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Tetraspanins Stimulate Protein Synthesis in Myeloma Cell Lines

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ABSTRACT

Intensive protein synthesis is a unique and differential trait of multiple myeloma (MM) cells. Previously we showed that tetraspanin (CD81, CD82) overexpression in MM cell lines attenuated Akt/mTOR cascades, activated UPR, and caused autophagic death, suggesting breach of protein homeostasis. Here, we explored the role of protein synthesis in the tetraspanin-induced MM cell death. Contrary to attenuation of the major metabolic regulator, mTOR we determined elevated steady-state levels of protein in CD81N1/CD82N1 transfected MM lines (RPMI-8226, CAG). Elevated levels of immunoglobulins supported increased protein production in RPMI-8226. Changes in cell morphology consistent with elevated protein synthesis were also determined (cell, nuclei, and nucleoli sizes and ratios). Increased levels of phospho-rpS6 and decreased levels of phospho-AMPK were consistent with increased translation but independent of mTOR. Involvement of p38 and its role in tetraspanin induced translation and cell death were demonstrated. Microarray analyses of tetraspanin transfected MM cell lines revealed activation of protein synthesis signaling cascades and signals implicated in ribosome biogenesis (snoRNAs). Finally, we showed tetraspanins elevated protein synthesis was instrumental to MM cells' death. This work explores and demonstrates that excessive protein translation can be detrimental to MM cell lines and therefore may present a therapeutic target. Proteostasis is particularly important in MM because it integrates the high levels of protein production unique to myeloma cells with critically important microenvironmental cues. We suggest that increasing translation may be the path of least resistance in MM and thus may afford a novel platform for strategically designed therapy. J. Cell. Biochem. 113: 2500–2510, 2012.

KEY WORDS: MULTIPLE MYELOMA; PROTEIN SYNTHESIS; TETRASPANINS; RIBOSOME; rpS6; AMPK; snoRNAs

Multiple myeloma (MM) is an end stage B-cell neoplasia in which the malignant plasma cells accumulate in the bone marrow (BM) [Anderson, 2007]. Despite much progress in MM therapeutics the disease remains fatal due to its heterogeneity, intensive cell interaction with the BM microenvironment, and development of drug resistance [Anderson, 2007].

Contrary to their genetic heterogeneity, MM cells are unified by extensive protein synthesis and secretion that constitute unique and distinguishing features [Agnelli et al., 2007]. Accordingly, MM cells are characterized by expanded endoplasmic reticulum (ER) [Cenci and Sitia, 2007] and partial activation of unfolded protein response (UPR) pathways that contribute to the cells elevated protein production capabilities [Carrasco et al., 2007; Zismanov et al., 2009]. Evidence indicates that information transfer efficiency via translation (not only content) determines signal transduction, making control of protein synthesis/trafficking a basic cell characteristic [Agnelli et al., 2007]. In fact, protein synthesis deregulation has been linked to human cancers, MM included, with elevated global translation as well as increased synthesis of proteins integral to the malignant phenotype [Barnhart and Simon, 2007]. Studies also showed that oncogenic signals firstly affect translation then transcription [Yamasaki and Anderson, 2008].

Victoria Zismanov and Liat Drucker contributed equally to this study.

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Previously, we showed that MM cells and cell lines under express tetraspanin members, CD81 and CD82, compared with normal plasma and peripheral blood B cells [Tohami et al., 2004; Drucker et al., 2006; Tohami et al., 2007]. Tetraspanin proteins facilitate the spatial organization and localization of multi-protein complexes in distinct membranal micro domains that are important to exterior–interior cell signaling. As molecular coordinators, the tetraspanins are involved in many fundamental biological pathways and are correlated with the malignant process and prognosis [Lazo, 2007]. The importance of CD81 and CD82 to MM was shown by us in a study that reintroduced these tetraspanins in fusion vectors with eGFP (CD81N1, CD82N1) into MM cell lines [Tohami et al., 2007]. The tetraspanin overexpression resulted in significant autophagic cell death preceded by ER stress [Tohami et al., 2007; Zismanov et al., 2009]. Moreover, in another study we showed that reintroduction of tetraspanins caused an attenuation of both mTOR and Akt signaling cascades in the MM cell lines [Lishner et al., 2008]. These findings and the common knowledge that protein synthesis is controlled by multiple stimuli such as the metabolic cascade of mTOR and the mitogenic cue of PI3K/Akt [Kawauchi et al., 2009] led us to hypothesize that the tetraspanins may have affected metabolic control in our research model [Lishner et al., 2008].

Based on all these data, we speculated that protein synthesis regulated by these cascades, may have been perturbed by CD81/CD82 and involved in determining the fate of the transfected MM cells. Moreover, our initial expectation was to witness decreased synthetic activity based on the attenuated mTOR and Akt. Surprisingly, in the current study we show that the tetraspanins critically activated protein synthesis and related cascades.

MATERIALS AND METHODS

CELL LINES

MM cell lines RPMI-8226 (ATCC) and CAG (Professor J. Epstein, Littlerock, AR) [Borset et al., 2000; Dezorella et al., 2009] were cultured in RPMI1640 supplemented with 20% heat-inactivated FBS and antibiotics (Biological Industries) [Tohami et al., 2004, 2007; Lishner et al., 2008; Zismanov et al., 2009].

MATERIALS

Cycloheximide (CHX; 0.1 μg/ml; Sigma) was diluted in water. SB202190 (20 μM; Sigma) [Manthey et al., 1998] was diluted in DMSO.

TRANSIENT TRANSFECTION

Purified plasmids pEGFP-N1 (N1/Mock), CD81N1-eGFP (81N1), and CD82N1-eGFP (82N1) were separately introduced into RPMI-8226, CAG as described previously [Tohami et al., 2007]. Fluorescence (≥10,000 events/analysis) was analyzed by Flow Cytometer (FACS; EPICS-XL, Beckman Coulter) [Tohami et al., 2007]. Transfected/untransfected cells were harvested 18/24-h post-transfection and stained with propidium iodide (PI; 1 μg/ml) and enumerated by FACS (PI^-/PI^+). PI^- or $eGFP^+/PI^-$ were regarded surviving cell fraction, whereas PI^+ and $eGFP^+/PI^+$ were regarded as dead cells.

CELL SORTING

$eGFP^+$ and $eGFP^-$ cells expressing cells (18/24-h post-transfection) were collected using BD FACSAria™ cell sorter (Tel-Aviv University, Israel). $eGFP^-$ cells treated with transfection reagent only were used to calibrate $eGFP^+$ cells threshold.

rpS6 DETECTION

Fixated (4% formaldehyde) and permeablized (90% methanol) transiently transfected MM cells were assessed by FACS for ribosomal protein S6 (rpS6; ≥10,000 events/analysis). Rabbit anti-human phospho-S6 ribosomal protein (Ser235/236) and rabbit IgG-matched isotypes primary antibodies (Cell Signaling Technologies) followed by phycoerythrin (PE)-conjugated anti-rabbit secondary antibody (Dako) were used according to the manufacturers' instructions.

DiffQuik STAINING

$eGFP^+$ sorted cells were cytospinned onto glass slides (3 min in 500 rpm) and stained with DiffQuik (Sigma) according to manufacturer's instructions. Nikon "Labphot" light microscope was used for microscopic analysis. Nuclei and cells' areas were circled and measured with ImageJ software, which is designed for area measurement (10–20 cells/slide; n = 2–3 experiments).

QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)

Total RNA extracted from sorted cells (24-h post-transfection, mirVana miRNA isolation kit; Ambion) was reverse transcribed (500 ng, GeneAmp RNA PCR; Applied Biosystems) and amplified (Power SYBR Green; Applied Biosystems) according to manufacturer's instructions for SNORD21 (forward: 5′-CCTGTGGCTAATGACCTATTGAG-3′; reverse: 5′-CCTTGTCACACC ACCGATTGAAG-3′), SNORD31 (forward: 5′-CACCAGTGATGAGTTGAATACCG-3′; reverse: 5′-CAGCTCAGAAAATACCTTTCAGT C-3′); SNORD55 (forward: 5′-GTATGATGACAACTCGGTAATGC-3′; reverse: 5′-CAGCTCTCCAAGGTTGGCTTC-3′); and snU6 as control (forward: 5′-CTCGCTTCGGCAGCACA-3′; reverse: 5′-AACGCTTCAC GAATTTGCGT-3′) [Liao et al., 2010]. A standard sample dilution series was executed with unknown samples for SNORD21, 31, 55, and snU6 to verify results linearity.

IMMUNOGLOBULIN LIGHT CHAIN DETECTION

Mock/CD81N1/CD82N1 RPMI-8226 cells (10^6 cells/100 μl PBS) were fixed (FIX and PERM; Invitrogen) and incubated with anti-human Lambda (λ) light chains RPE or isotype control (Dako) according to manufacturer's instructions. $eGFP^+/\lambda^+$ cells were assayed by FACS for RPE mean fluorescence intensity (MFI).

WESTERN BLOTTING

Cells were lysed in lysis buffer [Zismanov et al., 2009] for 10 min on ice. Protein concentrations were determined using NanoDrop 1000

(Thermo Scientific). Protein samples of 300,000 cells mixed 1:5 with loading buffer were separated by SDS–PAGE and transferred to a PVDF membrane. Membranes were blocked (5% milk powder) and incubated with primary antibodies (overnight at 4°C). Rabbit anti-human phospho-AMPKα(Thr172), phospho-p38(Thr180/Tyr182), and total p38 (1:1,000 dilution) were from Cell Signaling Technology. Bound antibodies were visualized using peroxidase-conjugated anti-rabbit secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories; 75 min at 25°C) and ECL detection (Santa Cruz). Products were visualized with LAS3000 Imager (Fujifilm). Integrated optical densities of the immunoreactive protein bands were measured as arbitrary units employing Multi gauge software (Fujifilm). Results were normalized to cell number.

BIOINFORMATICS MICROARRAY DATA ANALYSIS

Affymetrix GeneChip® Human Gene 1.0 ST arrays (Affymetrix) were used for gene expression analysis according to instruction manual (http://media.affymetrix.com/support/technical/datasheets/gene_1_0_st_datasheet.pdf).

Microarray expression profiles were extracted from raw CEL files using Partek Genomic Suite (Partek® software, version 6.4 Copyright © 2009; Partek Inc., www.partek.com) and Expander tool [Sharan et al., 2003]. Data were normalized and summarized with the robust multi-average algorithm to allow for data comparison across the different arrays [Irizarry et al., 2003] followed by one-way analysis of variance (ANOVA). Genes were identified as differentially expressed with a cut-off $P < 0.05$ and 1.25-fold expression difference. Gene Ontology functional classification and pathway enrichment analysis of differentially expressed genes was assessed with: Panther: http://www.pantherdb.org; GENECODIS: http://genecodis.dacya.ucm.es; ToppGene: http://toppgene.cchmc.org; GATHER: http://gather.genome.duke.edu.

STATISTICAL ANALYSIS

Student's paired *t*-tests were used to analyze differences between cohorts except for area analysis of nuclei and cells in unpaired *t*-tests were used. Effects were considered significant when *P*-value was ≤ 0.05. Interaction between treatments was assessed with the attitude formula $q = P(A+B)/P(A) + P(B) - P(A) \times P(B)$. $q < 0.85$ indicated an antagonistic interaction; $q > 1.15$ indicated a synergistic interaction; and $1.15 > q > 0.85$ indicated an additive interaction. The formula was applied assuming tetraspanin transfection was treatment (A) and CHX treatment (B) [Su et al., 2004]. All experiments were conducted 3–7 separate times.

RESULTS

CD81/CD82 TRANSFECTED MM CELL LINES DISPLAYED ELEVATED STEADY-STATE PROTEIN LEVELS

Total protein levels per cell were measured in sorted CD81N1/CD82N1/Mock(N1) transfected MM cell lines (RPMI-8226, CAG). Increases in protein steady-state levels were demonstrated: ~150% in CD81N1/CD82N1 transfected RPMI-8226 24-h post-transfection; ~50% in CAG 18 h with CD81N1 and 24 h with CD82N1 post-transfection ($P < 0.05$; Fig. 1A).

CD81/CD82 TRANSFECTED RPMI-8226 EXPRESSED ELEVATED IMMUNOGLOBULIN LIGHT CHAIN LEVELS

We examined the levels of immunoglobulins that are an established correlate to synthesis rate in MM [Meister et al., 2007]. Specifically, we studied λ immunoglobulin light chain levels in transfected RPMI-8226 by flow cytometry (FACS) 24-h post-transfection (Fig. 1B). An increase in the λ light chain was determined in RPMI-8226 CD81N1 and CD82N1 compared to Mock transfected cells (40%↑ and 20%↑ in MFI of CD81N1 and CD82N1 transfected cells, respectively, $P < 0.05$), also supporting the observation that CD81N1/CD82N1 overexpression elevated protein levels.

CD81/CD82 TRANSFECTED MM CELL LINES PRESENTED ALTERED CELL MORPHOLOGY

We observed differences in tetraspanin transfected cell morphology that correspond to increased protein synthesis [Shaffer et al., 2004; Baserga, 2007]. Specifically, 24 h after tetraspanin transfection we detected enlarged nuclei in tetraspanin transfected MM cells using ImageJ software (detailed in Materials and Methods Section; RPMI-8226 ~20%↑; CAG/CD81N1 70%↑, CAG/CD82N1 40%↑, $P < 0.05$; Fig. 1C,E). We determined increased area ratios of nuclei/cells in both transfected cell lines (RPMI-8226 ~25%↑; CAG 30–40%↑, $P < 0.01$; Fig. 1D). Enlarged Golgi complexes were also visualized but not measured (Fig. 1E). FACS forward scatter characteristics demonstrated increased cell size in tetraspanin transfected MM cells relative to Mock controls (~20–50 arbitrary units↑ in RPMI-8226; ~25–100↑ in CAG, $P < 0.05$; Fig. 1F), despite higher levels of vector expression determined in the Mock transfected cells (MFI values: N1 18–24; CD81N1 4–5; CD82N1 8, $P < 0.05$).

ACTIVATION OF PROTEIN SYNTHESIS CASCADES EVIDENCED IN MICROARRAY ANALYSES OF CD81/CD82 TRANSFECTED MM CELL LINES

We determined activation of protein synthesis signaling cascades in cDNA microarrays analyses. Total RNA extracted from sorted N1/CD81N1/CD82N1 transfected MM cells (24-h post-transfection) of three independent experiments was hybridized to GeneChip Human Gene 1.0 ST array kit (Affymetrix). Hybridization and signal quantitation were performed according to Affymetrix's protocol by the Bioinformatics Unit at the Tel-Aviv University (Tel-Aviv, Israel). For comparison of global gene expression in the different transfections the array data were normalized to the median gene-expression levels determined in both cell lines (CAG and RPMI-8226)(18 chips). After normalization and summation, genes identified as differentially expressed in the tetraspanin transfected MM cell lines compared to respective Mock control ($P < 0.05$; fold change difference of 1.25) were further annotated for Gene ontology. Gene ontologies with $P < 0.05$ were considered significantly differential in the microarray experiment (Table I). Analyses of tetraspanin transfected MM cell lines differentially expressed genes with PANTHER classification system demonstrated a common activation of protein metabolism and biosynthesis in both RPMI-8226 and CAG with CD81N1 and CD82N1 ($P < 0.05$; Table I). Additional bioinformatics tools (GATHER, Toppgene, GeneCodis) depicted positive regulation of cell processes such as proliferation and production of hormones that are contingent

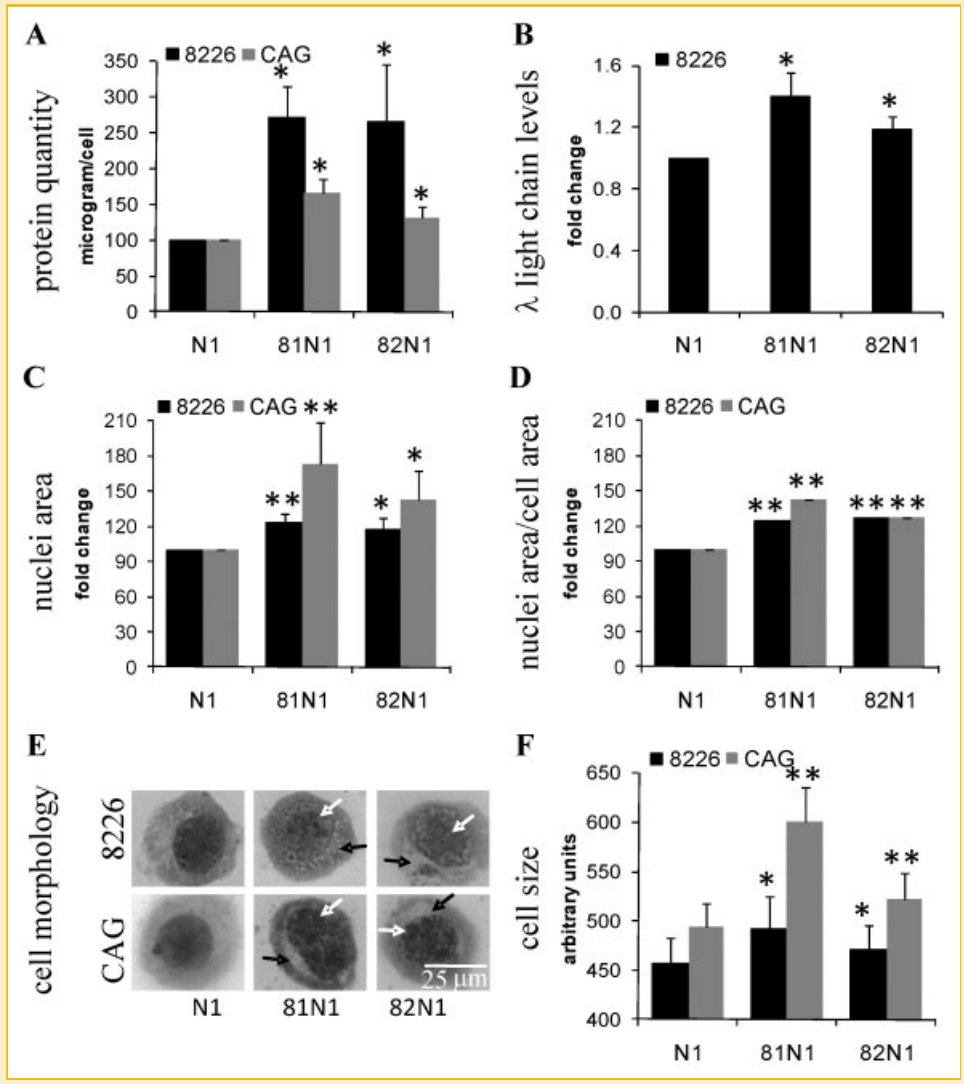

Fig. 1. Tetraspanins affected protein biosynthesis in MM cell lines. RPMI-8226 and CAG were transiently transfected with N1/Mock, CD81N1, or CD82N1 vectors (X-axis). Total protein levels (A) are presented as microgram protein/cell (mean ± SE) and normalized to Mock. RPMI-8226 lambda (λ) globulin light chain levels (B) are presented as fold change of Mock (mean ± SE). Nuclei and cell areas were measured from DiffQuik stained cytospin slides (C,D). Nuclei areas (C) and calculated ratios of nuclei to cells (D) are presented as fold change of Mock (mean ± SE). Photomicrographs of DiffQuik stained transfected cells are presented (E). White arrows indicate expanded and more numerous nucleoli and black arrows depict a larger more prominent Golgi complex. Cells of similar size were chosen to emphasize organelles' differences. Cell size (F) is presented as arbitrary units (mean ± SE) of cell dispersion forward scatter determined by FACS. Statistically significant differences are $^{*}P < 0.05$ and $^{**}P < 0.01$.

on protein production ($P < 0.05$; Table I). These results substantiate yet again that CD81N1/CD82N1 overexpression induced protein synthesis.

ACTIVATION OF RIBOSOMAL RNA PROCESSING EVIDENCED IN MICROARRAY ANALYSES OF CD81N1/CD82N1 TRANSFECTED MM CELL LINES

The Affymetrix microarrays analyses were expanded to additional signaling cascades related to protein synthesis. Particularly interesting were the distinct changes in expression of small nucleolar RNAs (snoRNAs) in the tetraspanin overexpressing MM cell lines. SnoRNAs are small evolutionarily conserved RNAs that localize to nucleoli where they promote rRNA biogenesis and facilitate translation [Matera et al., 2007]. We observed major elevations in more than 40 different snoRNAs in RPMI-8226 ($P < 0.05$) and a select group is listed in Table II ($P < 0.05$, <1.5-fold change). In CAG cells elevations in snoRNAs expression were evidenced as well but to a lesser extent ($P < 0.05$, <1.2-fold change) and with a smaller repertoire involved ($n = 5$; data not shown). The altered expression levels of select snoRNAs were validated by qPCR. Again, significant elevations were determined. Specifically, we observed increases in SNORD31 and SNORD55 levels in CD81N1 transfected RPMI-8226 ($P < 0.05$, 1.8- and 2.1-fold change, respectively) and SNORA21expression in CD82N1 transfected RPMI 8226 cells ($P < 0.05$, 2.7-fold change) compared to the mock transfected cells. These findings demonstrate a tendentious

TABLE I. Gene Ontology Analysis of Differentially Expressed Genes Reveal Increased Protein Synthesis in CD81N1/CD82N1 Transfected MM Cell Lines

Cell line	Vector	Bioinformatic tool	Biological process code	Biological process name	*P*-value	Term in query (# genes)	Term in genome (# genes)	Annotated (# genes)	Additional statistics
8226	CD81N1	Panther	GO:0019538	Protein metabolism	<0.05	42	3,080	148	
			GO:0006412	Protein biosynthesis	<0.05	7	419	148	
			GO:0030154	Cell proliferation and differentiation	<0.05	16	1,090	148	
			GO:0006445	Translation regulation	<0.05	1	849	148	
		Genecodis	GO:0008283	Cell proliferation	0.02	6	1,215	158	
			GO:0006412	Translation	0.01	4	419	158	
		Topgene	GO:0008283	Cell proliferation	0.04	15	1,215	181	None
			GO:0044085	Cellular component biogenesis	0.0006	21	1,268	181	FDR
			GO:0045750	Positive regulation of S-phase of mitotic cell cycle	0.02	1	3	181	None
		Gather	GO:0030890	Positive regulation of B-cell proliferation	0.03	1		202	Bayes 0
			GO:0042100	B-cell proliferation	0.03	15		202	Bayes 0
	CD82N1	Panther	GO:0019538	Protein metabolism	<0.05	29	3,080	75	
			GO:0006412	Protein biosynthesis	<0.05	8	419	75	
		Genecodis	GO:0006457	Protein folding	0.01	3	419	63	
		Topgene	GO:0090079	Translation regulator activity	0.04	1	12	94	None
			GO:0008494	Translation activator activity	0.02	1	7	94	
		Gather	GO:0006457	Protein folding	0.01	4	173	104	Bayes 1
CAG	CD81N1	Panther	GO:0019538	Protein metabolism	<0.05	19	3,080	57	
		Genecodis	GO:0008283	Cell proliferation	0.01	8	1,215	368	
		Topgene	GO:0045750	Positive regulation of S-phase of mitotic cell cycle	0.0001	2	3	145	None
			GO:0051246	Regulation of protein metabolic process	0.02	9	591	145	None
			GO:0045974	Regulation of translation, ncRNA mediated	0.01	1	5	59	None
			GO:0042466	Hormone biosynthetic process	0.04	2	48	145	None
			GO:0002378	Immunoglobulin biosynthetic process	0.01	1	2	145	None
		Gather	GO:0030890	Positive regulation of B-cell proliferation	0.004	1		64	Bayes 2
			GO:0042100	B-cell proliferation	0.03	4		64	Bayes 0
			GO:0006461	Protein complex assembly	0.003	4	2	64	Bayes 2
	CD82N1	Panther	GO:0019538	Protein metabolism	<0.05	11	3,080	29	
		Topgene	GO:0008283	Cell proliferation	0.029	3	1,215	13	None
			GO:0045787	Positive regulation of cell cycle	0.0005	2	63	13	FDR
			GO:0007049	Cell cycle	0.02	3	1,052	13	FDR
		Gather	GO:0007049	Cell cycle	0.002	5	1,052	35	Bayes 3
			GO:0008283	Cell proliferation	0.02	5	1,215	35	Bayes 1

and significant increase in ribosomal RNA processing that is in concordance with increased protein translation.

ELEVATED LEVELS OF ACTIVATED rpS6 PROTEIN ARE EVIDENT IN TETRASPANIN TRANSFECTED MM CELL LINES

The observation of elevated protein synthesis and particularly increased ribosomal RNAs in the tetraspanin transfected MM cell lines are unexpected in light of our previous publications regarding decreased phosphorylated mTOR [Lishner et al., 2008; Zismanov et al., 2009]. Thus, we asked if the mTOR downstream target, the rpS6, necessary for assembly of translation initiation complex is involved [Asnaghi et al., 2004; Ravitz et al., 2007]. In keeping with elevated protein synthesis and cell size [Meyuhas, 2008], we observed increased levels of phosphorylated rpS6 in CD81N/CD82N1 transfected CAG cells (25–30%↑, $P < 0.01$) and CD81N1 transfected RPMI-8226 cells (20%↑, $P < 0.05$) 24-h post-transfection compared to corresponding mock control (Fig. 2). This elevated phosphorylation of rpS6 is most probably attributed to a kinase other than mTOR in our model (elaborated upon in Discussion Section) [Cargnello and Roux, 2011].

DECREASED LEVELS OF PHOSPHORYLATED AMPK IN TETRASPANIN TRANSFECTED CAG

The AMP-activated protein kinase (AMPK) is a major regulator of cellular energy homeostasis positioned both upstream and downstream of mTOR (via Akt cascades) that coordinates metabolic signals [Fogarty and Hardie, 2010]. Upon activation, AMPK downregulates anabolic processes and activates catabolic processes. Recent reports described a synthesis promoting affect of AMPK that is independent of TSC2/mTOR signaling [Saha et al., 2010]. It was also shown that the negative control of AMPK can be disconnected from mTOR/p70S6K/rpS6 [Ginion et al., 2011]. Thus, we decided to explore whether AMPK may be involved in the tetraspanins contradictory affect on Akt/mTOR and rpS6/protein synthesis. Phosphorylated (Thr172) AMPK was assessed in immunoblots of sorted N1/CD81N1/CD82N1 transfected CAG cells 24-h post-transfection and normalized to cell number. A definite involvement of AMPK was determined with a decrease in phosphorylated and active AMPK (50%↓, $P < 0.01$), which is consistent with elevated energy consuming protein synthesis. The conflicting effects of CD81N1/CD82N1 on AMPK and mTOR support our earlier

TABLE II. Increased Expression of snoRNAs in CD81N1/CD82N1 Transfected RPMI-8226

Gene symbol	Fold change	*P*-value	Chromosome	Function
SNORA				
CD81				
SNORA9	1.64	0.018	7p13	
SNORA71A	1.58	0.020	20q22.23	
SNORA29	1.56	0.045	6q25.3	Involved in cell cycle[a]
SNORA71C	1.52	0.005	20q11.23	
SNORA4	1.51	0.004	3q27	Involved in ribosomal function and structure[a]
CD82				
SNORA48	1.67	0.037	17p13.1	Guides the sites of modification of uridines to pseudouridines[b]
SNORA21	1.61	0.017	17q12	Guides the pseudouridylation of residue U3797 of 28S rRNA[b]
SNORD				
CD81				
SNORD31	2.01	0.001	11q13	
SNORD15B	1.99	0.010	11q13.4	Involved in ribosomal function and structure[a]
SNORD25	1.85	0.026	11q13	
SNORD27	1.81	0.004	11q13	
SNORD32A	1.72	0.015	19q13.3	Involved in ribosomal function and structure[a]
SNORD22	1.55	0.016	11q13	
SNORD30	1.53	0.013	11q13	
SNORD55	1.53	0.025	1p34.1	Involved in ribosomal function and structure[a]
SNORD59A	1.51	0.002	12p13.3	
CD82				
SNORD3B-2	3.16	0.0003	17p11.2	
SNORD68	1.73	0.013	16q24.3	
SNORD32A	1.66	0.022	19q13.3	Acts as 2′-*O*-ribose methylation guide for ribosomal RNA[c]

Gray shading marks SNORs enriched localization to 11q13.
[a]Tafer et al. [2010].
[b]Kiss et al. [2004].
[c]Grigsby et al. [2010].

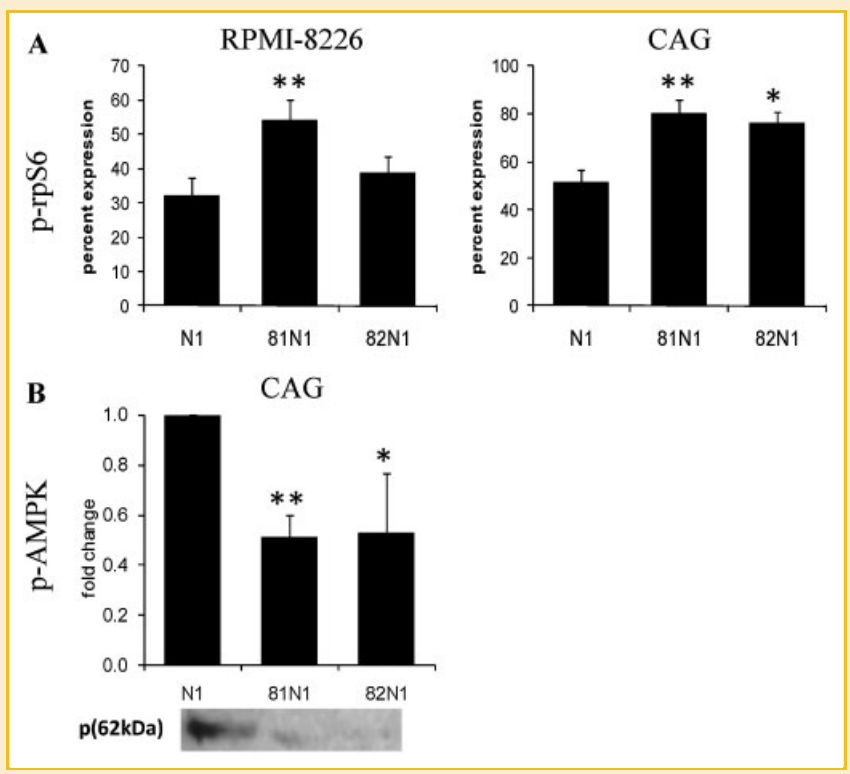

Fig. 2. Involvement of rpS6 and AMPK in MM cell lines response to CD81/CD82 transfection. (A) Levels of rpS6 in tetraspanin transfected MM cell lines were measured by FACS and presented as percent (mean ± SE) of $eGFP^{+}/rpS6^{+}$. (B) Representative immunoblot of p-AMPKα(Thr172) (300,000 cells/lane) in N1/CD81N1/CD82N1 transfected CAG and densitometry are presented (mean ± SE). p-AMPK were normalized to N1 and expressed as fold change. Statistically significant differences are $^{*}P < 0.05$ and $^{**}P < 0.01$.

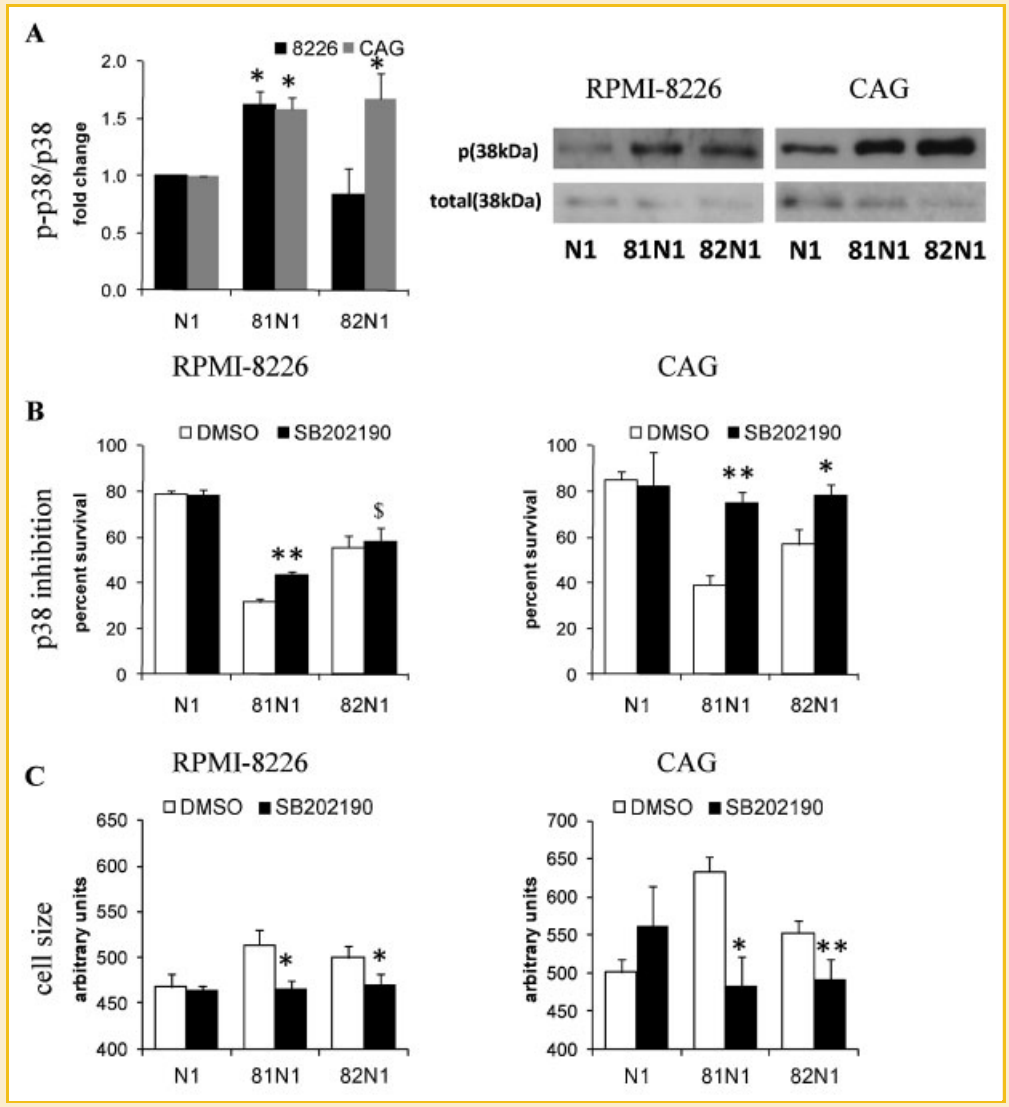

Fig. 3. Involvement of p38 in MM cell lines response to CD81/CD82 transfection. (A) Representative immunoblot of p38 and p-p38(Thr180/Tyr182) (300,000 cells/lane) in N1/CD81N1/CD82N1 transfected RPMI-8226 and CAG and densitometry are presented (mean ± SE). p-p38/p38 were normalized to N1 and expressed as fold change. (B) Cell survival of tetraspanin transfected MM cell lines with/without 20 μM p38 inhibitor SB202190 was measured by FACS. Results are calculated as the proportion of surviving transfected ($eGFP^+/PI^-$) cells in each sample and expressed as the mean relative percentage ± SE compared to total $eGFP^+$ Mock transfected cells. N1/81N1/82N1 transfections with treatment were normalized to untreated transfection and expressed as fold change. (C) Cell size of tetraspanin transfected MM cell lines with/without 20 μM p38 inhibitor SB202190 is presented as arbitrary units (mean ± SE) of cell dispersion forward scatter determined by FACS. Statistically significant differences ($^*P < 0.05$, $^{**}P < 0.01$) and antagonistic changes ($\$q < 0.85$) are indicated. At least three separate experiments in duplicate were conducted, and FACS recorded 10,000 events.

contention that mTOR most probably is not responsible for rpS6 phosphorylation and elevated protein synthesis in our model and that other signals are at play [Meyuhas, 2008].

CD81/CD82 TRANSFECTED MM CELL LINES DISPLAYED CRITICALLY ACTIVATED p38

A recent publication presented evidence that metabolic stress can lead to p38-mediated inhibition of mTOR signaling independently of AMPK [Kalender et al., 2011]. We studied whether p38 is activated in our research model. Indeed, elevated levels of phosphorylated (Thr180/Tyr182) p38 were detected in immunoblots of sorted N1/CD81N1/CD82N1 transfected RPMI-8226 (81N1: 60%↑, $P < 0.05$) and CAG (81N1: 60%↑, 82N1: 70%↑, $P < 0.05$) 24-h post-transfection (Fig. 3A). Next we asked ourselves if p38 may be involved in the tetraspanin induced cell death. We inhibited p38 (SB202190) in CD81N1/CD82N1 transfected MM cell lines and assessed the cells death rates by FACS 24-h post-transfection. A definite rescue of CD81N1 transfected RPMI-8226 (12%↑, $P < 0.05$) and CD81N1/CD82N1 transfected CAG was observed (36%↑ and 22%↑, respectively, $P < 0.05$; Fig. 3B). Importantly, the survival rates of CD81N1 overexpressing CAG cells were completely restored to normal (not significantly different than the mock transfected

cells). In CD82N1 transfected RPMI-8226 an antagonistic interaction between the tetraspanins effect and the p38 inhibitor was recorded ($q < 0.85$). This indicates that p38 is involved in the CD82N1-mediated RPMI-8226 cells death but its inhibition is not sufficient to reverse its affect. Based on the ability of p38 to promote proliferation [Cuenda and Rousseau, 2007; Cargnello and Roux, 2011] we wondered whether its activation affected the protein synthesis in the CD81N1/CD82N1 transfected cells. Thus, we compared the cell size (determined by FACS) of N1/CD81N1/CD82N1 transfected MM cell lines with and without SB202190. Indeed, the increased cell size of CD81N1/CD82N1 transfected MM cell lines (described above) was abrogated upon application of p38 inhibitor (a significant difference in cell size between SB202190 treated and untreated CD81N1/CD82N1 transfected RPMI-8226/CAG, $P < 0.05$; lack of significant difference between SB202190 treated CD81N1/CD82N1 transfected RPMI-8226/CAG compared to Mock transfected RPMI-8226/CAG (SB202190 treated and untreated), respectively; Fig. 3C).

PROTEIN SYNTHESIS IS INSTRUMENTAL TO THE TETRASPANIN-INDUCED MM CELL DEATH

In order to determine the relevance of protein synthesis to the death induced by CD81N1/CD82N1 in RPMI-8226 and CAG we inhibited protein production (CHX) for 6–8 h, 18 h after tetraspanin transfection and assessed cell fate. CD81N1/CD82N1 expression was selected for cell sorting of eGFP expressing cells, and analyses were limited to this specific population. Measurement of cell survival indicated an antagonistic interaction between tetraspanin overexpression and inhibition of protein synthesis ($q < 0.85$). Moreover, a definite though in some cases limited rescue of the transfected cells was also statistically evident in both cell lines transfected with CD81N1/CD82N1 and co-treated with CHX (8–10% in RPMI-8226, 11–34% in CAG; $P < 0.05$; Fig. 4). Special attention is called to the complete rescue of the 82N1 transfected CAG cells upon CHX administration (not significantly different than the CHX treated N1 CAG cells).

DISCUSSION

Increased rates of protein synthesis and altered metabolism are well-recognized characteristics of the malignant cells [DeBerardinis et al., 2008]. In fact, the initial view was that oncogenic signaling primarily affects transcriptional profiles, yet a growing body of evidence demonstrates that translational control is important to cancer progression as well [Yamasaki and Anderson, 2008].

Protein homeostasis within a cells' proteome (proteostasis) is sustained by the "proteostasis network" (PN) that includes pathways controlling protein synthesis, folding, trafficking, aggregation, disaggregation, and degradation; and a cell's proteome is the net result of balancing energetics and network capacity [Powers et al., 2009]. A balanced PN readapts innate cell biology to ever-changing conditions (internal and external), whereas failure to achieve equilibrium between protein demand and production underlies diseases, such as lysosomal storage diseases and cancer, as well as cell death [Powers et al., 2009]. Disruption of proteostasis activates stress response pathways that reduce global protein translation, increase folding efficiency, and induce protein clearance in an attempt to rebalance the cells' capacity of protein synthesis and its demand for specific proteins [Powers et al., 2009]. The premise of our study was that CD81N1/CD82N1 overexpression breached MM cell lines' proteostasis, based on activation of UPR and induction of autophagic death [Zismanov et al., 2009]. Previous work also demonstrated a decrease in active Akt and mTOR in our research model [Lishner et al., 2008]. Assessment of transfected cells' total protein uncovered increased levels that may have originated from elevated translation or decreased degradation. Evaluation of cell morphology (size, nucleoli, Golgi) presented additional evidence compatible with increased protein synthesis as did the higher levels of light chain immunoglobulin. These findings do not conform to the canonical role of Akt/mTOR signaling [Han et al., 2006]. Thus, we opted to examine the involvement of other major regulators/markers of protein synthesis and metabolic response. The rpS6 is a necessary component of the 40S ribosomal translation initiation

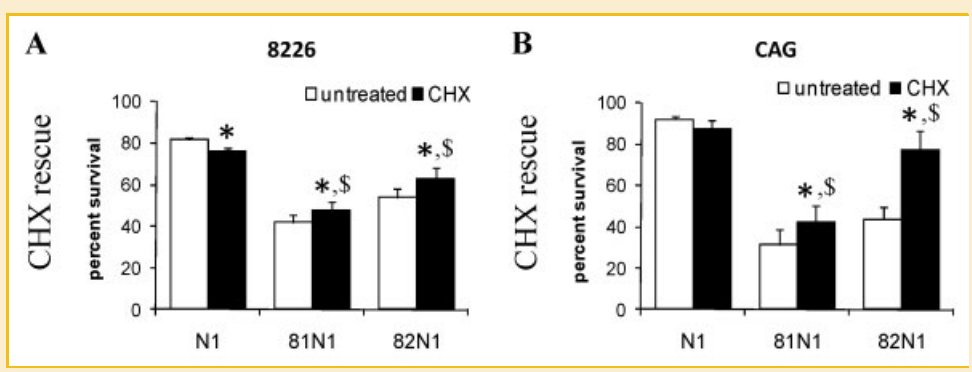

Fig. 4. Protein synthesis is instrumental to the tetraspanin induced MM cell death. Cell survival of CD81N1/CD82N1 transfected RPMI-8226 (A) and CAG (B) with/without 0.1 μg/ml cycloheximide (CHX) was measured by FACS. Results are calculated as the proportion of surviving transfected cells ($eGFP^+/PI^-$) in each sample and expressed as the mean relative percentage ± SE normalized to number of total $eGFP^+$ Mock transfected cells. Statistically significant differences ($^*P < 0.05$) and antagonistic changes ($: $q < 0.85$) are indicated. At least three separate experiments were conducted, and FACS recorded 10,000 events.

subunit and an established correlate of translation and cell size [Meyuhas, 2008; Hutchinson et al., 2011]. It is a recognized target of mTOR but also known to be regulated by several other kinases and phosphatases [Meyuhas, 2008; Cargnello and Roux, 2011; Hutchinson et al., 2011]. In accordance with its central and indispensible role in translation rpS6 was significantly activated in the tetraspanin overexpressing cells.

In an attempt to reconcile our present observations with previously reported decreased levels of active Akt and mTOR [Lishner et al., 2008] we decided to assess the activation state of two major sensors of stress and energy homeostasis that are also known to modulate protein translation rates, AMPK and p38 [Fogarty and Hardie, 2010; Kalender et al., 2011]. A decrease in the phosphorylated state of AMPK corresponds with the elevated anabolic cascade of translation but does not conform to the CD81N1/CD82N1 inhibited mTOR [Lishner et al., 2008; Zismanov et al., 2009]. A recent study showed that AMPK suppression caused elevated protein synthesis in mouse embryonic fibroblasts [Ng et al., 2011]. Furthermore, the study demonstrated that inhibition of protein synthesis promoted cell survival, thereby supporting our observation that elevated protein synthesis was instrumental to the MM cell death [Ng et al., 2011].

In contrast, the elevated active form of p38 complies with its role as a negative regulator of mTOR but is counterintuitive to the elevated protein synthesis. Accumulating data indicate that p38 has a much more pleiotropic function than initially perceived with a definite capability to induce protein synthesis under certain conditions [Cuenda and Rousseau, 2007]. Indeed, upon p38 inhibition the increased cell size of the CD81N1/CD82N1 transfected cells was eliminated. By blocking p38 we showed that its activity was instrumental to the tetraspanin induced cell death. These findings resemble the results with CHX that showed protein synthesis was involved in the tetraspanin induced cell death. It is thus possible that the p38 deleterious effect on the MM cell lines might have been due to its modulation of translation.

To the best of our knowledge this is the first report linking tetraspanins to global protein synthesis or mechanisms regulating the process. Previous studies described association of tetraspanin members with cell proliferation and production of specific proteins but did not make a more general connection to cells' proteostasis [Maecker, 2003; Saito et al., 2006; Hasegawa et al., 2007]. The specific connections of the tetraspanin overexpression to the elevated levels of the rpS6 protein, AMPK, are novel as well.

Data collected from the microarray analyses further supported manipulation of signaling cascades involved in translation. The microarray analyses also yielded new and exciting findings that depict an extensive shift in levels of snoRNAs primarily in RPMI-8226 and to a lesser extent in CAG. The snoRNAs are the most abundant of the non-coding RNAs in eukaryotes with over 200 unique species [Matera et al., 2007]. These evolutionally ancient RNAs consist of two classes that differ in the nucleotide modifications that they guide [Matera et al., 2007]. The C/D RNAs (SNORDs) direct 2′-*O*-ribose methylation and the H/ACA (SNORAs) guide the conversion of uridine to pseudouridine [Matera et al., 2007]. These functions are implemented in parallel and are essential for ribosome function [Matera et al., 2007]. Recent publications depict a role for snoRNAs in human pathologies including cancer [Liao et al., 2010; Gee et al., 2011]. Low snoRNA expression correlated with markers of aggressive pathology in breast cancer and head and neck squamous cell carcinoma [Gee et al., 2011]. On the other hand, high expression levels of specific snoRNAs were observed in non-small-cell lung cancer [Liao et al., 2010]. Mutations in specific snoRNAs are implicated in additional malignancies as well [Liao et al., 2010]. Yet, to the best of our knowledge there are no prior reports of snoRNAs in myeloma. It is noteworthy that 5 of 9 snoRNAs with elevated expression in CD81N1 transfected RPMI-8226 showed enriched localization to 11q13 (SNORD15B is localized to a different locus on the same chromosome; Table II). This is particularly interesting because it is a site of translocation involving the cyclin D1 gene frequently involved in MM [Bergsagel and Kuehl, 2005]. The reason for the association between these snoRNAs is presently unknown but suggests a common regulatory mechanism and warrants future studies [Gee et al., 2011]. This gene cluster is highly conserved, and is encoded within an intron of an mRNA gene "U22 host gene" (UNG). The function of snoRNAs was previously linked to translation [Tycowski et al., 1996; Frey et al., 1997] and suggested as potential cancer markers [Liao et al., 2010]. SnoRNAs are known to participate in ribosomal function and structure (SNORA4, SNORD55, SNORD32A, and SNORD15B), and in cell cycle (SNORA29; Table II) [Tafer et al., 2010]. SNORA21 is involved in modification of uridines to pseudouridines [Kiss et al., 2004], SNORA48 guides the pseudouridylation of residue U3797 of 28S rRNA [Kiss et al., 2004], SNORD32A functions as 2′-*O*-ribose methylation guide for rRNA [Grigsby et al., 2010] (Table II). SNORA4 was also specifically associated with eIF4A2 expression and translation initiation [Cheyssac et al., 2006]. We put forward that the altered regulation of multiple snoRNAs in association with the elevated protein synthesis and concomitant myeloma death suggests a novel therapeutic approach to be explored.

In summary, we suggest that the CD81N1/CD82N1 overexpression caused elevation in protein synthesis that in turn induced cellular stress (p38, FOXO, UPR, autophagy) [Lishner et al., 2008; Zismanov et al., 2009], decreased activity of metabolic gatekeepers Akt/mTOR [Lishner et al., 2008] and AMPK [Viollet et al., 2010], and finally caused autophagic death [Tohami et al., 2007; Zismanov et al., 2009]. Elevated protein synthesis is most likely attributed to changes in translation as a result of a changed balance of the key metabolic factors (Fig. 5). Current studies in our lab include assessment of the rate-limiting translation initiation factor eIF4E.

Available therapeutic approaches unbalance proteostasis by limiting protein synthesis and availability or affecting protein degradation. Our results present protein over production as a means to critically unbalancing myeloma cells' proteostasis. Future studies are warranted to establish whether this approach is generally applicable in cancer or specifically relevant to the highly taxed immunoglobulin synthesizing myeloma cells. Interestingly, it should be taken into consideration that increased global protein synthesis is an inherent trait of cancer and

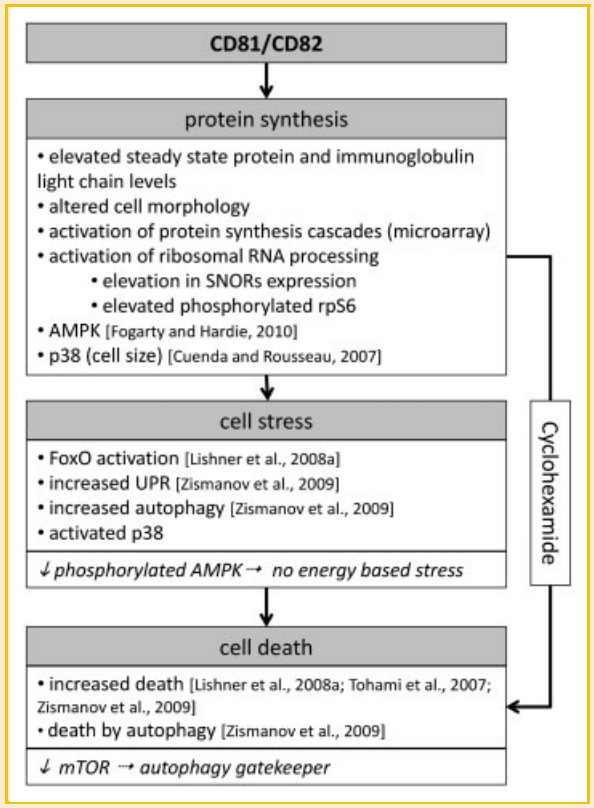

Fig. 5. Schematic summary of tetraspanins' effect on MM cell lines. CD81N1/CD82N1 overexpression caused elevation in protein synthesis that in turn induced cellular stress and finally caused autophagic death. The scheme is based on previous studies we have published [Cuenda and Rousseau, 2007; Tohami et al., 2007; Lishner et al., 2008; Zismanov et al., 2009; Fogarty and Hardie, 2010] and the current research.

therefore may present the path of least resistance where therapy is concerned.

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