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TITLE: IDENTIFICATION OF ATF5-INTERACTING, SH3-CONTAINING PROTEINS IN BREAST CANCER CELLS

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INTRODUCTION:

Growing evidence suggests that ATF5, a member of the ATF/CREB family of transcription factors, is a cancer-specific cell survival factor [1-3]. ATF5 is highly expressed in breast carcinomas and several other types of cancer cells; interference of ATF5 function in those cells causes cell death. Surprisingly, similar interference of ATF5 function in normal or non-cancer breast cells does not affect their survival [1, 2]. Consistent with a required role for ATF5 in survival of cancer cells, ATF5 is down-regulated in a number of cancer cells in response to trophic withdrawal, which induces cell death of those cells. Moreover, overexpression of ATF5 blocks such death [3, 4]. The mechanism underlying ATF5-regulated cellular functions is unknown. The overarching goal for this research is to identify the proteins that interact with ATF5 and that impact on ATF5's cancer-specific cell survival function. The purpose of this research is to understand how ATF5 acts as a cancer-specific cell survival factor in breast cancer cells and use this information to selectively destroy breast cancer.

BODY:

Task 1 - Identification of ATF5-interacting SH3-containing proteins in breast cancer cells

This was done following the two parallel strategies as described in SOW.

1. We constructed GST-ATF5(1-207) and carried out pull-down assays using cell extracts from MCF-7 and T47D. This approach, however, had not produced results as anticipated.
2. We purified native ATF5-containing protein complexes using the previously characterized C6-Flag-HA-ATF5 cell line and identified about 50 ATF5-interacting proteins.

ATF5-containing protein complexes were purified from C6-Flag-HA-ATF5 cell using tandem affinity purification (TAP) protocol [5, 6]. The purified proteins were resolved on SDS-PAGE and visualized by Coomassie/Cy2 staining (Fig. 1). Individual protein bands were excised from the gel and proteins in the gel were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). About 50 proteins were shown to have a confidence score >95%; most of these also have MW that is consistent with their migration position on the SDS-PAGE, indicating correct identification. Surprisingly, the majority of identified proteins seem to function in tightly related cellular processes that involve transcription, RNA processing, and translation regulation (Table 1), processes that fundamental to eukaryotic gene regulation. The fragmentation evidence for one of these proteins – B23 – is given in Fig. 2, showing unequivocal MS-identification of individual proteins. These potential ATF5-interacting proteins may hold the key to understand ATF5's cancer-specific cell survival function.

Task 2. Verification of interaction between ATF5 and identified SH3-containing proteins.

We performed IF analysis on over-expressed Flag-HA-ATF5 and endogenous ATF5 in MCF-7 and HeLa cells and observed a speckle pattern for ATF5, resembling the pattern for B23, PABP, and several hnRNP components listed in Table 1 that are part of the "splicing speckles" related to nucleoli stress sensing. Double-staining showed that ATF5 and B23 overlap neatly in these speckles, and co-IP analyses confirmed that ATF5 interacts with both B23 and GRP78 (Fig. 3). Although we had yet to determine whether these proteins interact with ATF5 via SH3 domains, the available information raises fascinating possibilities that ATF5, in addition of being a TF as

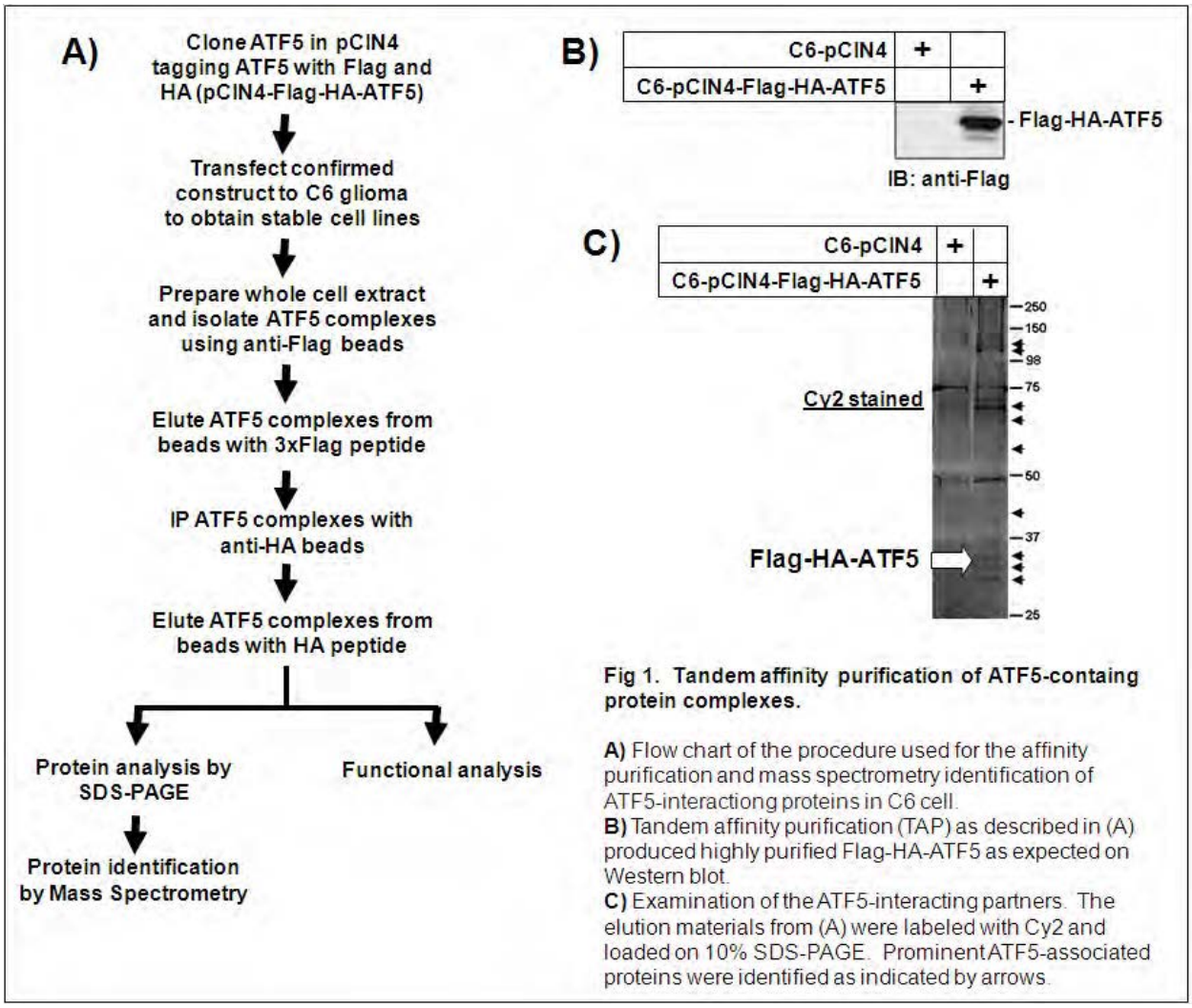

Fig 1. Tandem affinity purification of ATF5-containg protein complexes.

A) Flow chart of the procedure used for the affinity purification and mass spectrometry identification of ATF5-interactiong proteins in C6 cell.
B) Tandem affinity purification (TAP) as described in (A) produced highly purified Flag-HA-ATF5 as expected on Western blot.
C) Examination of the ATF5-interacting partners. The elution materials from (A) were labeled with Cy2 and loaded on 10% SDS-PAGE. Prominent ATF5-associated proteins were identified as indicated by arrows.

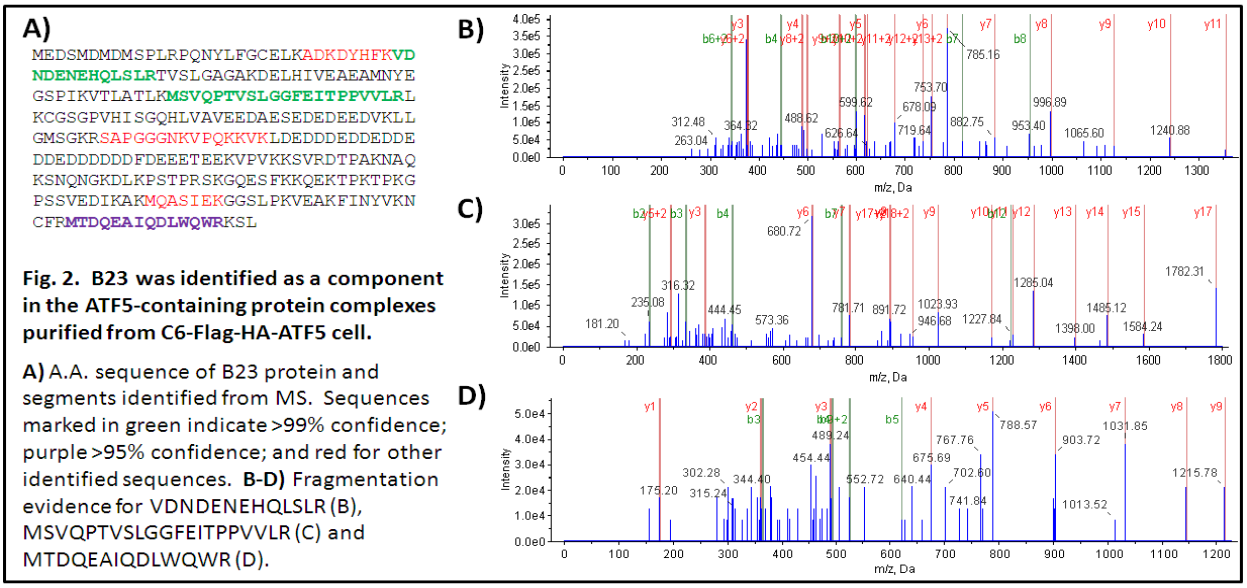

Fig. 2. B23 was identified as a component in the ATF5-containing protein complexes purified from C6-Flag-HA-ATF5 cell.

A) A.A. sequence of B23 protein and segments identified from MS. Sequences marked in green indicate >99% confidence; purple >95% confidence; and red for other identified sequences. **B-D)** Fragmentation evidence for VDNDENEHQLSLR (B), MSVQPTVSLGGFEITPPVVLR (C) and MTDQEAIQDLWQWR (D).

expected serves as a key component in functional RNP complexes and involved deeply in gene-specific RNA metabolism, and post-transcriptional/translational control.

Task 3. Identification of the critical region(s)/amino acid(s) that are important for interaction between ATF5 and identified SH3-containing proteins.

We had obtained evidence that B23 interacts with ATF5's C-terminal and impairs ATF5's transcriptional activity in CRE-dependent transcription, we are in the process to identify the critical amino acids that are responsible for interaction. We are also continuing to search the sequence of other identified proteins for potential SH3 domains.

Table 1. Potential ATF5-interacting proteins identified by MS. Score>2, confidence >99%; 1.3, >95%.

Score	Name(s) - Known Function
32.7	GRP78 precursor/BiP - ER stress; unfolded-protein response (UPR)
10.8	DDX3 - RNA helicase
8.27	P0/RPLP0 - pre-60S ribosomal particle formation; cell proliferation
6.12	PABP - mRNA 3'-tail binding; mRNA stability and translation initiation
5.89	MTHSP75/Mortalin - cell survival and proliferation
5.46	TAXREB107/LRP6 - housekeeping; drug resistance
5.29	Cl current inducer protein
4.96	hnRNP U - RNA processing, splicing
4.71	DDX17 - RNA helicase
4.42	hnRNP B1/A2 - RNA helicase
4.12	nucleophosmin/B23 - multiple function nucleo-cytoplasm shuttle protein
3.05	hnRNP D - RNA processing, splicing
3.06	eIF3 subunit 1a - protein translation; 5'-Cap bining
3.05	GAPDH - housekeeping; cell metabolism
2.66	TTP1/Cooperator of PRMT5 - histone modification and recognition
2.28	eIF2 subunit 1a - global control of protein translation; housekeeping
1.98	LARP7 - component of 7SK snRNP; coupling transcription and RNA splicing
1.75	EDG2/nascent polypeptide-associated complex alpha - stress response
1.73	ATF5 - bait in this experiment
1.7	RRP42 - RNA steability; exosome in AU-dependent RNA degradation
1.46	TAF15/RBP56/hTAFII68 - RNA binding; transcription activator; oncogene
1.44	PCM1 - RNA 5'-cap methyltransferase; elongation checkpoint
1.42	hnRNP E2/PCBP2 - RNA slicing; IRES-mediated translation; stress granule
1.42	eIF4G2 - cap-dependent translation
1.39	U2 snRNP auxiliary factor/U2AF1 - RNA plicing;
1.35	GTP binding protein 5
1.3	LL5 beta protein/Phldb1 - PI (3,4,5)-trisphosphate sensor

Task 4. Survival and apoptotic analysis (months 7-12):

1. We had found that B23 negatively regulates ATF5 abundance in MCF-7, Hep3B and C6 cells via the ubiquitin-26S proteosome pathway (Fig. 4). In addition, we found that B23 impacts on ATF5 regulation of CRE-dependent gene repression on R-Ras, HSP27, and 14-3-3eta, which contribute to ATF5-mediated cell proliferation in Hep3B cell. (Fig. 5)

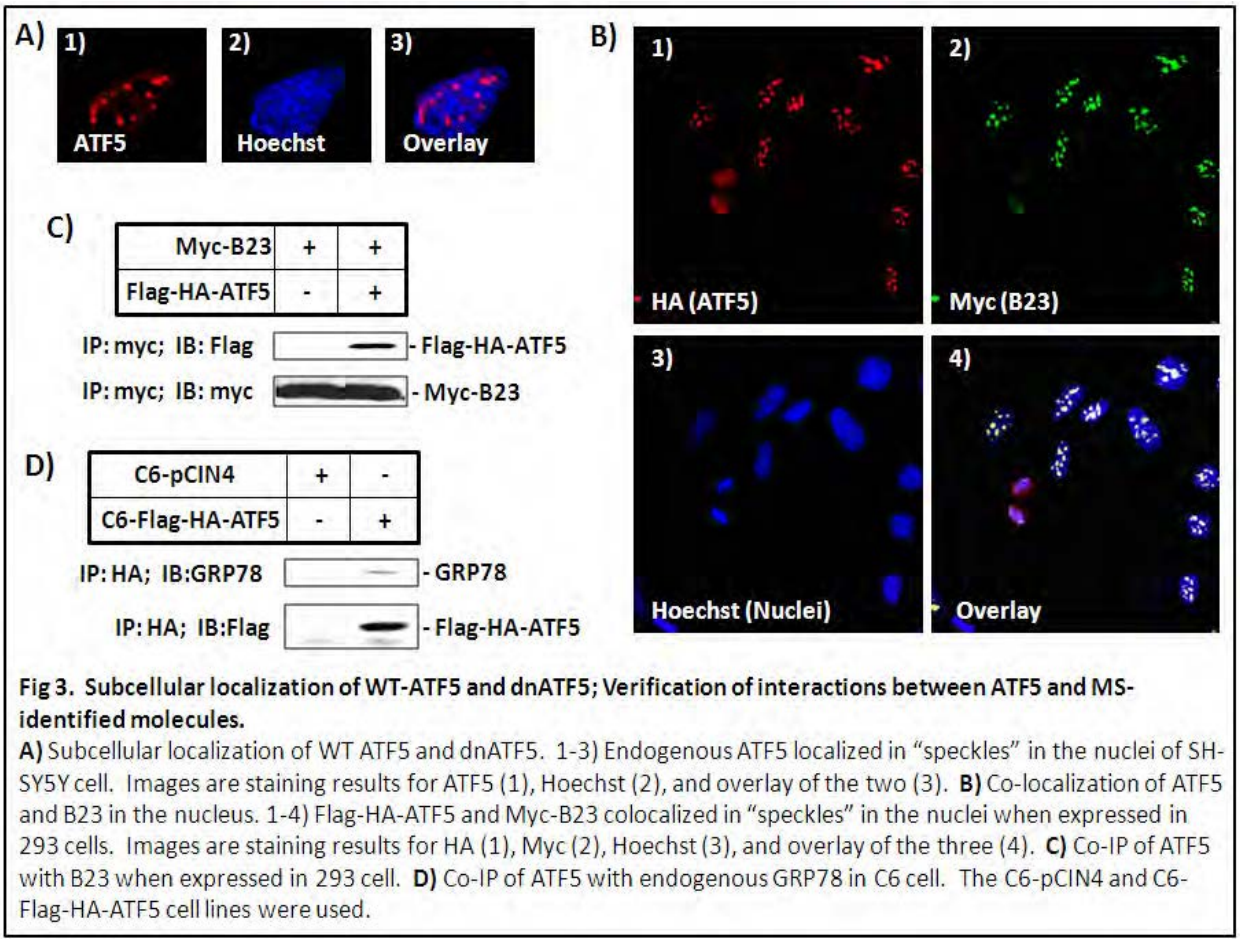

Fig 3. Subcellular localization of WT-ATF5 and dnATF5; Verification of interactions between ATF5 and MS-identified molecules.

A) Subcellular localization of WT ATF5 and dnATF5. 1-3) Endogenous ATF5 localized in "speckles" in the nuclei of SH-SY5Y cell. Images are staining results for ATF5 (1), Hoechst (2), and overlay of the two (3). **B)** Co-localization of ATF5 and B23 in the nucleus. 1-4) Flag-HA-ATF5 and Myc-B23 colocalized in "speckles" in the nuclei when expressed in 293 cells. Images are staining results for HA (1), Myc (2), Hoechst (3), and overlay of the three (4). **C)** Co-IP of ATF5 with B23 when expressed in 293 cell. **D)** Co-IP of ATF5 with endogenous GRP78 in C6 cell. The C6-pCIN4 and C6-Flag-HA-ATF5 cell lines were used.

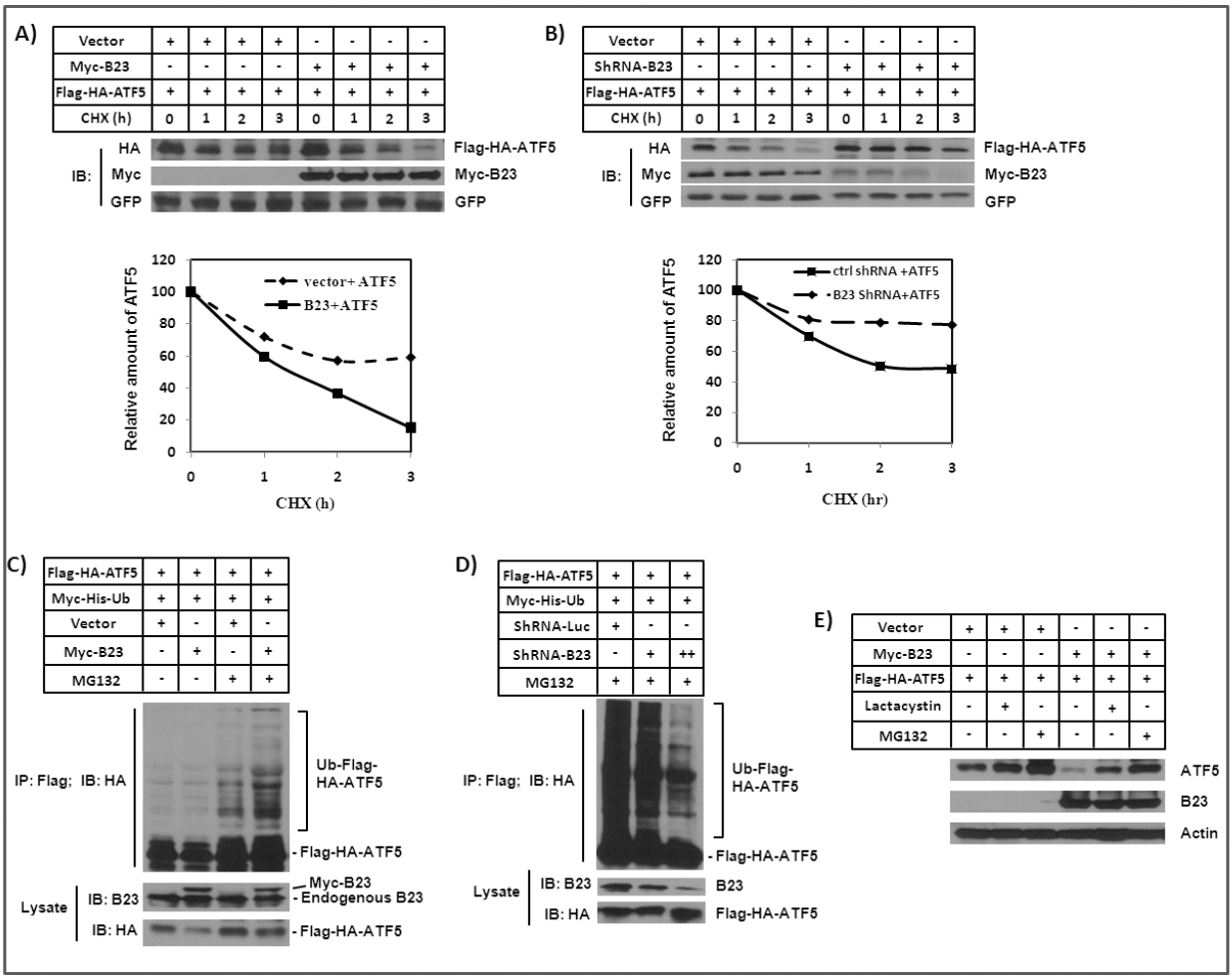

Fig. 4. B23 promotes degradation of ATF5 by polyubiquitination-proteasome pathway. A and B) HEK293T cells were transfected with indicated vectors. 24 h later, cycloheximide (CHX, **20μg/ml**) was added to the culture as indicated. Cell extracts were prepared 6 h later for western blot analysis using anti-HA, B23 and GFP antibodies. GFP was used as a control. **C, D and E)** HEK293T cell was transfected with indicated constructs and cell extracts were prepared as in (A) except CHX was replaced by MG132 or Lactacystin as indicated. Antibodies used for IP and IB were indicated.

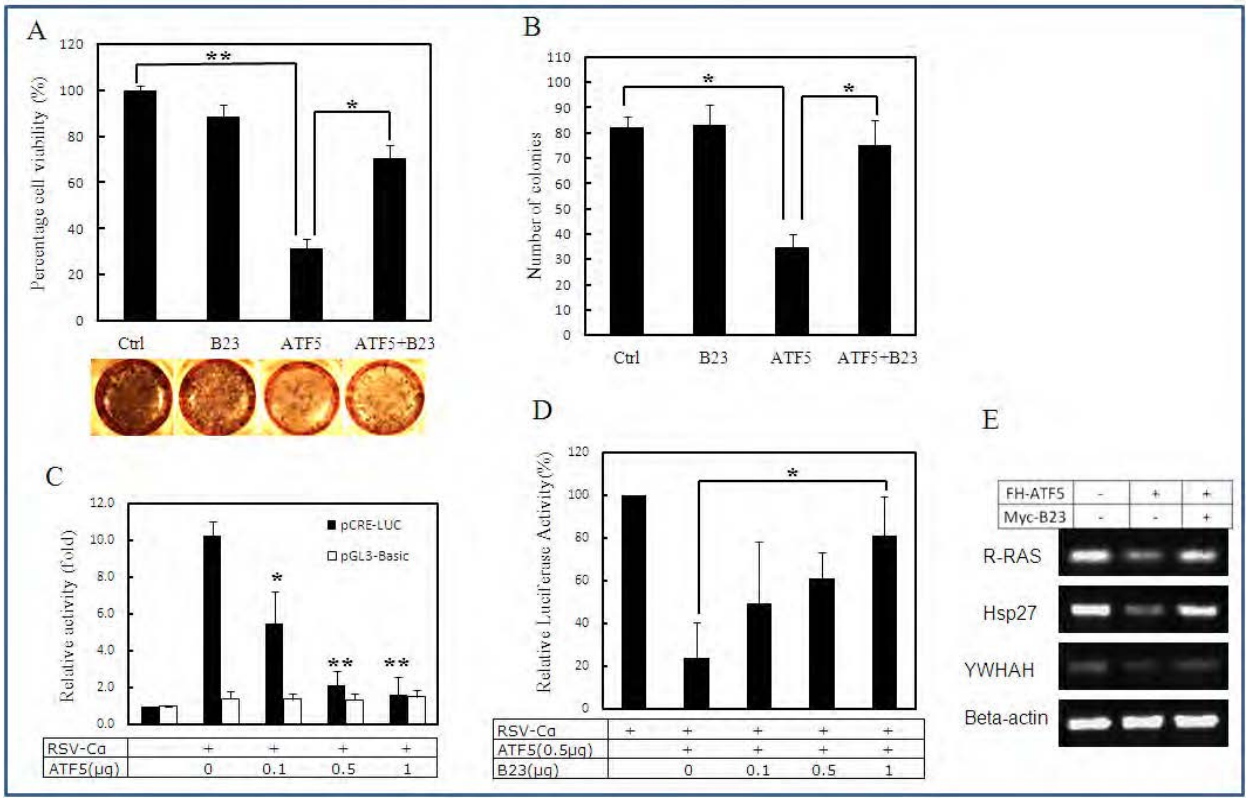

Fig. 5. B23 inhibits ATF5 function. A and B) Hep3B cell was transfected with indicated constructs and cell viability was determined 5 d later by MTT assay (A) and colony formation ability was determined 2 weeks later by crystal violet staining. Each sample was analyzed in triplicate. In B), Visible colonies containing >50 cells were scored. **C and D)** HEK293 cells were cotransfected with a reporter plasmid expressing luciferase under the control of the CRE sequence, renila, and indicated constructs. Twenty-four hours later, luciferase activities for the firefly and Renilla luciferase reporters were measured in the cell lysates. Luciferase activity was corrected against the Renilla activity in the same samples. Experiments were repeated three times with duplicate samples. Data are presented as mean ± SEM (n=3). *p<0.05, **p<0.01 two tailed student's test. E) Hep3B cell was transfected with indicated constructs and mRNA level for R-Ras, HSP27, and YWHAH(14-3-3eta) was determined by RT-PCR. β-actin was used as control.

KEY RESEARCH ACCOMPLISHMENTS:

1. Using TAP and LC-MS, we had identified about 50 ATF5-interacting proteins.
2. Confirmed the interaction between ATF5 and B23 and GRP78, two of the identified proteins.
3. B23 negatively regulates ATF5 protein stability via the ubiquitin degradation pathway.
4. B23-dependent regulation of ATF5 stability impacts on expression of ATF5 downstream targets R-Ras, HSP27, and 14-3-3eta, and cell proliferation of MCF-7, C6, and Hep3B cells.
5. ATF5 interacts with CBP/p300 and is acetylated at K29 by CBP/p300. ATF5 acetylation by CBP/p300 is required for ATF5-dependent Egr-1 expression, cell survival and proliferation.

REPORTABLE OUTCOMES:

1. A manuscript summarizing work on C6 differentiation in response to cycloheximide and the underlying mechanism is being submitted to BMC Cancer after revision.
2. Two manuscripts based on i) ATF5 regulation of Egr-1 and underlying mechanism and ii) B23 regulates ATF5 are being prepared.
3. A comprehensive list of ATF5-interacting proteins is obtained. This formed the foundation for another grant submitted to DOD and one to be submitted to NCI.
4. A presentation was given at Penn State University Cancer Institute on work related to ATF5 regulation of Egr-1 and mechanism.

CONCLUSION:

Using a combined TAP and LC-MS approach, we had identified about 50 ATF5-interacting proteins. The majority of these identified proteins function in tightly related cellular processes that involve transcription, RNA processing, and translation regulation, indicating a role for ATF5 in post-translational regulation.

During the rest of the funding period (we used about half of the one-year budget so far), there are 2 major tasks ahead. **First,** we will continue the planned research, as detailed in SOW, to identify SH3-containing ATF5-interacting proteins. This will be done by 3 alternative approaches. 1) Use GST-ATF5(1-207) to pull-down interacting proteins from MCF-7 and T47D cell extracts. 2) Use GST-ATF5(1-207) as bait to select ATF5-interacting proteins from an SH3 phage library. Both 1) and 2) are described in SOW but were not performed due to personnel changes and obvious success with TAP and LC-MS approach. 3) Adopt the TAP and LC-MS methodology for GST-ATF5(1-207) pulled-down materials to identify SH3-containing ATF5-interacting proteins. This will take the advantage of our experience in TAP/LC-MS and extend to the SH3-containing proteins. **Second,** in case that unforeseen difficulties are met in identify SH3-containing ATF5-interacting proteins, or, we can master enough man power to carry out additional experiments, we would recommend investigate one or two of the identified proteins in Table 1 for in-depth functional analysis. The benefits of this adjustment are clear. Research in this direction fits the overall goal of this grant to identify ATF5-interacting proteins that regulate ATF5's cancer-specific cell survival function. It builds on the achieved success on the TAP/LC-MS-identified proteins. It extends the research in a direction that shows extraordinary novelty and excitement.

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APPENDICES:

None.

SUPPORTING DATA:

None.