Spectrométrie de Masse Biologique et Protéomique
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Depicting the Molecular Effects of BCL2 Associated Athanogene 3 (BAG3) Silencing in Anaplastic Thyroid Cancer (ATC) Cells by a Quantitative Proteomics Approach

Liuu, Sophie [1]; Galdiero, Francesca[2]; Bello, Annamaria [2]; D'Andrea, Barbara[3]; Aiello, Concetta[3]; Calise, Celeste[3]; Chiappetta, Gennaro[2] ; Chiappetta, Giovanni[1]; Vinh, Joelle[1]

1. ESPCI Paris, PSL Research University, Spectrométrie de Masse Biologique et Protéomique (SMPB), CNRS USR 3149, 10 rue Vauquelin, F75231 Paris cedex05, France
2. I.N.T. G. Pascale,. Genomica Funzionale, via M.Semmola 80131, Napoli, Italy
3. Centro C.M.O., Torre Annunziata Napoli, Italy

Intro

The oncogenic properties of BAG3

Anaplastic thyroid cancer (ATC) is a rare aggressive tumor arising from the follicular cells of the thyroid gland. The average survival time is 4 to 9 months after the diagnosis and, at present, there are no curative therapies. Bcl-2-associated athanogene 3 (BAG3) is a member of the BAG family of co-chaperone proteins, and also known as a member of the HSP70 co-chaperones family. BAG3 abundance is constitutively high in ATC cells. And it was demonstrated involved in cancer maintenance inhibiting NFkB sequestering in the cytoplasm

IKKγ protein is a target of BAG3 regulatory activity in human tumor growth

Massimo Ammirante[a,b,1], Alessandra Rosati[b,c,1], Claudio Arra[c,d], Anna Basile[b,c], Antonia Falco[b,c], Michela Festa[b,c], Maria Pascale[b,c], Morena d'Avenia[b,c], Liberato Marzullo[b,c], Maria Antonietta Belisario[e], Margot De Marco[b,c], Antonio Barbieri[d], Aldo Giudice[d], Gennaro Chiappetta[e], Emilia Vuttariello[e], Mario Monaco[e], Patrizia Bonelli[d], Gaetano Salvatore[f], Maria Di Benedetto[f], Satish L. Deshmane[g], Kamel Khalili[g], Maria Caterina Turco[b,c,2], and Arturo Leone[b]

[a]Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology and Cancer Center, School of Medicine, University of California, San Diego, La Jolla, CA 92093-0723; [b]Department of Pharmaceutical Sciences (DiFarma), University of Salerno, 84084 Fisciano, Italy; [c]BioUniverSA S.R.L., 84084 Fisciano, Italy; [d]Animal Facility and [e]Functional Genomic Unit National Cancer Institute, "Tumor Institute Fond. Pascale," 80131 Naples, Italy; [f]Pathological Anatomy, Federico II University, 80131 Naples, Italy; and [g]Center For Neurovirology, Department of Neuroscience, Temple University, Philadelphia PA 19122

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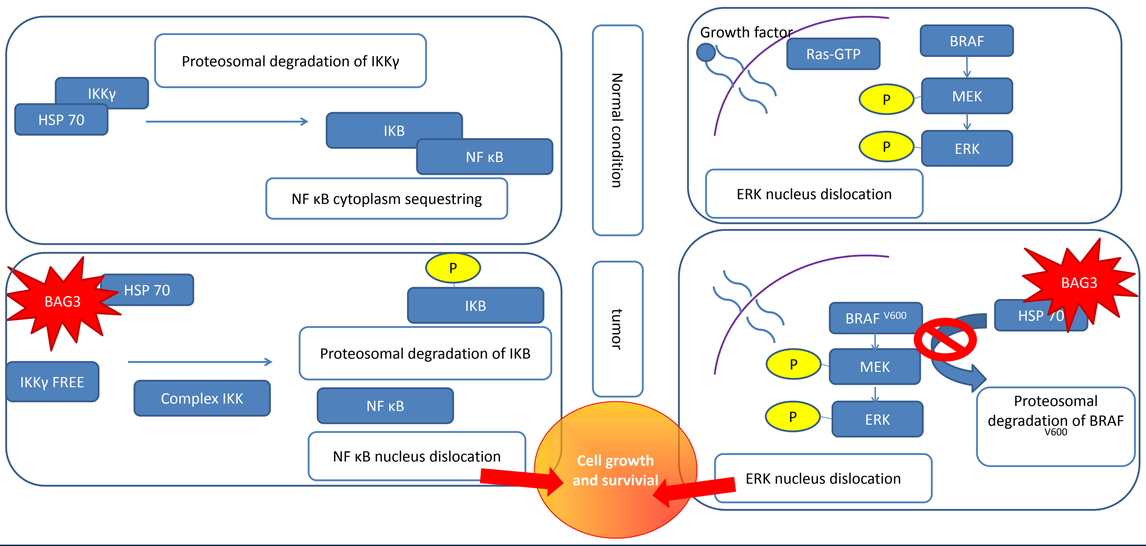

BAG3 silencing in xenograft

Mice xenograft models were obtained by subcutaneus injection of Human thyroid carcinoma cells (8505C) . The effects of BAG3 down-regulation on tumor growth were investigated treating mice with an adenovirus expressing a specific bag3 siRNA, by intratumor injection. Treatment with bag3 siRNA significantly reduced tumor growth and improved animal survival.

BAG3 Down-Modulation Reduces Anaplastic Thyroid Tumor Growth by Enhancing Proteasome-Mediated Degradation of BRAF Protein

Gennaro Chiappetta,* Anna Basile,* Claudio Arra, Daniela Califano, Rosa Pasquinelli, Antonio Barbieri, Veronica De Simone, Domenica Rea, Aldo Giudice, Luciano Pezzullo, Vincenzo De Laurenzi, Gerardo Botti, Simona Losito, Daniela Conforti, and Maria Caterina Turco

Functional Genomic Unit (G.C., D.C., R.P., V.D.S.), Animal Facility (C.A., A.B., D.R., A.G.), Thyroid and Parathyroid Surgery (L.P.), and Pathology Department (G.B., S.L.), National Cancer Institute, Fondazione G. Pascale, 80131 Naples, Italy; Department of Pharmaceutical Sciences (A.B., D.C., M.C.T.), University of Salerno, 84084 Fisciano, Italy; and Department of Biomedical Sciences (V.D.L.), University of Chieti-Pescara, 66100 Chieti, Italy

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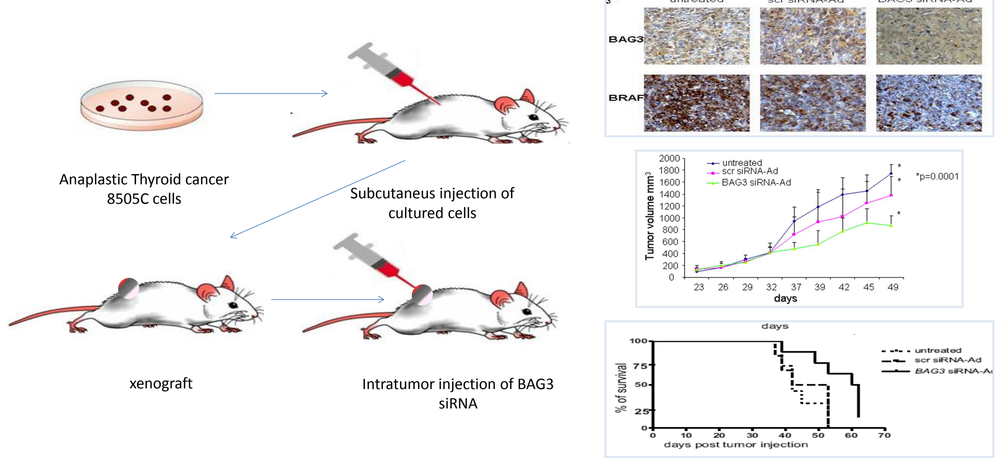


SILAC quantitative proteomics analysis of BAG3 silenced anaplastic thyroid cancer cells

Methods

Wild type 8505C were grown in $^{13}C_{10}$ Arg, $^{13}C_8$ Lys supplemented media and used as control. 8505C transfected with BAG3-siRNA and a scrambled (SCR) siRNA were grown in normal media.
Equal number of cells were pooled (*scheme 1)*. Protein extracts were digested by trypsin. The resulting peptides mixtures were analyzed by nanoLC-MSMS with a Q-Exactive (Thermo) mass spectrometer coupled to an RSLC (Thermo) chromatography system using a 75µmx50cm C18 column.

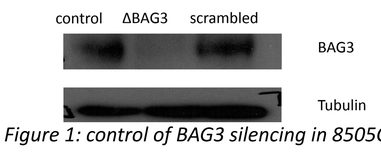
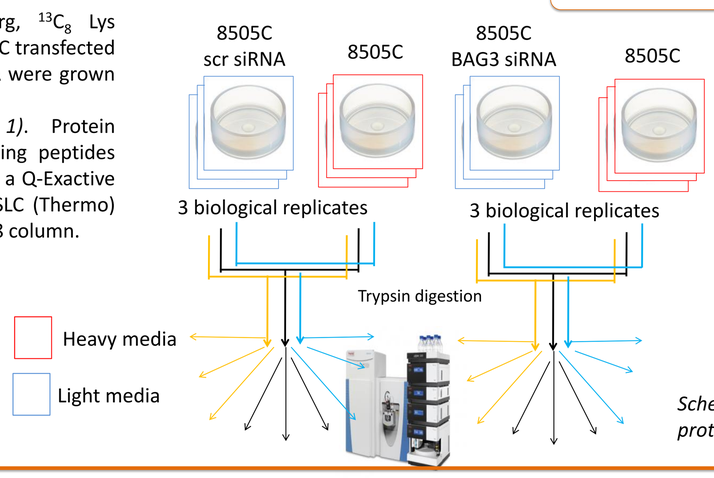
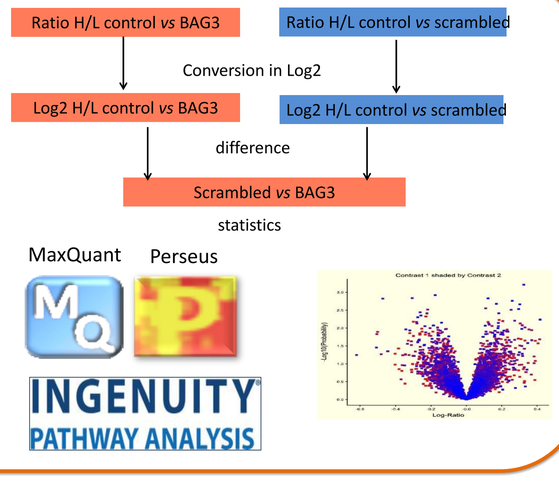


Figure 1: control of BAG3 silencing in 8505C cells



Scheme 1: Quantitative proteomics analysis workflow

Raw LC-MS/MS data were processed using MaxQuant 1.5.3.30 and Perseus 1.5.0.31 for protein sequence database research and quantitative proteomics data analysis. Statistical significant features were used to build a dataset that was submitted to INGENUITY PATHWAY ANALYSIS (IPA) in order to underline the biological effects of BAG3 silencing



Results

Among the 1167 quantified proteins, 37 were up-regulated proteins and 54 were down-regulated in ΔBAG3 choosing a p-value threshold of 0.003 (figure 2). Some proteins were already associated in literature with tumour progression, invasiveness and resistance to treatments. Supporting the anti-cancer proprieties of BAG3 silencing, 7 pro-apoptotic proteins are upregulated and 5 anti-apoptotic proteins are down regulated. Moreover, the increase of 9 anti apoptotic proteins suggests that 8505C cells developed a homeostatic balance adapting to BAG3 silencing.

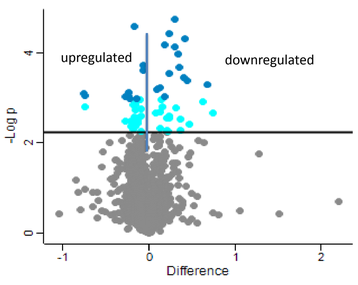


Figure 2: volcano plot related to BAG3 vs Scrambled protein expression profiles. Colored dots are related to proteins that significantly changed their abundance among the two conditions

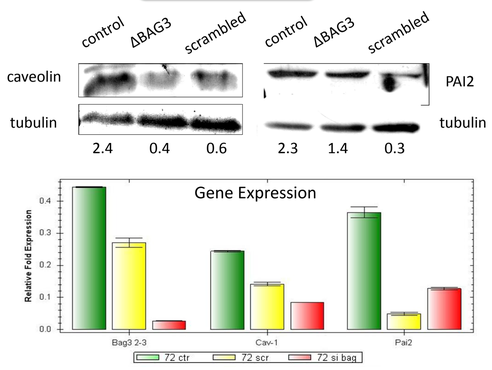


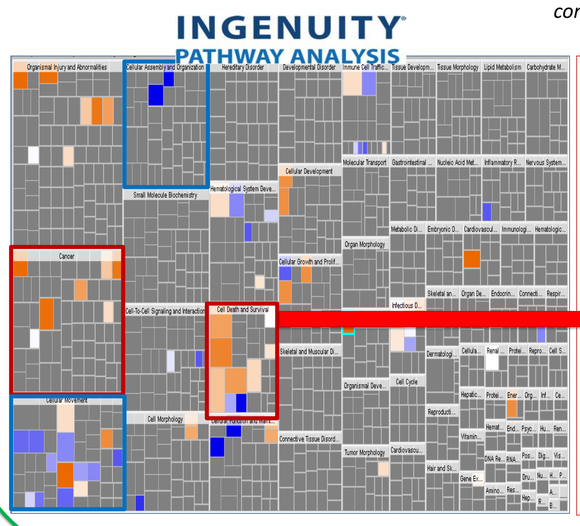
Figure 3: Validation of quantitative proteomics results by western blot and q-PCR analyses

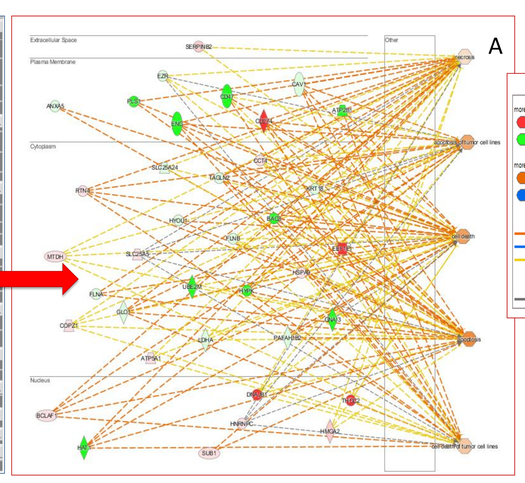
Proteomics data were validated with orthogonal methods monitoring expression profiles of BAG3, Caveolin, PAI2, chosen for the availability of the respective antibodies and RT-PCR reagents. qRT-PCR data and Western Blot analyses are in agreement with quantitative proteomics data (Figure 3: Caveolin downregulation PAI2 up regulation after BAG3 silencing) . Caveolin and PAI2 levels were monitored in human biopsies. They increased with thyroid cancers aggressiveness, revealing a their possible role in tumour development and maintenance.

Tables: analysis of human biopsies of different cancer tissues with increasing aggressiveness by immunodetection of PAI2 and caveolin

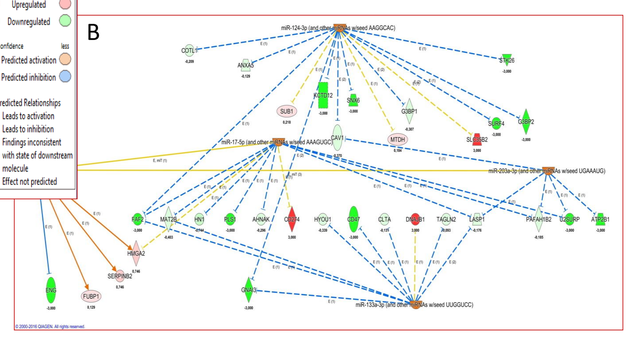
	CAV1 expression	
	+	-
Normal (n.6)	0	6
Goiter(n.7)	3	4
AD (n.6)	2	4
Papillary (n.14)	14	0
Follicular(n.6)	4	2
Anaplastic(n.12)	7	5

	PAI2 expression	
	+	-
Normal(n.6)	0	6
Goiter(n.7)	1	6
AD (n.6)	0	6
Papillary(n.14)	14	0
Follicular(n.6)	4	2
Anaplastic(n.13)	10	3





The list of proteins with expression profile variations was submitted to IPA. Considering the "Downstream Effect Analysis" of IPA, it the "Cell Death and Survival" functions increased while the "Cell Movement" and "Cell growth and proliferation" functions decreased (figure 4). This agrees with previous studies showing increased apoptosis in 8505C cells and in xenograft after BAG3 silencing. Moreover it shows the downstream molecular pathways affected after BAG3 silencing.



Upstream Regulator Analysis (URA) tool of IPA allows to predict the upstream molecules that could have a causal role in the observed proteome profiling. URA underlines the enrichment of 5 precursors distributed in two clusters. The first is characterized by the increased levels of 4 tumour suppressor miRNAs (miR-133a-3p, miR-203a-3p, miR17-5p, miR124-3p) related to the increase of cell death and decrease of invasiveness mechanisms. The second is the transcription factor TP63 whose level are predicted increased in association with the changes in protein expression profiles associated to the compensatory oncogenic adaptation mechanisms of 8505C cells to BAG3 silencing.

Figure 4: Pathway analysis of proteomics data with IPA. A) Downstream analysis B) Upstream analysis

Conclusions

Quantitative proteomics analysis is a powerful tool to depict the molecular mechanisms underlying BAG3 silenced phenotype, to understand why it is over-expressed in ATC and to study the feasibility of targeting BAG3 to induce cancer cells apoptosis. We showed that BAG3 silencing induces changes in oncosuppressor proteins that have a key role in apoptosis and cell migration. For example the levels Cav1 and PAI2, whose oncogenic/oncosuppressor features are still under debate, were found to be altered after BAG3 silencing. Their levels in different thyroid tissues increased in more aggressive thyroid cancer. All together these data suggest that CAV1 down-regulation in BAG3-silenced 8505C cells could have pro-apoptotic features while PAI2 up-regulation is an oncogenic adaptive response. Indeed our proteomics analysis showed a strong adaptive response of 8505C with increased levels of 9 anti-apoptotic targets such as HMGA2. The present quantitative proteomics analysis should be considered a discovery step necessary to set-up targeted proteomics approaches to further study the molecular networks underlying the BAG3 role in ATC.
At this stage, our study suggest the whole pathway could involve also mRNA molecules.