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14. ABSTRACT We investigated the role of the Discoidin Domain Receptor 1 (DDR1), a collagen receptor kinase, in Pancreatic Ductal Adenocarcinoma (PDAC). Hypothesis: DDR1 mediates the pro-malignant effects of collagen in PDAC progression. DDR1 mediates the crosstalk between mutant Kras "addicted" PDAC cells with collagen and activate signaling pathways that promote tumor cell survival and malignancy (MEK resistance). Aims: 1. Evaluate the Role of DDRs in Resistance to MEK Inhibition and their Effectiveness as Potential Therapeutic Targets in the KPC Mouse Model and 2. Establish the Anti-Tumor Effect of Single or Combinatorial Lethality of DDR1 Inhibition on Human PDX and Matched Organoid Cultures. Major Findings: We characterized DDR expression in multiple PDAC cell lines, with the majority displaying DDR1 but not DDR2 expression. DDR1 inhibition had no impact on cell proliferation or survival in the presence of soluble collagen I in PDAC cell lines with mutated Ras. However, inhibition was observed in PDAC cells with wild type Ras. In 3D collagen I gels in vitro, DDR1 expression significantly inhibited cell proliferation. Sensitivity to MEK inhibition was not correlated with levels of DDR expression in human PDAC cell lines. DDR1 inhibition increased the sensitivity of PDAC cell lines to Trametinib inhibition. DDR1b expression increased gemcitabine sensitivity in MiaPaCa-2 cells cultured in 3D collagen I gels. DDR1 is critical for tumor development and progression in the KPC mouse model of PDAC because DDR1 deficiency restricts tumor growth and metastases. DDR1b significantly promoted tumor growth of human MiaPaCa-2 cells when implanted within collagen 1 in mice. DDR1b expression and activation in these tumors is associated with activation of Src/PDPK1/AKT activity.

15. SUBJECT TERMS
Pancreatic cancer, discoidin domain receptors, collagen, drug resistance, tumor growth

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1. INTRODUCTION

Pancreatic Ductal Adenocarcinoma (PDAC) is the ninth most common cancer but its dismal survival rate elevates it to the third leading cause of cancer-related death in the United States, and is on track to become the second most common cause of cancer related death before 2020. Chemotherapy and radiation therapy have little impact on PDAC, leaving surgery as the most effective treatment. Unfortunately, only ~20% of patients that undergo surgery survive past five years.

One of the hallmarks of PDAC is the intense pro-fibrotic response (collagen deposition). The extensive fibrosis in PDAC occupies a large area of the tumor mass, and is characterized by a dense matrix rich in interstitial collagen, hyaluronic acid, high number of stromal cells, hypovascularity, and hypoxic conditions, all of which have been postulated to contribute to the aggressive nature of PDAC tumors and their resistance to cytotoxic therapies. Collagen, the major component of fibrotic stroma, has been shown to elicit some of the pro-malignant effects of the desmoplastic stroma on the PDAC cells, including migration, invasion, survival, and drug resistance via regulation of epithelial-to-mesenchymal transition (EMT), protease production, and activation of TGF-β signaling, just to mention a few. These data point to an important pro-malignant effect of collagens in PDAC that may facilitate disease progression and resistance to treatment. Consistent with this notion, recent studies have postulated that therapies targeting the PDAC stroma or the crosstalk between PDAC cells and the collagen matrix may represent promising approaches for the treatment of PDAC.

The Discoidin Domain Receptors (DDRs) are unique RTKs because they are the only kinase receptors that recognize collagens as their ligands. Upon collagen binding, DDRs activate signaling pathways that regulate cell proliferation, migration, survival, and differentiation. Importantly, DDRs are emerging as new players in cancer progression because they mediate the interactions of tumor cells with their immediate collagen environment. The DDR family comprises two distinct members, DDR1 and DDR2, which undergo receptor autophosphorylation in response to fibrillar collagens (DDR1 and DDR2) or non-fibrillar collagen IV (DDR1), with distinctive activation kinetics and downstream effectors. DDRs regulate tumor cell migration and invasion, cell survival, and drug resistance. DDRs have been implicated in drug resistance to targeted therapies, and thus targeting DDRs may aid to increase drug sensitivity. Thus, DDRs are good candidates for mediating the pro-survival effects of the PDAC fibrotic stroma.

New therapeutic approaches are urgently required to target the pro-oncogenic signaling networks activated in PDAC. In excess of 90% of PDAC harbor oncogenic Kras mutations that drive tumorigenesis and disease progression. Mutant Kras signals via the downstream components the RAS/RAF/MEK/ERK pathway, and major efforts have been invested to target Kras and its effectors. Direct inhibition of Kras has proven to be challenging. Thus, most approaches target downstream effectors such as MEK1/2 for the treatment of Kras-ERK driven PDAC. However, single agents targeting Ras and/or RTK downstream effectors have yielded disappointing results. In the case of MEK inhibitors, feedback reactivation of ERK or PI3K signaling was shown to be mediated in part by compensatory RTK activation pathways (kinome reprograming) leading to MEK inhibitor resistance.

Kinome reprograming is a process in which cancer cells can rewire their signaling networks to restore ERK activity and override the actions of MEK inhibition by reactivating MEK2, resulting in c-myc degradation, and transcriptional activation of several RTKs. Importantly, studies in breast cancer cells showed that DDR1 is one of the RTKs that appears to compensate for the inhibition of MEK1/2 in AZD6244-resistant triple negative breast cancer cells. Consistently, downregulation of DDR1 restored MEK inhibitor sensitivity. As we described in our application, we searched for resistance to AZD6244, a MEK1/2 inhibitor, in several human PDAC cell lines harboring mutant Kras using the COSMIC database (http://cancer.sanger.ac.uk/cosmic), and compared the extent of MEK inhibitor resistance in the cells in relation to the expression levels of DDR1. Interestingly, we observed a direct relationship between higher levels of DDR1 and resistance to AZD6244, suggesting that DDR1 expression is associated with MEK inhibitor resistance. MiaPaCa-2 cells, a mesenchymal PDAC cell line, which only express DDR2, is the most sensitive to AZD6244. Based on this preliminary, yet interesting association, and the potential role of DDR1 in kinome reprograming we hypothesized that DDR1, but not DDR2, expression may be part of the genomic make up of Kras-mutated PDAC tumors displaying greater MEK inhibitor resistance. Thus, a combination of DDR1 and MEK inhibition may produce synthetic lethality in MEK-dependent mutated Kras-driven PDAC tumors thriving within the collagen-rich environment.

During the funding period of this award, we examined the role of DDR1 in regulating PDA sensitivity to MEK inhibition under various conditions. We have also characterized further the role of DDR1 in mediating collagen-initiated signaling utilizing tumor extracts from xenografts of human PDA cells implanted within a collagen 1 (COL1) scaffold. Together, these analyses revealed unique pathways regulated by DDR1/COL1 interactions that could potentially impact PDAC survival and drug resistance.

2. KEYWORDS

Discoidin domain receptors, pancreatic cancer, receptor tyrosine kinases, collagen, chemotherapy, drug resistance, MEK inhibitors, kinome reprograming

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Specific Aim 1: Evaluate the Role of DDRs in Resistance to MEK Inhibition and their Effectiveness as Potential Therapeutic Targets in the KPC Mouse Model.

Major Task 1: Evaluate roles of DDRs in KPC cell lines in *in vitro* studies

Major Task 2: Evaluate role of DDRs in KPC cell lines in the orthotopic syngeneic mouse model

Major Task 3: Evaluate the therapeutic effect of a pan-DDR kinase inhibitor (Compound A) in the KPC model of pancreatic cancer

Specific Aim 2: Establish the Anti-Tumor Effect of Single or Combinatorial Lethality of DDR1 Inhibition on Human PDX and Matched Organoid Cultures.

> **Major Task 4:** Evaluate Compound A in Organoids from Primary Tumor Lines Derived from PDAC Patients (PDX, currently in hand)
>
> **Major Task 5:** Evaluate the Therapeutic Response of human PDX to DDR plus MEK Inhibition

- **What was accomplished under these goals?**

1) **Major activities:**

NOTE: All the activities described here were conducted from the start of the award in August 2018 to July 31, 2021, which includes the Non-Cost Extension period from August 1, 2020, to July 31, 2021.

a. Analyses of DDR1 expression in human and mouse PDAC cell lines
b. Generation of human PDAC cell lines with upregulated or downregulated DDR1 expression
c. Analyses of DDR1 function in PDAC cell lines: cell proliferation, survival, migration
d. Analyses of DDR expression in KPC tumors in mice
e. Impact of DDR1 on Trametinib (MEK inhibitor) and Gemcitabine sensitivity in human PDAC cell lines
f. Analyses of DDR1 expression and activation in MiaPaCa2 xenografts grown in the presence of collagen type I.
g. Analyses of signaling pathways in the MiaPaCa-2 xenografts
h. Analyses of DDR1 impact on the KPC tumor model of PDAC progression

2) **Specific objectives:**

a. To investigate the effects of DDR1 on PDAC malignant behavior in cultured cells. We studied the impact of DDR1 and collagen on PDAC sensitivity to Trametinib or Gemcitabine in combination with DDR1 inhibitors on the proliferation and survival of PDAC cell lines. Examine effects of DDR1 activation and signaling (MAPK and PI3K/AKT pathways).

b. Examine the role of DDR1 in PDAC progression in the KPC model of PDAC. Characterize the pathological consequences of DDR1 ablation on PDAC growth and metastasis. Determine effects on the tumor microenvironment, particularly on the development of fibrosis, as determined by collagen levels and organization.

c. Determine the impact of DDR1 on the growth of MiaPaCa-2 cells when embedded within a collagen matrix.

d. Unveil signaling pathways activated in MiaPaCa-2 xenografts expressing DDR1b.

3) Significant results or key outcomes:

Major Task 1:

To test the role of DDR1 in regulation of MEK inhibitor sensitivity, we examined DDR1 expression in selected human PDAC cell lines (**Figure 1**). MiaPaCa-2 and PANC1 cells, which are reported to be sensitive to the MEK inhibitor AZD6244, express low levels of DDR1. In contrast, resistant cell lines usually express high levels of DDR1. To examine the role of DDR1 in MEK inhibitor response, we decided to utilize MiaPaCa-2 and PANC-1 with expression of recombinant DDR1. Thus, we generated human MiaPaCa-2 and PANC-1 cells overexpressing DDR1.

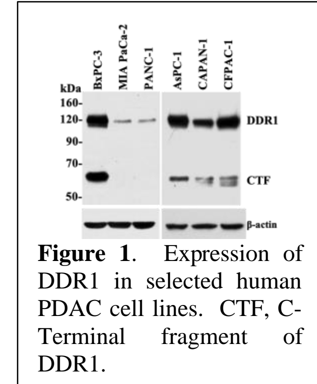

Figure 1. Expression of DDR1 in selected human PDAC cell lines. CTF, C-Terminal fragment of DDR1.

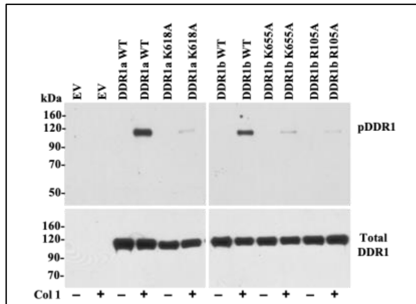

Figure 2. Expression and activation of DDR1 (DDR1a or DDR1b) wild type (WT) and mutants in human MiaPaCa-2 cells. EV, empty vector control cells. pDDR1: phosphorylated DDR1.

Figure 2 shows the expression and activation of two DDR1 isoforms, DDR1a and DDR1b, in the human MiaPaCa-2 cell line. These stable transfectants were generated to express wild type (WT) DDR1a or DDR1b or DDR1 mutants. Specifically, the K618A (DDR1a) and K655A (DDR1b) mutants, which are unable to display kinase activity (referred to as kinase dead, KD), and the mutant R105A, which dampens binding to collagen, resulting in lack of ligand-stimulated activation.

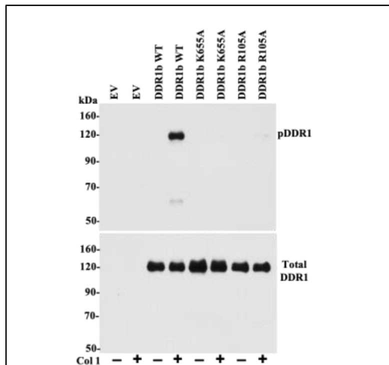

Figure 3. Expression and activation of DDR1b wild type (WT) and mutants in human PANC-1 cells. EV, empty vector control cells. pDDR1: phosphorylated DDR1.

Figure 3 shows the expression and activation of DDR1b, WT and K655A, and R105A mutants in human PANC-1 cells. As shown in **Figures 2 and 3**, WT DDR1 isoforms were activated in response to collagen I (COL1), a ligand for DDR1. In contrast, the K618A, K655A, and R105A mutants were not responsive, as expected. These figures also show that the level of total recombinant DDR1 expression in the transfectants is comparable.

Next, we tested the response of the human PDAC cell lines to MEK inhibition. To this end, we used Trametinib (Selleck Chemicals Cat. #: S2673), an FDA approved MEK inhibitor. First, we verified that the inhibitor inhibits constitutive ERK phosphorylation in MiaPaCa and ASPC-1 cells. These studies showed complete ERK1/2 phosphorylation by Trametinib, as expected (**Figure 4**).

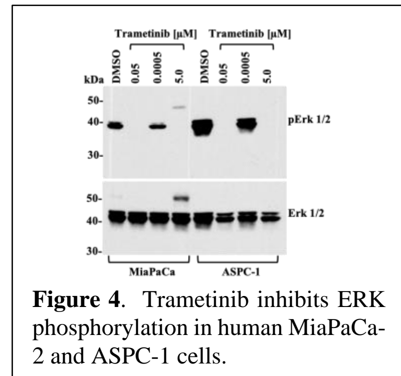

Figure 4. Trametinib inhibits ERK phosphorylation in human MiaPaCa-2 and ASPC-1 cells.

Next, we determined the sensitivity of ASPC-1, BXPC-3, CFPAC-1 and MiaPaCa-2 cell lines to Trametinib. With the exception of MiaPaCa-2 cells, the other cell lines express high levels of DDR1 (Fig. 1). The cells were incubated for 72 h with various doses of Trametinib in complete media and then analyzed with the MTT assay. No collagen was added in this experiment. As shown in **Figure 5**, CPFAC-1 cells were highly resistant to Trametinib with an IC50 of 149 nM. In contrast, ASPC-1, BXPC-3, and MiaPaCa-2 cells displayed a comparable IC50: ~5-10 nM. From these preliminary results we concluded that sensitivity to Trametinib is not directly related to the levels of DDR1 expression in the absence of collagen stimulation.

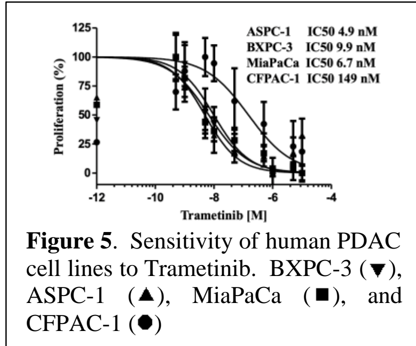

Figure 5. Sensitivity of human PDAC cell lines to Trametinib. BXPC-3 (▼), ASPC-1 (▲), MiaPaCa (■), and CFPAC-1 (●)

Because CFPAC-1 cells express DDR1 and are highly resistant to Trametinib when compared to the other tested PDAC cell lines, we utilized two DDR1 silencing shRNAs (shDDR1 44b and 45b) to downregulate DDR1 in these cells. These stable cell lines with control non-silencing shRNA or shDDR1 were treated with various Trametinib concentrations in the presence of collagen I to activate the receptor. As shown in **Figure 6**, DDR1 downregulation with two different shRNA constructs reduced the IC50 of Trametinib by 5-7-fold compared to cells with the non-silencing shRNA, suggesting <u>that expression of DDR1 is associated with increased resistance to Trametinib, at least in CFPAC-1.</u>

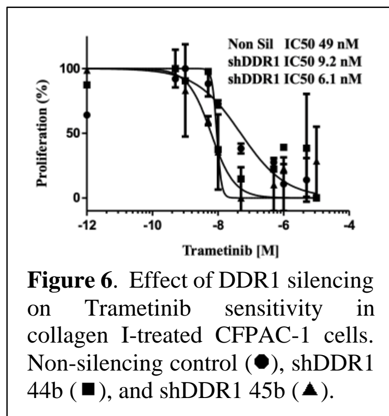

Figure 6. Effect of DDR1 silencing on Trametinib sensitivity in collagen I-treated CFPAC-1 cells. Non-silencing control (●), shDDR1 44b (■), and shDDR1 45b (▲).

Based on our results with CPFAC-1 cells, which under our conditions exhibit the most resistance phenotype to Trametinib (IC_{50} = 149 nM), we decided to use these cells to test whether the selective DDR1 inhibitor RO8807 in combination with Trametinib will reduce the resistance of CFPAC cells to MEK inhibition. R08807 is a potent DDR1 inhibitor (binding IC_{50} = 0.026 μM, phosphorylation IC50 = 0.018 μM) developed by Roche and provided to our lab via a Material Transfer Agreement. In contrast, R08807 displays a binding IC50 = 2.3 μM for human DDR2 (Roche data). As shown in **Figure 7**, using MiaPaCa-2 cells overexpressing recombinant human DDR1b (these cells are DDR1 deficient), R08807 inhibits the activation of DDR1b in response to collagen I with an IC_{50} between 0.05-0.1 μM, under our conditions. R08807 was also effective inhibiting the phosphorylation of endogenous DDR1 after collagen I stimulation in other PDAC cell lines including CFPAC-1, PANC-1 cells (data not shown).

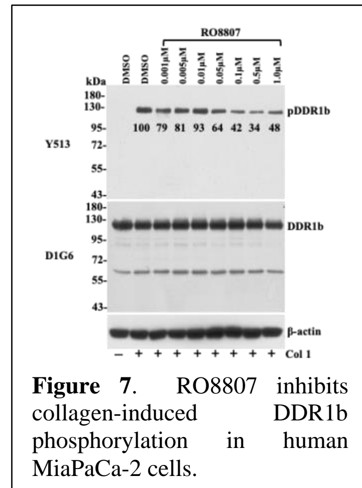

Figure 7. RO8807 inhibits collagen-induced DDR1b phosphorylation in human MiaPaCa-2 cells.

Next, we tested the combination of Trametinib with RO8807 in various human PDAC cell lines expressing endogenous DDR1. ASPC-1 and CFPAC-1 cells display mutated Kras whereas BXPC-3 harbors a wild type Kras. The cells were seeded in 96-well plates in medium supplemented with 2% FBS. The next day, half of the medium was replaced with fresh medium containing 0.1 μM (final concentration) of R08807 in DMSO, alone or in combination with Trametinib (at the IC_{25} concentration foe each cell line, as indicated in the legend of Fig. 8). Each well also received 20 μg per ml of collagen type I to induce DDR1 activation. Control wells received DMSO or 0.02N acetic acid. After 3 days, cell proliferation was evaluated using the XTT assay as described by the manufacturer. As depicted in **Figure 8**, in the absence of collagen, Trametinib alone inhibited the proliferation of ASPC-1 and BXPC-3 cells by 47 and 30% when compared to controls. As expected, CFPAC-1 cells were resistant to Trametinib. R08807 alone had minimal impact on cell proliferation, suggesting that DDR1 inhibition alone is not sufficient to alter the proliferation of these cell lines. The combination of Trametinib and RO8807, in the absence of collagen, did not alter the effects of Trametinib alone.

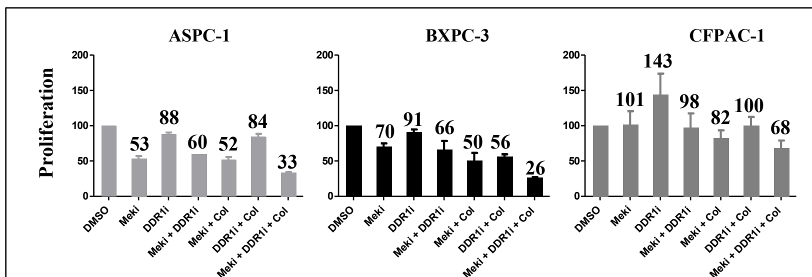

Figure 8. Effect of Trametinib (MEKi) and RO8807 (DDR1i), alone or in combination, on cell proliferation in the presence or absence of soluble collagen I. Trametinib was used at IC_{25} concentrations (determined earlier), as follows: 1.2 nM for ASPC-1, 2.2 nM for BXPC3 and 24 nM for CFPAC-1 cells. RO8807 was used at a concentration of 0.1 μM. Numbers on top of the bars indicate the percentage of cell proliferation relative to the controls (100%), which was evaluated by the XTT assay.

In the presence of collagen type I, sensitivity of Trametinib was not altered in ASPC-1 cells but collagen + Trametinib elicited a stronger effect (~20%) on cell proliferation over Trametinib alone in BXPC-3 and CFPAC-1 cells, suggesting that collagen synergized with Trametinib to inhibit cell proliferation. R0887 inhibited growth of BXPC-3 by ~40% only in the presence of collagen. Thus, in wild type Kras-expressing PDAC cells DDR1 activation is associated with inhibition of cell proliferation. These studies also revealed that the combination of Trametinib and RO8807 increased lethality in the presence of collagen in both ASPC-1 (from 60 to 33%) and BXPC-3 (from 66 to 26%) cells when compared to the combination in the absence of collagen. Thus, taken together these studies suggested that inhibition of activated DDR1 by RO8807 DDR1 inhibitor increased the sensitivity of these cells to Trametinib, and thus a DDR1 inhibitor together with a MEK1 inhibitor may be an effective combination for inhibition of PDAC cell proliferation in cells growing on collagen type I. Consistently, reduced DDR1 expression in CFPAC-1 cells increased resistance to Trametinib (Fig. 6).

We analyzed the impact of Trametinib, RO8807, and another DDR1 inhibitor, DDR1-IN-1, on DDR1 and ERK activation in CFPAC-1 cells stimulated or not with collagen 1. As shown in **Figure 9**, both RO887 and DDR1-IN-1 inhibited collagen-mediated DDR1 activation, as expected. Interestingly, both DDR1 inhibitor increased pERK, with DDR1-IN-1 showing the higher effect, regardless of COL1 stimulation. Trametinib had no impact on DDR1 activation (lane 3 vs 4) but completely abolished ERK phosphorylation. Thus, DDR1 inhibitors appear to

increase pERK, consistent with their lack of effect on cell proliferation (Fig. 8). However, under these conditions, Trametinib dampened pERK regardless of the status of DDR1 activation. Conversely, we did not detect a noticeable effect of Trametinib on the levels of DDR1 activation. Thus, while the combination of Trametinib + RO8807 promoted inhibition in cell proliferation (shown in Fig. 8), this effect was not accompanied by a clear impact on DDR1 activation from that observed without Trametinib. At present, these effects of DDR1 inhibition on ERK phosphorylation remain unclear. However, we are continuing to pursue this area beyond the funding period of this award.

Figure 9. Effect of Trametinib, RO8807, and DDR1-IN-1 alone or in combination, on DDR1 and ERK activation in CFPAC-1 cells the presence or absence of collagen I (COL1). Serum-starved CFPAC-1 cells were incubated with 2 μM each of either RO8807 or DDR1-IN-1. Thirty min later, COL1 (50 μg/ml) were added to selected wells. One hour later, Trametinib (150 nM) or DMSO was added, and the cells were incubated for an additional 1 h. Cells were lysed and the lysates resolved by SDS-PAGE followed by immunoblot analysis for DDR1 and ERK. Number in lanes represent relative levels of ERK or pERK.

We also tested a DDR1-neutralizing monoclonal antibody referred to as Ab 49, a generous gift from Dr. Marco Prunotto (University of Geneva). This mAb effectively blocks DDR1 activation in response to COL1 (data not shown). Therefore, we tested its impact on colony formation (survival) using several human PDAC cell lines expressing endogenous DDR1 (ASPC-1, CAPAN-1 and CFPAC-1). Cells were cultured in COL1-coated in the presence of various concentrations of Ab 49 or IgG1 control. These data showed no significant effect of Ab 49 on colony number when compared to control IgG1 (**Figure 10**, only CFPAC-1 data is shown). A similar lack of effect was observed in ASPC-1 and CAPAN-1 cells (data not shown). Thus, targeting endogenous DDR1 did not alter the survival of PDAC cell lines under the experimental conditions used. These data agreed with the lack of effect of a small molecule tyrosine kinase inhibitor of DDR1 (R0887) on cell proliferation in the presence of COL1 (Fig. 8). Thus, based on these results, we concluded that endogenous DDR1 activation is not critical for proliferation of PDAC cell lines *in vitro* even in the presence of collagen. Yet, overexpression of recombinant DDR1 in PDAC cell lines with very low levels of endogenous DDR1 (MiaPaCa-2, PANC-1, Fig. 1) significantly inhibits cell proliferation when the cells are cultured within a 3D COL1 matrix (**Fig. 11**). We have found that expression of either DDR1b or DDR1a reduced cell proliferation when MiaPaCa-2 cells are cultured in the presence of soluble COL1 (Fig. 11, left panel) or within a 3D COL1 gel (Fig. 11, right panel). This inhibitory effect was dependent on DDR1 kinase activity because a kinase dead receptor (a or b isoform) had no impact on cell proliferation. These studies demonstrated that in culture conditions DDR1 elicits an anti-proliferative effect in the presence of COL1 in MiaPaCa-2 cells that requires receptor activity.

Figure 10. Effect of anti-DDR1 mAb (Ab 49) on colony formation by CPFAC-1 cells. After seeding in 6-well COL1-coated plates, the cells were grown in full media supplemented with Ab 49 or IgG1 (added every other day). After 12 days in culture, the cells were fixed and stained, and colonies were counted under a microscope.

Because the standard of care for the treatment of PDAC patients includes Gemcitabine (GEM), an inhibitor of DNA synthesis, we examined the impact of DDR1b expression in GEM sensitivity. We hypothesized that due to the reduced proliferation of DDR1-expressing PDAC cells in COL1 (Fig. 11) this would result in increased resistance to GEM. To test this hypothesis, we used MiaPaCa-2 cells with or without DDR1b expression, which were cultured within a 3D COL1 gel. Cells ($1x10^4$ cells per well) were mixed with 80 µg of neutralized collagen type I (2 mg/ml final concentration) and seeded in a 96-well plate. After 1h at 37 ˚C, media (DMEM +2% serum) supplemented without or with various concentrations of GEM were added to the wells. After 3 days, cell proliferation was assessed using the XTT assay. Surprisingly, expression of DDR1b in MiaPaCa-2 cells reduced the IC_{50} of Gemcitabine by 1.6-folds (from 0.0146 to 0.0088 µM) when cells were cultured within a 3D COL1 matrix, as shown in **Figure 12**. Thus, under the experimental conditions, DDR1b expression increased Gemcitabine sensitivity in MiaPaCa-2 cells. Under reduced proliferative capacity in response to DDR1 expression, MiaPaCa-2 cells were more sensitive to GEM. Thus, while DDR1 contributed to Trametinib resistance (Fig. 8), it increased sensitivity to GEM. These results suggest that the ability of DDR1 to reduce PDAC proliferation in response to COL1 *in vitro*, predisposes the cells to resist the inhibitory effects of Trametinib. Thus, under conditions that restrict cell growth signals, *i.e.*, DDR1 expression in COL1-rich microenvironments, the efficacy of MEK inhibition is compromised. In contrast, the opposite occurs with GEM, namely DDR1 expression is associated with enhanced sensitivity to GEM. At present, the mechanisms for this differential effect of DDR1 on cytotoxic (GEM) vs. targeted (MEK inhibition) therapies when cells are in contact with COL1 is unclear and requires more studies.

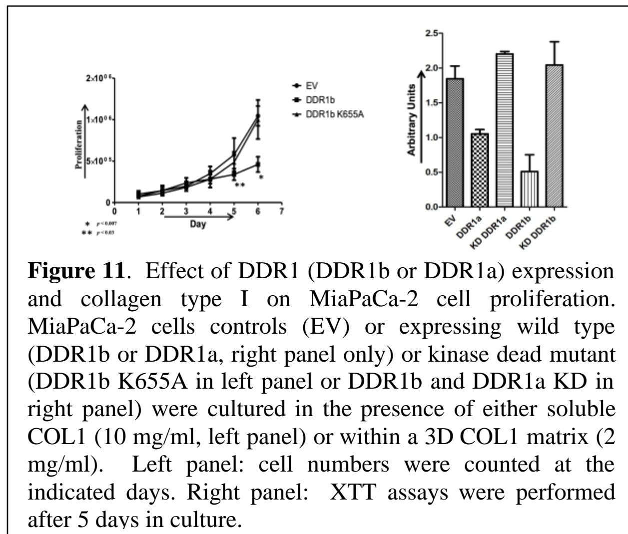

Figure 11. Effect of DDR1 (DDR1b or DDR1a) expression and collagen type I on MiaPaCa-2 cell proliferation. MiaPaCa-2 cells controls (EV) or expressing wild type (DDR1b or DDR1a, right panel only) or kinase dead mutant (DDR1b K655A in left panel or DDR1b and DDR1a KD in right panel) were cultured in the presence of either soluble COL1 (10 mg/ml, left panel) or within a 3D COL1 matrix (2 mg/ml). Left panel: cell numbers were counted at the indicated days. Right panel: XTT assays were performed after 5 days in culture.

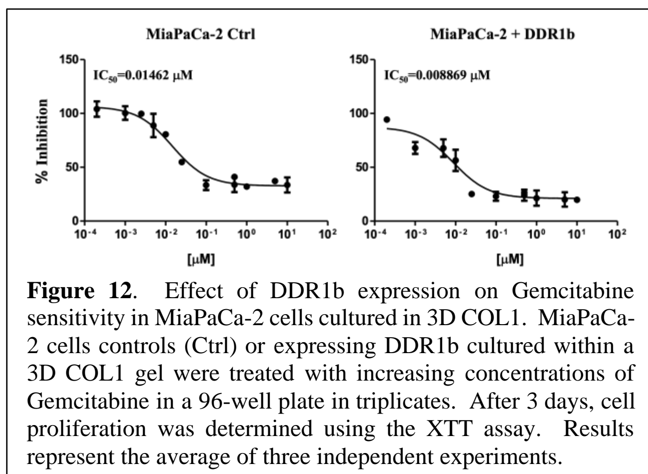

Figure 12. Effect of DDR1b expression on Gemcitabine sensitivity in MiaPaCa-2 cells cultured in 3D COL1. MiaPaCa-2 cells controls (Ctrl) or expressing DDR1b cultured within a 3D COL1 gel were treated with increasing concentrations of Gemcitabine in a 96-well plate in triplicates. After 3 days, cell proliferation was determined using the XTT assay. Results represent the average of three independent experiments.

In summary, these studies produced the following outcomes:

1. DDR1 is the most ubiquitous DDR expressed by human PDAC cell lines in culture, regardless of Ras mutational status.

2. Two PDAC cell lines, MiaPaCa-2 and PANC-1, among those tested, express low levels of

DDR1 and thus were engineered to express recombinant DDR1b or DDR1a, wild type and mutants. Studies were conducted to characterize expression and activation of DDR1 in these cells.

3. We characterized the ability of DDR1 inhibitors, a tyrosine kinase small molecule inhibitor and a neutralizing mAb, to inhibit DDR1 phosphorylation in response to collagen stimulation.

4. The DDR1 inhibitors had no impact on cell proliferation or colony formation in the presence of COL1 in PDAC cell lines with mutated Ras. However, inhibition was observed in BXPC-3 cells (wild type Ras). Thus, Ras mutational status in PDAC cells may impact DDR1 inhibitor sensitivity for cell growth *in vitro*.

5. Expression of DDR1 in DDR1-deficient MiaPaca-2 and PANC-1 cells significantly inhibits cell proliferation in cells cultured within a 3D COL1 matrix.

6. Trametinib, a MEK inhibitor, inhibits the proliferation of PDAC cell lines expressing endogenous DDR1, regardless of DDR1 level.

7. R08807, a DDR1 inhibitor, increased the sensitivity of PDAC cell lines to Trametinib inhibition in the presence of COL1 suggesting that a combination of these inhibitors may increase PDAC lethality.

8. DDR1b expression increased GEM sensitivity in MiaPaCa-2 cells cultured in 3D COL1.

Major Task 2:

At the time we were planning the studies of these Tasks, Dr. Crawford (co-PI in this award) was already crossing DDR1-deficient mice with mice harboring specific K-Ras and P53 mutations in the pancreatic cells (KPC model). KPC mice develop PDAC, which progress to a highly metastatic cancer and thus it resembles the human disease. Thus, these mice provided an opportunity to examine the impact of DDR1 in PDAC progression. Other funds were used for the cost of the animal experiments. The results of these comprehensive studies, supported in part by this award, were published in the ***American Journal of Pathology, Volume 190, Issue 8, August 2020, Pages 1622-1624; PMID: 32339496***. From Ruggieri et al AJP 190, 1622-1624, 2020.

Pancreatic ductal adenocarcinoma (PDA) and chronic pancreatitis are characterized by a dense collagen-rich desmoplastic reaction. Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase activated by collagens that can regulate cell proliferation, migration, adhesion, and remodeling of the extracellular matrix. To address the role of DDR1 in PDA, Ddr1-null ($Ddr1^{-/-}$) mice were crossed with the $Kras^{G12D/+}$; $Trp5^{R172H/+}$; $Ptf1a^{Cre/+}$ (KPC) model of metastatic PDA. $Ddr1^{-/-}$; KPC mice progress to differentiated PDA but resist progression to poorly differentiated cancer compared with KPC control mice. Strikingly, severe pancreatic atrophy accompanied tumor progression in $Ddr1^{-/-}$; KPC mice. To further explore the effects of Ddr1 ablation, Ddr1-/- mice were crossed with the $Kras^{G12D/+}$; $Ptf1a^{Cre/+}$neoplasia model and subjected to cerulein-induced experimental pancreatitis. Similar to KPC mice, tissue atrophy was a hallmark of both neoplasia and pancreatitis models in the absence of Ddr1. Compared with controls, $Ddr1^{-/-}$ models had increased acinar cell dropout and reduced proliferation with no difference in apoptotic cell

death between control and $Ddr1^{-/-}$ animals. In most models, organ atrophy was accompanied by increased fibrillar collagen deposition, suggesting a compensatory response in the absence of this collagen receptor. Overall, these data suggest that DDR1 regulates tissue homeostasis in the neoplastic and injured pancreas.

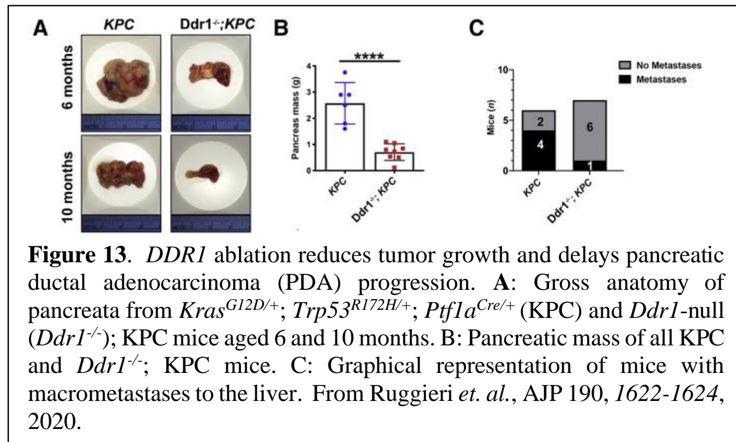

Figure 13. *DDR1* ablation reduces tumor growth and delays pancreatic ductal adenocarcinoma (PDA) progression. **A**: Gross anatomy of pancreata from $Kras^{G12D/+}$; $Trp53^{R172H/+}$; $Ptf1a^{Cre/+}$ (KPC) and *Ddr1*-null ($Ddr1^{-/-}$); KPC mice aged 6 and 10 months. B: Pancreatic mass of all KPC and $Ddr1^{-/-}$; KPC mice. C: Graphical representation of mice with macrometastases to the liver. From Ruggieri *et. al.*, AJP 190, *1622-1624*, 2020.

These studies demonstrated the critical role that DDR1 plays in the progression of PDAC using a relevant mouse model that resembles in many aspects the development and progression of human PDAC. *DDR1* loss significantly reduced the tumor mass and the progression of the tumor to a metastatic phase, as shown by the reduced number of metastases in the liver (**Fig. 13**). Collectively, these data suggested that DDR1 is essential for pancreatic tissue homeostasis and consequently plays a role in support of PDAC progression through the interacts with collagens, possibly by promoting a fibrotic response that is conducive to tumor progression. Altogether, our data indicated that DDR1 is an important signaling factor during pancreatic injury, tumor development, and tumor progression. These analyses were consistent with the scientific goal of Task 2: "Evaluate role of DDRs in the KPC model" but utilizing instead the spontaneous model.

At the time we were examining the impact of DDR1 and Trametinib on the proliferation of human PDAC cell lines (Task1), we were conducting other studies (not supported by this award) to examine the roles of DDRs in PDAC malignancy in mice using collagen gels. Because DDRs are collagen receptors and PDAC thrives within a collagen-rich fibrotic stroma, we decided to test the impact of DDRs on the growth of PDAC cells inoculated with COL1. To this end, we developed a method whereby tumor cells are subcutaneously (s.c.) implanted in mice within a solution of fibrillar COL1 (2 mg/ml). Because COL1 forms a scaffolding gel upon grafting, this approach generates a tumor milieu that partially resembles the rich COL1 environment encountered by tumors cells within fibrotic environments. We chose human MiaPaCa-2 cells, which were engineered to express human DDR1b or DDR2. Using this model, we found that the pro-tumorigenic effects of DDRs are potentiated by the presence of COL1 *in vivo*. This pro-tumorigenic effect of DDRs was discovered by inoculating MiaPaCa-2 cells expressing recombinant DDR1b or DDR2 with or without a solution of COL1 (2 mg/ml), the major collagen type present in PDAC tumors. As depicted in **Figure 14**, these studies showed that DDRs significantly accelerated tumor growth of human MiaPaCa-2 cells only when the cells were co-inoculated with COL1. In contrast, presence of COL1 had no effect on the growth of control

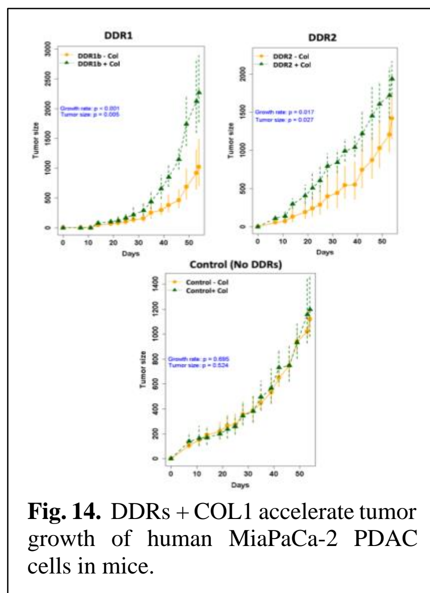

Fig. 14. DDRs + COL1 accelerate tumor growth of human MiaPaCa-2 PDAC cells in mice.

MiaPaCa-2 cells (without recombinant DDR expression) (Fig. 14, lower panel). Thus, presence of COL1 was not sufficient to confer a growth advantage without DDR expression. Hence, only the combination of DDRs and COL1 drives the accelerated growth of human MiaPaCa-2 tumors in mice. Thus, as opposed to the inhibitory effects of DDRs on cell proliferation *in vitro* (within a 3D COL1 matrix), *in vivo* DDRs support the growth of MiaPaCa-2 cells.

The tumors generated in these experiments were harvested and subjected to analyses of DDR expression and activation by immunoblot analyses. We found that tumors expressed and activated the DDRs *in vivo*, as depicted in **Figure 15** (only DDR1b tumors are shown). The tumor tissues were also analyzed for various signaling pathways including the MAPK/PI3K pathway, which demonstrated increased AKT phosphorylation at Thr308, a major activation site phosphorylated by the kinase PDPK1 (discussed below). We also found that DDR1b + COL1 tumors (referring to tumors generated by MiaPaCa-2 cells expressing DDR1b and inoculated with COL1; Mia/DDR1b) display high levels of phosphorylated Src at Y416, when compared to control tumors (no expression of DDR1b + COL1; Mia/EV) (**Figure 16**). These data indicate that DDR1b expression is associated with enhanced Src activity, which, considering its role in oncogenesis, may mediate the pro-tumorigenic effects of the DDR1b/COL1 axis in MiaPaCa-2 cells.

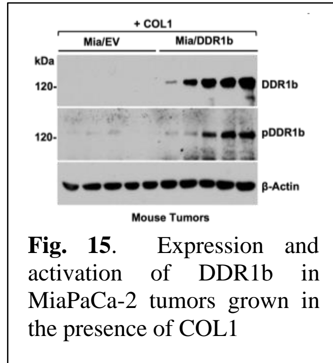

Fig. 15. Expression and activation of DDR1b in MiaPaCa-2 tumors grown in the presence of COL1

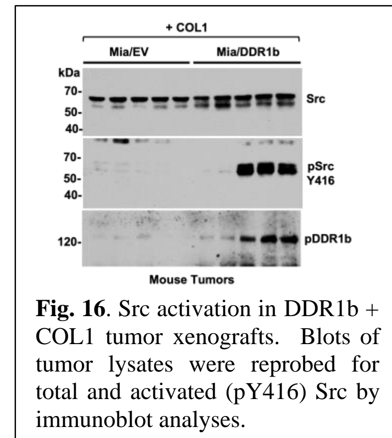

Fig. 16. Src activation in DDR1b + COL1 tumor xenografts. Blots of tumor lysates were reprobed for total and activated (pY416) Src by immunoblot analyses.

Evidence indicate that Src plays a key role in the activation of PDPK1 (also known as PDK1), a serine/threonine kinase belonging to the family of AGC kinases, which is master regulator of key signaling pathways. In collaboration with Dr. Coppe (University of San Francisco), we found that MiaPaCa-2 DDR1b + COL1 tumor extracts display high levels of PDPK1. This collaboration was conducted in part during the non-extension cost period of this award and involved *in vitro* analyses of the MiaPaCa-2 tumors. The data points to a role for a DDR1-Scr-PDPK1 axis in PDAC progression. We are currently testing the expression and role of this axis in PDAC models with the ongoing collaboration with Dr. Howard Crawford (co-PI in this award) and Dr. Coppe. Thus, our data unveiled a new signaling pathway driven by DDR1 and COL1 that promotes the tumorigenicity of MiaPaCa-2 cells *in vivo*. This pathway is now being explored in PDAC tumors.

In summary, these studies produced the following outcomes:

1. Performed analyses of tissues derived from *Ddr1*$^{-/-}$ and *Ddr1*$^{-+/+}$ KPC mice, which demonstrated that DDR1 deficiency in the KPC model of PDAC progression inhibits tumor growth and metastases.

2. The analyses conducted focused on unveiling the characteristics (in tissue sections and extracts) of pancreas derived from *Ddr1*$^{-/-}$ and *Ddr1*$^{-+/+}$ KPC mice. These studies provided mechanistic insight into the ability of DDR1 to support PDAC progression in the KPC model.

3. Conducted analyses of tissues derived from tumors generated by MiaPaCa-2 cells $\pm$ DDR1b + COL1 to determine potential mechanisms.

4. Identified an association between DDR1b expression and activation by COL1 and a Src/PDPK1 activity pathway, which is associated with enhanced tumorigenicity within a COL1-rich matrix.

Major Tasks 3-5: Due to our focus on the *in vitro* studies with the human PDAC cell lines attempting to identify the combination of DDR1 inhibition with MEK inhibition, and the data generated from the xenograft models, these studies were delayed, and eventually could not be completed. See Section 5 for the change in scope.

4) **Other achievements:**

Scientific: We conducted in *vitro* studies analyses of genes regulated by DDR1b in the presence of collagen by performing RNAseq analyses and phosphoproteomics. For instance, we found that DDR1 expression in MiaPaCa-2 tumors is strongly associated with downregulation of several genes encoding for receptor type phosphatases, including PTPRG, PTPRK, and PTPRF. This finding is noteworthy because it is well established that DDR1 displays a unique kinetic of receptor phosphorylation characterized by its slow and sustained activation when stimulated with collagen. This is in striking contrast with other members of the receptor tyrosine kinase (RTK) family, which are rapidly phosphorylated and dephosphorylated upon ligand binding. The reason(s) for the sustained and slow DDR1 activation remains a significant gap in knowledge in the field of RTKs and raises important questions as to the significance and impact of this unique phosphorylation kinetics in DDR1 function. This is an area we are pursuing. Together, the genomic and proteomic analyses are being examined in conjunction with the kinase activity results obtained in collaboration with Dr. Coppe. Thus, we are building a comprehensive network of pathways activated by the DDR1/COL1 axis in PDAC cells. These ongoing analyses are revealing promising targets of DDR1 in pancreatic tumors. We are currently following these results by validating some of the promising targets of DDR1 action. We believe these studies will shed light into the action of DDR1 in PDAC. In another series of studies, we have also identified the impact of COL11, another key collagen from the PDAC stroma which has been shown to promote malignancy, on DDR1 activation. Specifically, we found that COL11 is a potent inducer of DDR1 phosphorylation in PDAC cell lines when compared to COL1. These data suggest that presence of COL11 within the stroma of PDAC may super-activate DDR1, which in turn may elicit pro-malignant activity. These novel finding identifies DDR1 as a potential mediator of the pro-oncogenic signals elicited by COL11. We are currently following up with these findings.

Funding: Some of the data generated with this award was used to obtain funding from the Sky Foundation, a private foundation focusing on pancreatic cancer.

- **What opportunities for training and professional development has the project provided?**

Nothing to report.

- **How were the results disseminated to communities of interest?**

We are preparing a manuscript with the new data generated. We hope to disseminate our results in the cancer field, particularly in the area of tumor microenvironment and PDAC.

- **What do you plan to do during the next reporting period to accomplish the goals?**

This is the final report. However, we are pursuing the findings obtained with this award.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

Our goal is to submit a publication of our studies. We believe these results will provide evidence on the pro-malignant effects of DDR1 in PDAC cells in contact with COL1. Importantly, we demonstrated that DDR1/COL1 can

- **What was the impact on other disciplines?**

Our findings not only are relevant for PDAC but for other tumors known to thrive within collagen-rich matrices including sarcomas, breast cancer, and others. Therefore, our studies may be relevant for other tumor types. They also be relevant for understanding how collagen signals in cells, which is significant in the field of cell biology, biochemistry, and diseases involving pathological regulation of extracellular matrices.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

Nothing to report.

5. CHANGES/PROBLEMS

Major Task 3-5: These studies were delayed and eventually not conducted due to our focus on the studies with the human PDAC cell lines described in Task 1 and the studies of Task 2. We had also some issues with the proposed KPC mouse model of PDAC driven by the Ptf1a-Cre allele to the Pdx1-Cre allele, due to prevalent brain tumor formation in the former model, which delayed progress and thus these studies could not be accomplished within the funding period.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report. This is a Final Report.

- **Changes that had a significant impact on expenditures**

Nothing to report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

- **Significant changes in use or care of human subjects**

Nothing to report.

- **Significant changes in use or care of vertebrate animals.**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. PRODUCTS

- **Publications, conference papers, and presentations**

Ruggeri JM, Franco-Barraza J, Sohail A, Zhang Y, Long D, Pasca di Magliano M, Cukierman E, Fridman R, Crawford HC. Discoidin Domain Receptor 1 (DDR1) is Necessary for Tissue Homeostasis in Pancreatic Injury and Pathogenesis of Pancreatic Ductal Adenocarcinoma. Am J Pathol. 2020 Aug;190(8):1735-1751. PMID: 32339496

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project? See Note below Table**

Name	Project Role	Nearest Person Months Worked	Contribution to the Project	Funding Support
Rafael Fridman	PI	0.24	Design of experiments and data analyses	This grant
Anjum Sohail	Research Scientist	3		This grant
Howard Crawford (University of Michigan)	Co-I	0.24	Design of experiments and data analyses	Subcontract
Daniel Paglia (University of Michigan)	Research Technician	3	Establishment and maintenance of organoid cultures	Subcontract

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

- **What other organizations were involved as partners?**

Organization Name: Hoffmann-La Roche
Location of organization: Basel, Switzerland
Partner's contribution to the project: Supplied antibodies for DDR1 and a small molecule inhibitor for DDR1.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES

Nothing to report.