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Functional analysis of the human RNASET2 gene: non-cell autonomous tumor suppression and putative stress-induced cell autonomous roles

Tutor: Prof. Roberto Taramelli

Co-tutor: Prof. Francesco Acquati

Tesi di dottorato di:

Marta Lualdi

Dipartimento di Scienze Teoriche e Applicate

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INTRODUCTION

NATURE AND CURRENT EXPLANATIONS OF HUMAN CANCERS

Literally, "neoplasm" means "new growth". One of the most cited definition of tumor is that given from the British pathologist R.A. Willis, in 1952: "A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimulus which evoked the change" (Willis, 1952).

From an histopathological point of view, tumors often retain the distinctive structures that characterize the tissue/organ of origin. Solid tumors tipically contain a parenchyma of proliferating neoplastic cells and a supporting tissue, or stroma.

Because tumor size typically increases with time, pathologists have postulated that the underlying cause of tumor formation must have been excessive cell proliferation in the parenchyma (Willis, 1967). Within this conceptual frame, cancer has been interpreted as a cell-centered problem, and hence, the aim of cancer research has been to understand how a normal cell becomes a cancer cell. However, as Boveri already remarked in 1914, a major problem in the study of carcinogenesis is that it is not possible to identify a neoplasm "*in statu nascendi*". Consequently, different theories of carcinogenesis have emerged. Most of them centered at the cellular level of biological organization and described cancer as a problem of cell proliferation or cell differentiation. Others, by contrast, focused on the tissue level of biological organization.

In this context, the **somatic mutation theory of carcinogenesis** (**SMT**) has been the prevailing paradigm in cancer research for the last 50 years (Curtis, 1965). Its main premise is that cancer derives from a single somatic cell that has accumulated multiple DNA mutations over time. This implies that cancers are monoclonal, deriving from aberrant proliferation of a single mutated cell (Weinberg, 1998). A second implicit premise is that the default physiological condition of cells in multicellular organisms is quiescence, where "default state" means the state in which cells are found when they are freed from any active control (Alberts, 2002). A third premise of this theory considers that cancer is a disease of cell proliferation and that cancer-causing mutations

occur in genes that control cell proliferation and/or the cell cycle (Alberts, 2001).

The rise of the SMT as the mainstream theory of carcinogenesis was supported by the following findings: (i) a considerable number of carcinogenic chemicals were found to be mutagenic; (ii) specific viral genes enabled *in vitro* cell transformation and development of tumors after injection in some animal models; (iii) DNA fragments from chemically transformed cells were in turn able to transform recipient normal cells and the DNA sequences involved were identified as mutated versions of endogenous genes (the so-called proto-oncogenes). Thus, oncogenes were considered to host "gain of function" mutations that led the cells to enhanced and de-regulated proliferation.

However, the study of rare hereditary cancers, representing 5% of all human tumors, revealed that the DNA defects transmitted along the germ line were due to deletions in specific genes. Unlike the case of oncogenes, these deletions implied a "loss of function". The first of these anti-oncogenes (later re-named "tumor suppressor genes") was retinoblastoma (RB) gene, that was soon implicated in cell cycle regulation.

Many different types of genetic variation can indeed influence the propensity for neoplastic transformation of a normal cell. They are known to influence DNA repair, cell cycle-checkpoints control, epigenetic imprinting or apoptotic proneness (Hanahan, 2000). Six **hallmarks of cancer** have been proposed by Hanahan and Weinberg in 2000 (Hanahan, 2000), which represent distinctive capabilities of tumor cells that enable tumor growth and metastatic dissemination.

The most important trait of cancer cells is their ability to **sustain chronic proliferation**. Indeed, tumor cells can (i) produce growth factors themselves and (ii) send signals to the supporting stroma inducing production of growth factors (Bhowmick, 2004). Alternatively, receptor signaling can be de-regulated by the overexpression of receptor proteins or by the constitutive activation of signaling pathways operating downstream of these receptors (Lemmon, 2010).

A second hallmark of tumor cells is their ability to **evade programs that negatively regulate cell proliferation**. Many of these programs depend on the action of tumor suppressor genes. The two prototypical anti-oncogenes encode the retinoblastoma-associated (RB) and the TP53 proteins, which

operate within the regulatory circuits that govern the decision of a cell to proliferate or, alternatively, activate senescence and apoptotic death programs (Sherr, 2002).

Third, tumor cells acquire the ability to **escape programmed cell death by apoptosis**, which is a natural barrier to cancer development. Cancer cells have evolved a lot of different strategies to circumvent apoptosis. Once again, most common is the loss of the critical damage sensor TP53, but the same result may be achieved by either overexpressing anti-apoptotic regulators (Bcl-2, Bcl-xL) or downregulating pro-apoptotic factors (Bax, Bim, Puma) (Evan, 1998).

Cancer cells also require **unlimited replicative potential** in order to generate tumors. Normal cells are able to pass through only a limited number of divisions and then enter senescence. The immortalization of tumor cells is due to their ability to maintain telomeric DNA at lengths sufficient to avoid triggering senescence or apoptosis; this is achieved by upregulating expression of telomerase or, less frequently, by an alternative recombination-based telomere maintenance mechanism (Shay, 2000).

Moreover, like normal tissues, tumors require nutrient and oxygen supplies as well as the ability to eliminate metabolic wastes and carbon dioxide. Thus, during tumor progression an **angiogenic switch** is activated, causing normally quiescent vascolature to continually sprout new vessels that help sustaining tumor growth (Hanahan, 1996).

Finally, tumor cells often acquire the ability to **locally invade tissues and/or metastasize**. Typically, alterations in cancer cell shape as well as in attachment to other cells and to the extracellular matrix occur. The invasion-metastasis cascade seems to involve discrete steps: (i) local invasion, (ii) intravasation into nearby blood and lymphatic vessels, (iii) transit through the lymphatic and hematogenous systems, (iv) extravasation, (v) micrometastases formation and (vi) colonization with development of macroscopic tumors (Talmadge, 2010).

Recently, two other distinctive characteristics of cancer cells have been proposed to be functionally important for tumor development and might therefore be added to the list of core hallmarks (Hanahan, 2011). The first involves **reprogramming of cellular energy metabolism** in order to support continuous cell growth and proliferation. The second involves active **evasion** by cancer cells **from attack and elimination by immune cells**.

Although the majority of carcinogenesis theories are strictly cancer cell-centered, in recent years tumors have increasingly been recognized as organs whose complexity must be taken into account to understand tumor biology.

Oncogenes and tumor suppressor genes are highly conserved in all mammals, as well as the pathways in which they are involved. If their pro-oncogenic mutation would be the rate-limiting step in tumor development, the frequency of tumors might be expected to increase with the number of cells in an animal. We know this is not the case. This concept is known as "Peto's paradox": a huge animal like the whale, that has 10^{17} total cells, doesn't have more but rather less tumors than the mouse, with 10^{9} total cells.

Moreover, in view of the large number of genetic and epigenetic changes that can initiate and promote tumor development, cancer can be seen nevertheless as a relatively rare disease. Two of three people never develop clinically manifest cancer and even the majority of heavy smokers remain tumor-free (Klein, 2007). These and other similar observations suggest the presence of a **systemic control** which plays an important role in tumor development. Within this framework, the reductionist view of the tumor as a collection of cancer cells must be revised and the biology of tumor development should be investigated in the context of the **tumor microenvironment** (Hanahan, 2011).

From a theoretical point of view, among the proposed organicist theories of carcinogenesis, the **tissue organization field theory** (**TOFT**) is the most known and evidence-supported. The TOFT is based on two main premises, which are not compatible with those proposed in the SMT: (i) proliferation rather than quiescence is the default state of cells in multicellular organisms and (ii) carcinogenesis acts initially by disrupting the normal interactions that take place among cells which compose the stroma and parenchyma of an organ (Sonnenschein, 2000).

In carcinomas and adeno-carcinomas, which represent the majority of human neoplasms, the proposed model is that disruption of these interactions allows the epithelial cells to exercise their constitutive property to proliferate (hyperplasia). Next, the tissue organizational pattern would become altered (dysplasia), leading to carcinoma *in situ* formation. Within this framework, somatic mutations might represent an epiphenomenon and don't have a causal role in tumor initiation and progression.

Central to this dynamic process is its **reversibility** (Clark, 1991). The neoplastic phenotype can be experimentally reversed through cell-cell interactions as demonstrated for embryonal carcinoma cells injected into blastocysts (Illmensee, 1976), hepatocellular carcinoma cells injected into normal livers (McCullough, 1998), or by modification of the extracellular matrix components (Bissell, 2001). Of course, this reversibility negatively correlate with both the tumor stage and the severity of the histoarchitecture compromission.

Because of the high grade of heterogeneity within both the tumor mass and the stromal compartment, little is known about the mechanisms that govern the interactions between cancer cells and the microenvironment. Different cell types are known to be present in the tumor surroundings: fibroblasts (cancer-associated fibroblasts, CAFs), endothelial cells, pericytes, immune inflammatory cells (tumor-associated macrophages, TAMs) and progenitor cells of the tumor stroma. All of these cells could have either a promoting or an inhibitory activity on tumor growth, depending on the presence of specific signaling molecules which induce different cell polarization (Hanahan, 2011).

The documented genetic instability of tumor cells, coupled with their rapid replication, results in the emergence of resistant clones under the selective pressure of conventional cytotoxic therapy. By contrast, the non-neoplastic stromal cells do not display marked genetic instability, thus being a better target for anti-cancer therapy (Tarin, 2012). Within this framework, the detailed investigation of the molecular interplay between cancer cells and tumor microenvironment is mandatory because of its important therapeutic implications.

ROLE OF THE RNASET2 GENE IN HUMAN CANCERS

In the past two decades, a solid body of epidemiological data was collected reporting associations between chromosomic alterations in the peritelomeric region of the long arm of human chromosome 6 and a wide range of solid and hematological tumors, i.e. ovarian carcinoma (Saito, 1992) (Tibiletti, 1996), breast cancer (Chappell, 1997), endometrial cancer (Tibiletti, 1997), gastric carcinoma (Queimado, 1995), hepatocellular tumor (Souza, 1995), colorectal cancer (Honchel, 1996), renal cell carcinoma (Morita, 1991), melanoma (Millikin, 1991), B-cell non-Hodgkin lymphoma (Gaidano, 1992) and acute lymphoblastic leukemia (Hayashi, 1990).

Particularly, researchers have put a lot of efforts into investigation of molecular mechanisms which subtend ovarian cancers growth. From an epidemiological point of view, **ovarian cancer** is the most lethal gynecological cancer. Ovarian carcinomas, accounting for 90% of all ovarian tumors, are a heterogeneous group of neoplasms. Pathologists currently employ a morphology-based classification system to divide ovarian carcinomas into four major subgroups, based on tumor grade and type of differentiation: serous (70%), endometrioid (10-15%), clear cell (10%) and mucinous (3%) carcinomas (Cho, 2009). Currently, very little is known about how the cancer initiates and progresses. The ovary has long been considered the primary origin of this tumor. However, precursor lesions have not been identified in the ovary and new evidence has emerged to propose the fallopian tube as a different source of ovarian cancer (Kurman, 2010) (Crum, 2007). Within this frame, uncovering the molecular pathways involved in ovarian carcinoma initiation and progression could improve diagnostic success and consequently reduce death rate.

Loss of heterozygosity (LoH) studies have been conducted in the last decades on ovarian tumors and ovarian cancer cell lines, showing LoH regions on human chromosomes 1, 6, 9, 10 and 11 (Nakayama, 2007) (Tapper, 1997) (Suehiro, 2000). Particularly, three different *consensus* regions of loss in human chromosome 6 were identified: 6q21-23.3 (Orphanos, 1995), 6q25.1-25.2 (Colitti, 1998) and 6q.26-27 (Saito, 1992) (Tibiletti, 1996). The **human RNASET2 gene** maps within the latter chromosomic region. It spans 27 kb, it is a single-copy gene and it is composed by nine exons and eight introns (Tibiletti, 1998). It codes for the unique human member of the extracellular Rh/T2/S ribonuclease family. T2 RNases are ubiquitous highly evolutionary-conserved glycoproteins endowed with multiple biological functions in diverse organisms (McClure, 1990).

The human RNASET2 protein is composed by 256 aminoacids and primary sequence analysis revealed the presence of three distinct protein segments: (i) a signal peptide for secretion at the N-terminus (aa. 1-24), (ii) a central core that undergoes N-glycosilation and in which resides the two conserved active-site segments (CASI and CASII) indispensable for the catalitic activity of the protein and (iii) a C-terminal de-structured portion (Campomenosi, 2006).

RNASET2 protein exists in three different intracellular isoforms: the full-lenght 36 kDa form, which is the only one secreted in the extracellular space, and the 31 and 27 kDa isoforms, which originate from proteolytic cleaveges at the C-terminus of the full-lenght protein. A sub-cellular fractionation experiment in RNASET2-overexpressing HEY4 cells has shown that both the 31 and the 27 kDa forms were present in the lysosomal fraction. This observation is consistent with the hypothesis of a functional role of RNASET2 as an acid ribonuclease in the acid lysosomal compartment (Campomenosi, 2006). As for the ribonuclease activity of RNASET2 protein, it shows a base preference for poly-A and poly-U syntetic polynucleotides, with respect to poly-G and poly-C, at pH 5.0 (Campomenosi, 2006).

Because of the chromosomic location of human RNASET2 gene in a region frequently rearranged in tumors, we investigated the putative tumor suppressive role of this gene.
Using the ovarian carcinoma as an experimental model, no mutations were found in the coding sequence of the RNASET2 gene in human primary ovarian tumors and tumor cell lines (Acquati, 2001). Nevertheless, RNASET2 down-regulation at the mRNA level was reported in both primary ovarian tumors and ovarian tumor cell lines, with respect to normal tissue/cells. In order to understand the cause of the lower RNASET2 expression levels in tumors, a promoter methylation analysis was performed. As a result, no significant differences in promoter methylation were reported between tumor and normal samples, suggesting that other epigenetic mechanisms could be responsible for RNASET2 hypoexpression in tumors (Acquati, 2001).
Using both ovarian carcinoma and malignant melanoma as experimental models, we have demonstrated a marked RNASET2-mediated tumor suppression *in vivo*. Indeed, RNASET2-overexpressing tumor clones turned out to be strongly suppressed in their tumorigenic and metastatic potential after injection in immunodeficient mice (Acquati, 2001) (Monti, 2008).
On the basis of this evidence, we can consider RNASET2 as a class II tumor suppressor gene: it is hypoexpressed in tumors, despite the absence of structural alterations in the sequence of the gene.
To address the role of the catalytic activity of RNASET2 protein in the observed *in vivo* tumor suppression, Hey3Met2 ovarian tumor clones overexpressing a

catalytically inactive form of the protein were generated. The catalytically-dead form of the protein was obtained by replacement of the two key Histidine residues in CAS sytes (H65 and H118) with two Phenylalanine residues.

Strikingly, RNASET2-mediated tumor suppression turned out to be independent of the ribonuclease activity of the protein. Indeed, using xenograft models in nude mice, RNASET2 H65/118F-overexpressing Hey3Met2 clones resulted to be suppressed in their tumorigenicity as well as their RNASET2 wild type-overexpressing counterpart (Acquati, 2005).

Moreover, a close histological examination of xenograft tumors sections revealed a consistent infiltrate of host cells in tumors derived from the injection of RNASET2-overexpressing Hey3Met2 clones. Further histological analysis with specific cell-surface markers demonstrated that the monocyte-macrophage cell lineage was predominant in this infiltrate. Particularly, M1-polarized macrophages, which have an anti-tumoral activity, were more represented than M2-polarized macrophages, which have a pro-tumoral activity (Acquati, 2011).

To further investigate the role of the monocyte-macrophage cell population in the RNASET2-mediated tumor suppression, we employed a new experimental model *in vivo*, in which the monocyte-macrophage lineage was depleted by clodronate treatment in RAG-γ chain mice. We observed that the RNASET2-mediated tumor suppression activity was drastically impaired in clodronate-treated mice if compared to the untreated control mice (Acquati, 2011). According to this view, the tumor microenvironment and particularly the monocyte-macrophage cell population seems therefore to play a crucial role in RNASET2-mediated tumor suppression that we have demonstrated to occur *in vivo*.

Supporting this hypothesis, a significant body of evidence has been recently collected strongly suggesting a role for T2 RNases in innate immune response. As an example, the T2 ribonuclease omega-1 secreted from *Schistosoma mansoni* eggs has been found to be the major soluble factor in conditioning dendritic cells (DCs) to promote Th2 lymphocyte differentiation (Steinfelder, 2009).

In spite of this strong tumor suppression activity *in vivo*, RNASET2 overexpression in tumor cells seems to have no effect on several *in vitro* cancer-related parameters, such as proliferation rate, clonogenic capability, cell-adhesion, apoptotic rate and anchorage-independent growth (Acquati, 2011).
Within this atypical framework, RNASET2 could be ranked in the novel class of "*tumor antagonizing genes*" or "*malignancy suppressor genes*" (Islam, 2000). These genes are endowed with an asymmetric tumor suppressive function, carried out *in vivo* but not *in vitro*. Most importantly, central to their anti-tumor function is the interaction with the tumor microenvironment, which plays a pivotal role in the *in vivo* tumor suppression (Klein, 1976).

T2 FAMILY OF RIBONUCLEASES AND STRESS-RESPONSE

Ribonucleases (RNases) are ubiquitous enzymes whose main function is RNA metabolism. Being involved in a wide range of important and conserved cellular functions, RNases are ancestral enzymes with a pivotal role in determining cell life or death.
Recently, a number of genome sequencing projects have highlighted the presence of several non-coding RNAs which are frequently involved in both the regulation of gene expression and the control of cell proliferation, differentiation and development (Mattick, 2004). Consequently, RNases too were supposed to have an important role in such biological processes. Indeed, the human RNases Onconase and BS-RNase have proven to be strong inhibitors of cell proliferation and the former has been included in clinical trials for the treatment of mesothelioma, a rare tumor with no effective treatments to date (Ardelt, 1991) (D'Alessio, 1993). Within this framework, RNases could be an important part of novel therapeutical strategies for treatment of tumors.

Traditionally, ribonucleases are classified on the basis of their base specificity, structure, function, optimal pH for activity and origin. Following the most common categorization criteria, RNases are classified as basic or acid. In the first group we find T1-RNase and A-RNase families, while in the second group we find almost only the T2-RNase family (Irie, 1999).
RNases belonging to the T2 family are endoribonucleases either located in cellular compartments involved in the secretory pathway or secreted directly

from the cells (Deshpande, 2002). The name "T2" originated from an RNase of *Aspergillus oryzae* that was discovered by Sato and Egami (Sato, 1975), who proposed a mechanism for T2 RNases that released 3'-adenylic acid from RNA degradation. Enzymes of the T2 family have a molecular mass around 25 kDa and they generally have no base specificity (Irie, 1999). In their protein structure, all members of this family contain two highly-conserved active-site segments (CAS I and CAS II) which are indispensable for the catalytic activity of these enzymes (Mattick, 2004).

A member of the T2 family of ribonucleases can be found in almost every organism examined including plants, fungi, bacteria, viruses and animals (Deshpande, 2002). This high rate of conservation throughout evolution suggests that these ribonucleases could have an important ancestral function. Moreover, due to gene duplication events occured during evolution, many organisms possess multiple T2 ribonuclease genes, which have diverged to take on specialized functions (Taylor, 1991).
T2 RNases are involved in a wide range of activity and their sub-cellular location is also extremely variable: lysosomes, vacuoles, free cytoplasm and nucleus (Deshpande, 2002).
Some plant ribonucleases of the T2 family have been well characterized. In tomato, two S-like RNases (RNaseLE and RNaseLX) have shown hyperexpression at both the mRNA and the protein levels during senescence (Lers, 2006). Another S-like RNase, AhSL28 from *Anthirrhinum*, increases with both senescence and phosphate starvation treatments (Liang, 2002). RNaseLE in tomato, RNS1 in *Arabidopsis* and RNaseNW in tobacco are also induced in response to wounding and during pathogen attack, suggesting an ancestral role for these enzymes in general stress-response and defence (Kariu, 1998) (Kock, 2004) (Kurata, 2002).
Orthologues of T2 RNases have been found in organisms beside plants. As well as plant enzymes, however, they were also found to be involved in stress-response processes.
In response to environmental stress, eukaryotic cells reprogram their translational machinery to allow the selective expression of proteins required for viability in the face of changing conditions. During stress, mRNAs encoding constitutively expressed "housekeeping" proteins are redirected from

polysomes to discrete cytoplasmic foci known as "stress granules" (SGs), a process that is synchronous with stress-induced translational arrest (Anderson, 2002). mRNAs within SGs are not degraded, making them available for rapid translation re-initiation in cells that recover from stress. Although this process of stress-induced mRNA stabilization is poorly understood, it likely involves the inactivation of one or more mRNA decay pathways. Two major mechanisms of mRNA degradation are active in eukaryotic cells. In the first pathway, deadenylated transcripts are degraded by a complex of 3'-5' exonucleases known as the exosome (Decker, 2002). The second pathway entails the removal of the 7-methyl-guanosine cap from the 5'-end of the transcript by the DCP1-DCP2 complex, allowing 5'-3' exonucleolytic degradation by XRN1. In yeast, but data were confermed also in mammalian cells, components of the 5'-3' decay pathway are concentrated at discrete cytoplasmic foci known as "processing bodies" (PBs) (Sheth, 2003).

Recent studies have described a novel aspect of stress-induced response in eukaryotic cells wherein cytosolic tRNAs are separated into half molecules by cleavage in the anticodon loop. Such mechanism has been observed in plant, yeast and human systems, in particular during oxidative stress (Thompson, 2008). Interestingly, T2 ribonucleases has shown to take part to this stress-response strategy.

As an example, the T2 RNase Rny1p from *Saccharomyces cerevisiae* has been shown to cleave tRNAs during periods of oxidative stress (MacIntosh, 2001); in fact, during treatment this RNase was released from the vacuole and cleaved tRNAs only in the affected cells (Thompson, 2009). When Rny1p was overexpressed in yeast cells, the latter developed an hypersensitivity to oxidative stress which led to reduced viability. Surprisingly, this death-promoting function of Rny1p turned out to be independent of its catalytic activity. More surprisingly, human RNASET2 overexpression in yeast cells led to reduced viability, too (Thompson, 2009). Within this framework, increasing cytoplasmic Rny1p would be a stress signal to the cell. Following a small Rny1p increase, tRNAs destruction might be sufficient for stress relief. By contrast, following a more prominent increase, cell-death program is triggered.

As is widely recognized, stress, in particular metabolic stress, is of paramount importance for cancer initiation and progression. As an example, tumor cells

have to cope with acidification of both the intra- and the extra-cellular environments, with nutrient deprivation and with hypoxia (Hanahan, 2011). There is plentiful scientific literature reporting human tumor suppressor genes which play a pivotal role in stress-response: TP53 is among the most known (Schetter et al., 2012).

Within this framework, we set up to define whether human RNASET2 also plays a role in stress-response in mammalian cells. While investigating the intracellular trafficking of RNASET2, we have recently found that the protein shows co-localization with processing-bodies upon metabolic stress (Vidalino, 2012). This is the first evidence for a putative RNASET2 involvement in RNA metabolism in response to stress conditions.

AIM OF THE PROJECT

The human RNASET2 gene maps in 6q27, a chromosomal region which has been frequently found to be deleted or rearranged in a wide range of solid and hematological tumors. Using ovarian carcinoma as an experimental model, we have demonstrated a role for this gene in tumor suppression. Indeed, *in vivo* xenograft models in immunodeficient mice have revealed a significant RNASET2-mediated suppression of tumorigenicity, which was not dependent on the ribonuclease activity of the protein. On the other hand, we found that the overexpression of RNASET2 in ovarian tumor cell lines had no effect on several *in vitro* cancer-related parameters. We thus ranked RNASET2 in the class of "tumor antagonizing genes" or "malignancy suppressor genes", whose function is carried out only *in vivo* and it is mainly related to the tumor microenvironment. Within this conceptual framework, a close histological examination revealed a consistent infiltrate of host macrophages in xenograft tumors derived from the injection of RNASET2-overexpressing tumor clones. In the attempt to gain more insights into this microenvironmental contribution to RNASET2-mediated tumor suppression, we have recently demonstrated that, following selective *in vivo* depletion of the monocyte/macrophage cell lineage in immunodeficient mice, the RNASET2-mediated tumor suppression was impaired.

In order to gain more insights into this non-cell autonomous RNASET2 function, the first aim of my Ph.D. project was to **investigate the putative occurrence of a functional cross-talk between extracellular RNASET2 protein and the tumor microenvironment**.

My working model is the following: RNASET2 protein could activate an host immunocompetent response against the tumor by means of (i) the production and secretion of signaling molecules, endorsed with immunological functions, by the RNASET2-overexpressing cancer cell itself, or (ii) a direct interaction between secreted RNASET2 protein and immunocompetent cells.

As for the aforementioned first point, I have examined genes whose expression level is modulated by RNASET2 both *in vitro* and *in vivo*. More precisely, I have determined the gene expression profile of cultured RNASET2-overexpressing ovarian tumor cell clones and compared it to that of xenograft tumors deriving from the injection of the same tumor clones into mice.

As for the second point, I have first investigated the cell-surface binding of RNASET2 protein on different tumor cell lines and on both native and M1/M2-polarized human macrophages. Then, I have examined the mechanisms of a putative chemotactic activity of RNASET2 protein on both primary monocytes and pro-myelocytic cell lines.

Being T2 ribonucleases so highly-conserved throughout all the *phyla*, from viruses to mammalian cells, we reckoned that RNASET2 could be endowed with an ancestral cell-autonomous activity, in particular within the context of a general stress-response. Indeed, an important role in counteracting nutrient starvation and pathogen attacks has been recently demonstrated for several members of the RNase T2 family in plants, fungi and yeasts. Since we have recently identified RNASET2 protein as a novel component of processing-bodies, determining whether human RNASET2 plays a stress-response role in mammalian cells could thus be of great interest.
To shed light on this issue, the second aim of my Ph.D. project was to **investigate the putative cell autonomous role of human RNASET2 in general stress-response processes**.
To this end, I have challenged different tumor cell lines with a variety of stress-inducing chemicals or treatments and then I have examined changes in both RNASET2 protein expression levels and sub-cellular localization patterns.

RESULTS (Part I)

As previously mentioned, the principal aim of my Ph.D. project has been to gain more insights into the non-cell autonomous tumor suppressive function of RNASET2 gene. Particularly, I have been involved in investigation of the putative occurrence of a functional cross-talk between extracellular RNASET2 protein and the tumor microenvironment.

The rationale of my experimental work was based on two main hypothesis, which are not mutually exclusive, about the mechanisms by which RNASET2 carries out its oncosuppressive role *in vivo*: (i) RNASET2-overexpressing tumor cells could produce and secrete signaling molecules, endorsed with immunological functions, which in turn activate an host immunocompetent response against the tumor; (ii) RNASET2 protein secreted by the tumor cell could directly interact with immunocompetent target cells and recruit them at the tumor site (**Figure 1**).

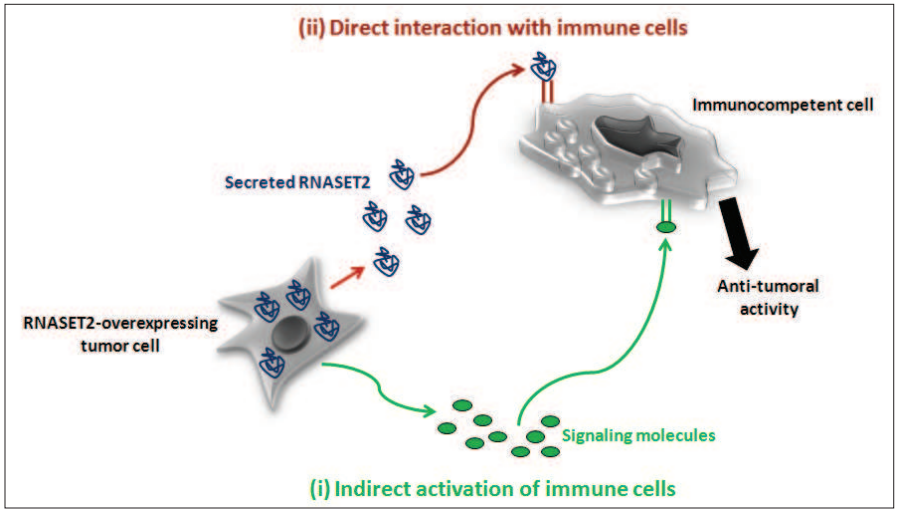


Figure 1 - Schematic representation for both the direct and the indirect model of cross-talk involving RNASET2-overexpressing tumor cells and innate immune cells.

Within this conceptual framework, I'm presenting experimental data concerning both the aforementioned hypothesis.

RNASET2 overexpression in the tumor cell induces changes in gene expression profile, both *in vitro* and *in vivo*

I decided to start my experimental investigations within the hypothesis of an indirect cross-talk involving RNASET2-overexpressing tumor cells and innate immune cells. More precisely, I assessed whether the RNASET2-overexpressing cancer cells showed changes in their gene expression pattern which could be consistent with the secretion of signaling molecules, such as cytokines and chemokines, involved in both recruitment and functional activation of innate immune cells.

Although the best experimental setting for a tumor antagonizing gene is the *in vivo* context, I started my investigation with an *in vitro* setting, using the RNASET2-overexpressing Hey3Met2 ovarian cancer model in which we had previously demonstrated a strong *in vivo* tumor suppression (Acquati, 2005), together with a marked recruitment of immune cells at the tumor site (Acquati, 2011).

Within this experimental context my principal aim was to detect upregulation of signaling molecules involved in innate immunity in RNASET2-overexpressing cells, with respect to control cells. Particularly, I have first defined the gene expression profile of RNASET2-overexpressing Hey3Met2 cell clones and compared it with that of control Hey3Met2 clones. To this end, an Agilent Whole Human Genome Oligo microarray was performed with total RNA from Hey3Met2 clones stably-overexpressing either the wild type or the catalitically inactive (H65/118F) form of the RNASET2 protein, both of them being effective in tumor suppression. Total RNA from Hey3Met2 clones stably transfected with the empty vector was used as a control. As a result, sixty-five genes were found to be modulated by RNASET2 and were further organized by hierarchical clustering (**Figure 2**) (Acquati, 2011).

In order to define the cellular processes/pathways affected by RNASET2, all differentially-expressed genes were cross-referenced to the Gene Ontology database, which in turn did not show any significant enrichment, probably due to the small number of genes analyzed. Of note, quite unexpectedly, among the sixty-five modulated genes we have found no genes encoding signaling molecules involved in innate immunity.

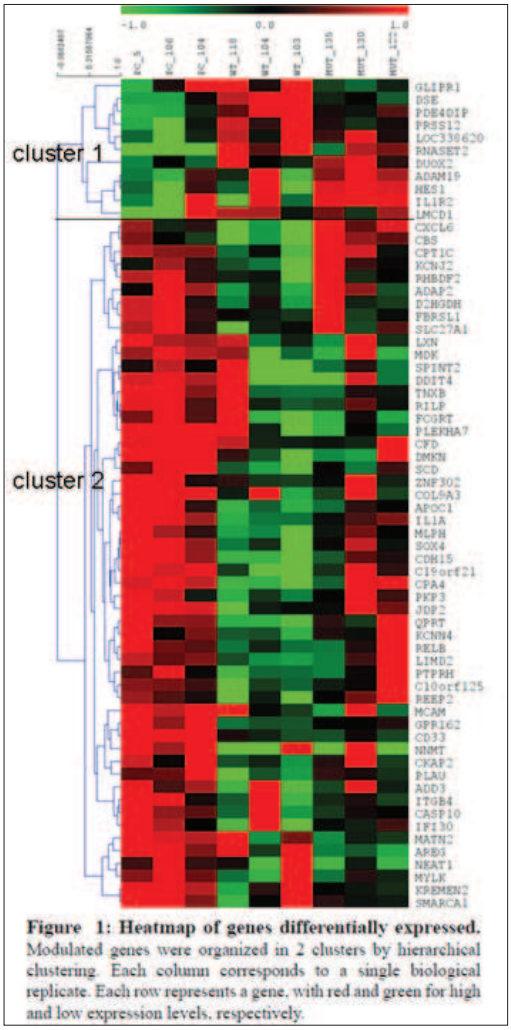


Figure 1: Heatmap of genes differentially expressed. Modulated genes were organized in 2 clusters by hierarchical clustering. Each column corresponds to a single biological replicate. Each row represents a gene, with red and green for high and low expression levels, respectively.

Figure 2 - Image from: Acquati, F. (2011). Molecular signature induced by RNASET2, a tumor antagonizing gene, in ovarian cancer cells. *Oncotarget 2*, 477-484

Nevertheless, among the sixty-five modulated genes we selected a set of thirteen RNASET2-responsive genes for further validation, mainly because of their involvement in cancer-related processes such as cell-adhesion, migration, proliferation and differentiation (**Table 1**) (Acquati, 2011). In fact, besides our data demonstrating a non-cell autonomous tumor suppressive function for the RNASET2 gene, we could not completely rule out the hypothesis that RNASET2

could also carry out a cell autonomous role that we had not previously investigated.

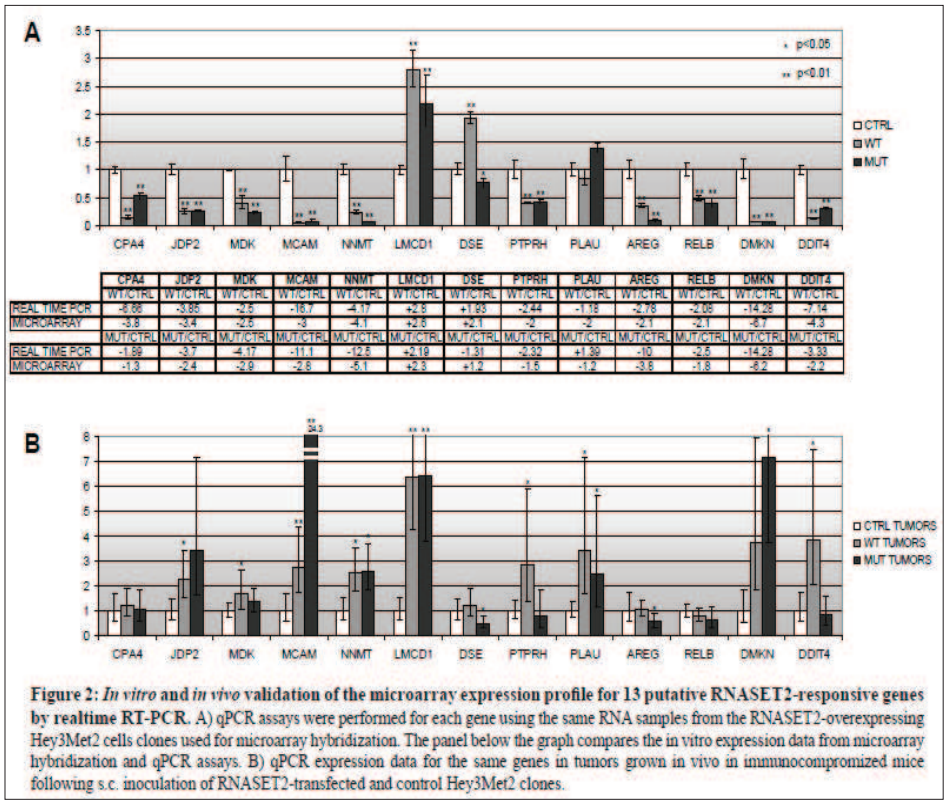
Table 1: List of RNASET2-modulated genes selected on the basis of fold change, GO analysis and biological relevance.

GENE SYMBOL	GENE DESCRIPTION	FOLD-CHANGE WT	FOLD-CHANGE MUT	GENE ONTOLOGY
MDK	midkine (neurite growth promoting factor 2)	-2.5	-2.9	heparin binding; glycosaminoglycan binding
MCAM	melanoma cell adhesion molecule	-3	-2.8	cell adhesion;motility
AREG	amphiregulin	-2.1	-3.8	growth factor activity-cell invasion
NNMT	nicotinamide N-methyltransferase	-4.1	-5.1	cell migration
PLAU	plasminogen activator,urokinase	-2.0	-1.2	wound healing; fibrinolysis and degradation of extracellular matrix.
DDIT4	DNA-damage-inducible transcript 4	-4.3	-2.2	
CPA4	Carboxypeptidase A4	-3.8	-1.3	peptidase activity;zinc ion binding.
RELB	v-rel reticuloendotheliosis viral oncogene homolog B	-2.1	-1.8	DNA binding; transcription factor activity; transcription regulator activity
JDP2	Jun dimerization protein 2	-3.4	-2.4	DNA binding;transcription factor activity;transcription regulator activity
DMKN	dermokine	-6.7	-6.2	Rab GTPase binding
PTPRH	protein tyrosine phosphatase receptor type H	-2	-1.5	phosphoprotein phosphatase activity;transmembrane receptor protein phosphatase activity
DSE	dermatan sulfate epimerase	+2.1	+1.2	racemase and epimerase activity;chondroitin-glucuronate 5-epimerase activity
LIMCD1	LIM and cysteine-rich domains 1	+2.6	+2.3	zinc ion binding;transcription factor binding;transcription regulator activity

Table 1 - Image from: Acquati, F. (2011). Molecular signature induced by RNASET2, a tumor antagonizing gene, in ovarian cancer cells. *Oncotarget 2*, 477-484

In order to validate changes in the expression of these thirteen genes, I performed real time RT-PCR (qPCR) assays on the same RNA samples used for microarray hybridization. As a result, the pattern of gene expression changes observed following microarray hybridization was confirmed by qPCR for most tested genes (**Figure 3A**) (Acquati, 2011).

On the basis of these results, we asked whether some of these genes could have any relevance in RNASET2-mediated tumor suppression *in vivo*. To this end, since we could not perform an *in vivo* microarray analysis, total RNA extracted from xenograft tumors was employed for qPCR analysis of the expression pattern of the selected thirteen genes. As shown in **Figure 3B**, the RNASET2-mediated changes in the expression levels that we have previously observed *in vitro* were not confirmed for most tested genes in the *in vivo* setting. Indeed, significant changes in the expression pattern that were found to be in agreement with those observed *in vitro* could be reported for just three genes, namely LMCD1, DSE and RELB.



	CPA4	JDP2	MDK	MCAM	NNMT	LMCD1	DSE	PTPRH	PLAU	AREG	RELB	DMKN	DDIT4
	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL	WT/CTRL
REAL TIME PCR	-6.66	-3.85	-2.5	-16.7	-4.17	+2.8	+1.93	-2.44	-1.18	-2.78	-2.06	-14.28	-7.14
MICROARRAY	-3.8	-3.4	-2.5	-3	-4.1	+2.6	+2.1	-2	-2	-2.1	-2.1	-6.7	-4.3
	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL	MUT/CTRL
REAL TIME PCR	-1.89	-3.7	-4.17	-11.1	-12.5	+2.19	-1.31	-2.32	+1.39	-10	-2.5	-14.28	-3.33
MICROARRAY	-1.3	-2.4	-2.9	-2.6	-5.1	+2.3	+1.2	-1.5	-1.2	-3.8	-1.8	-6.2	-2.2

Figure 2: *In vitro* and *in vivo* validation of the microarray expression profile for 13 putative RNASET2-responsive genes by realtime RT-PCR. A) qPCR assays were performed for each gene using the same RNA samples from the RNASET2-overexpressing Hey3Met2 cells clones used for microarray hybridization. The panel below the graph compares the in vitro expression data from microarray hybridization and qPCR assays. B) qPCR expression data for the same genes in tumors grown in vivo in immunocompromized mice following s.c. inoculation of RNASET2-transfected and control Hey3Met2 clones.

Figure 3 - Image from: Acquati, F. (2011). Molecular signature induced by RNASET2, a tumor antagonizing gene, in ovarian cancer cells. *Oncotarget 2*, 477-484

Taken together, these results provide a clear indication that investigations of the molecular mechanisms by which tumor antagonizing genes carry out their biological functions necessitate a thorough comparison between the *in vitro*

and the *in vivo* expression patterns. Thus, LMCD1, DSE and RELB genes represent *bona fide* candidate effector genes for RNASET2-mediated tumor suppression *in vivo* and are worth to be analysed in depth. However, these results clearly indicate that the employment of an *in vitro* approach to investigate biological properties of a gene which exerts its antitumoral function only in the *in vivo* context could not be the right choice. Thus, at least within the described experimental framework, the hypothesis of an indirect mode of action of RNASET2 protein in tumor suppression seems to be not so plausible.

After the investigation of the indirect model of cross-talk between RNASET2-overexpressing tumor cells and innate immune cells, I focused my experimental work on the hypothesis of a **direct** interaction between RNASET2 protein and immunocompetent cells (**Figure 4**).

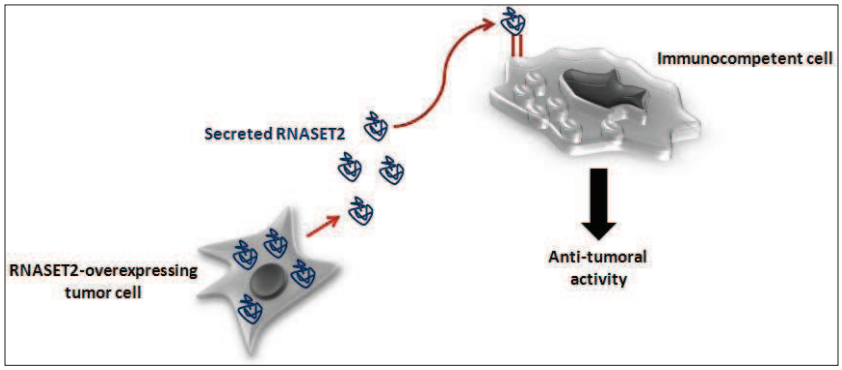


Figure 4 - Schematic representation for the direct model of cross-talk involving RNASET2-overexpressing tumor cells and innate immune cells.

Central to this concept is the observation that the RNASET2 protein is secreted in the extracellular space and has therefore the potential to directly interact with a target cell. According to our previous data showing a consistent infiltration of host macrophage cells in RNASET2-overexpressing xenograft tumors, cells from the monocyte-macrophage lineage could reasonably represent potential target cells for this protein. Within this frame, I have first investigated the cell surface binding of RNASET2 protein in different target cells and then I assessed the chemotactic activity of RNASET2 protein on monocyte-macrophage cells.

RNASET2 protein binds to the tumor cell surface and could be internalized

In order to gather evidence for a direct interaction between RNASET2 protein and the cell surface, I have first investigated the issue of a cell-surface binding of endogenously-produced RNASET2 protein on tumor cells. To this end, an *in vivo* cell-binding assay was firstly performed in the human cervical carcinoma-derived HeLa cell line model, which showed an intermediate RNASET2 expression level when compared to other cancer cell lines. The assay consisted in avidin-mediated precipitation of biotinylated cell surface proteins, followed by a Western blot analysis of the pulled-down proteins (**Figure 5**). As a result, endogenously produced RNASET2 actually proved to bind to the cell surface of human HeLa cancer cells.

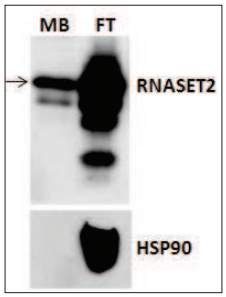


Figure 5 - Cell surface binding of RNASET2 protein on HeLa cells. HeLa cell surface proteins were labeled with biotin, then cells were lysed and the labeled proteins were pulled-down using an avidin-functionalized resin. A Western blot analysis was performed with both anti-RNASET2 and anti-HSP90 primary antibodies on both pulled-down proteins (membrane bound MB) and column flow through (FT). HSP90 was only found in the FT, as expected for an exclusively intracellular protein. All of the three RNASET2 protein isoforms were found in the FT, while the secreted 36 kDa form was also found in the membrane-bound pulled-down proteins pool (black arrow).

Therefore, I decided to test the cell surface binding of endogenously-produced RNASET2 protein in other human tumor cell lines, including our experimental model: ovarian carcinoma cells. To this end, a flow cytometry analysis was performed using a fluorophore-conjugated anti-RNASET2 antibody. As a result, a significant membrane RNASET2-relative fluorescent signal was detected in a human ovarian cancer cell line (OVCAR3), in two human colon cancer cell lines (RKO and HTC-116) and in a human fibrosarcoma-derived cell line (8387) (**Figure 6**).

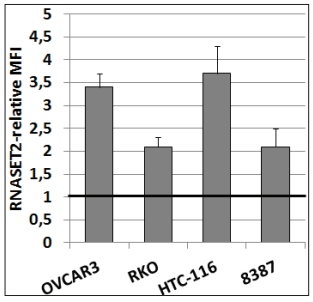


Figure 6 - Cell surface binding of RNASET2 protein on tumor cells. Briefly, cells were harvested and incubated for 30 minutes in the dark with fluorophore-conjugated anti-RNASET2 primary antibody. Each bar represents RNASET2 relative MFI (membrane fluorescence intensity) value, that is the ratio between RNASET2 MFI and isotypic control (anti-human rabbit IgG) MFI. A ratio equal to 1 (black baseline) means no RNASET2-specific membrane signal.

Finally, I have employed a different experimental approach to confirm RNASET2 binding to the plasma membrane and to investigate the possibility of the internalization of the surface-bound protein. We have recently established in our laboratory an RNA interference-mediated silencing of RNASET2 in the human ovarian cancer cell line OVCAR3, which is a poorly tumorigenic cancer cell line with high RNASET2 endogenous levels (Acquati 2012, submitted) (**Figure 7**).

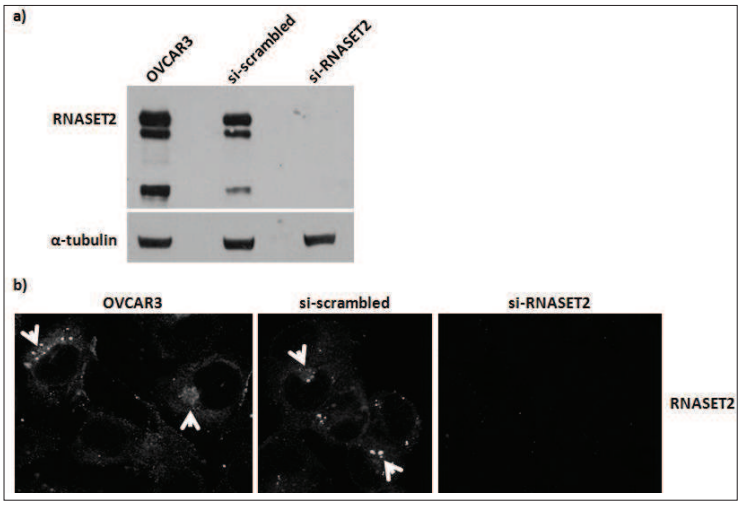


Figure 7 - RNASET2 knockdown in the human ovarian cancer cell line OVCAR3. a) Western blot analysis of RNASET2 protein expression in parental OVCAR3 cells and in either si-scrambled or si-RNASET2 stably-transfected OVCAR3 clones. RNASET2 protein resulted completely absent in silenced OVCAR3 cells. **b)** Indirect IF assay showing cytoplasmic RNASET2 staining in OVCAR3 cells and confirming RNASET2 knockdowm in silenced OVCAR3 clones. Particularly, a peri-nuclear localization is highlighted (white arrows). Confocal microscopy images. 40X.

Using this model, I have performed an indirect immunofluorescence assay with an anti-RNASET2 antibody on stably RNASET2-silenced OVCAR3 cells after a 24 hours exposure to a conditioned medium rich in RNASET2. Particularly, cells were either permeabilized or left untreated after fixation, in order to confirm not only the cell surface binding, but also investigate the possibility of the internalization of the membrane-bound protein.

As a result, only a surface RNASET2 signal was obtained in the unpermeabilized cells. In the permeabilized RNASET2-silenced OVCAR3 cells, by contrast, a cytoplasmic signal was also detectable, consistent with the hypothesis of RNASET2 binding to the cell-surface which is then followed by the internalization of the protein within the cancer cell itself (**Figure 8a**). Of note, the same results were obtained after a 24 hours exposure of RNASET2-silenced OVCAR3 cell clones to recombinant RNASET2 protein produced in the baculovirus/insect cells heterologous expression system (**Figure 8b**).

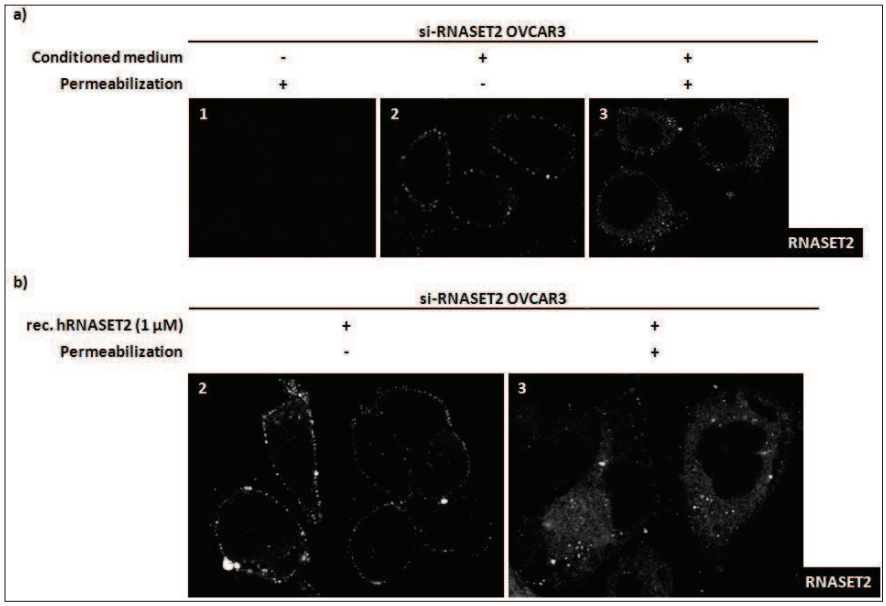


Figure 8 - Surface binding and internalization of exogenously-provided RNASET2 protein in RNASET2-silenced OVCAR3 cells. RNASET2-silenced OVCAR3 cells plated on coverslips were incubated for 24 hs with either a conditioned medium rich in RNASET2 (**a**) or the recombinant RNASET2 protein at the final concentration of 1 µM (**b**). After incubation, cells were either fixed and permeabilized or only fixed and immunostained. In untreated RNASET2-silenced cells, no RNASET2 signal was detectable (**a1**). A cell surface staining was detectable in unpermeabilized cells (**a2**, **b2**). A cytoplasmic staining was also observable after permeabilization, thus suggesting the internalization of the membrane-bound protein (**a3**, **b3**).

Taken together, these data demonstrate that endogenously-produced RNASET2 protein could bind the tumor cell surface. Moreover, using our RNASET2-silenced ovarian cancer cell model, I also demonstrated that exogenously-provided RNASET2 protein could be internalized following the binding to the tumor cell surface.
Noteworthy, as previously mentioned, the target cell of RNASET2 function is likely to be the innate immune cell, rather than the cancer cell. However, these results demonstrating both the cell surface binding and the internalization of RNASET2 protein within the cancer cells suggest the existence of a common, likely receptor-mediated, binding/internalization pathway for RNASET2 protein, which could be cell-type independent.

RNASET2 protein binds to human macrophage cell surface in a dose-dependent manner

After investigating the cell surface binding of RNASET2 protein on tumor cells, I focused my work on the cell-type which could more likely represent the target of RNASET2. According to this view, extracellular RNASET2 could be directly involved in recruiting innate immune cells at the tumor site and these immunocompetent cells could eventually mediate the effective tumor suppression that we have observed in mice after injection of RNASET2-overexpressing tumor clones.
To shed light on this issue, I have first investigated the cell surface binding of exogenously-provided RNASET2 protein on both native and M1/M2 polarized human macrophages. To this end, after exposure to increasing concentrations of recombinant human RNASET2 protein purified from *Pichia pastoris*, a flow cytometry analysis was performed to detect the cell surface binding using a fluorophore-conjugated anti-RNASET2 antibody. As a result, a significant and dose-dependent binding to the cell surface was detected on both native and *in vitro* M1/M2 polarized human macrophages, thus suggesting a receptor-mediated interaction between RNASET2 protein and innate immune cells (**Figure 9**).

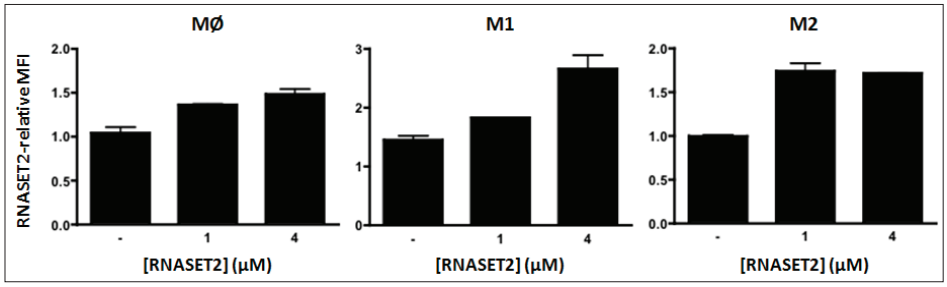


Figure 9 - Cell surface binding of recombinant human RNASET2 on human macrophages. Briefly, monocytes isolated from buffy coats were differentiated in macrophage cells and M1 or M2 polarization was induced with specific cytokines. Therefore, cells were seeded in 96-wells plates and incubated with recombinant human wild-type RNASET2 protein at 4°C for 3 hours. Then, cells were harvested and incubated for 30 minutes in the dark with fluorophore-conjugated anti-RNASET2 primary antibody. Each bar represents RNASET2 relative MFI (membrane fluorescence intensity). MØ: native human macrophages. M1: M1-polarized human macrophages. M2: M2-polarized human macrophages.

The observed RNASET2 protein binding to macrophage cells could suggested that, when secreted in the extracellular space from the cancer cell, RNASET2 could have a chemotactic role in recruiting immunocompetent cells at the tumor site.

RNASET2 protein is endowed with chemotactic activity on both human primary monocytes and the U937 cl.10 promyelocytic cell line

In order to gain more insights into the mechanisms of interaction between RNASET2 protein and innate immune cells, I decided to assess the chemotactic activity of the protein on human primary monocytes isolated from peripheral blood of healthy donors.

To this end, I have first purified recombinant RNASET2 protein, both wild-type and catalytically-inactive (H65/118F), expressed in two different heterologous systems: baculovirus/insect cells (BEVS) and *Pichia pastoris*. Recombinant RNASET2 protein purification was performed in two steps: (i) an affinity chromatography on Ni-NTA agarose columns, which exploited the C-terminal 6xHis tag of the protein, and (ii) a SEC/FPLC polishing step (**Figure 10**). Subsequently, I have assessed the protein preparations in order to exclude the presence of contaminant endotoxins, which are known to interfere in migration assays on monocytes, using a qualitative LAL-test.

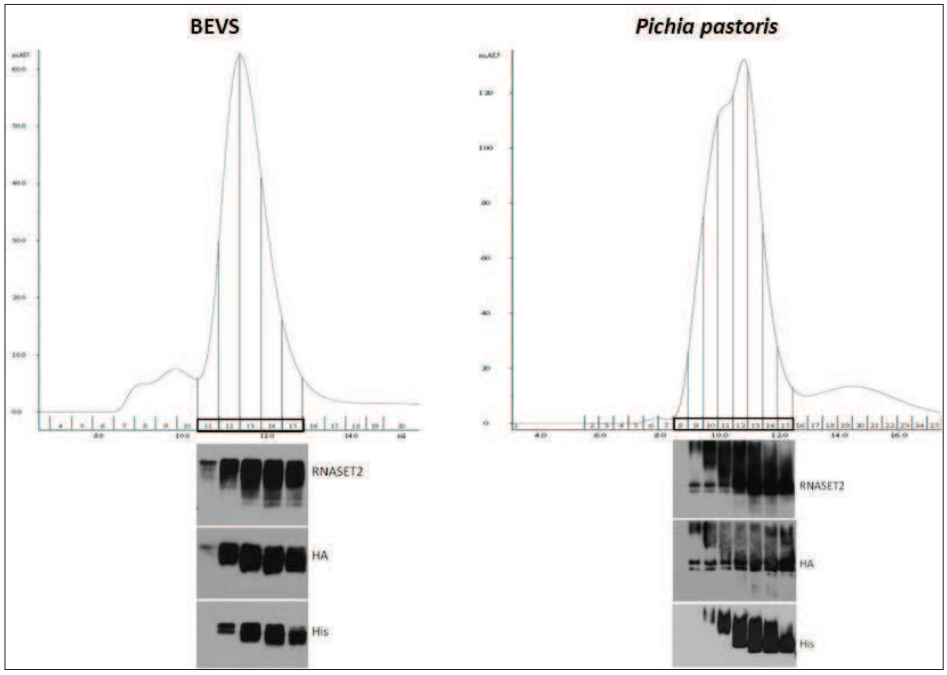


Figure 10 - Recombinant human RNASET2 protein purification. Both wild-type and H65/118F recombinant RNASET2 proteins produced in two heterologous expression systems were affinity-chromatography purified and then subjected to a SEC/FPLC polishing step. Chromatograms show the elution peaks for the protein. Recombinant RNASET2 protein produced in *Pichia pastoris* is hyperglycosylated with respect to that produced in BEVS. Below the corresponding peaks is the Western blot analysis of each eluted fraction. Three different primary antibodies were used to assess both the presence and the integrity of the purified protein: anti-RNASET2, anti-HA tag and anti-His tag.

The endotoxin-free recombinant RNASET2 protein was then tested as a chemotactic factor on human monocytes isolated from buffy coats. To this end, I first performed pilot migration tests in blind-well chambers, in which monocytes were exposed to increasing concentrations of recombinant wild-type human RNASET2 protein. As a result, I obtained migration responses to RNASET2 protein reminiscent of a bell-shaped curve, which is the expected trend for a chemokine (**Figure 11**). According to these results, the RNASET2 protein concentration of 0.227 µM, corresponding to 8.172 mg/l, was chosen for the following experiments.

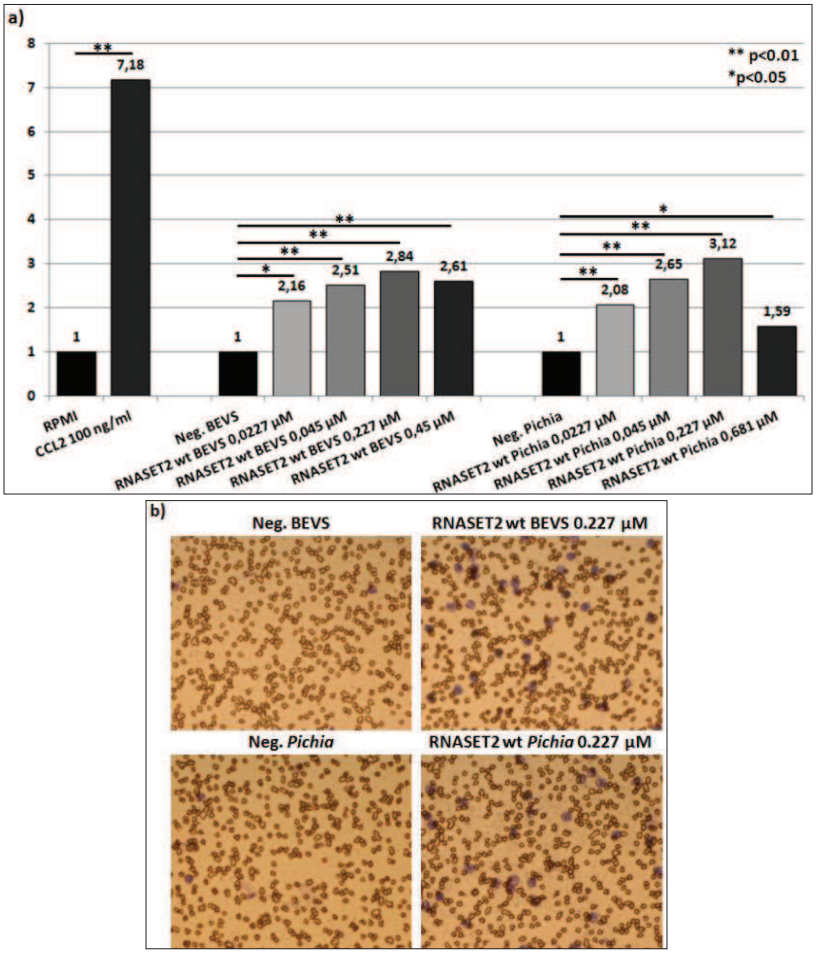


Figure 11 - Recombinant human wild-type RNASET2 protein exerts a chemotactic activity on human monocytes from buffy coats. Peripheral blood mononuclear cells (PBMC) were gradient-separated from buffy coats and monocytes were isolated by plating. Chemotaxis assays were performed in blind well chambers. Briefly, chemoattractants diluted in serum-free RPMI medium were placed in the lower well of the chamber, while cells were seeded in the upper well. The two wells are separated by a porous filter, which allows cells to migrate in response to the chemoattractant. After a three-hours incubation, non-migrating cells were scraped, filters were fixed and cells were stained with hematoxylin/eosin and counted using a light microscope. **a)** Bars represent the relative migration mean values from three independent experiments. Serum-free RPMI medium was used as negative control, while the CCL2 chemokine was used as positive control for monocytes migration. The recombinant protein elution/storage buffer was used as negative control for migration in response to recombinant RNASET2 protein (Neg. BEVS and Neg. *Pichia*). Statistical analysis of data was performed with bilateral Student's *t*-test. A bell-shaped migration curve was obtained for both the BEVS and the *Pichia pastoris* recombinant human wild-type RNASET2 protein. **b)** Four representative light microscopy images. Filter pores (small dark brown rounds) and migrating monocytes (violet) are observable. 40X.

Of note, in performing migration assays on monocytes isolated from buffy coats I have recorded a large variability from one test to another. This is most likely due to the fact that monocytes from different patients are markedly heterogeneous, thus making difficult to obtain reproducible results. In order to overcome this problem and standardize the method, I decided to perform migration assays with RNASET2 protein in a human promyelocytic cell line, rather than in freshly-isolated human monocytes. To this end, I have first evaluated the RNASET2 endogenous expression levels in four different promyelocytic cell lines (**Figure 12**). Then, in order to allow the establishment of an RNASET2 gradient within the migration chamber, the cell line which showed the lowest RNASET2 protein expression levels (U937 cl.10) was selected for the following experiments.

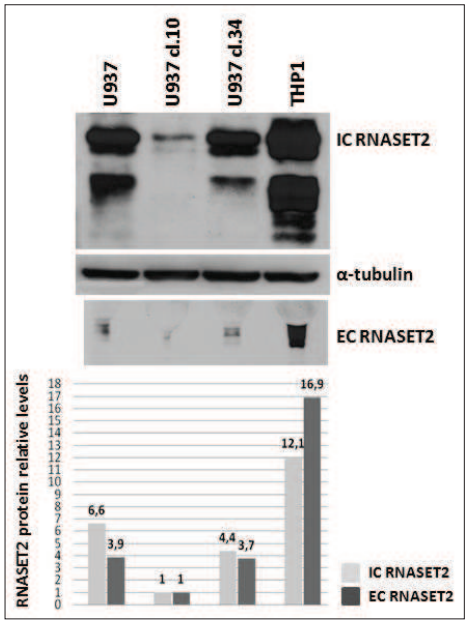


Figure 12 - RNASET2 protein endogenous levels in four human promyelocytic cell lines. A Western blot analysis was performed on both total protein extracts (IC RNASET2) and supernatants (EC RNASET2). α-tubulin was used for normalization. U937 cl.10, a human promyelocytic cell line derived from histiocytic lymphoma, showed the lowest RNASET2 protein levels.

Thus, I have performed migration assays on U937 cl.10 cell line using both wild-type and H65/118F recombinant human RNASET2 protein produced both in BEVS and in *Pichia pastoris* heterologous expression systems. As a result, I

observed a consistent migration in response to both the wild-type and the catalytically-inactive form of RNASET2 protein (**Figure 13**).

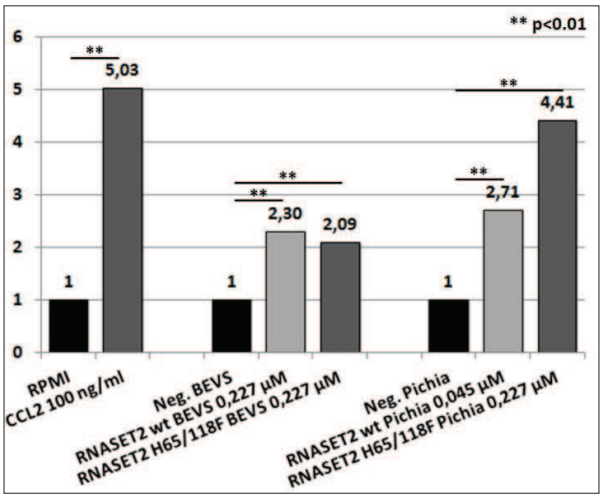


Figure 13 - Recombinant human RNASET2 protein, both wild-type and catalytically-inactive, exerts a strong chemotactic activity on U937 cl.10 promyelocytic cell line. Chemotaxis assays were performed in blind well chambers. Bars represent the relative migration mean values from two independent experiments. Serum-free RPMI medium was used as negative control, while the CCL2 chemokine was used as positive control for cell migration. The recombinant protein elution/storage buffer was used as negative control for migration in response to recombinant RNASET2 protein (Neg. BEVS and Neg. *Pichia*). Statistical analysis of data was performed with bilateral Student's t-test.

Taken together, these results strongly suggest that extracellular RNASET2 protein could actively behave as a chemokine in recruiting cells from the monocyte-macrophage cell lineage at the tumor site.

Since the RNASET2 protein proved to directly interact with monocyte-macrophage cells and also showed a chemotactic activity on this cell population, I decided to investigate the occurrence of receptor-mediated binding of the RNASET2 protein to the target cell surface. Intracellular signaling pathways in response to chemokines are in most cases triggered by the binding of the signaling molecule to a cell-surface G-protein coupled receptor (GPCR) (Murphy, 1994). I thus replicated the migration assays on U937 cl.10 cells following pre-treatment of cells with pertussis toxin (PTx), which is known to inhibit the GPCRs function by means of ADP-ribosylation of G_i proteins. As a

result, PTx pre-treatment drastically impaired U937 cl.10 cells migration in response to recombinant wild-type human RNASET2 protein (**Figure 14**).

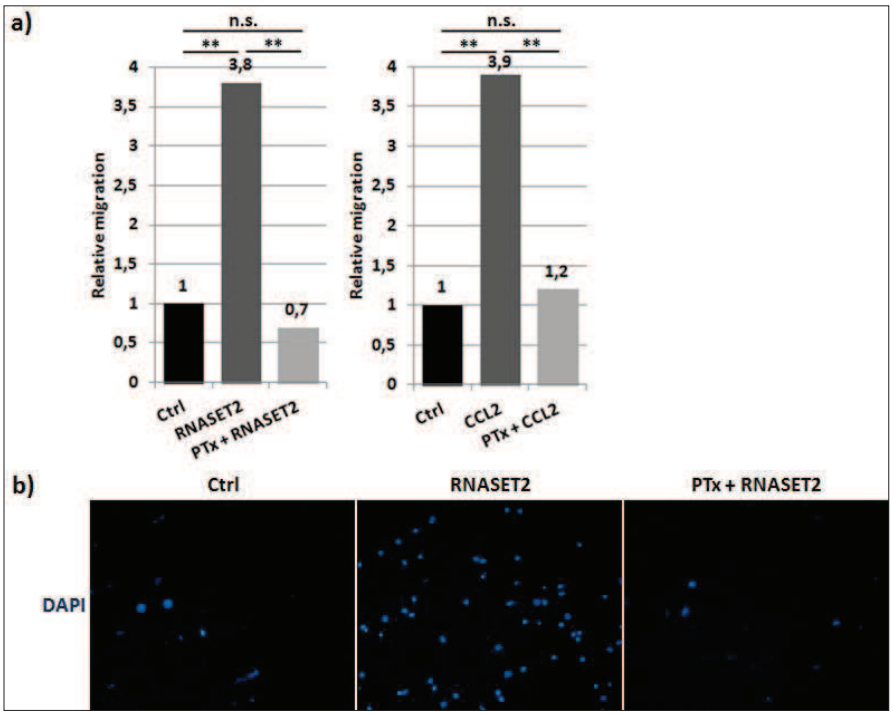


Figure 14 - Human RNASET2 protein exerts a chemotactic activity on U937 cl.10 cell line by means of a GPCRs-mediated cell-surface binding. a) U937 cl.10 cells were either pre-treated with 1 µg/ml PTx for three hours or left untreated. *In vitro* migration assays were performed in blind well chambers. Recombinant wild-type human RNASET2 protein produced in BEVS and CCL2 chemokine were used as chemoattractants, at the final concentrations of 8.172 µg/ml and 100 ng/ml, respectively. Statistical analysis was performed with bilateral Student's *t*-test. **p<0.01. **b)** Fluorescence microscopy images of filters. After fixation, nuclei were stained with DAPI. 40X.

On the basis of this body of evidence, I propose that RNASET2 could exert its tumor-antagonizing role by means of a direct receptor-mediated interaction with the monocyte-macrophage cell population, whose functional activation could be finally responsible for the RNASET2-mediated tumor suppression that we have observed in the *in vivo* settings.

RESULTS (Part II)

RNASET2 protein levels increase following stress induction

As I have previously argued, T2 ribonucleases are highly-conserved enzymes throughout all the *phyla*, from viruses to mammalian cells. In particular, these RNases are well known to increase their expression levels in plants, fungi and yeasts in response to stress challenges such as nutrient starvation, chemically-induced cellular stresses and pathogen attacks (Deshpande, 2002). Being the T2 ribonucleases stress-response role so highly conserved, we reckoned that a similar function could be hypothesized for mammalian cells as well.

In order to start to investigate this issue, we took advantage of some information we have recently collected concerning the subcellular co-localization of RNASET2 protein with processing-bodies markers (Vidalino, 2012). As already mentioned, PBs are transient cytoplasmic structures mainly associated with the mRNA decay process (Kedersha & Anderson, 2007).

Thus, we decided to set up a panel of experiments aimed at uncovering whether human RNASET2 plays a stress-response role in mammalian cells. To this end, I have challenged different tumor cell lines with a variety of stress-inducing chemicals or treatments and then I have examined changes in both RNASET2 protein expression levels and sub-cellular patterns of localization.

I have performed *in vitro* stress induction assays using a standardized cancer cell line model (HeLa) and two ovarian cancer cell lines, Hey3Met2 and SKOV3, as an experimental model. I decided to challenge these cell lines with metabolic stress, oxidative stress, osmostress, heat shock, UV light, aminoacid starvation and chemical hypoxia. After each treatment and following the proper recovery time, I have assessed RNASET2 protein levels by Western Blot analysis in stress-exposed cells with respect to untreated cells.

As a result, I observed significantly higher RNASET2 protein levels in treated Hey3Met2 and SKOV3 cells, in response to different stresses. Particularly, we detected specific patterns of RNASET2 protein isoforms overexpression in response to different stress inductions; indeed, the 31 kDa and the 27 kDa isoforms were significantly more represented in treated cells with respect to control cells following clotrimazole treatment, heat shock, aminoacid starvation and hypoxic stress (**Figure 15** and **Table 2**).

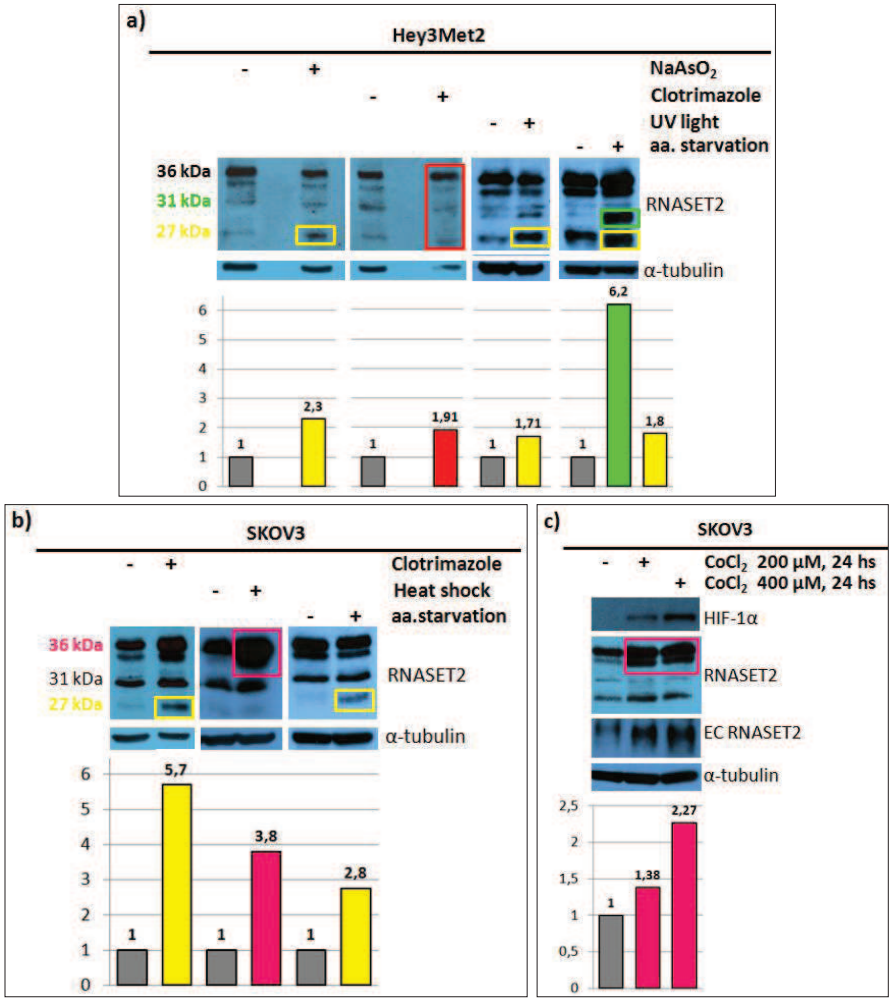


Figure 15 - Specific human RNASET2 protein isoforms overexpression in response to stress induction. A Western Blot analysis was performed on total protein extracts obtained from both treated and control cells following stress induction. Signals quantification was performed with ImageJ software. α-tubulin was used for signal normalization. **a)** An overall higher RNASET2 protein level was observed in clotrimazole-treated Hey3Met2 cells (red bar). The 27 kDa RNASET2 isoform was induced in response to metabolic stress, UV-light exposure and aminoacid starvation (yellow bars). The 31 kDa RNASET2 isoform was induced in response to aminoacid starvation (green bar). **b)** The full-length 36 kDa RNASET2 protein was induced in SKOV3 cells in response to heat shock (pink bar). The 27 kDa RNASET2 isoform was induced in response to clotrimazole treatment and aminoacid starvation (yellow bars). **c)** In $CoCl_2$-treated SKOV3 cells we observed significant induction of the 36 kDa RNASET2 protein isoform, at both the intracellular (pink bars) and the extracellular (EC RNASET2) levels. HIF-1α was used as an hypoxia marker.

		$NaAsO_2$		Clotrimazole	D-sorbitol		Heat shock		UV light		Aminoacid starvation	$CoCl_2$			
												200 µM		400 µM	
	Untreated	Treated	Treated + rec.		+FBS	-FBS	Treated	Treated + rec.	Treated	Treated + rec.		12 hs	24 hs	12 hs	24 hs
HeLa	1	+1,44	+1,32	-1,33	-1,59	+1,88	+1,04	-1,18	-1,20	+1,19	-2,5	-1,28	+1,14	-1,49	-1,09
Hey3Met2	1	+2,30 §	+2,20 §	+1,91	+1,03	-1,22	-1,12	+1,00	+1,71 §	+2,02 §	+6,2 $^{\#}$ +1,81 §	-1,09	-1,01	+1,21	-1,19
SKOV3	1	-1,28	-1,39	+5,7 §	+1,31	+1,03	+1,2 *	+3,8 *	+1,24	+1,13	+2,76 §	+2,11 *	+1,38 * + 2,29 **	+4,95 *	+2,27 * + 2,97 **

Table 2 - Human RNASET2 protein levels change following stress induction. Treated VS untreated cells relative fold-changes are reported for each cell line. Western Blot signals quantification was performed with ImageJ software. Mean values from two/three independent experiments are reported. RNASET2 protein level in untreated cells was arbitrarily based to 1. Metabolic stress: 500 µM $NaAsO_2$ for 45 minutes, followed by a 120-minutes recovery period. Oxidative stress: 20 µM clotrimazole for 60 minutes, in serum-free medium. Osmotic shock: 1 M D-sorbitol for 30 minutes in either serum-free or complete medium. Heat shock: 42°C for 20 minutes, followed by a 24-hours recovery period. UV-light exposure: 312 nm (UVB) for 1 minute, followed by a 30-minutes recovery period. Aminoacid starvation: 48-hours incubation with DMEM with 4.5 g/l glucose and $NaHCO_3$, without Methionine, Cysteine and Glutamine. Hypoxic stress: 200-400 µM $CoCl_2$ for either 12 or 24 hours. Positive fold-changes are in light and dark green; negative fold-changes are in light and dark red. * Fc value referred to 36 kDa isoform. ** Fc value referred to extracellular 36 kDa isoform. $^{\#}$ Fc value referred to 31 kDa isoform. § Fc value referred to 27 kDa isoform.

Moreover, where I have observed overexpression of the intracellular 36 kDa form of the RNASET2 protein, I have also recorded a concomitant increase in the secreted protein level (**Table 2**). RNASET2 expression turned out to change in response to several stresses in HeLa cells as well, although the observed pattern was significantly different from that I have observed in the above mentioned ovarian cancer cell lines (**Table 2**).
These preliminary results are clearly compatible with a role for human RNASET2 as a wide-range stress response gene.

RNASET2 protein re-localizes to P-bodies in response to hypoxic stress conditions

I have demonstrated that RNASET2 protein levels increase in response to different stress-inducing treatments. According to this descriptive observation, I decided to deeply investigate the functional significance of the observed RNASET2 increase in response to stress, particularly in the context of P-bodies dynamics. Indeed, as previously mentioned, we have recently demonstrated a co-localization between RNASET2 protein and PBs markers (Vidalino, 2012). In

order to better clarify this issue, I'm presenting here some preliminary experiments and results.
I have challenged SKOV3 cells with $CoCl_2$ treatment, which induces chemical hypoxia, and then I have processed cells for double indirect immunofluorescence in order to assess the re-localization of RNASET2 protein to PBs. To this end, a primary anti-RNASET2 antibody was employed, together with a primary anti-DCP1 antibody, which is a well-known PBs marker protein. As a result, I have detected a marked co-localization signal for RNASET2 and DCP1 proteins in treated SKOV3 cells when compared to the untreated cells (**Figure 16**).

RNASET2 knockdown leads to increase in P-bodies and stress-granules number

Afterwards, I decided to better investigate the functional role of RNASET2 protein in RNA metabolism and particularly in P-bodies (PBs) and stress-granules (SGs) dynamics. Briefly, SGs are cytoplasmic phase-dense structures that occur in cells exposed to environmental stress and are composed of stalled translation pre-initiation complexes (Kedersha & Anderson, 2007).
In order to shed light on this issue, I have employed the RNASET2 knock-down experimental model that we have obtained in the human ovarian cancer cell line OVCAR3. Particularly, I have first assessed whether RNASET2 knockdown could *per se* influence PBs number and size, without administering any specific stress induction. Using indirect immunofluorescence with a PBs-specific marker (RCK/p54), I was able to detect a significant increase in P-bodies number and size in RNASET2-silenced OVCAR3 clones when compared to control clones (**Figure 17a**). Therefore, using the same experimental approach, I have investigated changes in SGs sub-cellular signal and I have obtained results in the same direction as for PBs (**Figure 17b**).
A possible explanation for the observed findings could be that cells lacking RNASET2 have a basal cellular stress level higher than cells in which RNASET2 protein is normally expressed. According to this view, I decided to assess whether OVCAR3 cells lacking RNASET2 showed RNA accumulation at the cytoplasmic level. To this purpose, an RNA-specific fluorescent dye was employed but no differences have been noticed in cytoplasmic staining of RNASET2-silenced OVCAR3 clones when compared to control clones (**Figure**

17c). Finally, according to our previous data demonstrating a lysosomal subcellular location for RNASET2 enzyme (Campomenosi, 2006), I decided to investigate whether cells lacking RNASET2 showed changes at the lysosomal compartment level. No changes in the staining pattern of lysosomes were observed in RNASET2-silenced OVCAR3 cells with respect to control clones (**Figure 17d**).

Taken together, these data suggest that human RNASET2 could play an housekeeping role in orchestrating defense mechanisms against stress conditions at the cellular level.

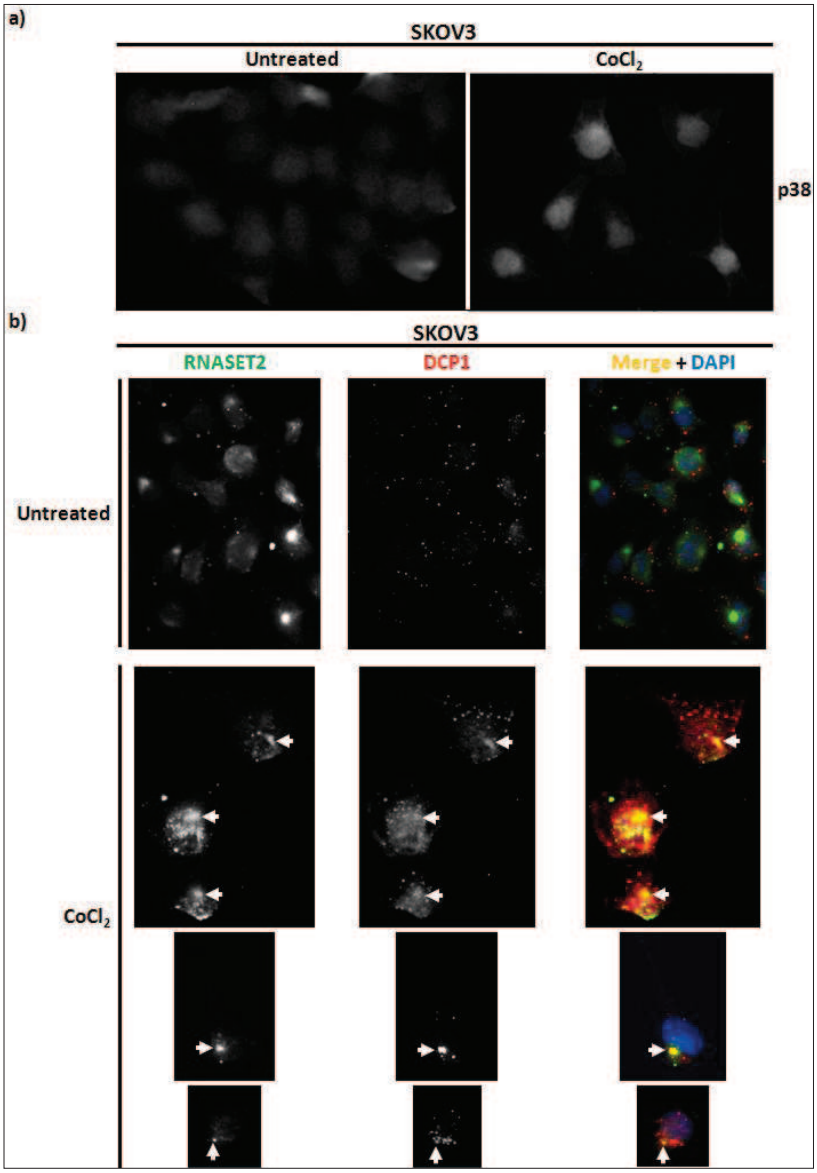


Figure 16 - RNASET2 protein re-localizes to P-bodies in response to hypoxia. SKOV3 cells were seeded on coverslips and treated with 400 µM $CoCl_2$ for 24 hours. After treatment, cells were fixed, permeabilized and processed for indirect immunofluorescence. The p38 MAP-kinase, which is known to translocate to the nucleus in response to hypoxic stress, was used as an experimental control (**a**). A double indirect immunofluorescence was performed to assess RNASET2 re-localization to P-bodies. DCP1 protein was used as PBs marker. No significant co-localization signal was detected in untreated SKOV3 cells (**b**, upper panel) while a marked co-localization (white arrows) was detected following hypoxia-inducing treatment (**b**, lower panels). Fluorescence microscopy images. 40X.

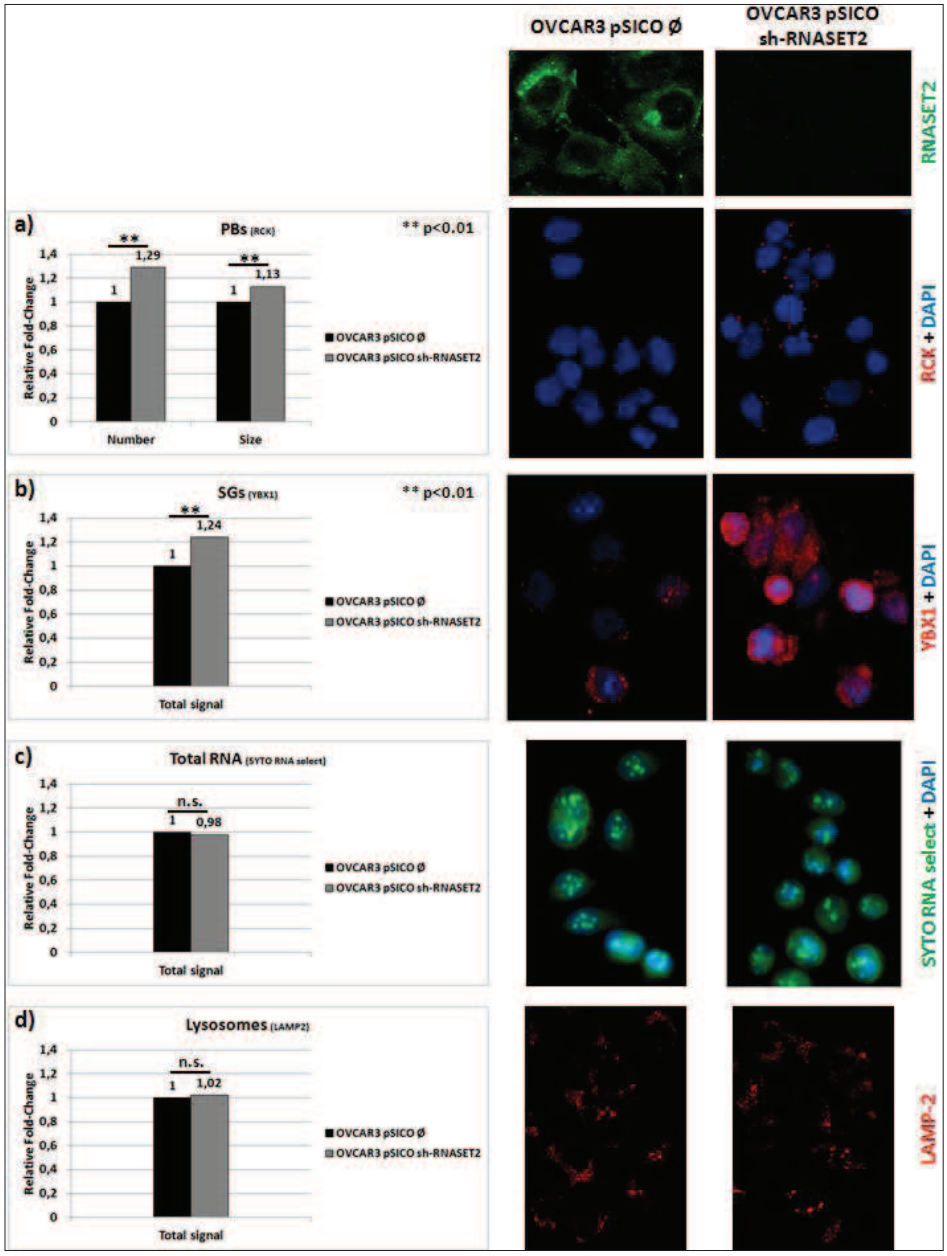


Figure 17 - OVCAR3 cells lacking RNASET2 show increase in PBs and SGs number. Two independent RNASET2-silenced OVCAR3 cell clones were employed and compared to two independent control OVCAR3 cell clones. Briefly, cells were seeded on coverslips, indirect immunofluorescence was performed and fluorescence microscopy images (**a**, **b**, **c**) or confocal microscopy images (**d**) were acquired. Signal quantification was performed with the "Analyze particles" function of the ImageJ software and normalized on counted cell number (DAPI signal,

where present). Bars represent the relative fold-changes calculated. Approximately 500 cells for each of the four clones were counted. Statistical analysis of data was performed with bilateral Student's *t*-test. RCK, YBX1 and LAMP-2 are marker proteins for PBs, SGs and lysosomes, respectively. SYTO RNA select is an RNA-specific fluorescent dye. A significant increase in PBs (**a**) and SGs (**b**) was observed in RNASET2-silenced OVCAR3 clones with respect to control clones. No changes in both total RNA amount (**c**) and lysosomal staining pattern (**d**) were observed.

MATERIALS AND METHODS

CELL CULTURES

Primary cells

Human monocytes were isolated from peripheral blood using Lympholyte®-H (CEDARLANE), a density gradient separation medium specifically designed for the isolation of viable lymphocytes and monocytes.

Native human macrophages were differentiated from monocytes with a six-days exposure to 100 ng/ml MCSF. M1-polarization was induced with 100 ng/ml LPS + 20 ng/ml IFN-γ. M2-polarization was induced with 20 ng/ml IL-4. Culture medium: RPMI-1640 + 10% heat-inactivated FBS + 1% L-glutamine. Culture conditions: 37°C, 5% CO_2, humidified atmosphere.

Cell lines

Adhesion growth: Hey3Met2 (human ovarian cancer); HeLa (human cervical carcinoma); OVCAR3 (human ovarian cancer); RKO (human colon cancer); HTC-116 (human colon cancer); 8387 (human fibrosarcoma); SKOV3 (human ovarian cancer). Culture medium: DMEM/DMEM-F12 + 10% FBS + 1% L-glutamine.

Suspension growth: U937 (human histiocytic lymphoma); U937 cl.10 (human histiocytic lymphoma, HIV-permissive clone); U937 cl.34 (human histiocytic lymphoma, HIV non-permissive); THP-1 (human acute monocytic leukemia). Culture medium: RPMI-1640 + 10% heat-inactivated FBS + 1% L-glutamine. Culture conditions: 37°C, 5% CO_2, humidified atmosphere.

Stably-transfected cell clones

Hey3Met2 empty pcDNA3; Hey3Met2 pcDNA3-hRNASET2 w.t.; Hey3Met2 pcDNA3-hRNASET2 H65/118F. Culture medium: DMEM-F12 + 10% FBS + 1% L-glutamine + 350 µg/ml G418.

OVCAR3 empty pSICO; OVCAR3 pSICO-siSCRAMBLED; OVCAR3 pSICO-siRNASET2. Culture medium: DMEM-F12 + 10% FBS + 1% L-glutamine + 1.5 µg/ml puromycin.

Culture conditions: 37°C, 5% CO_2, humidified atmosphere.

ANTIBODIES AND FLUORESCENT DYES

Primary Abs: polyclonal rabbit anti-hRNASET2 (Dabio, Germany); monoclonal mouse anti-hHSP90 (SIGMA-ALDRICH); monoclonal mouse anti-HA tag (Roche, Milan, Italy); monoclonal mouse anti-His tag (Santa Cruz Biotechnology);

monoclonal mouse anti-αtubulin (SIGMA-ALDRICH, T-9026); monoclonal mouse anti-DCP1 (Abnova, H00055802-M06); monoclonal mouse anti-RCK/p54 (Santa Cruz Biotechnology); polyclonal rabbit anti-p38 (Cell Signaling Technology); monoclonal mouse anti-YBX1 (Abnova); monoclonal mouse anti-LAMP2 (Santa Cruz Biotechnology). Secondary Abs: goat anti-mouse IgG HRP-conjugated (PIERCE); goat anti-rabbit IgG HRP-conjugated (PIERCE); goat anti-mouse IgG TRITC-conjugated (SIGMA-ALDRICH); goat anti-rabbit IgG FITC-conjugated (SIGMA-ALDRICH). Fluorescent dyes: 4',6-Diamidino-2-phenylindole dihydrochloride (SIGMA-ALDRICH); SYTO®RNASelect™ Green Fluorescent Cell Stain (Molecular Probes, S32703).

STRESS-INDUCING CHEMICALS AND REAGENTS

Sodium (meta)arsenite $NaAsO_2$ (SIGMA-ALDRICH, S-7400); Clotrimazole (SIGMA-ALDRICH, C-6019); D-sorbitol (SIGMA-ALDRICH, 240850); Dulbecco's Modified Eagle's Medium-high glucose-without L-methionine, L-cysteine and L-glutamine (SIGMA-ALDRICH, D-0422); Cobalt(II) chloride $CoCl_2$ (SIGMA-ALDRICH, 60818).

MICROARRAY GENE EXPRESSION ANALYSIS

The Agilent Whole Human Genome Oligo Microarray was performed in collaboration with the Institute for Health and Consumer Protection, Molecular Biology and Genomics Unit of the Joint Research Centre (Ispra, Varese, Italy). The *in vitro* microarray analysis was performed on total RNA extracted from Hey3Met2 cell clones (TRI reagent, SIGMA-ALDRICH). The GO analysis was done at GO FAT level of biological process and molecular function, whereas Expression Analysis Systematic Explorer (EASE) biological theme analysis was carried out online using DAVID (http://david.niaid.nih.gov).

QUANTITATIVE REAL TIME RT-PCR

Total RNA was extracted from Hey3Met2 cells and xenograft tumors with TRI reagent (SIGMA-ALDRICH), was subjected to DNase treatment and was reverse transcribed with random examers using the High-capacity cDNA RT kit (Applied Biosystems). Primer sequences were designed with the Primer express program (Applied Biosystems). Real-time RT-PCR was performed on ABI PRISM 7000 with the Power SYBR-green PCR Master Mix (Applied Biosystems), following the manufacturer's instructions. Amplification reactions were performed in triplicate. Following a polymerase activation step at 95°C for 10 minutes, samples were denatured at 95°C for 15'' and annealed/extended at

60°C for 1 minute, for 40 cycles. Fluorescent signals generated during PCR amplification were monitored and analyzed with ABI PRISM 7000 SDS software (Applied Biosystems). Comparison of the amount of each gene transcript among different samples was made using β-actin as reference. The amount of target RNA, normalized to the endogenous reference gene, was calculated by means of the difference-in-threshold-cycle parameter ΔC_t.

CELL SURFACE PROTEINS ISOLATION

In order to obtain biotinylation and isolation of cell surface proteins for Western blot analysis, the Pierce®Cell Surface Protein Isolation Kit (THERMO SCIENTIFIC) was employed, according to the manufacturer's instructions. Briefly, cells were first labeled with EZ-Link Sulfo-NHS-SS-Biotin, a thiol-cleavable amine-reactive biotinylation reagent. Subsequently, cells were lysed with a mild detergent and the labeled proteins were then isolated with NeutrAvidin Agarose. The bound proteins were released by incubating with SDS-PAGE sample buffer containing 50 mM DTT and processed for Western Blot analysis.

WESTERN BLOT ANALYSIS

Cells for intracellular extracts were mechanically scraped in PBS + 5mM EDTA and resuspended in lysis buffer (0.5% Igepal, 0.5% Triton X-100 in PBS + 5mM EDTA) supplemented with protease inhibitors (PMSF, benzamidine, aprotinin, and leupeptin). Quantification of total proteins was performed with Bradford reagent (BIO RAD), using bovine serum albumin as standard. For the SDS-PAGE analysis, 30-70 µgs of intracellular lysate were loaded. Immunoblot analysis was performed using standard procedures and detected with a chemiluminescent substrate (Super-signal *West Dura* extended duration kit, Thermo Scientific). When necessary, acquired images were quantified using the "Analyze gels" tool of the ImageJ software.

FLOW CYTOMETRY ANALYSIS

The flow cytometry analysis was performed in collaboration with the Laboratory of Immunopharmacology of the Humanitas clinical institute, IRCCS (Rozzano, Milan, Italy). The Zenon Rabbit IgG Labeling Kit (Molecular Probes) was employed in order to produce an antibody conjugate anti-RNASET2/fluorophore, following the manufacturer's instructions. Briefly, the following protocol was performed for the staining of the cells. Cell suspension (1×10^6 cells/mL) was mixed with the Zenon labeling mixture and incubated for

30 minutes at room temperature. Then, cells were washed once with PBS, centrifuged, resuspended and analyzed using appropriate instrument parameters.

In order to perform flow cytometry analysis for binding of the exogenously-provided RNASET2 protein, the same protocol was used after a three-hours incubation at 4°C with the recombinant RNASET2 protein or the appropriate isotypic control.

INDIRECT IMMUNOFLUORESCENCE ASSAYS

Cells were grown on coverslips for 24 hours and then treated or directly processed for indirect immunofluorescence. Cells were fixed either in 3% paraformaldehyde or Methanol, depending on the primary antibody employed. After fixation, cells were permeabilized using Triton X-100 and incubated in a 10% goat serum blocking solution in PBS 1X. Primary and secondary antibodies incubations were performed in diluted blocking solution (3% goat serum in PBS). Cell nuclei were counterstained with DAPI and coverslips were mounted on microscope slides using Vectashield mounting medium (Vector). Fluorescence/confocal microscopy images were acquired. Image quantification analysis was performed using the "analyze particles" tool of the ImageJ software. Statistical analysis was performed using an independent two sample Student's *t*-test (unequal sample size, equal variance).

RECOMBINANT PROTEIN PURIFICATION FROM SUPERNATANTS

Recombinant wild-type and mutant proteins were both secreted in the extracellular medium using the endogenous RNASET2 secretion signal and could therefore be easily purified from supernatants of both BEVS and *Pichia pastoris* systems. Supernatants were 10/15-fold concentrated using Centricon Plus 70 (MILLIPORE) and dialyzed against 50 mM Tris–HCl, 100 mM KCl, at pH 8.0 (Base solution) in order to improve subsequent binding to the Ni-NTA agarose resin (Qiagen). Binding to the resin was allowed to proceed in falcon tubes, after addition of 10 mM imidazole, at 4°C for 2 hs before applying onto an empty poly-prep chromatography column (Bio Rad). After washes with 20 column volumes of wash solution (Base solution + 50 mM imidazole), elution was performed with four column volumes (one volume at a time) of base solution + 250 mM imidazole. Presence of the protein in eluted fractions was checked by SDS–PAGE analysis followed by Coomassie staining. Protein

containing fractions were pooled and dialyzed against 10 mM potassium phosphate, 0.2 mM β-mercaptoethanol, pH 6.6 and then stored at 20°C in small aliquots. A second step of protein purification was performed by SEC/FPLC, in collaboration with the Laboratory of functional post-genomic and protein engineering of the University of Insubria (Varese, Italy). The LAL-PYROGENT single test vials kit (LONZA) was employed in order to exclude any endotoxin contamination of the final protein preparations.

CHEMOTAXIS ASSAYS

The Lympholyte®-H reagent (CEDARLANE) was used for the isolation of human monocytes from buffy coats. This is a density gradient separation medium specifically designed for the isolation of viable lymphocytes and monocytes from human peripheral and cord blood. It allows the elimination of erythrocytes and dead cells from human blood and also removes the majority of granulocytes. The resulting cell population consists of a high and non-selective recovery of viable human lymphocytes and monocytes. Monocytes were further isolated from lymphocytes by plating.

In vitro migration assays on both primary monocytes and promyelocytic cell lines were performed in blind well chambers (NeuroProbe). A polycarbonate filter with 3 µm pores (NeuroProbe) separated the upper and the lower chamber, in order to allow migrating cells to get through the pores. Cells were incubated in serum-free RPMI-1640 medium for 24 hours before starting the test. Then, cells were resuspended in serum-free medium at a final density of $6x10^5$ cells/ml and were placed in the upper compartment of the chambers (500 µl/well). Chemoattractant agents were diluted in serum-free medium and were put in the lower compartment of the chambers (200 µl/well). Chambers were incubated for 3 to 21 hours at 37°C, in order to allow migration to occur. After incubation, non-migrated cells were scraped and filters were fixed, stained with haematoxilin-eosin (Diff-Quik staining protocol-Medion Diagnostics) and mounted on microscopy slides (7,5 µg/ml DAPI in Vectashield mounting medium). Nuclei were counted and relative migration values were calculated. As negative control, the protein storage buffer diluted in serum-free RPMI-1640 medium was used. Statistical analysis was performed using bilateral Student's *t*-test for unpaired data.

PERTUSSIS TOXIN (PTx) TREATMENT

U937 cl.10 cells were harvested and resuspended at a final density of 1 x 10^6 cells/ml in serum-free RPMI-1640 medium. Treatment with 1 µg/ml PTx (CalBiochem) for 90 minutes was performed at 37°C, 5% CO_2. After incubation, cells were washed twice in PBS and then resuspended for chemotaxis assay.

STRESS-INDUCING TREATMENTS

Stress-inducing treatments were performed following protocols suggested by Kedersha and Anderson (Kedersha & Anderson, Mammalian stress granules and processing bodies, 2007). The following table summarizes the employed procedures.

Treatment/Reagent	Conditions	Recovery time	Effects	Comments
Metabolic stress Sodium (meta)arsenite, $NaAsO_2$	500 µM, 45'	120'	Induces both SGs and PBs	/
Oxidative stress Clotrimazole	20 µM, 60'	/	Induces SGs	Requires serum-free media
Osmostress D-sorbitol	1 M, 30'	/	Induces SGs	Duration of exposure is more important than degree of hypertonicity
Heat shock	42°C, 20'	24 hs	Induces SGs early, may induce PBs later	Cells adapt and SGs can disappear within one hour
UV-light	312 nm (UVB), 1'	30'	Induces SGs	/
Aminoacid starvation DMEM with 4.5 g/l glucose and $NaHCO_3$, without Methionine, Cysteine and Glutamine	48 hs	/	Induces SGs	/
Hypoxia Cobalt(II) chloride, $CoCl_2$	200-400 µM, 12-24 hs	/	Induces both SGs and PBs	/

DISCUSSION

RNASET2 is an atypical tumor suppressor gene. In our recent works we have demonstrated that RNASET2 overexpression in ovarian cancer cells is associated with a strong suppression of their tumorigenicity *in vivo*, without affecting any *in vitro* cancer-related growth parameter (Acquati, 2005) (Acquati, 2011). Noteworthy, the control of tumor growth apparently relies on the establishment of a cross-talk between RNASET2-overexpressing cancer cells and the tumor microenvironment, where the monocyte/macrophage lineage turned out to represent the most likely target of RNASET2 activity (Acquati, 2011). These biological properties of RNASET2 gene allowed us to record it as a member of the "tumor antagonizing/malignancy suppressor genes" class (Klein, 2009). This family of genes is characterized by an asymmetric tumor-suppressive activity, which is only carried out in the *in vivo* context. Central to this concept is the existence of a microenvironmental control of tumorigenesis (Bissell & Hines, 2011) (Nelson & Bissell, 2006).

Thus, the first and principal aim of my Ph.D. project was to further **investigate the putative occurrence of a functional cross-talk between extracellular RNASET2 protein and immunocompetent cells**. This could be achieved by means of at least two different mechanisms, which represent my working hypothesis: (i) an indirect course of action by the RNASET2-overexpressing cancer cell, which involves the production and secretion of signaling molecules endorsed with immunological functions, or (ii) a direct interaction between secreted RNASET2 protein and immunocompetent cells.

As for the first hypothesis, some important premises must be taken into account in order to correctly interpret my experimental choices.

We have recently demonstrated that RNASET2-overexpressing ovarian tumor Hey3Met2 cells were strongly inhibited in their tumorigenic potential *in vivo*, but they showed no differences in cancer-related growth parameters *in vitro* when compared to control cells. More in detail, we have assessed the following five *in vitro* assays: proliferation rate, colony formation, cell adhesion, anchorage-independent growth and apoptotic rate (Acquati, 2011). By contrast, a consistent infiltration of host immune cells was observed in xenograft tumors derived from the injection of RNASET2-overexpressing

Hey3Met2 cells (Acquati, 2011). Within this conceptual and experimental framework, we have therefore attributed to the RNASET2 gene a *non-cell autonomous* role for tumor suppression and tentatively set aside the possibility that RNASET2 could exert its antitumoral function in a *cell autonomous* way. Consequently, we decided to perform an *in vitro* microarray gene expression analysis in order to compare RNASET2-overexpressing Hey3Met2 clones with control clones. The rationale for this assay was to look for changes in the expression levels of signaling molecules involved in innate immunity in RNASET2-overexpressing tumor cells. On the other hand, this assay had the potential to investigate at a deeper level the occurrence of putative cell-autonomous roles for RNASET2, which could still not be completely rule out, due to the limited range of *in vitro* assays previously carried out. Indeed, a solid body of evidence from two RNASET2 orthologues, namely Rny1p from *S. cerevisiae* and ACTIBIND from *A. niger*, has recently demonstrated that important RNASET2-mediated processes actually take place *within* cells (Thompson, 2009) (Roiz, 2006).

As the experimental data showed, no changes were observed in the *in vitro* expression pattern of genes involved in innate immunity. In the context of a non-cell autonomous model for RNASET2-mediated tumor suppression, this peculiar result is prone to two different interpretations: (i) the hypothesis of an *indirect* role of RNASET2 protein in *in vivo* tumor suppression could still be plausible, but not detectable by the microarray technology in the present experimental setting, which was carried out in cells cultured *in vitro*, or (ii) a direct role of extracellular RNASET2 in tumor suppression should be envisioned.

Within this frame, using an opposite and complementary *in vivo* experimental model, our research group has recently gathered evidence strongly supporting the latter interpretation. More in detail, using an RNA-interference approach we have established a total abolition of RNASET2 expression in the poorly tumorigenic human ovarian cancer cell line OVCAR3. Following the injection of RNASET2-silenced OVCAR3 cells in nude mice, we have observed the appearance of tumor masses significantly larger than those observed after injection of control OVCAR3 cells. Interestingly, when an *in vivo* analysis of the gene expression profile for human genes was performed on xenograft tumors, a significant change in the expression levels of genes involved in leukocytes

activation was observed. Moreover, when the same analysis was performed in order to investigate the expression profile for mouse genes, significant changes in gene categories such as "immune response", "defense response", "innate immune response" and "acute inflammatory response" were recorded (Acquati 2012, submitted).

As long as the occurrence of a putative *cell autonomous* role for RNASET2 is concerned, the microarray hybridization assay unveiled sixty-five genes which turned out to be modulated by RNASET2. We thus selected among these genes a set of thirteen candidates on the basis of their involvement in cancer-related processes and we further validated expression changes by RT-qPCR, in both the *in vitro* and the *in vivo* contexts. The qPCR analysis thus confirmed the pattern of gene expression changes for most tested genes *in vitro*. By contrast, the RNASET2-mediated changes in the expression levels of the thirteen genes that we have observed *in vitro* were not confirmed in xenograft tumors, with the exception of LIMCD1, DSE and RELB genes.

Thus, these three genes represent *bona fide* candidate effector genes for RNASET2-mediated tumor suppression *in vivo* and are worth to be analysed in depth. Interestingly, two of them (RELB and DSE) show a plausible link with cellular functions related to tumor rejection by the immune system. RELB gene, in fact, is involved in the NF-kB pathway and is clearly associated with adaptive immune responses mediated by dendritic cells (Clark G. J., 1999). This is particularly interesting in the light of a recent work demonstrating a role for the omega-1 T2 ribonuclease from *Schistosoma mansoni* in priming dendritic cells for Th2 polarization of T-lymphocytes (Steinfelder, 2009). As for the DSE gene, it was originally identified on the basis of the ability of its protein product to be recognized with high efficiency by a subset of cytotoxic T-lymphocytes in certain tumors (Nakao, 2000).

Finally, LIMCD1 gene encodes for a poorly characterized protein involved in cardiac hypertrophy (Frank, 2010); however, the related LIMD1 gene has been recently reported to display functional properties reminiscent of tumor antagonizing genes (Klein, 2007). Taken together, the gene expression profiling data seem to provide a support for the hypothesis that the RNASET2-mediated tumor suppression observed *in vivo* is likely mediated by a direct, *non-cell autonomous* role.

In my opinion, these data deserve some general comments concerning the relevance of the methodological approach employed in the investigation of an experimental subject. In fact, the higher the sensitivity of the method employed, the higher the amount of information that we can obtain. And this is not a merely quantitative problem. Indeed, employing five different *in vitro* experimental assays we have observed no differences between RNASET2-overexpressing tumor cells and control tumor cells and we have reasonably concluded that RNASET2 gene behaves as a tumor antagonizing gene, acting in an asymmetric non-cell autonomous way. By contrast, employing the much more sensitive microarray/qPCR approach we have uncovered three genes that showed a congruent change in the expression pattern between the *in vitro* and the *in vivo* settings and whose function could be important in RNASET2-mediated tumor suppression. This finding is in sharp contrast with the asymmetric nature of tumor suppressive function attributed to tumor antagonizing genes.
Thus, is RNASET2 gene a tumor antagonizing/malignancy suppressor gene? Maybe the right answer is that the strict categorization of a gene within a particular class is like putting the gene within a sealed cage. In my opinion, a more reasonable classification of genes could be represented by a cage with an open door, from which genes could either enter or go out depending on the experimental approach that we employ to investigate their function. What is objectively documented is the fact that RNASET2 gene is endowed with a strong antitumor activity, both related to and dependent on the tumor microenvironment context.

Being the tumor microenvironment so important in the RNASET2-mediated tumor suppression *in vivo*, I decided to focus my investigation on the issue of a direct interaction between the RNASET2 protein and innate immune cells. This hypothesis is supported by two main observations: (i) the RNASET2 protein is secreted from cells in the extracellular space, where a direct interaction with a target cell could occur, and (ii) the recruitment and functional activation of immunocompetent cells belonging from the monocyte-macrophage lineage is necessary for the RNASET2-mediated tumor suppression to occur *in vivo* (Acquati, 2011).

Searching for an RNASET2 target cell, I started anyway my experimental investigations performing cell surface binding assays on tumor cells, as they were easily available in the laboratory and as we can reasonably hypothesize the existence of a common binding/internalization pathway for RNASET2 protein, which could be cell-type independent. Thus, to our aim tumor cells, which are known to express a wide range of transmembrane receptor proteins for different types of signaling molecules (Hanahan, 2011), could represent an adequate experimental model.
Thus, using three different *in vitro* experimental approaches I was able to demonstrate that endogenously-produced RNASET2 protein could bind the tumor cell surface. Moreover, using the previously described RNASET2-silenced OVCAR3 cell model, I have also observed that exogenously-provided RNASET2 protein could be internalized following the binding to the tumor cell surface.

On the basis of these encouraging results, I decided to focus my investigation on the cell-type which could more likely represent the target of the RNASET2 protein tumor suppressive function: immunocompetent cells, particularly those belonging to the monocyte-macrophage lineage. To shed light on this issue, I have first investigated the cell surface binding of exogenously-provided RNASET2 protein on both native and M1/M2 polarized human macrophages, where the latter are known to exert an anti- and pro-tumor function within the tumor microenvironment context, respectively (Galdiero, 2012). As a result, a dose-dependent RNASET2 binding to the cell surface was detected.
Thus, it is tempting to hypothesize the existence of a cell surface receptor for RNASET2 protein in innate immune cells. Within this frame, enlightening new evidences have been very recently collected by Everts et al. concerning the omega-1 T2 ribonuclease secreted by *Schistosoma mansoni* eggs, which has recently been shown to condition mammalian dendritic cells to prime Th2 responses (Steinfelder, 2009). Mechanistically, the researchers have demonstrated that omega-1 protein is bound and internalized via its glycans by the mannose receptor and subsequently impairs protein synthesis by degrading both rRNAs and mRNAs (Everts, 2012). Being human RNASET2 protein also glycosylated, one of our next aims would be the investigation of a similar mechanism in human macrophage cells.

Another recent finding concerning the fine structural characterization of human RNASET2 protein, which has been performed by Thorn and colleagues, deserves some comments. Indeed, a potential tumor necrosis factor receptor-associated factor 2 (TRAF2) binding motif has been identified in positions 222-225 (PKQE). TRAF2 motif is known to bind the TNFα receptor and modulate TNFα action on the MAP kinase and NF-kB pathways (Thorn, 2012). Intriguingly, this motif could either represent an additional possibility for RNASET2 to enter the cell or could play a putative role in protein-protein contacts mediated by RNASET2.

The observed RNASET2 protein binding to macrophage cells strongly suggests that RNASET2 protein could be endowed with a chemotactic role in recruiting immunocompetent cells at the tumor site. To shed light on this issue, I decided to perform *in vitro* migration assays on human primary monocytes. Using increasing concentrations of recombinant endotoxin-free human RNASET2 protein as a chemoattractant, I obtained migration responses reminiscent of a bell-shaped curve, which is the expected trend for a chemokine whose binding to the cell surface is receptor-mediated. Because of the high variability recorded in performing migration assays on primary monocytes, I decided to employ a promyelocitic cell line model (U937 cl.10 cells) for chemotaxis assays. Thus, I have performed migration assays on U937 cl.10 cells using both wild-type and catalytically inactive H65/118F recombinant human RNASET2 protein, produced both in BEVS and in *Pichia pastoris* heterologous expression systems. As a result, I have observed a consistent migration in response to both the form of RNASET2 protein.
In my opinion, this is a very intriguing result. In fact, these data demonstrate that the catalytic activity of RNASET2 protein is totally dispensable for its chemotactic function, as we have previously demonstrated to occur for the *in vivo* tumor suppressive activity. This evidence has important outcomes, both experimental and theoretical.
Firstly, among the different *in vitro* experimental approaches that we have employed in the investigation of RNASET2 anti-tumor activity, chemotaxis assays are the first in which we have been able to observe results in a totally congruent fashion with those obtained *in vivo*. Within this frame, chemotaxis assays could represent a solid experimental tool for further *in vitro*

investigation of the RNASET2-mediated tumor suppression that occurs in the *in vivo* context.
Secondly, the apparently dispensable catalytic activity for functional behavior of RNASET2 suggests that this protein could be included in the class of "moonlighting proteins". As proposed by Jeffery in 1999, the "one gene-one protein-one function" theory must be carefully revised, since an increasing number of proteins are being identified as multifunctional. Moonlighting proteins form a special class of multifunctional proteins: they perform multiple autonomous and unrelated functions, often without partitioning these functions into different protein domains. Striking examples are enzymes, which in addition to their catalytic function are involved in fully unrelated processes such as autophagy, protein transport or DNA maintenance (Jeffery, 1999). The human RNASET2 ribonuclease seems to behave in this way.

Since the RNASET2 protein proved to directly interact with monocyte-macrophage cells and also showed a chemotactic activity on this cell population, I decided to start to investigate the occurrence of a receptor-mediated binding of the RNASET2 protein to the target cell surface. Intracellular signaling pathways in response to chemokines are in most cases triggered by the binding of the signaling molecule to a cell-surface G-protein coupled receptor (GPCR) (Murphy, 1994). On the basis of this information, I decided to perform migration assays on U937 cl.10 cells following pre-treatment with pertussis toxin (PTx), which is known to inhibit the GPCRs function by means of ADP-ribosylation of G_i proteins (Schratzberger, 1996). As a result, PTx pre-treatment drastically impaired U937 cl.10 cells migration in response to recombinant wild-type human RNASET2 protein, thus confirming the hypothesis of a receptor-mediated cell surface binding between the RNASET2 protein and the target cell.

As a final remark concerning this first part of my Ph.D. project, I think it is reasonable to propose that RNASET2 could exert its tumor-antagonizing role by means of a direct receptor-mediated interaction with the monocyte-macrophage cell population. Consequently, the recruitment and the functional activation of such cells within the tumor microenvironment could be

responsible for the RNASET2-mediated tumor suppression that we have demonstrated to occur *in vivo*.

In spite of the large amount of data that we have gathered demonstrating the RNASET2 activity in controlling tumor suppression, we have very little knowledge concerning the RNASET2 protein sub-cellular trafficking. This is not an irrelevant issue, since a deeper investigation of these aspects of RNASET2 biology could allow us to get more insights into putative cell autonomous functions of this moonlighting protein.

In order to start to investigate this issue, we took advantage of some data that we have recently collected concerning the subcellular co-localization of RNASET2 protein with processing-bodies (PBs) markers (Vidalino, 2012). PBs are transient cytoplasmic structures mainly associated with the mRNA decay process, which are induced by cellular stress (Kedersha & Anderson, 2007). Interestingly, it has been recently demonstrated that PBs are spatially, compositionally and functionally linked to stress granules (SGs), which represent cytoplasmic aggregates of stalled translational pre-initiation complexes that accumulate in cells during stress (Kedersha, 2005).

Moreover, an important function in general stress-response mechanisms has been demonstrated for several members of the T2 family of RNases. Indeed, RNS2 from *Arabidopsis thaliana* is essential for rRNAs recycling in conditions of nutritional stress (Hillwig, 2010). Similarly, yeast Rny1p is released from vacuoles in response to oxidative stress and has a fundamental role in tRNAs decay (Thompson, 2009). Being the T2 ribonucleases stress-response role so highly conserved throughout the *phyla*, we reckoned that a similar function could be hypothesized for mammalian cells as well.

On the basis of these experimental and theoretical premises, the second aim of my Ph.D. project has been the **investigation of the putative role of human RNASET2 protein in general stress-response processes**.

To this end, I have challenged different tumor cell lines with a variety of stress-inducing chemicals or treatments and then I have first examined changes in RNASET2 protein expression levels. As a result, I have observed significantly higher RNASET2 protein levels in treated cells, in response to different stresses. Particularly, I have shown that the overexpression of a specific form

of RNASET2 protein (36 kDa, 31 kDa or 27 kDa) was typically observable in response to different stress inductions.
According to this descriptive observation, I have deeply investigated the functional significance of the observed RNASET2 increase in response to stress, particularly in the context of P-bodies dynamics. As a result, I have detected a marked co-localization signal for RNASET2 and DCP1 proteins in $CoCl_2$-treated SKOV3 cells when compared to the untreated cells, thus confirming RNASET2 protein as a novel component of P-bodies.
Finally, I have better investigated the functional role of RNASET2 protein in RNA metabolism employing the RNASET2 knock-down experimental model that we have obtained in the human ovarian cancer cell line OVCAR3. Within this experimental framework, I was able to detect a significant increase in P-bodies number and size in RNASET2-silenced OVCAR3 clones when compared to control clones. Therefore, using the same experimental approach, I have detected changes in SGs sub-cellular signal in the same direction as for PBs. A possible explanation for the observed findings could be that cells lacking RNASET2 have a basal cellular stress level higher than cells in which RNASET2 protein is normally expressed.

Taken together, these results are very interesting and in my opinion deserve some comments.
First, both the 31 kDa and the 27 kDa isoforms of RNASET2 protein, which originate from proteolytic cleavages at the C-terminus of the full-length 36 kDa protein, are exclusively intracellular forms that likely reside within lysosomes (Campomenosi, 2006). The stress-induced overexpression of these RNASET2 protein isoforms indicates a likely cell autonomous role of the RNASET2 protein in stress response which could rely on its catalytic activity and could be reminiscent of the mechanism recently described to occur in the yeast *Saccharomyces cerevisiae*. Indeed, in response to oxidative stress the Rny1p T2 ribonuclease is released from yeast vacuole into the cytosol, where it cleaves tRNAs (Thompson, 2009). Since it has been demonstrated that human lysosomal proteins are also released into the cytosol following oxidative stress (Guicciardi, 2004), we could postulate the existence of a similar mechanism involving RNASET2 protein in mammalian cells. Thus, an indepth investigation of this issue is among our next experimental aims.

A second important consideration brings the discussion back to the non-cell autonomous role of RNASET2 in tumor suppression and suggests a link between the stress response role and the antitumor activity of this gene. In fact, we have also observed an increase in the expression and secretion of the full-length 36 kDa protein following specific stress-inducing stimuli such as $CoCl_2$ treatment, which induces chemical hypoxia. It is well-known that, in order to grow and establish a solid tumor mass, single tumor cells have to develop the ability to counteract different kinds of physical, mechanical and chemical stresses, for instance hypoxia. Within this complex frame, it is tempting to postulate that RNASET2 protein could act as a stress "sensor" during tumor development. Indeed, in the early stages of tumor progression RNASET2 protein might be induced and secreted by the cancer cell in the extracellular space, where it might act as an "ALARMIN". The term "alarmin" was coined by Oppenheim and Yang in order to identify a heterogeneous class of molecules, which (i) are released and/or secreted by cells in response to danger and (ii) are endowed with the ability to alert innate and adaptive immune defense mechanisms. Interestingly, this molecules tipically display both chemotactic and activating effects on antigen presenting cells (APCs), such as dendritic cells and macrophages (Oppenheim & Yang, 2005).
Strongly supporting our hypothesis of an alarmin function for RNASET2 protein during tumor development are some recent data demonstrating that the RNASET2 transcript levels are tipically higher in early stages tumors than in later stages tumors (unpublished results of our group).

Thus, as final remarks concerning this second part of my Ph.D project, it might be argued that human RNASET2 could play a cell autonomous role in orchestrating defense mechanisms against stress conditions, as epitomized by both the overexpression of the intracellular forms of RNASET2 protein in response to stress and the co-localization with PBs marker proteins.
On the other hand, at the higher complex tumor microenvironment level, human RNASET2 could act as an alarmin, which could be overexpressed and secreted during the early stages of tumor progression in order to activate innate immune defense mechanisms against the tumor.

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