Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Institute for Cancer Research

2. **Reporting Period (start and end date of grant award period):** 1/1/2011 – 6/30/2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Maria Minko Gill

4. **Grant Contact Person's Telephone Number:** 215-728-2659

5. **Grant SAP Number:** 4100054848

6. **Project Number and Title of Research Project:** 02 - DUSP6 Regulates the Response to EGFR Inhibitors in Cancer

7. **Start and End Date of Research Project:** 1/1/2011 – 12/31/2013

8. **Name of Principal Investigator for the Research Project:** Igor Astsaturov, M.D., Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 456,242

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Astsaturov, Igor	PI	10% year 1; 8% year 2	$35,151.98
Bagnyukova, Tetyana V	Postdoctoral Associate	100% year 1; 79% year 2	$117,855.49
Restifo, Diana	Scientific Technician I	19% years 1-2	$11,296.06
Gabitova, Linara	Graduate Student	35% year; 50% year 2; 25% year 3	$28,581.34
Banina, Evgeniya	Visiting Scientist	50% year 3	$9,239.36
Serebriiskii, Ilya G	Assistant Research Professor	5% years 1-2 ; 7% year 3	$17,522.40

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds awarded:
R21 - Targeting transcription as treatment for pancreatic cancer	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	February 2014	$490,875	Not Funded
R01 - Synergistic targeting of cholesterol metabolism and EGFR signaling in cancer	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	March 2014	2,231,250	Confirmed-to be awarded

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

We are planning to expand our interest in targeting EGFR-RAS-MAPK-MYC cascade in pancreatic cancer. Our focus will be on synergistic targeting of MYC and EGFR-RAS-MAPK signaling. We anticipate submitting grant applications to support this research in the near future.

12. Future of Research Project. What are the future plans for this research project?

DUSP6 is frequently deleted or silenced in pancreatic adenocarcinoma, the most lethal form of human cancer. As a negative regulator of ERK signaling, DUSP6 loss indicates previously underappreciated dependency of pancreatic cancer on MAPK signaling. Targeting components of MAPK cascade downstream of KRAS has proven difficult to achieve

substantial tumor growth suppression. Our research will focus on the feedback mechanism mediated via the MYC-regulated expression of RTKs that is responsible for rapid compensation of the inhibitory effects to maintain MAPK cascade activity. Several strategies geared towards MYC inactivation or combined targeting of transcription and MAPK cascade will be tested.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female		1		1
Unknown				
Total		**1**		**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic		1		1
Unknown				
Total		**1**		**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White		1		1
Black				
Asian				
Other				
Unknown				
Total		**1**		**1**

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes____X_____ No_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

Tetyana Bagnyukova, Ph.D. - Previous affiliation: FDA-National Center for Toxicological Research, Jefferson, AR.

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The grant provided salary support to Dr. Tetyana Bagnyukova, who was a highly productive lab member and contributed substantively to a number of other projects with CURE funded investigators:

1. Targeting C4-demethylating genes in the cholesterol pathway sensitizes cancer cells to EGF receptor inhibitors via increased EGF receptor degradation. Sukhanova A, Gorin A, Serebriiskii IG, Gabitova L, Zheng H, Restifo D, Egleston BL, Cunningham D, Bagnyukova T, Liu H, Nikonova A, Adams GP, Zhou Y, Yang DH, Mehra R, Burtness B, Cai KQ, Klein-Szanto A, Kratz LE, Kelley RI, Weiner LM, Herman GE, Golemis EA, Astsaturov I. Cancer Discov. 2013 Jan;3(1):96-111. doi: 10.1158/2159-8290.CD-12-0031. Epub 2012 Nov 2. PMID: 23125191.
2. Synthetic lethal screen of an EGFR-centered network to improve targeted therapies. Astsaturov I, Ratushny V, Sukhanova A, Einarson MB, Bagnyukova T, Zhou Y, Devarajan K, Silverman JS, Tikhmyanova N, Skobeleva N, Pecherskaya A, Nasto RE, Sharma C, Jablonski SA, Serebriiskii IG, Weiner LM, Golemis EA. Sci Signal. 2010 Sep 21;3(140):ra67. doi: 10.1126/scisignal.2001083.

Secondly, the funding provided by this grant allowed my lab to develop over 30 patient-derived xenograft models of pancreatic and other carcinomas. This invaluable resource is being actively used by the Fox Chase investigators to conduct “xenopatient” trials of novel compounds aimed to treat lung and pancreatic cancer.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes___X_____ No_____

If yes, please describe involvement with community groups that resulted from the research project:

Our interest in understanding the role of DUSP6 and the MAPK pathway in the pathogenesis of pancreatic cancer has led to a highly productive relationship with PANCAN and AACR to raise funds and increase awareness of the challenge of pancreatic cancer.

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not

print as boxes (☐) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

We report that some of the results of this research were published in the 2013 issue of the British Journal of Cancer (as referenced in section 20).

We have demonstrated that:
1. *DUSP6 depletion sensitizes cancer cells to signaling inhibitors and DNA-damaging agents via induction of apoptosis*;
2. *Depletion of DUSP6 sensitizes A431 xenografts to cetuximab*;
3. *DUSP6 depletion activates signaling proteins in the DNA damage response (DDR) pathway*.

In our project, we have focused on studying the mechanisms involved in the DUSP6-mediated regulation of the DNA damage response:

1. DUSP6 depletion activates signaling proteins in the DNA damage response (DDR) pathway.

Activation of the DDR pathway (Fig. 1A) in the nuclei is manifested by formation of foci containing activated DDR proteins (Polo & Jackson, 2011). To address if DNA repair process is influenced by DUSP6, we assessed DNA breaks by TUNEL assay in the FaDu cancer cell line following siRNA knockdown of DUSP6 and found constitutively higher number of TUNEL-positive cells following DUSP6 depletion (Fig. 1B), both in vehicle or CPT11 treated cells, suggesting this response to loss of DUSP6 activity is independent of any exogenous DNA damaging stimulus. We also determined increased formation of nuclear foci containing phosphorylated H2AX, ATM, and 53BP1 in DUSP6-depleted A431 cells compared to non-targeting control shRNA (Fig. 1C). These foci were further enhanced by treatment of DUSP6-depleted A431 cells with CPT11 and erlotinib. In accord with decreased viability of multiple carcinoma cell lines following DUSP6 siRNA transfection, we found consistent upregulation of pH2AX and pATM foci in DUSP6-depleted cancer cell lines (Fig. 1B, D) under basal conditions, or following treatment with CPT11 or erlotinib. We used Western blot as an alternative method to assess DDR pathway activity (not shown). Here, silencing of DUSP6 with siRNA in FaDu cells caused significant increase in pH2AX under basal conditions as well as following treatment with erlotinib or CPT11. DUSP6 silencing also accentuated CHEK2 phosphorylation although these changes were less pronounced.

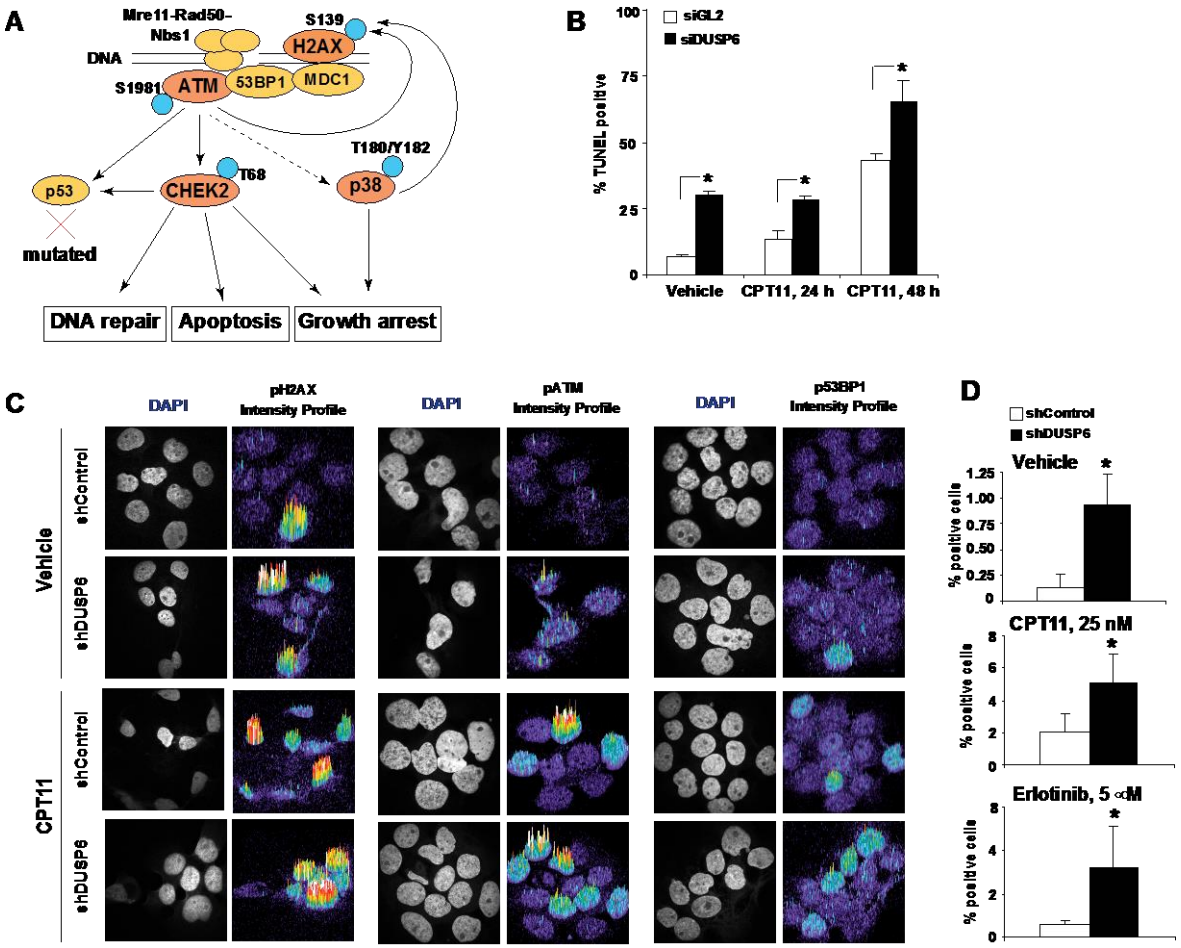

Figure 1. DUSP6 deficiency causes intrinsic and drug-stimulated DNA damage.
(A) Simplified scheme of the ATM/CHEK2/p38 pathway. **(B)** siRNA depletion of DUSP6 in FaDu cells increases DNA breaks by TUNEL Guava flow cytometry assay. siDUSP6 vs. siGL2 at the corresponding drug concentrations; *, $p < 0.05$, Fisher's test. **(C)** DNA damage response in shRNA-modified A431 cells after overnight treatment with 25 nM CPT11. **(D)** Formation of P53BP1 foci in shRNA-modified A431 cells following overnight incubation with vehicle, 25 nM CPT11, or 5 mM erlotinib. Bars are mean of three independent experiments ± S.E.M.; *, $p < 0.05$, Fisher's test.

2. DUSP6 depletion induces cell cycle checkpoint delays and increases the intrinsic rate of cellular DNA damage.

Persistent presence of DNA breaks typically triggers G1/S checkpoint activation mediated by the ATM/CHEK2 pathway (Zhao *et al*, 2002) and delays DNA replication in the S-phase (Falck *et al*, 2001). If so, the observed slow growth of xenografts and cell lines in vitro in which DUSP6 was stably depleted with shRNA might reflect a slowed cell cycle, due to extensive triggering of DNA damage checkpoints (Fig. 2A-C). To evaluate the cell cycle-specific effects of DUSP6 loss, we synchronized shRNA-modified A431 cells in the S phase (with thymidine), and then tracked recovery and progression. Following synchronization in the S-phase with thymidine, DUSP6-depleted cells showed a marked delay in the S-phase up to nine hours after transfer to

thymidine-free full growth medium. The DUSP6-depleted cells arrested in the early M phase (with nocodazole, not shown) or in the S-phase with CPT11 showed similar, although less pronounced, effects on subsequent rate of progression from the G1 to the S phase in DUSP6-depleted cells.

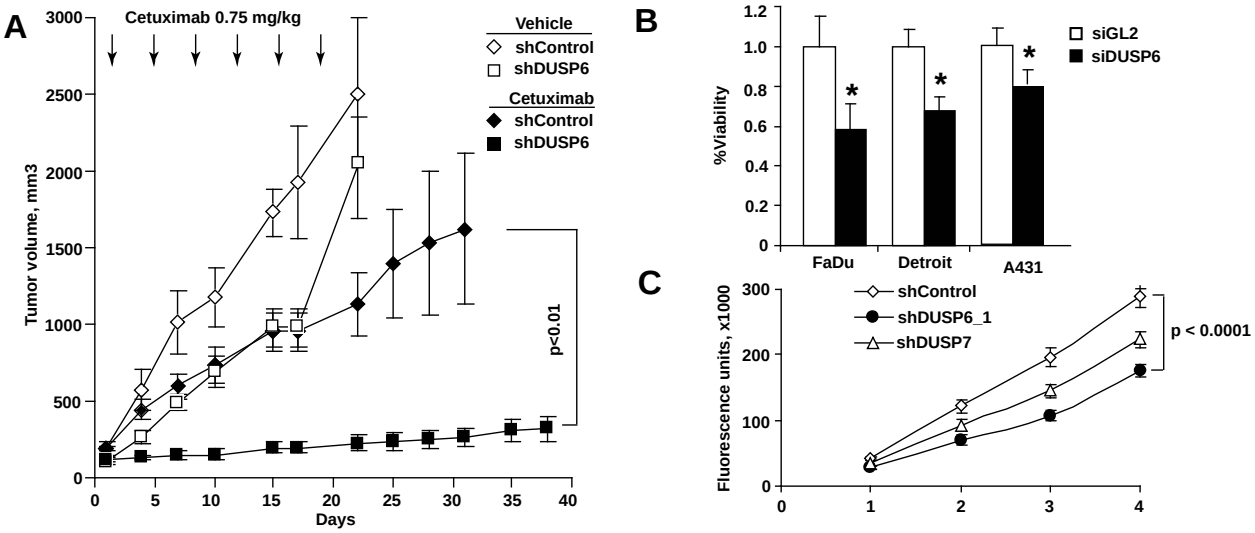

Figure 2. DUSP6 regulates growth and sensitivity to multiple cytotoxic agents. (A) Silencing of DUSP6 sensitizes A431 xenografts to cetuximab. The results are tumor volumes of xenografts of shRNA-modified A431 cells treated with cetuximab (closed symbols) or vehicle (open symbols); means ± SD, *p* value (3) <0.0001. **(B)** DUSP6 depletion decreases viability of cancer cell lines under basal conditions, the viability (CellTiter Blue) was measured 72 h post-transfection. **(C)** A431 cells made deficient in DUSP6 demonstrate delayed growth. Shown are mean results of three experiments ±S.E.M., *, *p* <0.01. Viability data of four independent experiments were normalized to mock-treated cells expressing corresponding shRNA; means ± S.E.M., shDUSP6 versus to non-silencing shRNA control at the corresponding drug concentrations; *p*-values were determined by Wilcoxon's test.

3. *DUSP6 activity in DDR in the context of EGFR pathway signaling*.

Striking results were observed with sensitization to the EGFR inhibitor cetuximab *in vivo* (Fig. 2A). We previously determined that increased sensitivity to erlotinib of DUSP6-depleted cells was associated with increased apoptosis, reflected by increased annexin V (not shown) and elevated Poly (ADP-ribose) polymerase **(**PARP) and caspase 3 cleavage, and staining. Importantly, DUSP6 depletion profoundly increased the sensitivity of A431 cells to the inhibition of the EGFR pathway *in vivo*, based on analysis of subcutaneously implanted A431 xenografts containing integrated shDUSP6 or control shRNA (Fig. 2A). Both control and DUSP6-deficient cells formed palpable tumors at 8-10 days post implantation, although shDUSP6 A431 tumors grew more slowly compared to controls (Fig. 2A). Treatment of control-depleted tumors slightly delayed growth, and growth resumed after cessation of cetuximab treatment. By contrast, cetuximab treatment suppressed growth of shDUSP6 tumors by 83% versus controls (TGI 83%, p<0.0001), with this effect lasting at least three weeks after the last dose of cetuximab (Fig. 2A).

The well-validated target of DUSP6 is ERK1/2 (Camps *et al*, 1998). We explored whether enhanced activation of ERK1/2 might either potentiate the consequences of DUSP6 inhibition, or act independently, in increasing phosphorylation of proteins in the DDR pathway. To test this idea, we created a catalytically inactive DUSP6 mutant by replacing the critical cysteine-293 with serine (C293S mutant) (Levinthal & Defranco, 2005). For this, we co-transfected HEK-293T cells with plasmids expressing wild type or catalytically inactive DUSP6 constructs and a constitutively active form of the ERK1/2 activator MEK-DD (Boehm *et al*, 2007) (Fig. 3). Expression of the C293S mutant of DUSP6 independently elevated the basal level of pATM foci-positive cells, similar to the use of a DUSP6 shRNA, implying a dominant negative activity. By contrast, exogenous expression of wild type DUSP6 reduced basal levels of pATM foci by ~50%. In this system, MEK-DD independently caused an increase in the number of ATM-positive foci, accompanied by an induction of ERK phosphorylation (Fig. 3). In combination with MEK-DD, the wild type DUSP6 eliminated the MEK-DD increase of ATM foci (Fig. 3A), and suppressed ERK phosphorylation by more than ten-fold (Fig. 3B), while the C293S mutant of DUSP6 further increased the number of such cells with ATM foci (Fig. 3B).

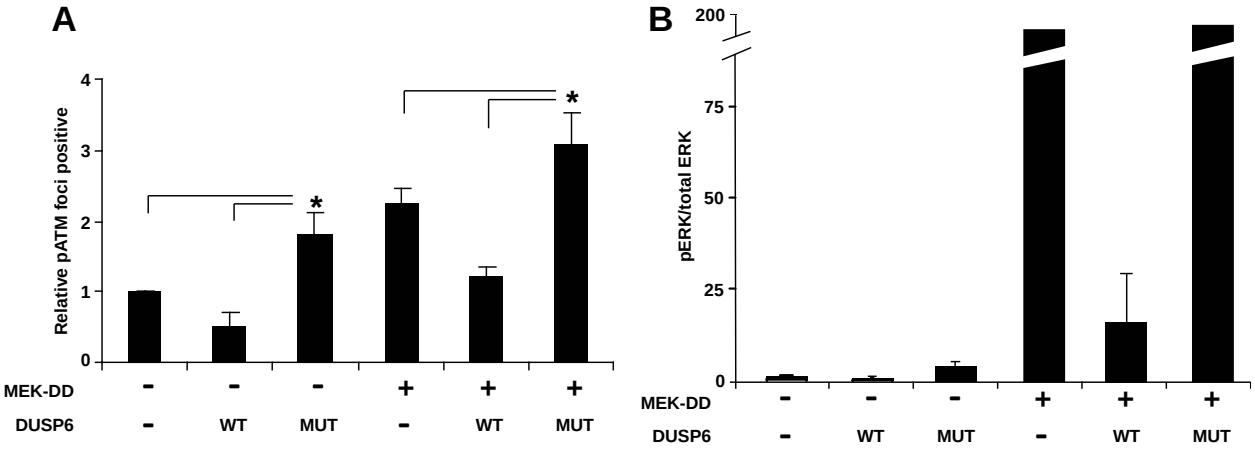

Figure 3. DUSP6 inactivation induces DNA damage response via ERK signaling. (A) pATM foci formation assessed by confocal immunofluorescence in HEK-293T cells transfected with pLEX plasmids expressing either wild type (WT) or C293S mutant (MUT) DUSP6. To activate ERK phosphorylaton, HEK-293T cells were co-transfected with either an empty vector (pBabe) or constitutively active MEK expression plasmid (pBABE-MEK-DD mutant). **(B)** Western blot assessment of ERK1/2 phosphorylation in HEK-293T cells transfected with DUSP6 and MEK-DD plasmid constructs. In (A, B), HEK-293T cells were collected 48 hours post transfection and analyzed by immunofluorescence (A) or Western blot (B). Shown are averaged results of 3 independent experiments; *bars*, standard deviations; *, $p < 0.05$, Student's *t*-test.

Stated Aims 2&3:

Aim 2. Model the DUSP6-p38-CHK2 interaction network.

Aim 3. Investigate the impact of DUSP6 expression on outcomes in head and neck squamous cell carcinoma (HNSCC).

Aim 2 was to decipher the potential mechanism responsible to DUSP6 deficiency-mediated sensitization to apoptosis in cancer cell lines. In the absence of any experimental data, we planned to model the interaction network of DUSP6-MAPK cascade and establish testable linkages to the ATM-CHK2 pathway. We hypothesized that the conserved genetic and physical interactions (using the yeast and human orthologs at the level of functional modules) would define and evaluate the DUSP6 effects on cell cycle checkpoints, growth and viability in DUSP6-silenced or DUSP6-overexpressing cells.

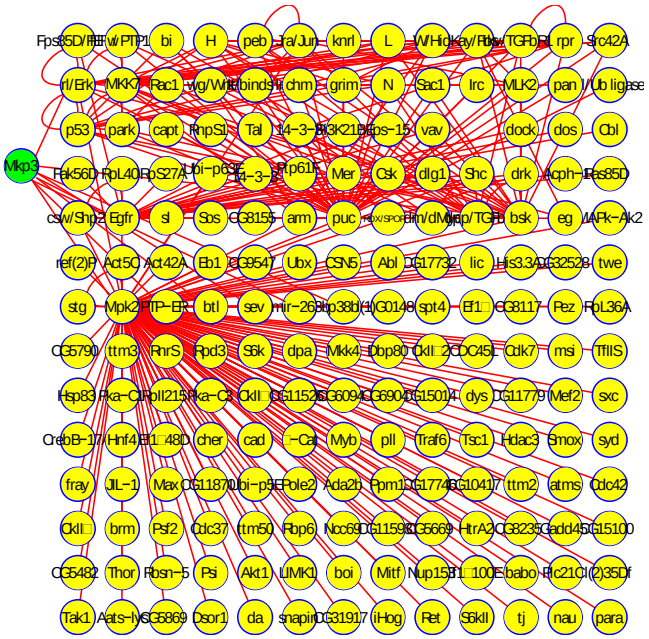

Figure 4. DUSP6/MKP3 interactome identifies multiple interactions.

While this approach (Fig. 4) was logical and could lead to identification of the testable mechanistic connections, we chose to use experimental data as a starting point for our investigations. For this, we assessed the alteration of signaling pathways in drug-sensitized DUSP6-depleted cells in an unbiased fashion: we measured levels of phosphoproteins by solid phase antibody array. We compared protein phosphorylation in cells made deficient of DUSP6 or DUSP7 with specific shRNA, or with a non-targeting control shRNA, in cells grown in 1% fetal bovine serum (FBS) treated for 3 hours with vehicle or 1 μM erlotinib (Fig. 5A). Following quantification of data, we observed significant increase in phosphorylation relative to control-depleted cells for a limited number of proteins (Fig. 5A) in shDUSP6 cells relative to shDUSP7 or control shRNA. Two proteins, the AMP-activated protein kinase AMPKa1 and the MAPK kinases MEK1/2, were elevated under basal conditions; however, since these changes were similar in both shDUSP6 and shDUSP7 cells, we excluded these proteins from further consideration as relevant to the DUSP6-specific phenotypes affecting cell viability and drug sensitization. Notably, we again did not observe very significant changes in the phosphorylation status of the EGFR downstream signaling effectors ERK1/2 and AKT (Fig. 5A), again suggesting that additional phosphatases compensate for DUSP6 deficiency, and that DUSP6 depletion exerts at least some important biological effects outside of the canonical EGFR

pathway.

As candidates for mediators of such effects, we observed that depletion of drug-sensitizing DUSP6 but not DUSP7 increased levels of phosphorylated MAPK14/p38 kinase (T180/Y182), and phosphorylated CHEK2 (T68), both in the presence or absence of erlotinib treatment, suggesting they might be relevant to both the sensitization and the effect on basal cell viability (Fig. 5A). These array results were subsequently further confirmed by direct Western blotting (Fig. 5B), which showed consistently elevated levels of phosphorylation of p38 and CHEK2 in DUSP6-silenced but not in control or DUSP7-depleted cells. Increased phospho-p38 (Bulavin *et al*, 2001; Reinhardt *et al*, 2007) and phospho-CHEK2 (Reinhardt & Yaffe, 2009) are sensitive biomarkers of cellular DNA damage response (Fig. 1), and are frequently activated in cancer cells. However, no previous study has identified a role for DUSP6 in regulating activation of the DNA repair machinery.

To more broadly explore whether DUSP6 silencing intrinsically activates signaling proteins involved in DDR, and at what level in the pathway DUSP6 might be active, we investigated additional DDR pathway effectors in multiple cancer cell lines. ATM activation in response to DNA damage (Matsuoka *et al*, 2007) mediates DNA repair, apoptosis and cell cycle regulation through phosphorylation of a variety of downstream targets including CHEK2, TP53, 53BP1, and H2AX (Kastan & Lim, 2000; Rogakou *et al*, 1998; Shiloh, 2006). Silencing of DUSP6 increased the activation-associated S1981 phosphorylation of ATM (Fig. 1) and the S139 phosphorylation of histone H2AX (Fig. 1), even in the absence of drug treatment, thus suggesting an intrinsic effect of DUSP6 on the DNA repair machinery.

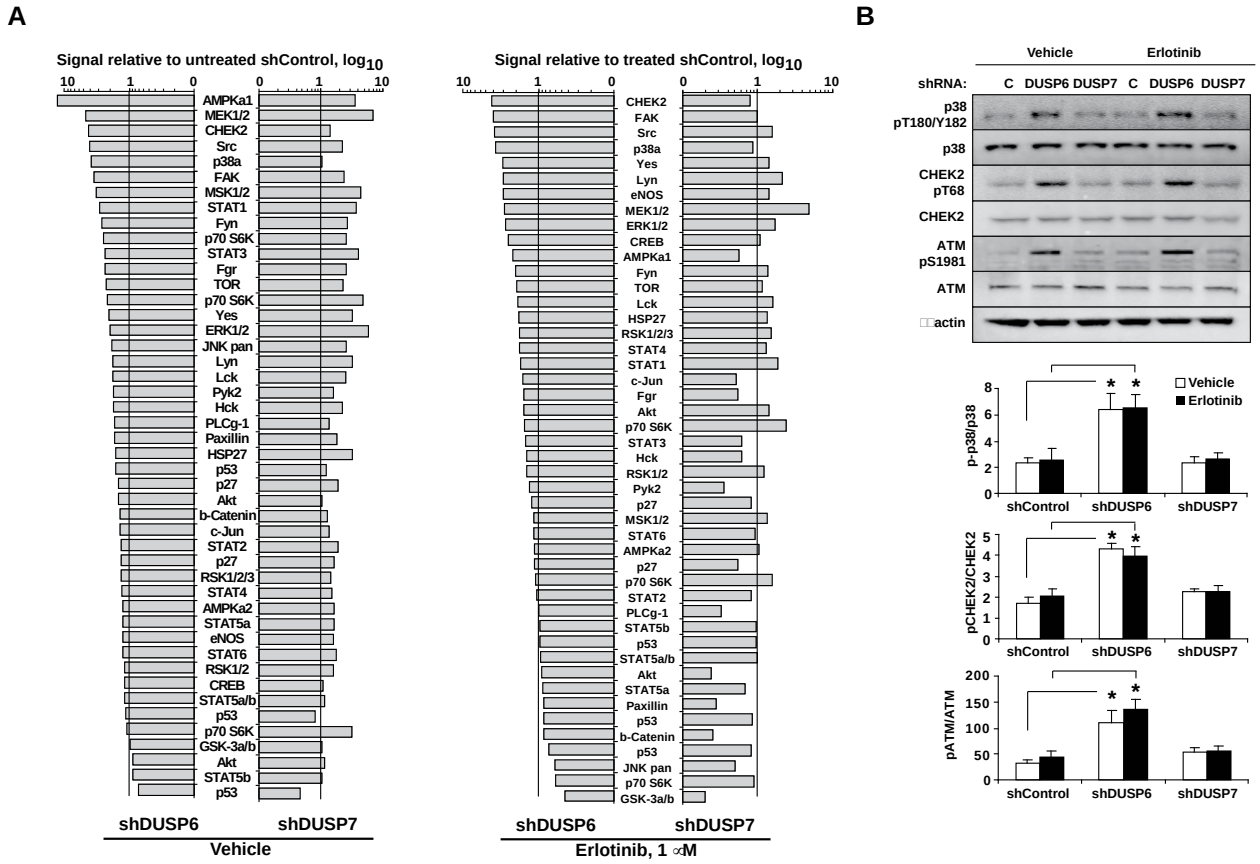

Figure 5. Effects of DUSP6 depletion by phosphoproteomic analysis. (A) Averaged results of reverse phase antibody array assessment of 46 signaling proteins in A431 cells stably expressing shDUSP6, shDUSP7, or non-silencing shRNA control. Cells were grown in 1%FBS-DMEM and treated with vehicle (left panel) or 1 µM erlotinib (right panel) for 3 hours. The data are relative increase in phosphorylation signal. **(B)** Western blot analysis of phosphorylation of p38, CHEK2, and their upstream effector ATM. Top, a representative image; bottom, summary of results quantified from the top. Bars represent averaged results of three independent experiments, mean ± S.E.M. DUSP6-depleted cells versus to non-silencing control at the corresponding drug concentrations; *, p <0.05, Wilcoxon's test.

In **Aim #3**, we attempted to correlate expression of DUSP6 with head and neck cancers responsiveness to EGFR targeting or chemotherapy agents with specific activity in G2/M phase of the cell cycle (inhibitors of topoisomerase I and II, cisplatin, doxorubicin, or ionizing radiation). However, <u>our extensive attempts to validate available anti-DUSP6 antibody reagents did not produce satisfactory results</u>.

Contrastingly, DUSP6 is commonly deleted or epigenetically silenced in pancreatic adenocarcinoma (PDAC). This indicates the centrality of KRAS-MAPK-DUSP6 signaling axis in PDAC. Therefore, we chose to focus on studies of pancreatic ductal adenocarcinoma (PDAC) to probe into the mechanism that could lead to dismantling of this signaling axis. In further studies, we focused on: 1) development of a panel of patient-derived xenograft models (PDX);

and 2) discovery of new drug targets to deregulate KRAS-MAPK signaling in PDAC.

Following our successful publication on DUSP6, we made the next logical step into the investigation of therapeutic targets in pancreatic cancer. Our focus on this type of tumor is two-fold: 1) the centrality of KRAS-MAPK-MYC signaling axis is further supported by the loss of DUSP6 in these tumors; 2) the grave nature of pancreatic cancer needs urgently novel therapy strategies, one of which we believe should be based on the disruption of the critical MAPK pathway.

Our preliminary results for this new direction of our studies are as follows:

1. Small molecule screen for inhibitors of primary PDAC cells. Our goal was to identify the most effective inhibitors of PDAC tumor growth. To avoid artifacts often associated with use of cell lines cultured for many passages on plastic, we adapted an innovative, recently described approach to screen primary pancreatic tumor cells. Briefly, PDAC surgical samples are dispersed to individual cells, treated with Rho inhibitors, and cultured for five to six passages on a fibroblast feeder system; this results in many features of in vivo tumors. In collaboration with Dr. Louis M. Weiner (Lombardi Cancer Center, Washington, DC), we conducted six parallel chemosensitivity screens of PDAC cells from six independent patients, using a focused library of 867 clinically relevant drugs (the NCI Clinical Collection and the FDA-approved drug set). This screen assessed tumor cell viability at six drug concentrations ranging from 16 nM-10 μM. Ten of the top 16 hits inhibit the transcriptional machinery. Of these, only triptolide (consistently one of the most potent hits) has been previously considered for PDAC therapy. Other transcriptional inhibitors included actinomycin D, epirubicin (and other anthracycline antibiotics) and plicamycin.

2. Selective activity of triptolide in MYC-amplified tumors. We have developed and genotyped a set of patient-derived xenografts (PDXs) from pancreatic patients. Most standard therapies used for treatment of pancreatic cancer patients produce little or no effect in this system (Fig. 6), mimicking their poor performance in the clinic. In subsequent validation of hits from the primary screen for PDAC inhibitors, we noted that whereas two MYC-amplified cell lines grew as robustly as two non-MYC-amplified cell lines in the absence of drug treatment (Fig. 6B), there was a dramatic difference in their sensitivity to triptolide (Fig. 6). The two MYC-amplified models (PNX001 and YT037) showed <u>complete regression</u> by end of a 21-day treatment period, and mice remained tumor-free for at least 20 more days and exhibited no signs of toxicity, while the two lines lacking MYC amplification showed only transient response.

We predicted that MYC stability is one of the key determinants of activity of triptolide against PDAC. This would also predict that stabilized MYC would confer resistance to anti-MYC drugs. Activity of KRAS-ERK has been shown to regulate MYC half-life via phosphorylation. This may offer the opportunity to further deregulate KRAS-ERK-MYC axis via a combined targeting of MYC transcription and KRAS-ERK pathway signaling. We are going to test these possibilities in vitro and in vivo on our established platform of PDAC PDX models.

In sum, our results suggest that: 1) targeting the transcription process can cause rapid regression of some PDAC xenografts; 2) MYC is an indirect secondary target of triptolide inhibition of

ERCC3, and MYC inhibition can reciprocally cause loss of ERCC3; 3) stabilized phosphorylated MYC protein may be associated with resistance to transcriptional inhibitors; and hence, combined targeting of transcription and ERK pathway signaling may be synergistic in PDAC, particularly in the context of MYC-amplified, MYC-dependent tumors. Given that MYC is amplified in as many as 30% of PDACs, our proposed study could be of value to a significant subset of patients with PDAC, who would be highly responsive to triptolide used as a lower dose monotherapy or in conjunction with signaling inhibitors.

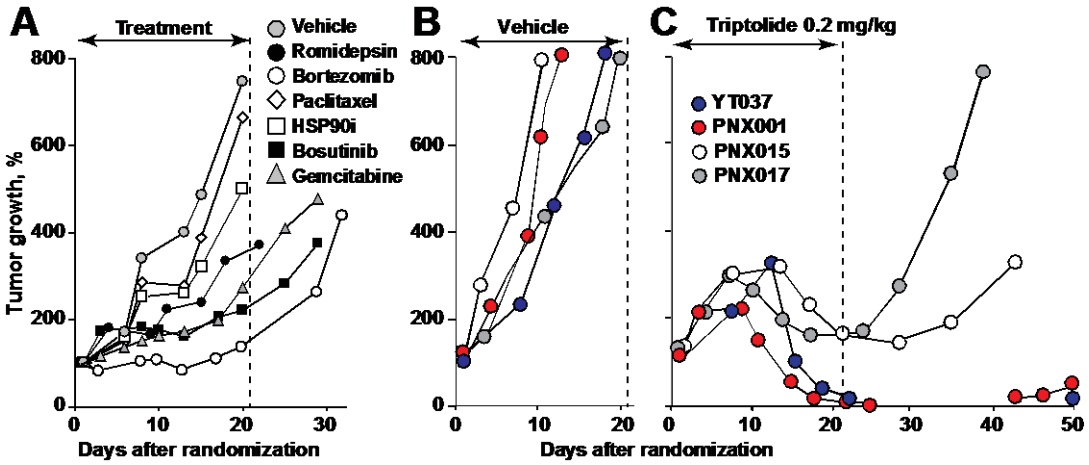

Figure 6. (A) 21-day therapy of PNX001 "xenopatient" shows limited efficacy of FDA-approved drugs. **(B)** Robust growth of control PDX tumors in vivo (vehicle) vs. **(C)** complete regression of MYC-amplified PDX models PNX001 and YT037 after 21-day treatment with triptolide. YT037 (blue circles) and PNX001 (red circles), n=5 mice, 10 tumors in each group] MYC-normal tumors showed only transient shrinkage. Lines are averaged volumes per group.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. DUSP6 regulates drug sensitivity by modulating DNA damage response.	Bagnyukova TV, Restifo D, Beeharry N, Gabitova L, Li T, Serebriiskii IG, Golemis EA, Astsaturov I.	British Journal of Cancer	February 2013	☐Submitted ☐Accepted ☒Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

We are in the process of submitting a paper on patient-derived xenograft testing of novel targets for the treatment of pancreatic cancer.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Our results provide novel insight into DUSP6 function in regulating genomic integrity and sensitivity to chemotherapy in cancer.

Background. Dual specificity phosphatase 6 (DUSP6) is a member of a family of mitogen-activated protein kinase (MAPK) phosphatases that dephosphorylates and inhibits activated ERK1/2. DUSP6 is dynamically regulated in development and in pathological conditions such as cancer.
Methods. Cancer cell lines were made deficient in DUSP6 by siRNA and shRNA silencing.

Sensitivity to anti-EGFR and chemotherapy agents was determined in viability and apoptosis assays and in xenografts established in SCID mice. Cellular effects of DUSP6 inactivation were analyzed by proteomics methods, and by markers of DNA damage response and cell cycle analyses.
Results. We determined that depletion of DUSP6 reduced viability of cancer cell lines and increased cytotoxicity of EGFR inhibitors in vitro and in vivo. These effects were also observed with other targeted and cytotoxic agents, implying broader activity. Subsequent phosphoproteomic analysis indicated DUSP6 depletion significantly activated CHEK2 and p38, which function in the DNA damage response (DDR) pathway. Pursuing this suggestion, we now establish that depletion of DUSP6 elevates levels of phosphorylated H2AX, ATM, and CHEK2, for the first time identifying a role for this protein in regulating DDR.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Igor Astsaturov, M.D., Ph.D.	Assistant Professor

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training and residency training if applicable.*)

INSTITUTION AND LOCATION	DEGREE	MM/YY	FIELD OF STUDY
I.M. Sechenov's Medical Academy, Moscow, Russia	M.D.	11/92	Medicine
Russian Hematological Research Center, Moscow, Russia	Residency	1992-1994	Internal Medicine/Hematology
Russian Hematological Research Center, Moscow, Russia	Ph.D.	12/97	Hematology/Oncology

A. Personal Statement

As a translational investigator, my long-term goal is to develop new therapeutic targets for cancer. My lab has developed expertise in Personalized Oncology based on derivation of tumor explants and cell lines directly from patients. Using systems biology integration of genomic data, we are poised to discover new synergistic drug combinations and drug targets in pancreatic as well as other cancers (including renal cell carcinoma). As a translational investigator, I am actively developing novel cancer treatment concepts experimentally and take these directly to early phase concept-testing clinical trials.

B. Positions and Honors

Positions and Employment

Postdoctoral Research Fellow, University of Toronto, Ontario, Canada	1998-2002
Internship and Residency, Internal Medicine, Good Samaritan Hospital, Baltimore, MD	2002-2005
Medical Oncology Fellow, Fox Chase Cancer Center, Philadelphia, PA	2005-2008
Assistant Member (Instructor), Fox Chase Cancer Center, Philadelphia, PA	2008-2009
Assistant Professor, Fox Chase Cancer Center, Philadelphia, PA	2009-date

Other Experience and Professional Memberships

University Clinic of Cologne, Germany	1994
The Royal Marsden Hospital, London, UK	1995
Johns Hopkins University, Baltimore, MD	2003
Leukemia Service, The Sidney Kimmel Cancer Center at Johns Hopkins University	2003
American Association for Cancer Research, Associate Member #125499	2005-date
American Society of Hematology. Associate Member	2005-date
American Society of Clinical Oncology Associate Member #70593	2005-date

Certification/Licensure

Unrestricted Medical License MD427094, Pennsylvania State Medical Board	2005
American Board of Internal Medicine Certification	2005-2015

Awards and Honors

MD Diploma with Honors, I.M. Sechenov's Medical Academy, Moscow, Russia	1985-1992
Training Award (ICRETT), International Union Against Cancer, Geneva, Switzerland	1995
George Soros International Science Educational Program Award, Moscow, Russia	1996-1997
Research Training Award, the Hospital for Sick Children, Toronto, Canada	2000-2001
AACR-OSI Scholar-in-Training Award	2007
Sol Sherry Research Award, Temple University, Philadelphia, PA	2007
Fox Chase-Genentech Scholar	2008-2010

C. Selected Peer-Reviewed Publications (in chronological order)

1. Cohen SJ, Konski AA, Putnam S, Ball DS, Meyer JE, Yu JQ, **Astsaturov I**, Marlow C, Dickens A, Cade DN, Meropol NJ. Phase I study of capecitabine combined with radioembolization using yttrium-90 resin microspheres (SIR-Spheres) in patients with advanced cancer. Br J Cancer. 2014 Jul 15;111(2):265-71.
2. Beeharry N, Banina E, Hittle J, Skobeleva N, Khazak V, Deacon S, Andrake M, Egleston BL, Peterson JR, **Astsaturov I**, Yen TJ. Re-purposing clinical kinase inhibitors to enhance chemosensitivity by overriding checkpoints. Cell Cycle. 2014 Jun 23;13(14).
3. Perkins J, Boland P, Cohen SJ, Olszanski AJ, Zhou Y, Engstrom P, **Astsaturov I**. Successful imatinib therapy for neuroendocrine carcinoma with activating Kit mutation: a case study. J Natl Compr Canc Netw. 2014 Jun;12(6):847-52.
4. Gabitova L, Gorin A, **Astsaturov I**. Molecular pathways: sterols and receptor signaling in cancer. Clin Cancer Res. 2014 Jan 1;20(1):28-34.
5. Bagnyukova T, Restifo D, Beeharry N, Gabitova L, Li T, Serebriiskii I, Golemis E, and **Astsaturov I**. DUSP6 regulates drug sensitivity by modulating DNA damage response. Br J Cancer 2013, Aug 20;109(4):1063-71.
6. Mehra R, Serebriiskii IG, Burtness B, **Astsaturov I**, Golemis E. Aurora kinases in head and neck cancer. Lancet Oncology, 2013, Sep;14(10):e425-35.
7. Sukhanova A, Gorin A, Serebriiskii IG, Gabitova L, Zheng H, Restifo D, Egleston, B.L., Cunningham, D., Bagnyukova, T., Liu, H., Nikonova, A., Adams, G.P., Zhou, Y., Yang, D.-H., Mehra, R., Burtness, B., Cai, K.Q., Klein-Szanto, A., Kratz, L.E., Kelley, R.I., Weiner, L.M., Herman, G.E., Golemis, E.A., **Astsaturov, I**. Targeting C4-demethylating genes in the cholesterol pathway sensitizes cancer cells to EGFR inhibitors via increased EGFR degradation. Cancer Discovery 2013, Jan;3(1):96-111.
8. Nikonova A.S., **Astsaturov, I.**, Serebriiskii, I.G., Dunbrack, R.L. Jr, Golemis, E.A. Aurora A kinase (AURKA) in normal and pathological cell division. Cell Mol Life Sci. 2012.
9. Gorin, A., Gabitova, L., **Astsaturov, I**. Regulation of cholesterol biosynthesis and cancer signaling. Curr Opin Pharmacol. 2012, Dec;12(6):710-6.
10. Dotan, E., Meropol, N.J., Zhu, F., Zambito, F., Bove, B., Cai, K.Q., Godwin, A.K., Golemis E.A., **Astsaturov, I.**, Cohen, S.J. Relationship of increased aurora kinase A gene copy number, prognosis and response to chemotherapy in patients with metastatic colorectal cancer. Br J Cancer. 2012; 106(4):748-55.
11. Chun YS, Cooper HS, Cohen SJ, Konski A, Burtness B, Denlinger CS, **Astsaturov I**, Hall MJ, Hoffman JP Significance of Pathologic Response to Preoperative Therapy in Pancreatic Cancer. Ann Surg Oncol. 2011 Dec;18(13):3601-7.
12. Wang S, **Astsaturov IA**, Bingham CA, McCarthy KM, von Mehren M, Xu W, Alpaugh RK, Tang Y, Littlefield BA, Hawkins LD, Ishizaka ST, Weiner LM.. Effective antibody therapy induces host-protective antitumor immunity that is augmented by TLR4 agonist treatment. Cancer Immunol Immunother. 2011 Aug 13.
13. **Astsaturov, I.A.**, Ratushny, V., Sukhanova, A., Skobeleva, N., Einarson, M.B., Tikhmyanova, N., Bagnyukova, T., Zhou, Y., Devarajan, K., Silverman, J.S., Sharma, C., Nasto, R.C., Jablonski, S.A., Serebriiskii, I.G., Weiner, L.M., Golemis, E.A. Synthetic lethal screen of an EGFR-centered network to improve targeted therapies. Sci. Signal. 3(140):ra67, 2010.