.

Anaplastic thyroid carcinoma (ATC) is the most aggressive type of thyroid cancer. ATC cells are extremely abnormal and spread rapidly to other

parts of the body. ATC make up only about 1% of all thyroid cancers and typically spreads beyond the thyroid by direct local extension. Metastases to regional nodes are also common but their presence is often masked by the presence of extensive soft tissue invasion. Distant metastases may be present in any site. No effective therapy is known for ATC and prognosis is very unfavourable with a mean survival time of less than one year.

Medullary thyroid carcinoma (MTC) develops from neuroendocrine C cells of the thyroid, producing calcitonin (CT). About 5 to 7% of all thyroid cancers are MTC that can be sporadic or familial as part of the Multiple Endocrine Neoplasia type 2 syndromes. MEN2 syndromes are inherited cancer disorders divided in three types: MEN2A, characterized by MTC, pheochromocytoma and parathyroid adenoma; MEN2B characterized by MTC, pheochromocytoma and additional tumors such as neuromas and ganglioneuromas of the gut; Familial Medullary Thyroid Carcinoma or FMTC whose only feature is MTC. MEN2 is inherited as a highly penetrant mendelian tract and this genetic transmission is due to gain-of-function mutations of the RET gene. MTC tends to metastasize to lymph-nodes and distant organs, the treatment consists in surgical removal of the lesion. Thus, MTC are fairly resistant to most chemotherapeutic agents.

1.2 Molecular Basis Of Well-Differentiated Thyroid Carcinoma

In well-differentiated thyroid carcinoma non-overlapping, activating events that involve the genes RET, NTRK1 (neurotrophic tyrosine kinase receptor 1), BRAF or Ras are detectable in nearly 70% of all cases.

The rearranged during tansfection (RET) proto-oncogene was isolated in 1985 and was the first activated receptor-tyrosine kinase to be identified in thyroid cancer (Takahashi et al 1988). The proto-oncogene, located on chromosome 10q11.2, encodes a transmembrane receptor-tyrosine kinase with four cadherin-related motifs in the extracellular domain. RET is normally expressed in the developing central and peripheral nervous systems and is an essential component of a signalling pathway that is required for renal organogenesis and enteric neurogenesis. Glial-derived neurotrophic factor (GDNF)-family ligands and GDNF-family receptor-alpha (GFRalpha) bind the extracellular domain of RET to induce tyrosine kinase autophosphorylation that activates several signalling pathways, including extracellular regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K), MAPK p38 and C-JUN kinase (JNK).

Gain-of-function mutations of RET are involved in sporadic and familial C-cell-derived medullary thyroid carcinoma, including multiple endocrine neoplasia 2A (MEN2A), MEN2B and familial medullary thyroid carcinoma. By contrast, chimeric oncogenes, designated RET/PTC, are implicated in the development of papillary carcinoma. Somatic chromosomal rearrangement leads to fusion of the 3'-terminal sequence of RET, which encodes the tyrosine kinase domain, with the 5'-terminal sequences of heterologous genes (Fig. 2).

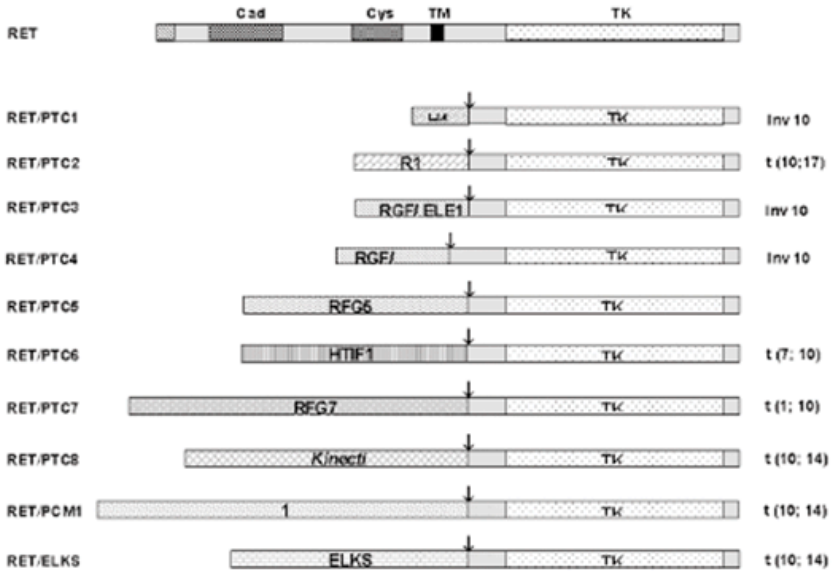

Figure 2. Chromosome 10q inversion in papillary thyroid carcinoma. *Schematic view of the paracentric inversion of chromosome 10q generating the transforming sequence RET/PTC. There are at least 10 different types of RET/PTC, all resulting from the fusion of the tyrosine kinase domain of RET to the 5' portion of different genes. RET/PTC1 and RET/PTC3 are the most common types, accounting for >90% of all rearrangements. The arrows indicate the breackpoints.*

Although wild-type RET is not normally expressed in follicular cells, RET/PTC chimeric oncoproteins, that lack a signal peptide and transmembrane domain, are expressed in the cytoplasm of follicular cells under the control of the newly acquired promoters. Ligand-independent tyrosine phosphorylation is induced by constitutive dimerization of the fusion proteins.

The reported overall prevalence of RET/PTC rearrangements in papillary carcinomas varies from 13–43%. More than 10 RET/PTC rearrangements have been described in sporadic and radiation-associated papillary carcinoma, Among them the most common forms are H4(D10S170)–RET (also known as RET/PTC1) and ELE1–RET (also known as RET/PTC3).

Rearrangements involving the RET gene are common in radiation-associated papillary thyroid cancer (PTC) and patients with a history of medical irradiation.

The RET/PTC1 type of rearrangement is an inversion of chromosome 10 mediated by illegitimate recombination between the RET and the H4 genes, which are 30 megabases apart. Despite the great linear distance between them, RET and H4 recombination might be promoted by their proximity in the nucleus. Spatial contiguity of RET and H4 within interphase nuclei may provide a structural basis for generation of RET/PTC1 rearrangement by allowing a single radiation track to produce a double-strand break in each gene at the same site in the nucleus (Nikiforova 2000) (Fig. 3).

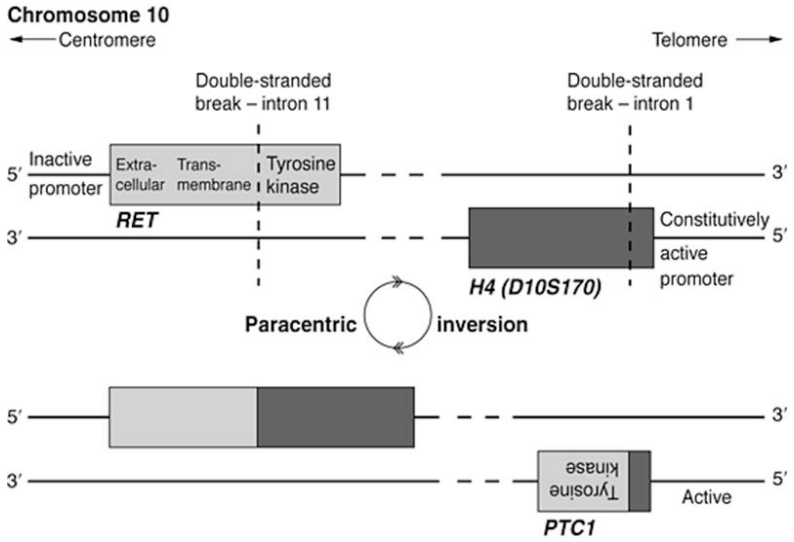

Figure 3. Schematic view of the paracentric inversion of chromosome 10q generating the transforming sequence RET/PTC1.

In RET/PTC1 rearrangement, the unscheduled expression of RET tyrosine kinase with its overespression, the deletion of negative regulatory domains of the receptor and constitutive oligomerization of PTC1 proteins are responsible for PTC1-transforming activity in the thyroid.
The amino terminal region of H4 (paragraph 1.3) is responsible for the dimerization of the PTC1 oncoprotein in vivo. This region, containing a putative leucine zipper, mediated dimerization and is essential for tyrosine hyperphosphorylation and the transforming activity of PTC1 (Tong et al 1997) (Fig. 4).

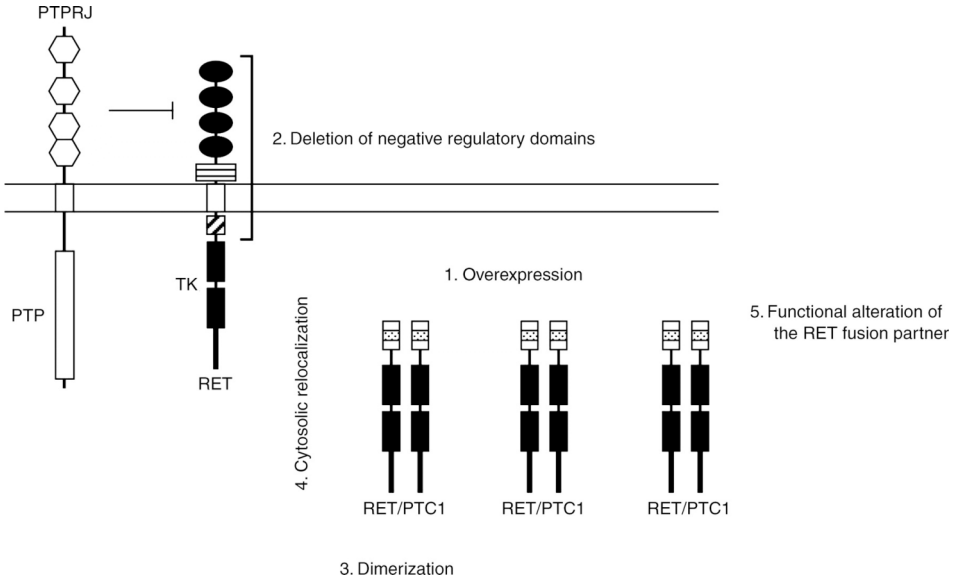

Figura 4 Mechanisms for RET/PTC1 activation. *The wild-type RET protein, the PTPRJ tyrosine phosphatase, and the rearranged RET/PTC1 oncoproteins are depicted.*

Mutations in BRAF were the most recently identified MAPK effector in thyroid cancer. The proto-oncogene form of BRAF situated on 7q24 encodes a serine/threonine kinase that transduces regulatory signals through the Ras–Raf–MEK–ERK cascade. Among point mutations of exon 15 in thyroid cancers, BRAFV600E is the most common alteration in sporadic papillary carcinoma. Gain of function mutations of BRAF are found in 29–69% of papillary thyroid carcinoma but not in follicular thyroid carcinoma, and in up to 13% of poorly differentiated thyroid carcinoma and 35% of undifferentiated thyroid carcinoma. BRAF mutations in papillary thyroid carcinoma correlate with distant metastasis and more advanced clinical stage, and occur at a significantly higher frequency in older patients.

The neurotrophic receptor-tyrosine kinase NTRK1 (also known as TRK and TRKA) was the second identified subject of chromosomal rearrangement in thyroid tumorigenesis. The NTRK1 proto-oncogene (which is located on chromosome 1q22) encodes the transmembrane tyrosine-kinase receptor for nerve growth factor. The activated receptor initiates several signal-transduction cascades, including ERK, PI3K and the phospholipase-Cgamma (PLCgamma) pathways. NTRK1 rearrangements, which show ectopic expression and constitutive activation of the tyrosine kinase that are analogous to RET rearrangements, have been noted in 5–13% of sporadic but only 3% of post-Chernobyl childhood papillary thyroid carcinomas.

Unlike other solid neoplasms, Ras is the least prominent participant in thyroid carcinogenesis. Three Ras proto-oncogenes are implicated in human tumorigenesis: HRAS (which is located on chromosome 11p11), KRAS (which is located on chromosome 12p12), and NRAS (which is located on chromosome 1p13). Mutations involving codon 61 of HRAS and NRAS have been reported with variable frequency in thyroid neoplasms. Ras mutations are more common in iodine-deficient than iodine-sufficient areas and in lesions with follicular architecture (including follicular carcinoma and follicular variant papillary thyroid carcinoma) than in typical papillary thyroid carcinoma. Interestingly, activating Ras mutations are rare in radiation-induced thyroid cancers of Chernobyl.

1.3 H4(D10S170)

The H4(D10S170) gene maps on the long arm of chromosome 10 at 10q21 (Grieco et al. 1990). The H4(D10S170) gene, also called CCDC6 (Coiled Coil Containig 6), contains 9 exons that encode for a transcript of 3 Kb and shows an open reading frame (ORF) of 585 aa. The H4 gene promoter, localized within 259 bp upstream of the ATG site, drives the gene expression ubiquitously in various human tissues including thyroid (Tong et al. 1995). Since the H4 gene is ubiquitously expressed, the specificity of the PTC-1 activation is due to the specificity for thyroid tissue of the somatic rearrangement of the *ret* proto-oncogene.

Sequence analysis of H4(D10S170) gene product shows extensive regions of alpha helices which have a high potential to adopt a coiled-coil conformation. Coiled-coils are formed by two or three alpha-helices that are strongly amphipathic and supercoil around each other, crossing at an angle of ca 20° (Lupas et al 1991). It has been shown that such regions can be involved in protein dimerization or oligomerization. The 'sticky' portion of H4(D10S170), that consists in 185 aa, from the aminoacid 53 to the 237, is only in part disrupted by the rearrangement and is responsible for RET/PTC1 dimerization and, hence, for constitutive activation of its tyrosine-kinase activity.

Moreover the N-terminal portion of H4 protein contains a poly-Gly region, while a poly-Pro region is present at the C-terminal. The C-terminal domain of H4 (amino acids 442-451) contains a putative SH3-binding sites domain, as defined by the PXXP motif (where X is any amino acid). SH3-binding domains play an important role in protein-protein interactions in numerous cellular processes, including clathrin-mediated endocytosis (McPherson 1999). For example, Grb2, amphiphysin, and PLCγ all bind to the SH3-binding domains of dynamin (Vidal et al 1998). The presence of a putative SH3-binding domain in H4(D10S170) gene product suggests that this protein might be a cytoskeletal one.

Some of the features of the H4 gene product are summarized in Table 1

Key	Position	Length	Description
REPEAT	106-134	29	1 EQEEEFISNT LFKKIQALQK EKETLAVNY
REPEAT	135-163	29	2 EKEEEFLTNE LSRKLMQLQH EKGELEQHL
REPEAT	164-192	29	3 EQEQEFQVNK LMKKIKKLEN DTISKQLTL
REPEAT	193-206	14	4; approximate EQLRREKIDL ENTL
REPEAT	207-235	29	5 EQEQEALVNR LWKRMDKLEA ETRILQEKL
REGION	106-235	130	5 X 29 AA tandem repeats EQEEEFISNT LFKKIQALQK EKETLAVNYE KEEEFLTNEL SRKLMQLQHE KGELEQHLEQ EQEFQVNKLM KKIKKLENDT ISKQLTLEQL RREKIDLENT LEQEQEALVN RLWKRMDKLE AETRILQEKL
COILED	53-237	185	PFRLEELTNR LASLQQENKV LKIELETYKL KCKALQEENR DLRKASVTIQ ARAEQEEEFI SNTLFKKIQA LQKEKETLAV NYEKEEEFLT NELSRKLMQL QHEKGELEQH LEQEQEFQVN KLMKKIKKLE NDTISKQLTL EQLRREKIDL ENTLEQEQEA LVNRLWKRMD KLEAETRILQ EKLDQ
COILED	253-332	80	(Potential) DSPENMMRHI RFLKNEVERL KKQLRAAQLQ HSEKMAQYLE EERHMREENL RLQRKLQREM ERREALCRQL SESESSLEMD
MOTIF	442-451	10	SH3-binding (Potential) PPPPPPPMQP

Table 1

It is not clear why RET/PTC1 has been found activated only in thyroid papillary carcinomas, since in vitro irradiation is able to induce its activation also in fibroblasts (Ito et al. 1993).

Recently, Nikiforova et al. (2000) suggested that the spatial contiguity of RET and H4 chromosomal loci might be responsible for the high frequency of RET/PTC1 radiation-induced rearrangements in thyroid human cells.

The H4/RET rearrangement is not the only one involving the H4(D10S170) gene, although it is the more frequent. A new chromosomal rearrangement involving the H4(D10S170) gene has been recently described

(Fig. 5). In two cases of atypical chronic myelogenous leukaemia (CML), the first 368 aa of H4(D10S170) fuse to the PDGFbetaR tyrosine kinase domain. The chromosomal event is a t(5;10) translocation. In the two cases of atypical CML in which the H4-PDGFbetaR transcript has been identified, the fusion gene codes for a 948 aa protein with most of the coiled-coil of H4(D10S170) and the transmembrane and the tyrosine kinase domains of the PDGFbetaR. The fusion protein oligomerization and constitutive activity is dependent on the coiled-coil domain of the H4(D10S170) gene. The reciprocal product of the translocation has not been found (Kulkarni et al. 2000; Schwaller et al. 2001).

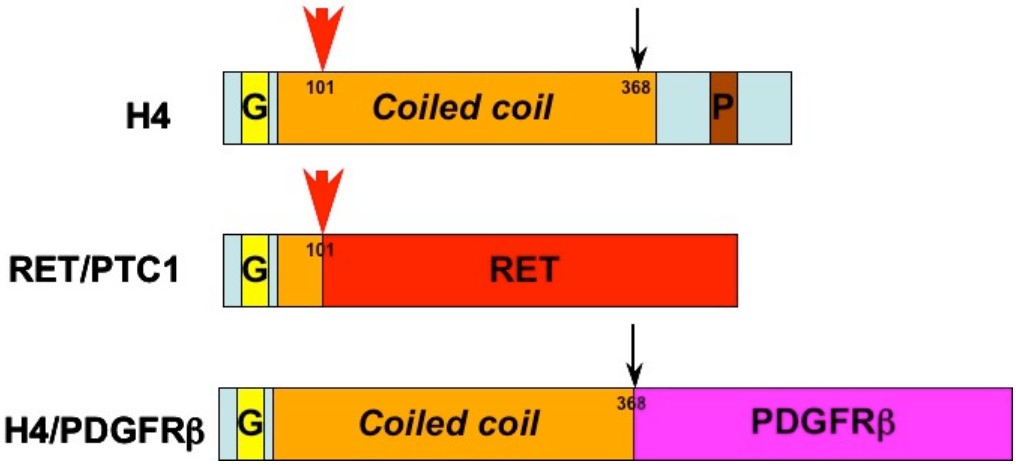

Figure 5 H4(D10S170) rearrangements. *The red arrow shows the RET/PTC1 and the black one shows the H4/PDGFbetaR breakpoint.*

In the various RET/PTC oncoproteins, little is known about the normal role of the 5' fusion partners. Some common features such as constitutive expression and the presence of dimerization domains have been observed for all the RET-fused genes identified so far. The ubiquitously expressed promoters of the 5'-fused sequences are responsible for the ectopic expression of RET in epithelial thyroid follicular cells, which do not normally express this protooncogene.

Although this indicates that disruption of the normal regulation of the RET kinase is critical to the transforming properties, less is known about whether disruption of the normal function of the 5' partner gene might also have an important role. To address this point, the identification of the normal physiological function of the heterologous RET fusion partners is required. Therefore, we have characterized the product of the first and most frequently observed RET-fused gene, H4(D10S170).

H4 protein is a nuclear and cytosolic protein (Celetti et al 2004) and its intracellular localization depends on several stimuli. Sequence analysis programs predict a putative Nuclear Localization Signal (NLS) in H4 amino acid sequence. The putative NLS of H4, that include the residues from aa 420 to 426, is a pattern starting with P and followed within 3 residues by a basic

segmant containing 3 K/R residues out of 4 (Merolla et al, manuscript in preparation) (Fig. 6).

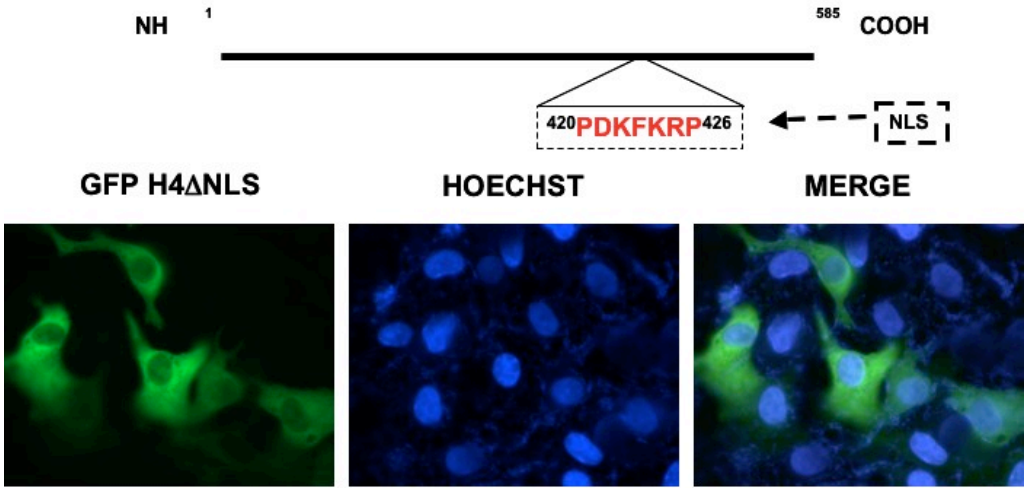

Figure 6 Putative NLS sequence in H4 protein. *Schematic representation of putative NLS in H4 sequence (upper panel). The loss of putative NLS sequence (GFP H4ΔNLS) delocalizes H4 gene product in the cytoplasm; nuclei were stained with Hoechst 33258; a merge image is also shown (lower panel).*

The H4 gene product is phosphorylated upon serum stimulation. We demonstrated that H4(D10S170) phosphorylation is ERK1 dependent and that S244 is the major site for this ERK1-dependent phosphorylation. This aa residue is embedded in the sequence (PXS/TP) and is located downstream the H4(D10S170) breakpoint generating RET/PTC1 oncogenes. This residue is also conserved in the course of the phylogenesis.

While overexpression of the H4(D10S170) gene was able to induce apoptosis of thyroid follicular epithelial cells, no proapoptotic function was observed for the S244 point mutant, thus suggesting that the H4(D10S170) protein phosphorylation by ERK1 is required for H4(D10S170)-induced apoptosis to occur. This was also suggested by the negative dominance exerted by the S244 point mutant on the H4(D10S170) proapoptotic activity.

No apoptosis was also observed by the overexpression of the H4(D10S170) truncated form corresponding to the portion of the protein included in RET/PTC1, while a negative-dominant effect on apoptosis was exerted by overexpressing this fragment together with the full-length H4(D10S170) protein. Furthermore, conditional expression of the H4(D10S170)-dominant negative truncated mutant protected cells from stress-induced apoptosis. The data showing a role in apoptosis induction exerted by H4(D10S170) and the ability of its truncated form to rescue the cells by stress-induced apoptosis suggest an explanation for the reported loss of H4(D10S170) expression in thyroid papillary carcinomas (Sheils et al 2000, Fusco et al manuscript in preparation). In the tumour initiation RET/PTC1, which is able

to form heterodimers with H4wt, might be able to modulate H4(D10S170) proapoptotic function negatively (Celetti et al 2004).

1.4 The ATM Gene Function in DNA Damage Response

DNA damage is a serious threat to cellular homeostasis because it compromises the stability and integrity of the cellular genome. Sequence alterations in DNA arise from normal genomic transactions, spontaneous chemical changes in DNA constituents, replication errors, and endogenous and exogenous agents that inflict damage on the DNA. The greatest challenge to genome stability comes from these last agents, which induce various types of DNA lesion. If not repaired, some of these lesions are extremely cytotoxic, whereas others are mutagenic with consequences ranging from malfunction of the cell to its malignant transformation.

DNA damage initiates various repair mechanisms that recognize and repair specific DNA lesions; genetic defects in crucial parts of these mechanisms lead to a group of human genetic disorders that are collectively called 'genomic instability syndromes'. These diseases are characterized by degeneration of specific tissues, sensitivity to particular DNA damaging agents, chromosomal instability and a marked predisposition to cancer. Of the many kinds of DNA lesion, double-strand breaks (DSBs) are particularly effective in triggering the DNA-damage response.

The extremely cytotoxic DSB is induced by ionizing radiation, radiomimetic chemicals and oxygen radicals formed in the course of normal metabolism, and can also follow replication fork stalling. DSBs are also part of normal genomic transactions, such as meiotic recombination and the maturation of the immune system genes via V(D)J recombination. Importantly, it has been recently shown that uncapped telomeres in senescent cells attract the same damage-response proteins that are recruited to DSBs and evoke an unregulated DNA-damage response. An ongoing DSB response has been also observed in precancerous cells and tumor tissues.

Eukaryotic cells use two main mechanisms to repair DSBs: nonhomologous end-joining (NHEJ), an error-prone ligation mechanism that acts throughout the cell cycle; and a high-fidelity process based on homologous recombination between sister chromatids, which is functional in the late S and G2 phases of the cell cycle.

The signaling network of DNA damage response affects numerous cellular systems. One of its hallmarks is the activation of cell-cycle checkpoints, which temporarily halt the cell cycle while the damage is assessed and repaired. Indeed, DSBs lead to profound changes in basic cellular processes such as gene expression, and protein synthesis, degradation and trafficking.

The most important process for the survival of a cell following ionizing radiation is the repair of DNA double strand breaks (DSBs) (Leskov KS et al, 2001). The product of the ATM gene which is mutated in the cancer prone disorder, Ataxia-teleangectasia (A-T), appears to be a major regulator of

cellular responses to ionizing radiation and plays an essential role in maintaining genome stability (Kastan, 2000; Shiloh, 2003).

ATM belongs to a conserved family of proteins termed the 'PI3K-like protein kinases' (PIKKs), most of which possess a serine/threonine kinase activity and all of which, as their name indicates, contain a domain with motifs that are typical of the lipid kinase phosphatidylinositol 3-kinase (PI3K).

Four mammalian PIKKs are involved in the DNAdamage response: DNA-PK, which has a role in the NHEJ repair pathway; ATM; ATR; and hSMG-1. Whereas ATM and DNA-PK primarily respond to DSBs, ATR mainly transduces the signal emanating from UV damage and stalled replication forks, but it also responds to DSBs, albeit later and with slower kinetics. The hSMG-1 kinase responds to both UV damage and DSBs.

Whereas ATM and ATR share substrates in the DSB response, they show selective substrate specificities in response to different genotoxic stresses and DSB inducers (Fig. 7).

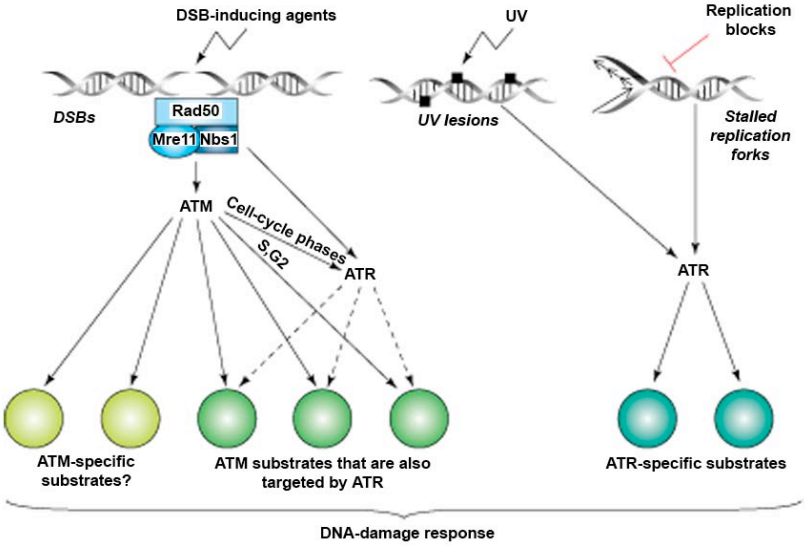

Figure 7 Separate and overlapping roles of ATM and ATR in responses to genotoxic stress. *The protein kinases ATM and ATR activate response networks after genotoxic stress by phosphorylating key proteins in various signaling pathways. The two protein kinases share substrates in the DSB response, but ATM functions upstream of ATR in some phases of the cell cycle during this response.*

ATM exists as an inactive dimer until DNA double-stranded breaks induce its activation by intermolecular autophosphorylation of Ser1981 of human ATM, leading to the generation of active monomers (Bakkenist and Kastan, 2003). ATM activation is facilitated by the interaction with the MRE11-RAD50-NBS1 complex that functions in DNA damage repair (Lee and Paull, 2004). Activated ATM triggers a series of downstream events, including phosphorylation of H2AX (γH2AX) and Brca1 to promote DNA damage repair and phosphorylation/stabilization of p53 leading to either cell cycle arrest (Shiloh 2003) or apoptosis.

2. AIMS OF THE STUDY

The aim of this study is to understand the role of H4(D10S170) gene in the signal transduction pathways activated by DNA damage and other cellular stress as critical determinant of cell survival and cellular transformation.

It has been reported a high frequency of H4 rearrangements in radiation-induced thyroid tumours, and recent observations suggested us that H4 protein could be phosphorylated by the phosphatidylinositol-3-OH kinase-like kinase (PI(3)KK) ATM after exposure to ionizing irradiation.

The purpose of the present investigation is then to verify:

1. if H4 interacts and is phosphorylated by ATM;
2. if phosphorylation at ATM-putative phosphorylation sites governs H4 intracellular localization;
3. H4 location and phosphorylation in A-T cells;
4. whether phosphorylation of H4(D10S170) could be important during cell cycle perturbations after DNA damage;
5. if the phosphorylation of H4(D10S170) is required for radio-resistant DNA synthesis;
6. the role of H4 in modulation of radiation sensitivity;
7. if the phosphorylation of ATM putative phosphorylation site/s is required for H4(D10S170) induced apoptosis in response to DNA damage;
8. if the overexpression of H4 proteins mutated at the ATM phosphorylation sites act in a dominant negative manner to inhibit the normal function of endogenous H4;
9. if the involvement of H4 in ATM-dependent DNA damage response is p53 dependent.

3. MATERIALS AND METHODS

3.1 Materials, antibodies

Etoposide [4′-Demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside), VP-16-213] is an antitumor agent that complexes with topoisomerase II and DNA to enhance double-strand and single-strand cleavage of DNA and reversibly inhibit religation.
Bleomycin (Blenoxane, Bleo, Blexane) is an antineoplastic antibiotic isolated from *Streptomyces verticillus*. Binds to DNA, inhibits DNA synthesis and causes DNA scissions at specific base sequences.
Hydroxyurea is an antineoplastic. Inactivates ribonucleoside reductase by forming a free radical nitroxide that binds a tyrosyl free radical in the active site of the enzyme. This blocks the synthesis of deoxynucleotides, which inhibits DNA synthesis and induces synchronization or cell death in S-phase.
Etoposide, Bleomycin and Hydroxyurea were obtained from Sigma Chemical, Co.
Anti-myc and secondary antibodies were from Santa Cruz Biotechnology.

3.2 Plasmids, GST Fusion Protein Production, Expression vectors.

pCDNA4TO-H4(D10S170) mutants (-T143A, -T191A, -T205A, -T323A, -T325, and -T434A) were obtained using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). H4(D10S170) cDNA fragments corresponding to nt 1231-1428 and to nt 450-1200 were amplified by PCR and then subcloned, respectively, in the BamHI-EcoRI and in the BamH1-SmaI restriction sites of pGEX2TK (Pharmacia). The resulting GST fusion proteins (GSTH4PR and GSTH4BS) were produced in BL21 bacteria and purified by glutathione-agarose beads.
pCDNA-Flag ATMwt and pCDNA-Flag-ATMKD have been generously provided by M.Kastan, and have been previously described.

3.3 Cell culture and transfection

TPC-1, 293T and SAOS2 cells were mantained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum; Hela and ataxia telangiectasia cells (B-Lymphocyte, GM01526; GM02184; Coriell Cell Repositories, Camden, NJ) were mantained in RPMI, supplemented with 15% foetal bovine serum. The Fugene reagent (Roche) was used to transfect cells accordingly to the manufacturer's instructions.

3.4 Antibody production

The anti-H4(C-17-V) antibodies were generated by immunization of rabbits with OVA-conjugated peptides (CYKALQEENRDLRKASV), aminoacids 84-99). The antibodies were affinity purified by using of the KLH-conjugated peptide.

3.5 Western Blotting

Western Blotting was performed as described (Laemli, UK, 1970; Towbin H, Staehelin, and Gordon J, 1979).
Blots were hybridized with antibodies to the indicated proteins and then with their corresponding species-specific horseradish peroxidase-conjugated secondary IgG and visualised using the ECL chemi-luminescence system (Amersham/Pharmacia)

3.6 In Vitro Kinase Assays

Cell extracts were prepared from 293T cells which had been transfected with FLAG-ATM. Cleared supernatants were immunoprecipitated with anti-Flag M2 antibody (Sigma Chemical, Co). In vitro kinase assay for ATM were performed as described earlier (Canman , Science, 1998). Immunoprecipitated Flag-ATM was confirmed by WB with anti-Flag antibody. Radiolabeled proteins were visualized and quantitated on PhosphorImager (Molecular Dynamics). For endogenous ATM kinase reactions, endogenous ATM was immunoprecipitated with ATM monoclonal antibody (NB104, Novus Biological, Inc), and in vitro kinase reactions were carried out.

3.7 Precipitation of ^{3}H thymidine labeled DNA with trichloroacetic acid

Cells were plated at 1 x 105/4 cm glass dish. One hour prior to harvesting cells were labelled with 1 mCi ml-1 3H thymidine. Cells were lysed in 25 mM Tris-HCl pH 8.0, 25mM EDTA, 0,5% SDS. 60% TCA was then added to a final concentration of 12%. Precipitated nucleic acids were collected and the amount of radioactivity was counted after the addition of Ecolume scintillant. Each experiment was carried out in triplicate.

3.8 Flow cytometry

Cells were harvested when subconfluent, fixed in 80% ethanol for 1 h at -20°C, rehydrated in PBS, and then treated with RNase A (100U/ml) for 30 min. Propidium iodide (50 μg/ml) was added to the cells for 30 minutes in the dark. Samples were analysed with a CYAN flow cytometer (Dako Corporation, San

Jose, CA, USA) using an argon-ion laser tuned to 488nm measuring forward and orthogonal light scatter, and red fluorescence measuring area and either peak of the fluorescent signal. Data were aquired using SUMMMIT® software and analysed with Modfit® software.

3.9 Mitotic cell population assessment

Cells were either untreated or irradiated with 10 Gy as indicated, then incubated for 1 hour at 37°C before fixation. Cells in mitosis were determined by staining with propidium iodide and antibody to phospho-histone H3 (P-H3) (Cell Signaling, Beverly, MA), followed by fluorescein isothyocyanate (FITC)-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) and the percentage of the M-phase cells was determined by flow cytometry.

3.10 Clonogenic assays

Cell lines were plated in triplicate at limiting dilutions into 6-well plates, incubated for 24 h, and then exposed to a range doses of ionizing irradiation (0-6Gy) followed by incubation for ten days. Prior to counting colonies, cells were fixed in 95% methanol and stained with crystal violet. A population of more than 50 cells was counted as one survived colony. The mean colony counts +/- standard errors are reported.

3.11 Indirect Immunofluorescence

The indirect immunofluorescence was performed as follow.
For immunofluorescence staining the H4(D10S170) gene product was detected with rabbit polyclonal antibody anti-H4 at 1:50 dilution applied 1h at 37 °C in a humidified chamber and a fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit secondary IgG antibody at 1:50 dilution (Jackson ImmunoResearch Laboratories Inc., West Grove, OK, USA).
For immunofluorescence staining, exogenous myc-H4wt and myc-H4T434A were detected with mouse monoclonal antibody anti-myc (Santa Cruz) at 1:50 dilution applied 1h at 37 °C in a humidified chamber and a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse secondary IgG antibody at 1:50 dilution (Jackson ImmunoResearch Laboratories Inc., West Grove, OK, USA).
After washing three times with PBS, the slides were washed with Hoechst 33258 (final concentration, 1 μg/mL; Sigma-Aldrich) to stain nuclei and then mounted in aqueous medium (Sigma, Milan, Italy).

The fluorescent signal was visualized with an epifluorescent microscope (Axioskop 2, Zeiss) interface with the image analyzer software Axiovision (Zeiss, Gottingen, Germany). FITC was irradiated at 488 nm and detected via a 505–560 nm band pass filter.

3.12 Bromodeoxyuridine incorporation

Cells were seeded on glass coverslips and bromodeoxyuridine (BrdUrd) was added to the cell culture medium at a final concentration of 100 μg/mL (BrdUrd Labeling and Detection kit, Boehringer Mannheim, Mannheim, Germany). After 1-hour incubation, cells were fixed with 70% ethanol/50 mmol/L glycine (pH 2.0). Coverslips were incubated with anti-BrdUrd mouse mAb and with a FITC-conjugated anti-mouse antibody. All coverslips were counterstained in PBS containing Hoechst 33258 (final concentration, 1 μg/mL; Sigma-Aldrich), rinsed in water and mounted in Moviol on glass slides. The fluorescent signal was visualized with an epifluorescent microscope (Axioskop 2, Zeiss) interfaced with the image analyzer software Axiovision (Zeiss, Gottingen, Germany).

3.13 RNA silencing

Small inhibitor duplex RNAs targeting human H4(D10S170) were designed with a small interfering RNA (siRNA) selection program available online at http://jura.wi.mit.edu/siRNAext/ and were chemically synthesized by PROLIGO (Boulder, CO). Sense strand for siRNA-345 targeting was (H4i-345) 5'-CAGGGCUGAGCAGGAAGAAGA AU-3', and sense strand for siRNA-419 targeting was (H4i-419) 5'-AAACCCUUGCUGUAAAUUAUG AG-3'. As a control, a not specific siRNA duplex containing the same nucleotides but in irregular sequence (scrambled) was used. The day before transfection, 1 X105 cells were plated in 60-mm dishes in medium supplemented with 10% fetal bovine serum and without antibiotics. Transfection was done using 100 nmol siRNA and Oligofectamine reagent (Invitrogen, Groningen, the Netherlands) following the manufacturer's instruction. 48 hours after transfection cells were exposed to genotoxic stress (etoposide, hydroxyurea, different doses of ionizing radiation) and BrdUdr incorporation.

3.14 MTT colorimetric assays

To determine cell viability, an MTT assay was performed as directed by the manufacturer (Roche). Briefly, the medium was removed from the cells and replaced with a fresh medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide (MTT) (Roche, 1 465 007) and incubated for 3 h at 37°C. The medium was removed and the coloured

precipitate formed by cleavage of MTT in living cells was solubilized with isopropyl alcohol containing 0.05 M HCl. Cell survival was determined by absorbance at 570 nm. The background was determined by absorbance at 690 nm.

4. RESULTS

4.1 DNA damage induces ATM-mediated phosphorylation of H4(D10S170) protein in vitro

Several putative phosphorylation sites for ATM are present in H4(D10S170) as predicted by ProfileScan primary sequence analysis and also conserved in philogenesis (Fig. 8).

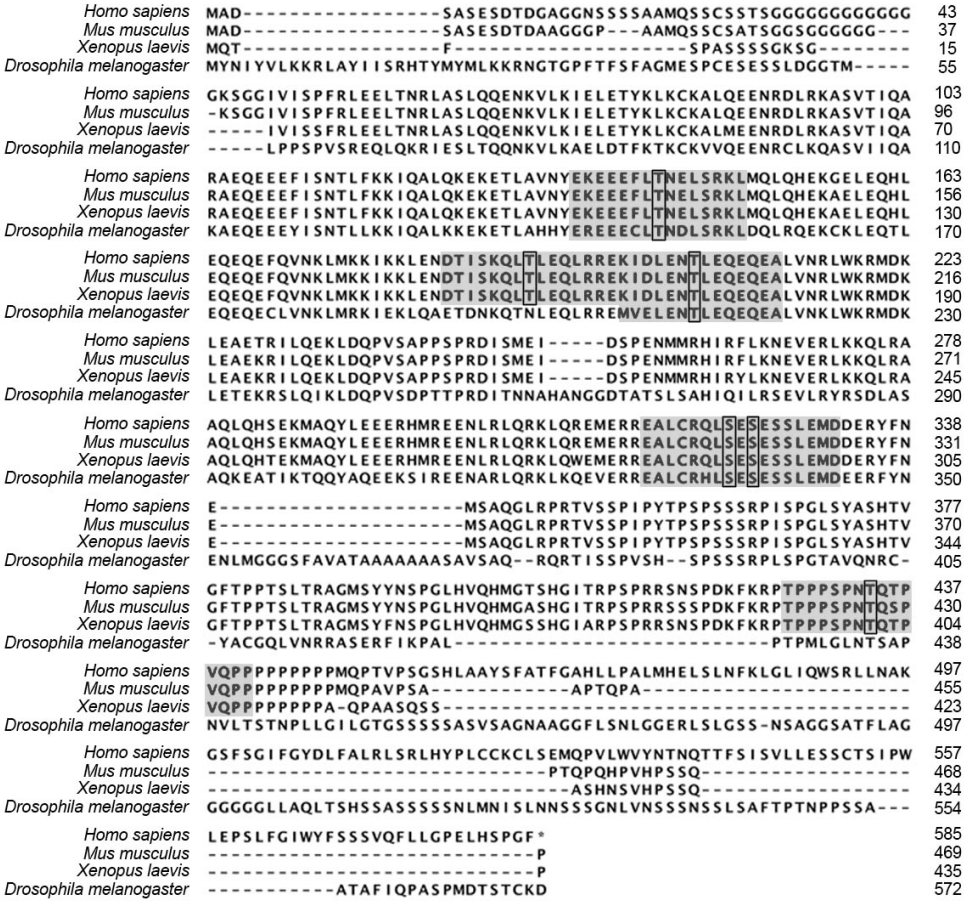

Figure 8 Alignment of H4 human protein sequence with Mus musculus, Xenopus laevis and Drosophila Melanogaster. *Aminoacid alignment of human, mouse, Xenopus and Drosophila H4(D10S170) proteins (Gen BankTM accession numbers: Homo Sapiens AAC60637; Mus Musculus XP_988138; Xenopus Laevis AAH45133; Drosophila Melanogaster NP_730234) were compared via a BLAST program (NCBI) as shown. Potential ATM family member consensus sequences (Kim, 1999) are highlighted in dark grey and predicted ATM phosphorylation sites are represented by the vertical hatched lines.*

H4(D10S170) protein was present in immunoprecipitates from unirradiated cells transfected with ATM (Fig. 9).

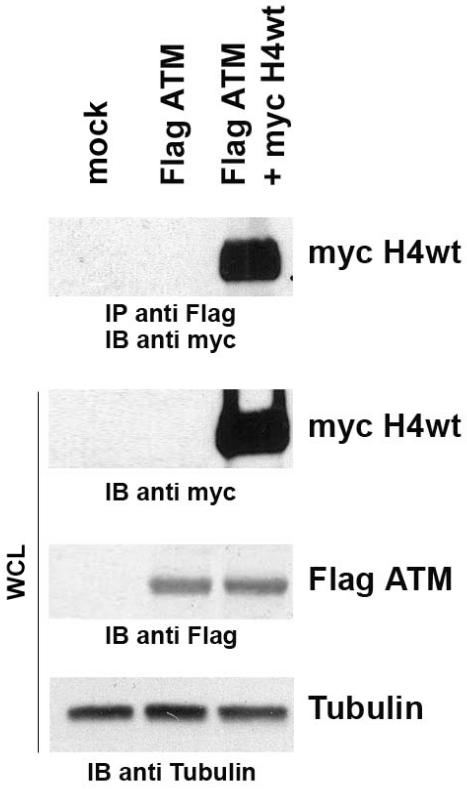

Figure 9 H4 co-immuno precipitates with ATM. *Extracts from 293T cells, transiently transfected with vector alone or expression vectors encoding Flag-ATM and myc-H4(D10S170)wt , were immunoprecipitated with the anti-Flag and blotted with anti-myc*

The requisite target sequence for phosphorylation by Atm (a serine or theonine followed by glutamine residue) (Kim et al, 1999; O'Neill et al 2000), was present in H4(D10S170) as Threonine at position 434 followed by a glutamine, Q. The GST-H4-PR (Proline-Rich region, nt 1231-1428) containing Threonine 434 (TQ) was good in vitro substrates for the wild type Atm kinase (Fig 1C). The caffeine pre-treatment impaired the H4-PR phosphorylation by wild-type ATM. A kinase-dead Atm was not able to phosphorylate H4 proline-rich, too (not shown).
Larger GST-H4 Bam-Sma fragment containing about 300 aminoacids surroundings threonine 191, threonine 205, serine 323 and serine 325 were then evaluated in these kinase assays. GST-H4 Bam-Sma was minimally phosphorylated by ATM. A GST-p53 peptide served as positive control and was efficiently phosphorylated by wild-type ATM (Fig. 10).

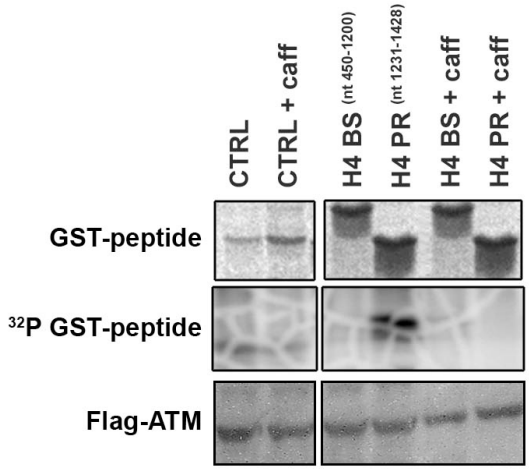

Figure 10 *Thirty minutes after irradiation (4 Gy) immunoprecipitated Flag-tagged WT-ATM was incubated in presence or absence of 1 mM caffeine with recombinant proteins consisting of several deletion mutants of human H4(D10S170) fused to GST. A p53-GST protein was used as positive control.*

4.2 ATM regulates H4(D10S170) through an increased half-life

To investigate the H4(D10S170) involvement in the DNA damage signalling pathway we assessed whether H4(D10S170) expression was induced by different genotoxic stresses. Cells treated with ionizing irradiation or etoposide or bleomycin (Fig. 11 and 12) showed increased levels of H4(D10S170) protein suggesting that the induction of H4(D10S170) protein may be a general response to DNA damage.

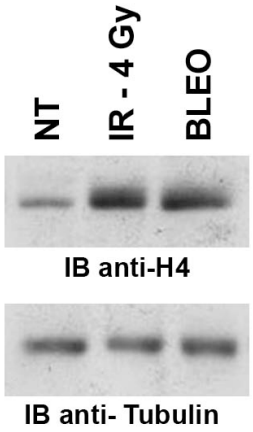

Figure 11 DNA damage increases H4(D10S170) half-life. *Lysates from HeLa cells exposed to 4Gy of IR for 90' or treated with bleomycin (BLEO, 5μg/ml for 6 h) or untreated (NT) were immunoblotted with anti-H4C17-V-ab. Anti α-tubulin was used for normalization.*

In myc-H4(D10S170) wild-type each of the six putative phosphorylation sites was altered (from serine/threonine to alanine) and the phosphorylation status and stabilization of the mutant derivatives assessed in cells treated with etoposide. The protein level of H4T434A was not increased in cells treated with etoposide but retained the constitutive level of expression observed with wild type H4(D10S170) in untreated cells (Fig. 12); similar effects were not observed with any other H4 mutants (data not shown).

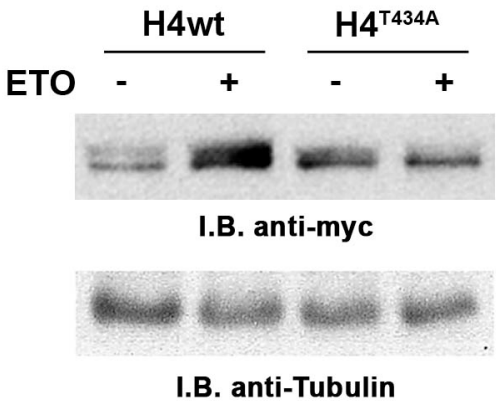

Figure 12 *HeLa cells transfected with 5 μg of expression vectors encoding H4(D10S170)wt, H4T434A or empty vector were treated with etoposide (10μM for 6h) and immunoblotted with anti-myc monoclonal antibody recognizing exogenous H4(D10S170).*

One possible explanation for our results on the regulation of H4(D10S170) by ATM is that ATM controls the abundance of H4 through an extended half-life. Although the half-life of H4 wt and H4T434A was similar in the absence of etoposide, the mutant was significantly less stable in presence of etoposide (Fig 13).

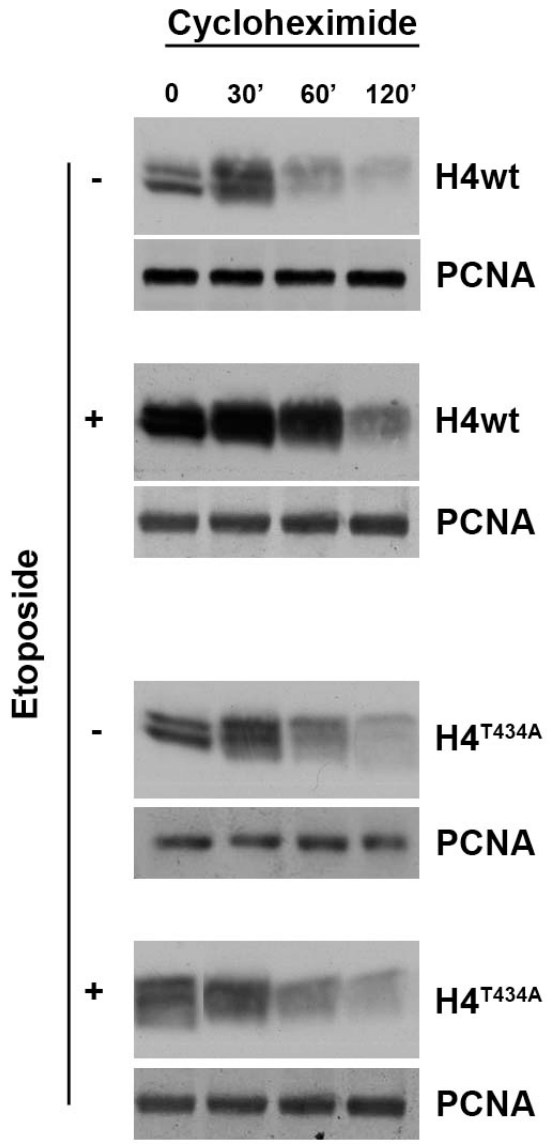

Figura 13 *HeLa cells were transfected as in fig. 10 and treated with or without etoposide (10μM) for 6h, as indicated. Cycloheximide (10mg/ml) was added and extracts were then immunoblotted with an anti-myc antibody at the indicated times. The level of PCNA is shown below each treatment as a loading control.*

As shown in figure 14, when cells were not treated with a DNA-damaging agents, the proteasome inhibitor MG132 led to significantly higher H4(D10S170) protein levels. Moreover, in the presence of MG132, levels of H4(D10S170) were not further increased upon IR or etoposide treatment (data not shown).

Thus, Threonine 434 is a functionally important residue in regulating H4(D10S170) stability in response to DNA damaging agents, etoposide, bleomycin and ionizing radiation.

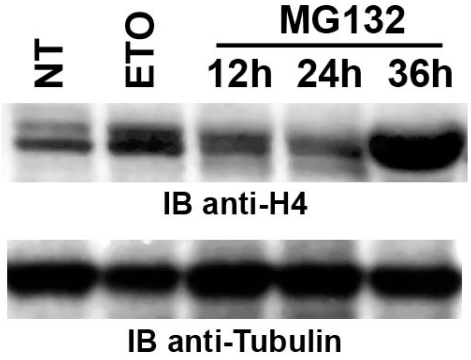

Figure 14 *Lysates from HeLa cells treated with etoposide (10μM for 6h), with MG132 (40μM at the indicated times) or untreated (NT) were immunoblotted with the anti-H4C17-V Ab.*

4.3 Phosphorylation of H4(D10S170) at Thr 434 governs intracellular localization

We investigated the mechanism through which the phosphorylation of Thr 434 may influence H4(D10S170) activity, and to this end we considered a role in regulating intracellular localization. In normal cells H4(D10S170) had a nuclear and cytosolic localization: we observed an increase in nuclear staining intensity occurring after etoposide treatment (Fig. 15c) correlating with the increased level of H4(D10S170) protein previously shown (Fig. 12). A similar effect was observed with exogenous H4, again exhibiting enhanced nuclear staining in cells treated with etoposide (Fig. 15g). Notably, the H4T434A was excluded from nuclei, being localized to the cytosol and treatment of cells with etoposide failed to alter its localization (Fig. 15i, k).

Therefore Thr 434 is able to regulate the intracellular localization of H4 in response to DNA damaging stresses.

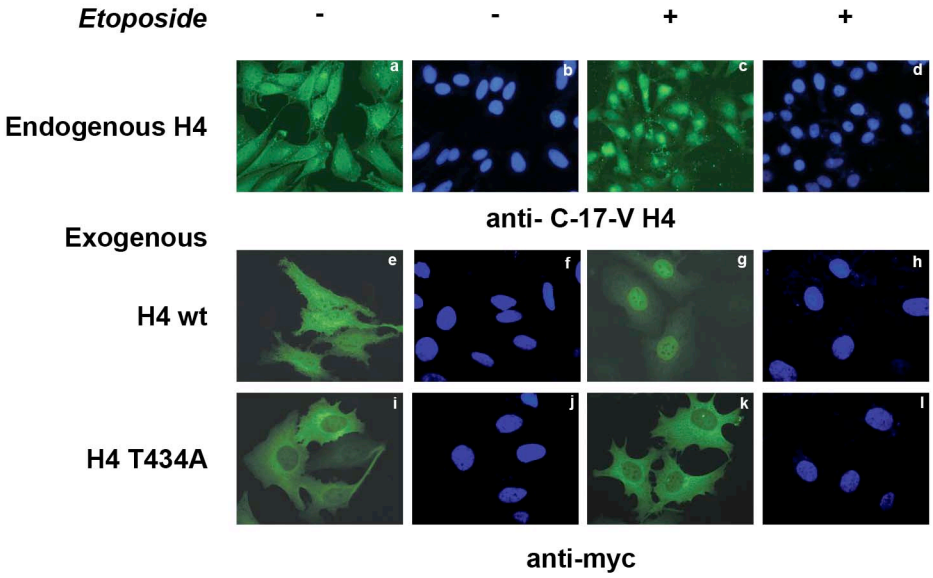

Figure 15 Intracellular localization of H4(D10S170)wt and H4T434A in HeLa cells after Etoposide treatment. *HeLa cells were immunostained with the anti-H4C17V antibody recognizing endogenous H4(D10S170) pre (a and b) or post (c and d) treatment with etoposide (10□M for 12h). HeLa cells were transfected with expression vectors encoding wild type H4(D10S170) (e-h) or H4T434A (i-l) fused to myc epitope and immunostained with anti-myc monoclonal antibody pre (e, f, i, j) or post (g, h, k, l) etoposide treatment (10μM for 12 h). Hoechst staining shown in b, d, f, h, j and l.*

4.4 H4 location and phosphorylation in A-T cells

Given the suggested role for ATM kinase in the phosphorylation and regulation of H4(D10S170) protein, we were interested in evaluating H4(D10S170) expression in ataxia teleangectasia cells. In ataxia teleangectasia lymphoblast cell lines [GM 01526 (A-T); GM 02184 (WT)] endogenous H4 localized to the cytoplasm and was excluded from the nucleus; etoposide treatment had no effect on this distribution (Fig. 16).

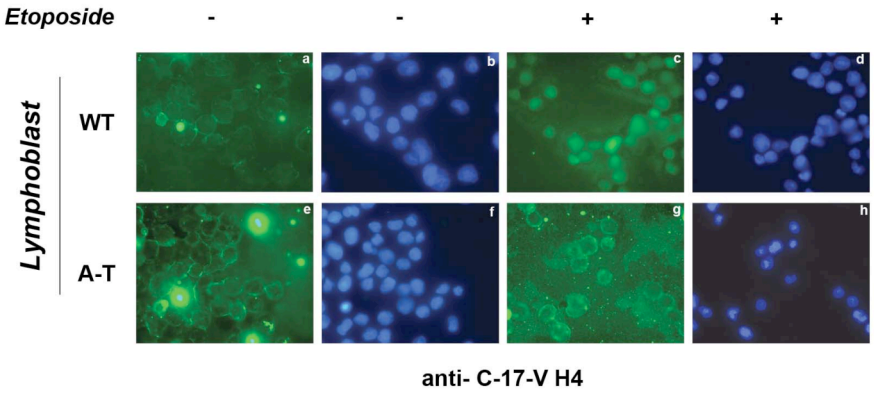

Figure 16 Intracellular localization of H4(D10S170)wt in ataxia-teleangectasia cells. *GM02184 (WT) and GM01526 (A-T) lymphoblast cells were immunostained for endogenous H4(D10S170) with anti-H4C17V Ab pre (a, b, e, f) or post (c, d, g, h) treatment with etoposide (10□M for 12 h). Hoechst staining is shown in b, d, f and h.*

No phosphorylation of the GST-H4-PR fusion protein was detected in A-T lymphoblasts up to 6 h after exposure to 10 Gy of IR, as shown in vitro kinase assay (Fig. 17) suggesting that the mechanism of threonine 434 phosphorylation following ionizing irradiation is defective in A-T lymphoblasts cells. In irradiated GM02184 normal lymphoblasts cells GST-H4-PR fusion protein was efficiently phosphorylated. Phosphorylation by ATM was specific since a caffeine (1mM) pre-treatment completely impaired this phosphorylation. Since in GM01526 ataxia teleangectasia cells ATM is missing these results further strengthen the hypothesis that ATM kinase phosphorylates Threonine 434 to regulate the intracellular location and activity of H4(D10S170).

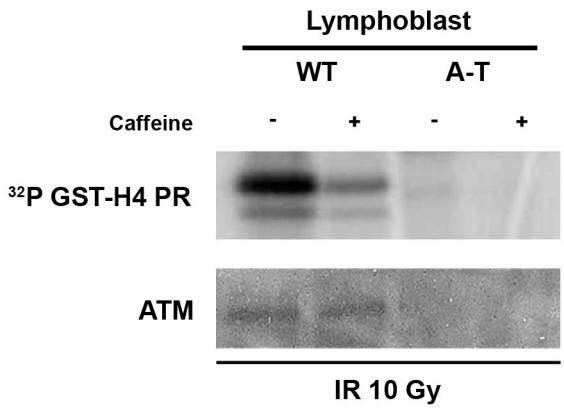

Figure 17 In vitro phosphorylation of H4(D10S170) in ataxia teleangectasia cells. *In ataxia teleangectasia lymphoblasts [GM02184 (WT) and GM01526 (A-T)] thirty minutes after irradiation (4Gy) immunoprecipitated endogenous ATM were incubated with GST-H4-PR (proline rich), in presence or not of caffeine (1mM).*

4.5 Expression of H4(D10S170) mutants increased cells survival following exposure to ionizing irradiation

In order to investigate whether H4(D10S170) can modulate the cell response to DNA damage the transient inhibition of DNA synthesis after 1 hour exposure to 10 Gy of ionizing irradiation in cells expressing wild-type or mutant H4(D10S170) was assessed. 293T cells transiently transfected with vectors expressing H4 wild-type or H4 mutants, like the H4(D10S170)-encoding portion of RET/PTC1 oncogene, H4(1-101), or H4(D10S170) with the threonine 434 mutated in alanine, H4T434A, were exposed to gamma radiation and analysed for 3H-thymidine incorporation: H4(1-101) and H4T434A expressing cells progressed to S-phase and had therefore incorporated radio-labeled thymidine. In contrast cells transfected with wild-type H4 incorporated less thymidine than control vector (Fig. 18). To investigate the potential role of these H4 mutants, we evaluated the survival of HeLa cells that transiently expressed the wild type or mutant H4(D10S170) proteins after two weeks from irradiation. The cells expressing either H4 mutant truncated at aminoacid 101 (H4 1-101) or H4(D10S170) mutated at Thr 434 (H4T434A), alone or together with H4wt, appeared radioresistant compared with cells transfected with control vector or H4(D10S170)wt (Fig. 19). Thus, similar to the truncated mutant H4(1-101) (Celetti, et al, 2004), the single point mutant H4T434A, heterodimerizing with H4wt, was able to coimmunoprecipitate efficiently the wt H4(D10S170) protein (Fig 20), behaving as dominant negative on H4wt protein function and conferring growth ability to the cell in long term colony assay.

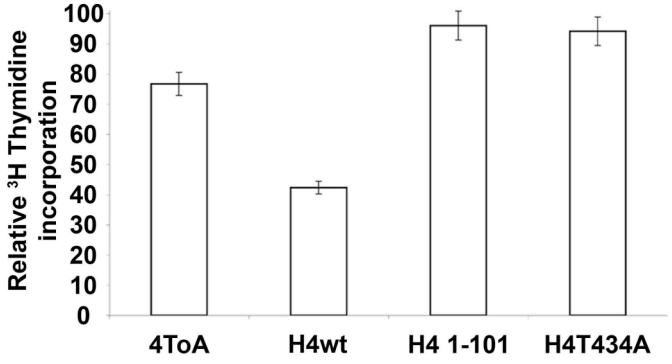

Figure 18 *Expression of H4 mutants increased ^{3}H Thymidine incorporation following IR exposure 293T cells transiently transfected with empty vector or H4(D10S170)wt, H4(1-101), H4T434A were assessed for inhibition of DNA synthesis 30 min after exposure to 10 Gy of ionizing irradiation. The average of at least triplicate samples, is reported. The graphic shows the ratio between 3H thymidine incorporation in irradiated over not irradiated cells (control). Error bars indicate s.e.m.*

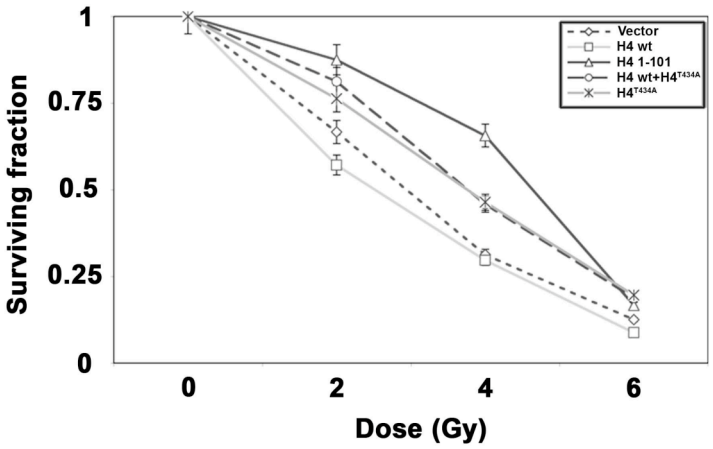

Figure 19 Expression of H4 mutants increased cell survival following IR exposure. *Expression of H4(D10S170) mutants increased radioresistance. HeLa cells expressing empty vectors, wild type, deletion or phosphorylation-site mutants constructs of H4(D10S170) were exposed to 0-6 Gy of ionizing irradiation and incubated for two weeks prior to fixation, staining and assessement of colony formation. The clonogenic survival assays were performed in triplicate. Error bars indicate s.e.m.*

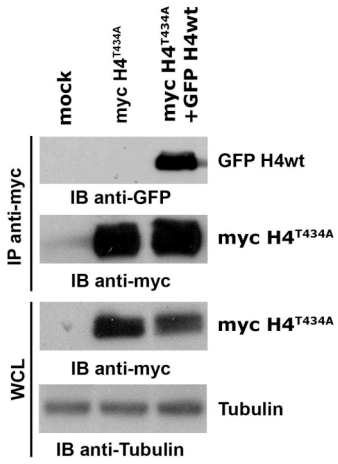

Figure 20 H4^{T434A} heterodimerizes with H4wt protein. *293T cells were transfected with the GFP empty vector (mock), GFP-H4wt, GFP-H4wt together with myc-H4T434A: the cell lysate were immunoprecipitated with anti-myc and blotted with anti GFP. Westerns blot stained with anti-myc or anti-tubulin were performed as controls.*

4.6 Phosphorylation of Thr 434 is required for apoptosis in response to DNA damage

To assess the functional significance of the interaction betweeen H4(D10S170) and ATM kinase we examined the involvement of ATM in H4-induced apoptosis. Transient expression of H4(D10S170) gene product was associated with induction of apoptosis in thyroid cells. TPC-1 cells, that harbours the RET/PTC1 rearrangement and have lost the expression of the normal unrearranged H4(D10S170) allele (Jossart et al 1996), transiently transfected with H4(D10S170)wt showed impaired apoptosis if pretreated with 50 nM wortmannin and 1 mM caffeine, known inhibitors of ATM kinase activity (Fig. 21). In addition, in TPC-1 cells, H4T434A expression had a dominant negative effect on H4wt-mediated apoptosis (Fig 21).

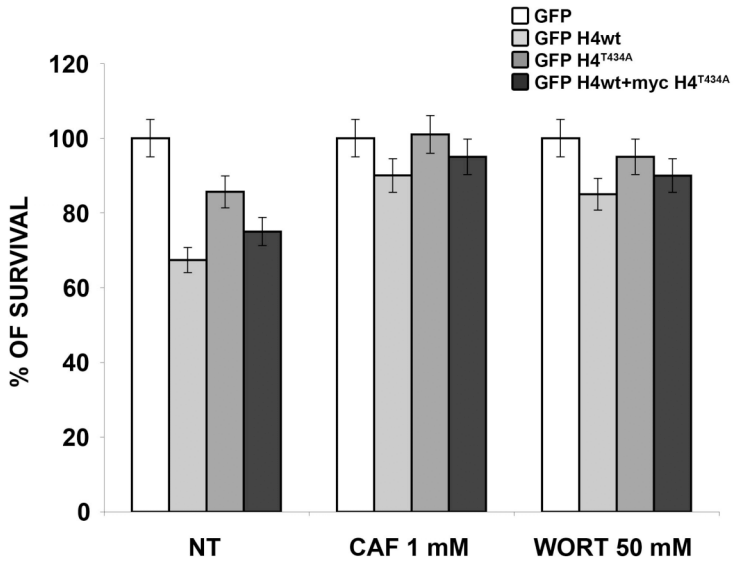

Figure 21 Percentage of survival evaluated by MTT analysis on TPC-1 cells expressing GFP, *GFPH4wt, GFP H4T434A, GFPH4wt together with mycH4T434A treated or not treated with 50 nm wortmannin or 1 mM caffeine. Values represent the mean of sextuplicate measurements and the plotted values represent the mean +/- s.e.m. of three independent experiments*.

Moreover, flow cytometry analysis showed that introduction of H4(D10S170)wt into etoposide-treated TPC-1 cells slightly increased the level of sub-G1 cells whereas introduction of the dominant negative H4T434A mutant reduced the level of apoptotic cells (Fig. 22). These results indicate a role for ATM kinase in contributing to H4-mediated apoptosis.

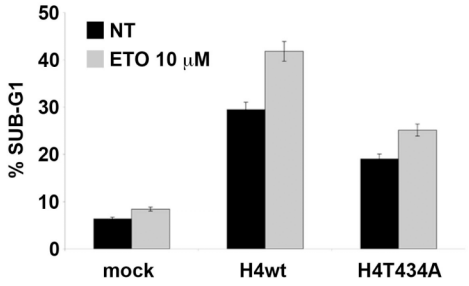

Figure 22 FACS analysis of TPC-1 cells expressing myc-H4wt, myc-H4T434A, and pcDNA vector alone, before and after the addition of Etoposide (10μM) for 12 hours. *The percentage of cell death was determined after 30 hours from transfection as subG1 population. The plotted values (lower panel) represent the mean +/- s.e.m. of three independent flow cytometry analysis.*

To rule out whether H4-apoptotic activity was p53 dependent, we used SAOS cells lacking the p53 gene product. In order to assess the function of Threonine 434 in regulating the DNA damage response, we compared the ability of wild type H4(D10S170) and H4T434A to enhance p53-dependent apoptosis in response to DNA damage. In SAOS2 cells, in which we previously observed H4-mediated cell death (Celetti et al 2004), the introduction of exogenous H4(D10S170) followed by treatment with etoposide caused cell cycle delay, increasing the G1/S population and the level of cells undergoing apoptosis (subG1) (Table 2). The presence of wild type p53 do not substantially modify the level of apoptotic cells. H4T434A failed to increase apoptosis either in presence of stress agents, either with p53, conferring a growth advantage to the cells (Table 2). These results support the idea the Thr 434 phosphorylation by ATM kinase is a functionally important event in the DNA damage response, and that it is independent by p53.

	Ctrl	p53	p53/Eto	H4wt/Eto	$H4^{T434A}$/Eto	p53/H4wt/Eto	p53/$H4^{T434A}$/Eto
Sub_G1	2,6	3,5	8,6	19,2	8,6	22,3	9,6
G1	45,7	31,4	32,6	37,1	48,2	35,3	42,4
S	23,6	68,4	67,4	56,1	29,2	56,6	32,6
G2	28,1	0,2	0	6,8	22,6	8,1	30,1

Table 2 In SAOS2 cells the introduction of exogenous H4(D10S170) followed by treatment with etoposide caused cell cycle delay. *Cell cycle distribution (%) of SAOS-2 cells transfected with the indicated constructs (Ctrl: mock transfection with pcDNA vector alone; Eto: Etoposide (20□M). Cells were transfected with 3 □g of p53, pcDNA-H4wt, and -H4T434A).*

4.7 Silencing of H4(D10S170) gene product increase cell survival, DNA synthesis and mitotic cell population after exposure to genotoxic stress

To determine H4(D10S170) role, we used small interfering RNAs (siRNAs) in the form of two independent, non-overlapping, 21-base pair RNA duplexes that target H4(D10S170) to inhibit its expression (Fig. 23).

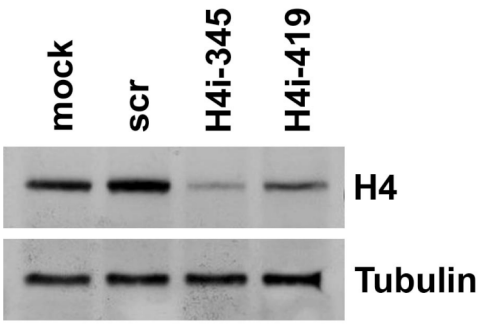

Figure 23 H4(D10S170) gene expression inhibition. *Lysates of 293T cells, transfected with siRNA oligos targeting H4(D10S170) or control oligos for 48 hours, were immunoblotted with anti-C17V-H4 and α-tubulin antibodies*

In the first instance we evaluated the percentage of survival on HeLa cells transfected with siRNA oligos and treated or not with several DNA damaging agents: the H4i-345 and H4i-419 oligos were able to confer growth advantage to the cells in comparison to the scrambled oligo (Fig 24).

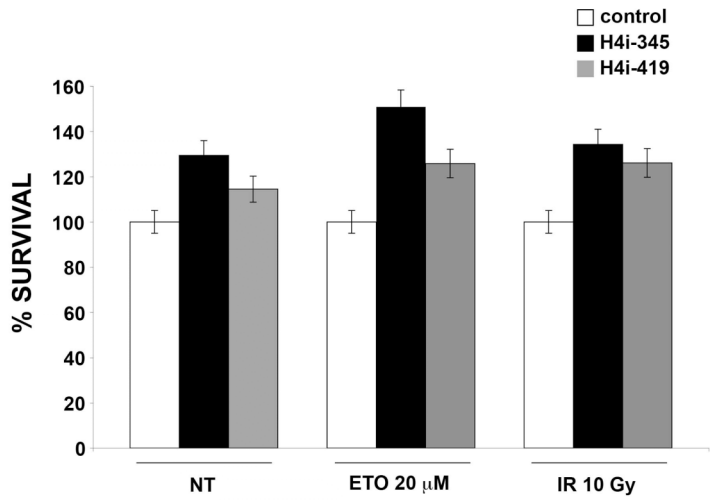

Figure 24 H4(D10S170) inhibition results in cell survival. *Percentage of survival evaluated by MTT analysis on HeLa cells transfected with siRNA oligos (H4i-345, H4i-419 and scrambled) and treated with 20 μM etoposide or 10 Gy of IR for 12h, as indicated.*

Replicative DNA synthesis was assessed in HeLa cells, 1 hour after 10 Gy doses of ionizing radiation were given: unlike the control cells, H4(D10S170)-inhibited cells showed increased BrdUrd incorporation (Fig. 25).

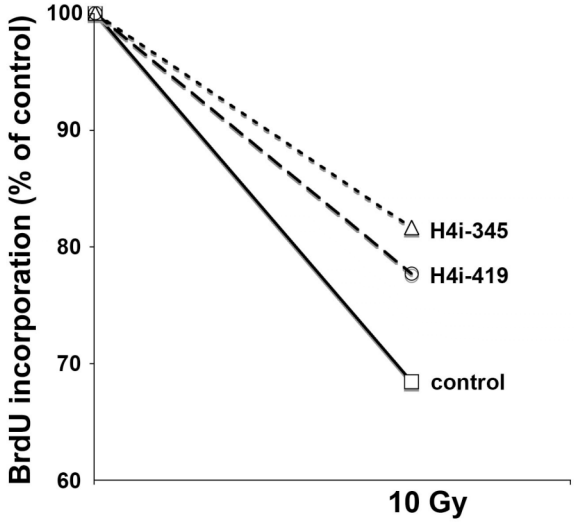

Figure 25 H4(D10S170) inhibition results in increased DNA synthesis following IR exposure. *For DNA synthesis BrdUrd incorporation was assessed 1 h after 10 Gy of IR in 293T cells transfected with siRNA oligos; the DNA synthesis in unirradiated cultures was set to 100% for cells transfected with scrambled oligos or siRNA oligos targeting H4(D10S170).*

Then, we irradiated H4(D10S170)-inhibited and control cells with 10 Gy of IR to assess the mitotic population. About double amount of H4(D10S170)-inhibited cells, with respect to the control cells, treated with 10 Gy entered into mitosis (Fig 26).

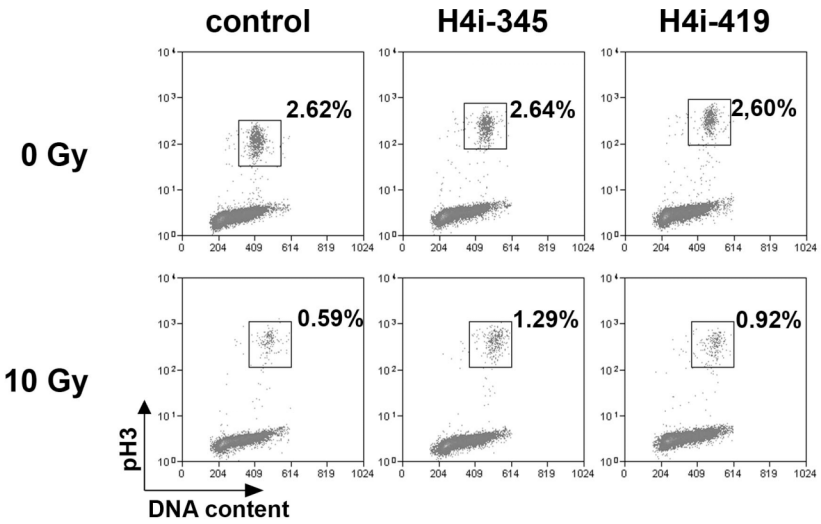

Figure 26 H4(D10S170) inhibition results in increased progression into mitosis after IR. *The mitotic percentage change of 293T cells transfected with siRNA oligos (H4i-345, H4i-419 and scrambled) 90 min after 10 Gy ionizing irradiation is shown. Mitotic cell percentage was determined by anti-phosphohistone-H3 hybridization followed by propidium iodide staining in bi-parametric flow cytometry analysis.*

5. DISCUSSION AND CONCLUSIONS

Signal transduction pathways activated by DNA damage and other cellular stresses are critical determinants of cell survival and cellular transformation. The predisposition of thyroid cells to chromosomal recombination has been ascribed to their relative resistance to cell death following DNA damage and to higher DNA end-joining enzimatic activity (Smith, 2003; Little, 2003). In the present study, we hypothesized that H4(D10S170) gene product might be involved in ATM-dependent DNA damage response on account of a high frequency of H4 rearrangements in radiation-induced thyroid tumours.

We intended to explore if H4(D10S170) interacted and was phosphorylated specifically by the ATM kinase, if H4(D10S170) was directly involved in the DNA-damage response pathway and if the H4 involvement in the radiosensitivity cell mechanisms contributed to the thyroid carcinogenesis after chromosome breackage.

Several observations suggest that H4 is regulated by ataxia teleangectasia mutated (ATM) after DNA damage. First, ATM deficient cells show no H4 phosphorylation, and no nuclear protein in response to γ-radiation compared with cells expressing WT-ATM. Second, caffeine and wortmannin treatments strongly inhibits γ-radiation-induced phosphorylation and H4-induced apoptosis. Third, the silencing of H4(D10S170) gene promotes radioresistant DNA synthesis and induces growth advantage to the cells, even in presence of genotoxic stresses. In this respect the dominant negative H4 1-101, the portion of H4 rearranged with RET in RET/PTC1 oncogene, can elicit proliferative effects and stimulate cell cycle progression in presence of ionizing radiation.

The fact that phosphorylation of H4(D10S170) is induced by DNA damage defines H4(D10S170) as a feasible effector protein in the cellular response to DNA damage. In fact, we identified the threonine 434 at COOH-terminus of H4(D10S170) as the ATM phosphorylation target site; moreover, we were able to ascribe the radioresistance DNA synthesis and the growth advantage that we observe with the truncated mutant 1-101 in presence of genotoxic stress to the lack of the threonine 434.

Strickingly, the silencing of H4 gene reproduced the same effects. These results enforced the dominant negative role exerted by H4 mutants.

No foci number differences were observed between scrambled and siRNA H4-transfected cells upon radiation exposure. Nevertheless, no H4 expression at sites of DNA damage have been reported neither colocalization with anti-phospho H2AX has been detected (data not shown). Taken together these results suggest that H4(D10S170) is an ATM substrate involved in the DNA damage-signalling pathway in mammalian cells but its role in hierarchical branched pathway for the recruitment of repair and signaling proteins to sites of DNA damage is unknown and is at moment under investigation. Several targets of ATM kinase that are involved in cellular response to ionizing

radiation have been characterized previously, including p53, Chk2, Mdm2, Nbs1, Brca1, p53BP1 and Smc1 (Kastan and Lim 2000). In this report we add the H4(D10S170) gene product to the list of bona fide ATM targets. Phosphorylation of threonine 434 site appears to be involved in both the IR-induced S phase checkpoint and in modulating radiation sensitivity. However, the mechanisms by which H4(D10S170) phosphorylation influences these processes remain to be worked out. The functional ramification of H4(D10S170) phosphorylation on DNA replication and on DNA recombination needs to be explored, as well. H4(D10S170) seems to have a somewhat general role in cellular stress responses because the data link it to response to diverse agents ranging from ionizing radiation to alkylating drugs and to oxidative stress as previously reported (Celetti et al 2004). In response to the agents other than ionizing radiation, H4(D10S170) could also be involved in stress pathway not involving ATM, and then establish a new link between DNA damage-induced signalling pathways and other protein complexes. This observation needs further investigation.

In conclusion, the results described here establish H4(D10S170) as a physiological target for ATM kinase in response to DNA damage signalling induced by etoposide treatment or ionizing radiation exposure and argue that ATM-mediated phosphorylation is involved in apoptosis mediated by H4(D10S170).

Therefore, the first 101 aminoacids of H4 in RET/PTC1 and the loss of H4(D10S170) function might have a synergistic effect in the transformation process of the thyroid cells driven by the RET kinase.

Moreover demonstrating its involvement in cellular responses to DNA damage, this study introduces the H4(D10S170) gene product in one of the most important fields in cancer biology. First, damage to cellular DNA causes cancer (we know this from epidemiological studies, from animal models and from the observation that many human-cancer-susceptibility syndromes arise from mutations in genes involved in DNA-damage responses). Second, DNA damage is used to cure cancer (most therapeutic modalities that we currently use to treat malignancies target the DNA, including radiation therapy and many chemo-therapeutic agents). Third, DNA damage is responsible for most of the side effects of therapy.

Considering the high frequency of gene rearrangements involving H4(D10S170) in neoplasia, the identification of the involvement of this gene in DNA damage and the future identification of the pathway involved will be useful for the establishment of new therapeutic approaches.

6. ACKNOWLEDGMENTS

At the end of my PhD programme, I would like to acknowledge the continuous help and encouragement that I received during my study and research.
I am thankful to Prof. Massimo Santoro who welcomed me in his group and offered me all his support to complete my PhD programme.
I am indebted to Prof. Alfredo Fusco for his incitement and all the stimulating discussions and I am also grateful to many members of the Department of Biologia e Patologia Cellulare e Molecolare in Naples for their friendly sustenance. All of them have been invaluable to my activity and have contributed to my good spirit during the past three years.
I spent an interesting and stimulating period in the laboratories of Prof. Carlo Maria Croce at the Ohio State University [Columbus, USA] and I wish to thank him and all his co-workers for their kindness and hospitality.
Words of thanks, however, would fail to express my indebtness to Dr. Angela Celetti and Prof. Giancarlo Vecchio, co-ordinator of the International Doctorate in Molecular Oncology and Endocrinology, whose continuous support, insights and suggestions have constantly stimulated my interest for research and whose influence is felt on almost every page of the present dissertation.
Finally, my gratitude goes to my family for the lovely encouragement.

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