



# **The Small Heat Shock Protein $\alpha$ A-Crystallin Is Expressed In Pancreas and Acts as Negative Regulator of Carcinogenesis**

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## **► To cite this version:**

Mi Deng, Pei-Chao Chen, Sisi Xie, Junqiong Zhao, Lili Gong, et al.. The Small Heat Shock Protein  $\alpha$ A-Crystallin Is Expressed In Pancreas and Acts as Negative Regulator of Carcinogenesis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 1802 (7-8), pp.621. <10.1016/j.bbadis.2010.04.004>. <hal-00600124>

**HAL Id: hal-00600124**

**<https://hal.science/hal-00600124v1>**

Submitted on 14 Jun 2011

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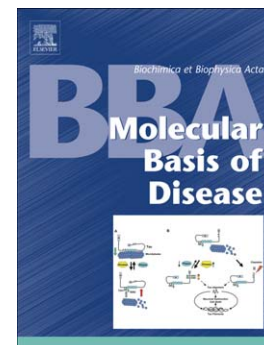
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PII: S0925-4439(10)00083-9  
DOI: doi: [10.1016/j.bbadis.2010.04.004](https://doi.org/10.1016/j.bbadis.2010.04.004)  
Reference: BBADIS 63091

To appear in: *BBA - Molecular Basis of Disease*

Received date: 16 January 2010  
Revised date: 14 April 2010  
Accepted date: 16 April 2010



Please cite this article as: Mi Deng, Pei-Chao Chen, Sisi Xie, Junqiong Zhao, Lili Gong, Jinping Liu, Lan Zhang, Shuming Sun, Jiao Liu, Haili Ma, Surinder Batra, David Wan-Cheng Li, The Small Heat Shock Protein  $\alpha$ A-Crystallin Is Expressed In Pancreas and Acts as Negative Regulator of Carcinogenesis, *BBA - Molecular Basis of Disease* (2010), doi: [10.1016/j.bbadis.2010.04.004](https://doi.org/10.1016/j.bbadis.2010.04.004)

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# The Small Heat Shock Protein AlphaA-Crystallin Is Expressed In Pancreas and Acts as A Negative Regulator of Carcinogenesis

(Short Title:  $\alpha$ A-Crystallin Is Down-Regulated in Pancreatic carcinoma)

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**ABSTRACT**

The small heat shock protein  $\alpha$ A-crystallin is a structural protein in the ocular lens. In addition, recent studies have also revealed that it is a molecular chaperone, an autokinase and a strong anti-apoptotic regulator. Besides its lenticular distribution, a previous study demonstrates that a detectable level of  $\alpha$ A-crystallin is found in other tissues including thymus and spleen. In the present study, we have re-examined the distribution of  $\alpha$ A-crystallin in various normal human and mouse tissues and found that the normal pancreas expresses a moderate level of  $\alpha$ A-crystallin. Moreover,  $\alpha$ A-crystallin is found significantly downregulated in 60 cases of pancreatic carcinoma of different types than it is in 11 normal human pancreas samples. In addition, we demonstrate that  $\alpha$ A-crystallin can enhance activity of the activating protein-1 (AP-1) through modulating the function of the MAP kinase, and also up-regulates components of TGF $\beta$  pathway. Finally, expression of  $\alpha$ A-crystallin in a pancreatic cancer cell line, miapaca, results in retarded cell migration. Together, these results suggest that  $\alpha$ A-crystallin seems to negatively regulate pancreatic carcinogenesis.

Key words: Small heat shock protein,  $\alpha$ A, Pancreas, AP-1, Smad2/3/5, TGF $\beta$   
Pancreatic Cancer, cell migration

## Introduction

Heat-shock proteins (HSPs) are implicated in multiple cellular functions including signaling transduction, protein degradation, survival promotion, exocytosis and endocytosis [1-5]. Recent studies have revealed that HSPs also play an important role in tumorigenesis [6-8]. The lens structural proteins,  $\alpha$ A- and  $\alpha$ B-crystallins are characterized as HSPs [9]. Besides their robust expression in mammalian lens [10-11], the two  $\alpha$ -crystallins are also expressed in non-lenticular tissues.  $\alpha$ B-crystallin is significantly expressed in retina, muscle, heart and brain, and acts to prevent neural degradation, heart fail, but promote carcinogenesis [4-5, 12-15].  $\alpha$ A-crystallin is also expressed in some non-lenticular tissues, such as retina, spleen and thymus [16]. However, the function of non-lenticular  $\alpha$ A-crystallin remains unknown.

Pancreatic cancer in human poses clinical challenges in diagnosis and treatment [17-18]. Among the various types of pancreatic malignancies, pancreatic duct adenocarcinoma is dominant and in addition, the mucinous adenocarcinoma, islet adenocarcinoma and acinic cell carcinoma are also observed with less frequency of occurrence [19-20]. At the molecular level, development of pancreatic malignancies is associated with canonical oncogenes and tumor suppressor genes, such as Ki-Ras, p16, p53, Smad4 and BRCA2, which are involved in several cross-talking cellular signaling pathways including TGF $\beta$ /SMAD, PI3K/AKT, and MAPK pathways [20-22].

In the present study, we present evidence to show that  $\alpha$ A-crystallin is expressed in the normal human and mouse pancreas at a moderate level. Moreover, analysis of  $\alpha$ A-crystallin in the tissue samples from normal human pancreas and 60 cases of pancreatic carcinoma reveals significant difference.  $\alpha$ A-crystallin is downregulated more than 10-fold in the pancreatic carcinoma of various types than

that in normal pancreas. To explore the possible role of  $\alpha$ A-crystallin in pancreatic carcinoma, we demonstrate here for the first time that  $\alpha$ A-crystallin positively regulates the DNA binding and transactivity of the activating protein-1 (AP-1). This upregulation of AP-1 activity is derived from changed MAPK activity, which can positively regulate c-Jun and c-Fos. Moreover, expression of  $\alpha$ A-crystallin in pancreatic cells enhances TGF $\beta$  signaling pathway. Finally, expression of  $\alpha$ A-crystallin in pancreatic cancer cells leads to retarded cell migration. Together, these results suggest that  $\alpha$ A-crystallin appears to suppress pancreatic carcinogenesis.

## **2. Materials and Methods**

### *2.1. Reagents and antibodies*

Anti- $\alpha$ A/B-crystallin antibodies were described before [23]. ERK2 kinase assay kit, and anti-phosphorylated Elk antibody were purchased from Cell Signaling, Inc. Anti-c-Jun antibody was a kind gift from Dr. Tom Curran's Laboratory. Anti-c-Fos, anti-Lamin B, and anti- $\beta$ -actin antibodies were purchased from Santa Cruz Biotechnology. Human normal pancreas and pancreatic carcinoma tissue samples were obtained from US Biomax, Inc.

### *2.2. Immunohistochemistry analysis of $\alpha$ A-Crystallin, measure of fluorescence intensity and HE staining*

Immunohistochemistry analysis was conducted as recently described [24]. Briefly, the sections were rehydrated in graded alcohols, heated up to boiling for 5 min in citrate buffer at pH 6. The sections were washed three times with PBS. For non-specific blocking, each section was incubated in 500  $\mu$ l of 5% normal goat serum (Sigma-Aldrich, CA) in PBS for 1 h at room temperature and then incubated

overnight in 400  $\mu$ l of diluted anti- $\alpha$ A-crystallin antibody (1:100) in a humidified chamber at 4 °C. The sections were then washed with PBS three times (5 min each) followed by incubation with 400  $\mu$ l of FITC-linked secondary antibody and DAPI (Vector Laboratories, CA) in blocking solution for 1 h in the absence of visible light. After incubation, sections were washed with PBS at room temperature 6 times (5 min each) and then observed under a Carl Zeiss Laser Scanning Confocal microscope. For negative controls, the sections were treated in the same way except that the primary antibody was replaced with normal serum IgG.

A quantitative measure of fluorescence intensity was conducted using confocal microscopy for assessment of the level of  $\alpha$ A-crystallin expression. During analysis of the obtained results, the designated values of 0 to 10, 10 to 100, 100 to 1000 and more than 1000 represent negative background, weak, moderate and strong expression of  $\alpha$ A-crystallin, respectively. The same immunohistochemical quantitative system was used for tissue microarray analysis by others [25]. Each core was measured individually. If more than one core was evaluated from each tissue the mean intensity and standard deviation were calculated and shown as Mean  $\pm$  SD.

For histology analysis, hematoxylin and eosin (HE) staining was conducted as recently described [26].

### 2.3. Cell culture and cell fraction extraction

The preparation of the pEGFP-C3 and pEGFP-C3- $\alpha$ A/ $\alpha$ B-crystallin expression constructs, and establishment of stable transfected cell lines were conducted as previously described [23-24]. The pEGFP- $\alpha$ TN4-1, pEGFP- $\alpha$ A- $\alpha$ TN4-1, pEGFP- $\alpha$ B- $\alpha$ TN4-1, pEGFP-Miapaca, pEGFP- $\alpha$ A-Miapaca and pEGFP- $\alpha$ B-Miapaca stable

clones were grown in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) containing 10% fetal bovine serum, 50 units/ml penicillin and streptomycin and 400 µg/ml neomycin as described before [23-24]. All cells were kept at 37 °C and 5% CO<sub>2</sub> gas phase. Cell fractions were extracted by differential sucrose gradient as previously described [23]. The cytoplasmic and nuclear extracts of vector (pEGFP)-, αA-crystallin (pEGFP-αA)- or αB-crystallin (pEGFP-αB)-transfected cells were used for western blot analysis. The nuclear extracts from parent, vector (pEGFP)-, αA-crystallin (pEGFP-αA)- or αB-crystallin (pEGFP-αB)-transfected cells were used for gel mobility shifting assays.

#### 2.4 Reverse transcription-linked polymerase chain reaction (RT-PCR)

Total RNA samples were extracted from αA-crystallin-transfected cells (pEGFP-αA-Miapaca), αB-crystallin-transfected cells (pEGFP-αB-Miapaca), vector transfected cells (pEGFP-Miapaca) and control parent pancreatic cancer cells (Miapaca) using TRIZOL reagent. Five µg of total RNA from each sample was used for cDNA synthesis. Several components of TGFβ signaling pathway were examined by RT-PCR using specific primer pairs listed in table 1. The PCR reaction is: 94°C for 5 min; 94°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec, in 30 cycles; 72°C for 5 min; keep at 4°C. The PCR products were examined by agarose gel electrophoresis and visualized under UV illumination.

#### 2.5. Gel mobility shifting assay

Gel mobility shifting assays were conducted as previously described [24, 27-29]. The following oligos were used: 5'-GTCCTTCATTACGTCACGCATAG-3' for conserved AP-1 binding site, 5'-GTCCTTCATTTCATTACGCATAG-3' for mutated



AP-1 binding site; Twenty  $\mu\text{g}$  of nuclear extracts or cytoplasmic extracts prepared from the pEGFP- $\alpha\text{TN4-1}$ , pEGFP- $\alpha\text{A-}\alpha\text{TN4-1}$ , pEGFP-Miapaca, and pEGFP- $\alpha\text{A-Miapaca}$  cells were incubated with  $1 \times 10^5$  cpm of  $^{32}\text{P}$ -labeled double-stranded synthetic oligonucleotides for 30 minutes at  $37^\circ\text{C}$  in binding shifting buffer [27-29]. For competition experiments, 50-fold of the non-labeled wild type or mutant double-stranded synthetic oligonucleotides were pre-incubated with the nuclear extracts for 30 minutes before the labeled probe was added into the reaction. For the pre-cleared experiments, 20  $\mu\text{g}$  of nuclear extract prepared from pEGFP- $\alpha\text{TN4-1}$ , pEGFP- $\alpha\text{A-}\alpha\text{TN4-1}$ , pEGFP-Miapaca, and pEGFP- $\alpha\text{A-Miapaca}$  cells was pre-incubated with 10  $\mu\text{g}$  antibody against c-Jun or/and c-Fos for 1 hour on ice, then mixed with protein A/G agarose and rotated for additional 1 hour at  $4^\circ\text{C}$ , and then the supernatant was recovered for incubation with  $1 \times 10^5$  cpm of  $^{32}\text{P}$ -labeled double-stranded synthetic AP-1 oligonucleotides for 30 minutes at  $37^\circ\text{C}$  in a binding shifting buffer. After the binding reactions, the mixtures were loaded onto 6% native PAGE and detected by autoradiography.

## 2.6. Kinase activity assay

Substrate Elk protein was incubated with different doses of ERK2 kinase, 0.1, 1, 5 or 10 ng or mock kinase, GSK3 $\beta$  in the kinase buffer for 30 min at  $37^\circ\text{C}$  in the presence of  $\gamma\text{-}^{32}\text{P-ATP}$ . For the effect of  $\alpha\text{A-crystallin}$  on ERK kinase, differential concentrations (0, 1 and 10  $\mu\text{g}$ ) of purified bovine  $\alpha\text{A-crystallin}$  were added to the kinase assay reactions. After reaction, the mixtures were loaded to SDS-PAGE and detected by western blot analysis using anti-phosphorylated-Elk antibody.

## 2.7 Western blot analysis

Preparation of total proteins from mouse tissues and various transfected cells and Western blot analysis of different protein samples were conducted as previously described [30-32].

### 2.8. Wound healing assay.

The three types of stable clones of pEGFP-Miapaca, pEGFP- $\alpha$ A-Miapaca and pEGFP- $\alpha$ B-Miapaca cells were seed in 6-well plates and cultured until 100% confluent. A straight scratch was made by using a 1 ml blue pipette tip to simulate the wound in each well. After PBS washing for 2 times, new DMEM medium was added for a continuous growth of another 48 hours. The wound healing process was recorded daily using the Leica Inverted Phase Contrast Fluorescence Microscopy with a 10x objective.

### 2.9. Quantitation and Statistical analysis

The mRNA gels were analyzed using the UN-SCAN-IT software from Silk Scientific Corporation (Orem, UT, USA). Total pixel data were averaged from three different groups of samples of each species after normalization against the background. The total pixels from the target band were divided by the total pixels from the corresponding  $\beta$ -actin. The student *t*-test was used to compare the medians of two unpaired groups.  $P < 0.05$  was considered significant [31].

## 3. Results

### 3.1. Expression of $\alpha$ A-crystallin in human non-lenticular tissues

Recent studies have shown that  $\alpha$ B-crystallin is implicated in promotion of carcinogenesis (6, 15, 33). Whether  $\alpha$ A-crystallin has similar functions remains to be

explored. For this reason, we have re-examined the tissue-specific expression of  $\alpha$ A-crystallin in human non-lenticular tissues using immunohistochemistry analysis of human multi-organ tissue array samples and confocal microscope measurement of the relative fluorescence density derived from immunohistochemistry staining. As shown in Fig. 1A, a strong lenticular expression of  $\alpha$ A-crystallin in the developing mouse eye was observed, which is consistent with previously reported result (10). In addition, we observed a moderate level of  $\alpha$ A-crystallin expression in human pancreas and to a less degree, in human liver (Fig. 1A). Similar pancreatic level of  $\alpha$ A-crystallin was observed in 7 out of 11 human samples examined (Fig. 1B). In pancreas,  $\alpha$ A-crystallin was specifically expressed in islets, acinus and interlobular tubes (Fig. 1A). In other non-lenticular ocular tissues,  $\alpha$ A-crystallin was also expressed at moderate level in retina and ciliary processes (data not shown). The relative mean values of fluorescence intensities in various non-lenticular tissues were measured with confocal microscope coupled with related software and were presented in Fig. 1B.

### 3.2. Expression of $\alpha$ A-crystallin in mouse tissues

To validate the observed results of  $\alpha$ A-crystallin expression in human samples, we next dissected 10 different tissues from adult mouse: lens, heart, thymus, kidney, brain, spleen, pancreas, lung, liver and testis, and analyzed the expression level of  $\alpha$ A-crystallin in these tissues. As shown in the top panel of Fig. 2, Western blot analysis revealed that besides its robust expression in the ocular lens (lane 1 contains only 0.5  $\mu$ g protein, rest of the lanes 100  $\mu$ g proteins),  $\alpha$ A-crystallin was detected in thymus and pancreas at a relatively strong level, then to a less degree in brain. The  $\alpha$ A-crystallin level in heart, kidney, spleen and liver was much lower than that in

thymus and pancreas. Finally, a trace of  $\alpha$ A-crystallin was also observed in testis. No  $\alpha$ A-crystallin was detected in lung. As comparison, we also examined the tissue distribution of  $\alpha$ B-crystallin in mouse. Besides the strongest expression in lens,  $\alpha$ B-crystallin was highly expressed in heart, thymus and brain, moderately expressed in kidney and lung. However, different from the tissue specific expression patterns of  $\alpha$ A-crystallin,  $\alpha$ B-crystallin was barely detectable in pancreas and spleen, and absent in liver and testis examined. Thus, our results confirm that the pancreas expresses a moderate level of  $\alpha$ A-crystallin. In addition, we also show that several other non-lenticular tissues also express low level of  $\alpha$ A-crystallin. These results are slightly different from the pattern of  $\alpha$ A-crystallin detected in different tissues from rat [16, 34].

### 3.3. Down-regulation of $\alpha$ A-crystallin in pancreatic tumors

Since both Hsp27 and  $\alpha$ B-crystallin are implicated in carcinogenesis [6-8, 15, 33, 36-37], we reason that  $\alpha$ A-crystallin may be also involved in regulation of carcinogenesis. To test this possibility, we compared the expression pattern of  $\alpha$ A-crystallin in human normal pancreatic tissues and various types of pancreatic carcinoma using the tissue array samples. As shown in Fig. 3A, expression of  $\alpha$ A-crystallin in human pancreatic cancer tissues was lower than that in human normal pancreas. Among the 11 normal pancreas samples examined, 7 samples displayed moderate expression level of  $\alpha$ A-crystallin (normal pancreas A in Fig.3A) and 4 samples relatively weak expression (normal pancreas B in Fig.3A). In contrast, among the 60 cases of pancreatic cancer samples, 4 had weak expression of  $\alpha$ A-crystallin signal (shown as pancreatic cancer #A of Fig.3A), 17 displayed barely

detectable  $\alpha$ A-crystallin signal and the remaining 39 samples displayed no detectable signals (shown as pancreatic cancer #B of Fig.3A).

To further characterize the expression of  $\alpha$ A-crystallin in different human pancreatic cancer, we divided the pancreatic cancer samples into 4 different types: duct adenocarcinoma (n=48), mucinous adenocarcinoma (n=3), islet adenocarcinoma (n=6) and acinic cell carcinoma (n=3). The fluorescence signal in each individual sample and the statistic data with the mean value of fluorescence intensity of  $\alpha$ A-crystallin expression in each type of pancreatic carcinoma were presented in Fig. 3B. The expression level of each normal pancreas and the statistical data derived from the normal pancreas were also included for comparison (Fig. 3B). Together, our results reveal that  $\alpha$ A-crystallin is significantly down-regulated in human pancreatic carcinoma comparing with normal pancreas.

#### *3.4. Expression of $\alpha$ A-crystallin in miapaca cells upregulates expression of c-Jun and c-Fos.*

It is well established that the transcriptional factor complex, activating protein-1 (AP-1) plays a significant role in tumorigenesis [38-42]. AP-1 is either a homodimer containing two Jun family members (c-Jun, Jun-B or Jun D) or a heterodimer containing one member from the Jun family and another from the Fos family (C-Fos, Fra-1 and Fra-2) [38]. To explore the possible function of  $\alpha$ A-crystallin in human pancreatic cancer, we overexpressed  $\alpha$ A-crystallin in both pancreatic cancer cells, miapaca and non-cancer cells,  $\alpha$ TN4-1, a SV40 large-T-transformed mouse lens epithelial cell line expressing very little endogenous  $\alpha$ A-crystallin. Then, we examined the AP-1 expression and activity in these transfected cells. As shown in Figure 4, expression and phosphorylation of c-Jun were significantly upregulated in

the nucleus of the miapaca cells expressing  $\alpha$ A-crystallin than in the nucleus of the vector-transfected cells, expression of c-Fos was also upregulated, though to a much less degree, in both nucleus and cytoplasm of the miapaca cells expressing  $\alpha$ A-crystallin than in the same fractions of the vector-transfected cells. Similar results were also obtained in the nucleus of the  $\alpha$ TN4-1 cells expressing  $\alpha$ A-crystallin (Supplementary Fig. 1).

### 3.5. Expression of $\alpha$ A-crystallin in miapaca cells upregulates AP-1 activity.

To determine whether the upregulated c-Jun and c-Fos actually led to increased AP-1 activity, we first conducted DNA binding assays using the oligos contained either the conserved AP-1 binding site (TGAGTCA) or the mutated site (ATCGGTA). As shown in Fig. 5A, the nuclear extract prepared from either parent miapaca cells (lane 13) or vector pEGFP transfected Miapaca cells can bind to the wild type AP-1 oligo (lane 2) which was competed off by cold wild type oligo (lane 3) but much less by the mutated oligo (Lane 4). The nuclear extracts pre-cleared by antibodies against c-Jun (lane 5), c-Fos (Lane 6) or both antibodies (lane 7) lost most of the AP-1 activity so that the formed AP-1 complex was either lost or appeared very weak. In contrast, the nuclear extracts pre-cleared by normal IgG only slightly attenuated the AP-1 complex (Lane 8). These results suggest that the specific band we detected between the nuclear extract from pEGFP-Miapaca cells and the wild type oligo is the AP-1/DNA complex. Next, we compared the AP-1 DNA binding activity in vector- and  $\alpha$ A-crystallin-transfected cells. As shown in lane 9 and 11 of Fig. 5A, miapaca cells expressing  $\alpha$ A-crystallin display significant upregulation of AP-1 activity. Similar results were obtained with the lens epithelial cells,  $\alpha$ TN4-1 expressing either only EGFP or EGFP- $\alpha$ A-crystallin (Supplementary Fig. 2). As a control for the specific

binding to the AP-1 site, we conducted the reaction using purified p53 protein and AP-1 oligo, lane 12 of Fig. 5A clearly showed that p53 did not bind to AP-1 oligo. To further confirm that the transactivity of AP-1 was indeed upregulated in miapaca cells expressing  $\alpha$ A-crystallin, we introduced two reporter genes into the two types of stable transfectants: pEGFP-Miapaca and pEGFP- $\alpha$ A-Miapaca. As shown in Fig. 5B, assayed with either pJun-Luc (the luciferase gene was driven by the Jun promoter), or pProlactin-1xAP-1-Luc (the luciferase gene was driven by a mini-prolactin gene promoter containing a copy of AP-1 binding site), the miapaca cells expressing  $\alpha$ A-crystallin showed statistically significant upregulation of luciferase activity, indicating the upregulation of AP-1 activity in  $\alpha$ A-crystallin expression miapaca cells (Fig. 5B). Thus, expression of  $\alpha$ A-crystallin in pancreatic cancer cells positively regulates AP-1 activity.

### 3.6. $\alpha$ A-crystallin positively regulates ERK2 MAPK.

It has been shown that AP-1 expression and activity can be enhanced by MAP kinases [44-48]. To explore how might  $\alpha$ A-crystallin regulate AP-1, we examined the possible modulation of ERK kinase activity by  $\alpha$ A-crystallin since ERK is a major MAP Kinase regulating AP-1 components in both expression and activation [44-48]. As shown in Fig. 6A, we found that Elk was dose-dependently phosphorylated by ERK2 in vitro. Moreover, in the presence of  $\alpha$ A-crystallin, phosphorylation of Elk by 0.1 or 1 ng ERK2 was significantly enhanced. Addition of 1  $\mu$ g of  $\alpha$ A-crystallin into the ERK2 kinase reaction with low level of ERK2 (1 ng) enhanced more than 10-fold Elk phosphorylation, reflecting the corresponding enhancement of ERK2 activity. Together, these results reveal that  $\alpha$ A-crystallin can positively regulate AP-1 activity through modulation of ERK activity. To further

confirm that  $\alpha$ A-crystallin enhances ERK2 kinase activity, we analyzed the ERK1/2 expression and activity in vector- and  $\alpha$ A-crystallin transfected stable lines of cells. As shown in Fig. 6C, both expression and activity of ERK2 were clearly upregulated in miapaca cells expressing  $\alpha$ A-crystallin than vector-transfected cells.

### 3.7. $\alpha$ A-crystallin regulates TGF- $\beta$ Signaling Pathway

To determine whether  $\alpha$ A-crystallin actively regulates TGF $\beta$  signaling pathway which plays key role in pancreatic carcinogenesis [20], we first examined expression of several genes in the TGF $\beta$  signaling pathway at the mRNA level. As shown in Fig. 7, TGF $\beta$  was increased about 2-fold when  $\alpha$ A-crystallin was overexpressed in Miapaca (Fig. 7A and 7B, similar result was observed in  $\alpha$ A-crystallin-transfected Capan-1 cells, data not shown). For the receptor, TGF $\beta$  receptor 1 and 2 are not detected and TGF $\beta$  receptor 3 displayed little change in the three types of cells (Fig. 7A and 7C). To further confirm the modulation of TGF $\beta$  signaling pathway by  $\alpha$ A-crystallin, we examined the protein expression levels of TGF $\beta$  and the downstream smads, as well as the phosphorylation status of the smads. As shown in Fig. 8, Western blot analysis revealed that TGF $\beta$  was also upregulated at the protein level. In addition, expression and phosphorylation of Smad 3 and Smad 5 were significantly upregulated. Thus,  $\alpha$ A-crystallin actively enhances TGF $\beta$  signaling pathway.

### 3.8. Retardation of cell migration in $\alpha$ A-crystallin-overexpressed cells

To explore how  $\alpha$ A-crystallin may regulate carcinogenesis, we conducted wound healing assay using the established stable cell lines: pEGFP-miapaca, pEGFP- $\alpha$ A-Miapaca and pEGFP- $\alpha$ B-Miapaca. When these cells with similar expression levels



were selected to conduct wound-healing assays (Fig. 9), it was found that miapaca cells expressing  $\alpha$ A-Crystallin displayed statistically significant retardation in cell migration. In contrast, the same cells expressing either the vector or  $\alpha$ B-Crystallin did not show such effect. Thus,  $\alpha$ A-Crystallin retards migration of pancreatic cancer cells, which is consistent with its downregulation in various types of pancreatic carcinoma (Fig. 3).

#### 4. Discussion

In the present study, we have demonstrated the following: 1)  $\alpha$ A-crystallin is expressed at moderate level in pancreas from human and mouse, a non-ocular tissue previously not documented; 2)  $\alpha$ A-crystallin is significantly downregulated in the pancreatic tumors than in the normal human pancreas; 3) Expression of  $\alpha$ A-crystallin in pancreatic carcinoma cells induces significant upregulation of the AP-1 components and activity, 4)  $\alpha$ A-crystallin can enhance activity of ERK2 MAP kinase, through which it can regulate AP-1 expression and activity; 5) Expression of  $\alpha$ A-crystallin in pancreatic carcinoma cells significantly enhanced the TGF $\beta$  signaling pathways; and 6) expression of  $\alpha$ A-crystallin in pancreatic cells led to obvious retardation of the cell migration. Together, our results suggest that  $\alpha$ A-crystallin appear to negatively regulate pancreatic carcinogenesis, which is contrast to the function of  $\alpha$ B-crystallin in breast cancer.

***$\alpha$ A-crystallin Is Expressed in Normal Pancreas Besides Other non-Lenticular Tissues***

$\alpha$ A-crystallin is first known as a lens structural protein [10-11]. It shares about 60% identity in amino acid sequence with  $\alpha$ B-crystallin, another lens structure protein [10-11]. Several lines of evidence have shown that although the two genes encoding  $\alpha$ A- and  $\alpha$ B-crystallins may arise from gene duplication, they have diverged significantly. First, during murine development, the initial turning on of the two genes occurs at different time. While  $\alpha$ B mRNA is first becoming detectable at E9.5, expression of  $\alpha$ A mRNA appears at E10.5 [10]. Such differential turning on time reflects the different control mechanisms in the promoters of the two  $\alpha$ -crystallin genes. Second, the two genes display distinct tissue-specific expression patterns. While  $\alpha$ A is highly restricted to lens during mouse embryonic developmental process,  $\alpha$ B-crystallin is expressed in the developing heart, nasal epithelium, and retinal pigment epithelium [10]. In the adult vertebrates, although both  $\alpha$ A and  $\alpha$ B are abundantly expressed in the lens, they display significantly difference in non-lenticular tissue expressions.  $\alpha$ B is strongly expressed in heart, skeletal muscle, kidney and brain [11-13]. In contrast,  $\alpha$ A is reported to be expressed at very low level in some non-lenticular tissues including spleen, thymus, heart, brain and liver [16-17]. In the present study, our results not only confirm the presence of a low level of  $\alpha$ A-crystallin in kidney and liver, but also reveal a moderate level of  $\alpha$ A-crystallin expression in a novel non-lenticular tissue, the normal human pancreas. Among the 11 normal human pancreas samples examined, 7 samples display consistently moderate level of  $\alpha$ A-crystallin expression and the remaining 4 samples also showed a low level of  $\alpha$ A-crystallin expression (Fig. 1). The pancreatic distribution of  $\alpha$ A-crystallin is further confirmed from analysis of mouse tissue samples (Fig.2).

In a previous study, Kato et al. [16] compared the relative abundance  $\alpha$ A-crystallin in both lens and non-lenticular tissues (ng  $\alpha$ A-crystallin/per mg total protein in organ). These authors found that in the adult rat (8–16 weeks), the lens contains 244  $\mu$ g  $\alpha$ A-crystallin/per mg total lens protein, and the spleen contains 16.6 ng  $\alpha$ A-crystallin/per mg total spleen protein, so the relative abundance of lens  $\alpha$ A-crystallin is about 14,698-fold. Using confocal fluorescence microscopy, we quantitated the relative abundance of  $\alpha$ A-crystallin in the developing mouse lens and the pancreatic tissue, and found the corresponding values are 807 $\pm$ 48 and 283 $\pm$ 67, respectively. Therefore, the human adult pancreatic signal of  $\alpha$ A-crystallin is about 1/3 of the developing mouse eye lens (Fig. 1). In mouse tissue, we estimate that  $\alpha$ A-crystallin in lens is about 500-fold higher than that in pancreas (see Fig.2, 200-fold difference in sample load  $\times$  2.5-fold difference in  $\alpha$ A-crystallin band in lens verse in pancreas). Together, our results demonstrate that the pancreas expresses a moderate level of  $\alpha$ A-crystallin.

#### ***$\alpha$ A-Crystallin Is Implicated in Carcinogenesis***

It is well established that small heat shock proteins are implicated in carcinogenesis [8]. One of the most studied members is Hsp27, which is involved in carcinogenesis of many different types of tissues [49-57]. Another member of the HSP family implicated in carcinogenesis is  $\alpha$ B-crystallin. Iwaki and Tateishi [58] first demonstrated the existence of  $\alpha$ B-crystallin in hamartomas of tuberous sclerosis. Then, it was found that concentrations of  $\alpha$ B-crystallin in prostatic carcinoma tissues were significantly higher than in benign prostatic hyperplasia [59]. In breast cancer cells,  $\alpha$ B-crystallin was found expressing constitutively in certain breast carcinoma

cell lines, and increased intensity of  $\alpha$ B-crystallin expression was correlated with shorter survival [33]. Moreover, expression of  $\alpha$ B-crystallin results in transformation of immortalized human mammary epithelial cells, induction of EGF- and anchorage-independent growth, and enhancement of cell migration and invasion [15]. Thus,  $\alpha$ B-crystallin is considered as a novel oncoprotein [15].

Both  $\alpha$ A- and  $\alpha$ B-Crystallins belong to the heat shock protein (Hsp) family [2]. Whether  $\alpha$ A-crystallin is also implicated in carcinogenesis remains to be explored. Compared with  $\alpha$ B-Crystallin, the limited distribution of  $\alpha$ A-crystallin in non-lenticular tissues may restrict its function in carcinogenesis [10-14]. Nevertheless, several recent studies suggest that  $\alpha$ A-crystallin may be also implicated in tumor development. First, in 4 cases of human sebaceous carcinoma of the eyelid examined, both  $\alpha$ A-Crystallin and  $\alpha$ B-Crystallin were found highly expressed [60]. Second, in the retinocytoma,  $\alpha$ A-crystallin was expressed in the cytoplasm of all tumor cells and the apoptotic index was significantly higher in those cases with weak signal of  $\alpha$ A-crystallin than in those that were strongly positive [61]. These results suggest that  $\alpha$ A-crystallin, acting like  $\alpha$ B-crystallin, seems to promote carcinogenesis. On the other hand, a recent study revealed that in 6 cases of retinoblastoma, preoperative chemotherapy induced strong expression of only Hsp27 and  $\alpha$ B-Crystallin but not  $\alpha$ A-Crystallin [62]. Moreover, the viable tumor cells survived contained high levels of Hsp27 and  $\alpha$ B-Crystallin but not  $\alpha$ A-Crystallin. Therefore, these results indicate that  $\alpha$ A-crystallin does not seem to act the same way as Hsp27 and  $\alpha$ B-Crystallin do in promoting carcinogenesis. Our present finding that in 60 different cases of pancreatic carcinoma, the expression level of  $\alpha$ A-crystallin was consistently down-regulated than that in 11 normal human pancreas samples also support the negative

regulation of carcinogenesis by  $\alpha$ A-crystallin. In addition, our results also suggest that  $\alpha$ A-crystallin may be used as one of the biomarkers for pancreatic cancer diagnosis.

### ***$\alpha$ A-crystallin May negatively Regulates Development of Pancreatic Tumors***

Pancreatic malignancy develops from intraepithelial neoplasia (PanIN), progressing through PanIN-1a, PanIN-1b, PanIN-2, and PanIN-3 to ductal adenocarcinomas (PDAC) [63-64]. During this process, key oncogenes and tumor suppressors such as Ki-RAS, p16<sup>INK4a</sup>, P53, DPC/SMAD4, and BRCA2 play very important roles [20-22, 65-68]. The carcinogenesis signals are mediated by major signaling pathways including MAPK pathways, sonic hedgehog and notch signaling pathways, and also TGF $\beta$  signaling pathways [20-22, 65-68]. In the present study, we present several lines of evidence to show that  $\alpha$ A-crystallin seems to negatively regulate development of pancreatic tumors. First, we demonstrate that compared with the physiological level in normal human pancreas,  $\alpha$ A-crystallin is significantly downregulated in 60 cases of pancreatic carcinomas of various types. Second, we show that pancreatic cancer cells expressing  $\alpha$ A-Crystallin display retarded cell migration. Finally, we show that pancreatic cancer cells expressing  $\alpha$ A-crystallin have enhanced expression levels of c-Jun and c-Fos, increased phosphorylation of c-Jun oncoprotein, and elevated level of AP-1 activities in both DNA binding and transactivation, which is derived from enhanced activity of the ERK2 kinase promoted by  $\alpha$ A-Crystallin (Fig. 6). As a result,  $\alpha$ A-crystallin positively regulates TGF $\beta$  signaling pathway (Figs.7 and 8) through MAP kinase, and thus negatively regulate development of pancreatic malignancy.

**Acknowledgements**

We thank Drs. J. Horvitz for the  $\alpha$ A/ $\alpha$ B-Crystallin antibodies and Dr. Tom Curran for the C-Jun antibody. This work is supported in part by the NIH grants 1R01 EY018380, and 1R01EY015765, and the Lotus Scholar Program Funds from Hunan Province Government and Hunan Normal University, and the Changjiang Scholar Team Award from the National Education Ministry of China to DWL.

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**Figure Legends:**

**Figure 1.** Expression of  $\alpha$ A-crystallin in human normal tissues. (A) Immunohistochemistry of  $\alpha$ A-crystallin in human lens, pancreas, liver, kidney, brain, heart, lung, esophagus, testis, ovary, and lymph node. Hematoxylin-Eosin staining (H.E. staining) results of various tissues were included for comparison (column 1). Nuclei were visualized by Hoechst staining (blue color in column 2).  $\alpha$ A-crystallin stained in green was derived from FITC (Column 3). Merge showed combination of Hoechst in blue and  $\alpha$ A-crystallin in green (Column 4). Mouse lens section from embryonic day 11.5 was stained as positive control for  $\alpha$ A-crystallin expression. Primary antibody was replaced by normal IgG in negative control (last row). Note that normal human pancreas displays moderate level of  $\alpha$ A-crystallin signal. Liver also displays  $\alpha$ A-crystallin signal but the intensity is less (see Fig. 1B for other tissues). Scale bar: 200  $\mu$ m. (B) Quantification of fluorescence intensity. The mean intensity of fluorescence was measured by Carl Zeiss Laser Scanning System LSM 510.

**Figure 2.** Western blot analysis of  $\alpha$ A- and  $\alpha$ B-crystallins in 11 tissues from normal adult mouse (4 weeks). Various tissues were dissected and total proteins were extracted from the 11 tissues indicated. For the lens sample, 0.5  $\mu$ g of total protein was used for Western blot analysis. For other samples, 100  $\mu$ g of total protein were used. The blot was probed with specific antibodies against  $\alpha$ A-crystallin,  $\alpha$ B-crystallin antibody,  $\beta$ -tubulin or  $\beta$ -Actin at a dilution of 1:1000 for the primary antibody and at a dilution of 1:1500 for the secondary antibody. Note that the mouse pancreas expresses a distinct level of  $\alpha$ A-crystallin but only a trace of  $\alpha$ B-Crystallin.

Both  $\beta$ -actin and  $\beta$ -tubulin are absent in lane 1 (lens) and lane 2 (heart) due to limited sample loaded (only 0.5  $\mu$ g lens protein in lane 1) or due to tissue characteristics (lacking  $\beta$ -actin and  $\beta$ -tubulin).

**Figure 3.** Expression of  $\alpha$ A-crystallin in human pancreatic cancer tissues. (A) Immunohistochemistry results of  $\alpha$ A-crystallin in human pancreatic cancer. As comparison, immunohistochemistry results of  $\alpha$ A-crystallin in two human normal pancreas samples were included. Hematoxylin-Eosin staining (H.E. staining) results of two normal and two cancerous pancreatic sections were included for comparison (column 1). Nuclei were visualized by Hoechst staining (blue color in column 2).  $\alpha$ A-crystallin stained in green was derived from FITC (Column 3). Merge showed combination of Hoechst in blue and  $\alpha$ A-crystallin in green (Column 4). Mouse lens section from embryonic day 11.5 was stained as positive control for  $\alpha$ A-crystallin expression. Primary antibody was replaced by normal IgG in negative control (last row). Scale bar: 200  $\mu$ m. Note that normal human pancreas sample A displays moderate level of  $\alpha$ A-crystallin signal, and normal human pancreas sample B shows weaker signals than the sample A. In the cancerous sample A, the level of  $\alpha$ A-crystallin signal was much weaker than that in normal human sample A and also slightly weaker than that in normal human sample B. (B) Quantification of fluorescence intensity. The fluorescence intensity was measured by Carl Zeiss Laser Scanning System LSM 510. Each spot indicates the value of fluorescence intensity from a single tissue sample. Each short solid line indicates mean value of fluorescence intensity of one type of pancreatic cancer. Specifically, mean intensity of normal pancreas is  $283 \pm 267$  (n=11 among which 3 samples have a value less than 100 and 8 samples have a value more than 200); mean intensity of pancreatic duct

adenocarcinoma is  $9 \pm 16$  (n=48); mean intensity of pancreatic mucinous adenocarcinoma is  $3 \pm 5$  (n=3); mean intensity of pancreatic islet adenocarcinoma is  $56 \pm 69$  (n=6) and mean intensity of pancreatic acinic cell carcinoma is  $128 \pm 55$  (n=3).

**Figure 4.** Expression of  $\alpha$ A-crystallin in pancreatic cancer cell line, Miapaca, upregulates expression of c-Jun and c-Fos. The stable clones of miapaca cells expressing pEGFP, pEGFP-H $\alpha$ A or pEGFP-H $\alpha$ B were grown to 95% confluence in MEM with 10% FBS and 400  $\mu$ g/ml G418, and then harvested for extraction of total proteins which were subjected to Western blot analysis for AP-1 components, c-Jun and c-Fos, in two different cellular fractions: cytoplasmic and nuclear portions. Note that miapaca cells expressing  $\alpha$ A-crystallin display upregulated levels of c-Jun and c-Fos protein. In addition, the phospho-c-Jun level was also enhanced in pEGFP-H $\alpha$ A-Miapaca cells.

**Figure 5.** Expression of  $\alpha$ A-crystallin in pancreatic cancer cell line, Miapaca, upregulates AP-1 DNA binding activity (A) and transactivity (B). A. Gel mobility shifting assay demonstrates the existence of AP-1 DNA binding activity in parent miapaca cell nuclear extract (Lane 13), pEGFP-Miapaca cell nuclear extracts (Lane 2 to 9), which was much lower than that in Miapaca cell nuclear extracts expressing  $\alpha$ A-crystallin (Lane 11) or  $\alpha$ B-crystallin (Lane 10). Nuclear extracts prepared from three types of cells were incubated with  $\gamma$ - $^{32}$ P-ATP-labeled oligos containing wild-type AP-1 binding site (up panel of Fig. 5A left part) under various conditions shown in the figure. Lane 1, gel mobility shifting assay with labeled wild type AP-1 oligo. Lane 2, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP-Miapaca nuclear extract. Lane 3, the same assay as in lane 2 except that 50-fold of

non-labeled wild-type AP-1 oligos was added into the reaction. Lane 4, the same assay as in lane 3 except that the non-labeled competing oligo contains a mutated AP-1 binding site (bottom panel of Fig. 5A left part). Lane 5, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP-Miapaca nuclear extract pre-cleared by anti-c-Jun antibody. Lane 6, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP-Miapaca nuclear extract pre-cleared by anti-c-Fos antibody. Lane 7, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP-Miapaca nuclear extract pre-cleared by anti-c-Jun antibody and anti-c-Fos antibody. Lane 8, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP-Miapaca nuclear extract pre-cleared by normal IgG. Lane 9, the same assay as in lane 2. Lane 10, the same assay as in lane 2 except that the nuclear extracts were from the pEGFP-H $\alpha$ B-Miapaca cells. Lane 11, the same assay as in lane 2 except that the nuclear extracts were from the pEGFP-H $\alpha$ A-Miapaca cells. Lane 12 (mock for AP-1 site specificity), the same assay as in lane 2 except that the nuclear extract was replaced with in vitro synthesized p53 protein. Note that p53 does not bind to AP-1 site. B. Luciferase assays to demonstrate that the AP-1 transactivity in pEGFP-H $\alpha$ A-Miapaca cells was significantly higher than that in pEGFP-Miapaca cells. The luciferase reporter gene driven by a mini-prolactin gene promoter without enhancer (pProlactin-Luc), or with 1 copy of AP-1 binding site (pProlactin-1xAP-1-Luc), or driven by the c-Jun promoter (pJun-Luc), together with a reference plasmid were separately transfected into pEGFP-Miapaca cells, pEGFP-H $\alpha$ A-Miapaca cells, or pEGFP-H $\alpha$ B-Miapaca cells. After 48 hours, the transfected cells were harvested for the assaying of luciferase activity. After calibration with the luciferase activity from the reference plasmid, the transactivity of AP-1 in each type of cells was presented. Note that as assayed by both constructs, pProlactin-1xAP-1-Luc and pJun-Luc, the AP-1 activity



in pEGFP-Miapaca cells were significantly lower than that in pEGFP-H $\alpha$ A-Miapaca cells ( $p < 0.005$  for pJun-Luc construct, and  $p < 0.05$  for pProlactin-1xAP-1-Luc construct). In contrast, the AP-1 activity in pEGFP-Miapaca cells is only slightly lower than that in pEGFP-H $\alpha$ B-Miapaca cells ( $p > 0.05$  in both cases).

**Figure 6.**  $\alpha$ A-crystallin enhances MAPK (ERK2) activity in the in vitro kinase assay.

A. The substrate of Elk-1 protein was phosphorylated by ERK2 kinase in the in vitro assay in the presence of ATP. Phosphorylation of Elk was detected by Western blot analysis as described above. Results of two different exposures were shown. Kinase reactions with GSK3 $\beta$  as kinase were also included as control for ERK2. B. Quantitative results of the Elk phosphorylation under different conditions. The relative pixel of each band was quantitated using the software from Silk Scientific Inc. Note that at the concentration of 0.1 and 1ng ERK2 kinase conditions, addition of 1 or 10  $\mu$ g of  $\alpha$ A-crystallin significantly enhances the ERK2 activity as reflected by the differential intensity of Elk phosphorylation. C. Western blot analysis of expression and activities of ERK1/2 in vector- or  $\alpha$ A-transfected miapaca cells. Western blot analysis was conducted as described in Fig.2. Note that both expression and activity of ERK2 was upregulated in miapaca cells expressing  $\alpha$ A-crystallin than vector-transfected cells.

**Figure 7.**  $\alpha$ A-crystallin modulates expression of TGF $\beta$  as demonstrated by RT-PCR analysis.

A. RT-PCR. Total cellular RNAs were extracted from vector-,  $\alpha$ A- or  $\alpha$ B-crystallin-transfected cells, then were used for RT-PCR assays as described in the experimental procedures. B. Quantitation of TGF $\beta$  mRNA level in vector-,  $\alpha$ A- or  $\alpha$ B-crystallin-transfected miapaca cells. C. Quantitation of TGF $\beta$  receptor 3 mRNA

level in vector-,  $\alpha$ A- or  $\alpha$ B-crystallin-transfected miapaca cells. Note that  $\alpha$ A-crystallin enhances expression of TGF $\beta$  mRNA but has little effect on TGF receptors.

**Figure 8.**  $\alpha$ A-crystallin modulates protein expression of TGF $\beta$ , and Smad3/5, as well as the phosphorylation status of Smad3/5. Western blot analysis was conducted as described in Fig. 2. Note that  $\alpha$ A-crystallin enhances expression of TGF $\beta$ , Smad3 and Smad5. Moreover,  $\alpha$ A-crystallin up-regulates the phosphorylation status of Smad3/5.

**Figure 9.** Demonstration that the pancreatic cancer cell line, miapaca, expressing the exogenous H $\alpha$ A-Crystallin display retarded cell migration. The stable transfected cell lines, pEGFP-Miapaca, pEGFP-H $\alpha$ A-Miapaca or pEGFP-H $\alpha$ B-Miapaca cells were seeded in 6-well plates and grown until 95% confluent. Then, two straight scratches were made by using a 1 ml blue pipette tip to simulate the wound healing in each well. After PBS washing for 2 times, new DMEM medium was added for a continuous growth of another 48 hours. The wound healing process was recorded daily using the Leica Inverted Phase Contrast Fluorescence Microscopy with a 10x objective. Note that the pEGFP-H $\alpha$ A-Miapaca cells display slower healing process than pEGFP-Miapaca and pEGFP-H $\alpha$ B-Miapaca cells, suggesting both slower cell migration and proliferation of pEGFP-H $\alpha$ A-Miapaca cells.

**Supplementary Figure 1.** Expression of  $\alpha$ A-crystallin in mouse lens epithelial cell line,  $\alpha$ TN4-1, also up-regulates expression of c-Jun and c-Fos. The stable clones of  $\alpha$ TN4-1 cells expressing pEGFP, or pEGFP-H $\alpha$ A were grown to 95% confluence in

MEM with 10% FBS and 400  $\mu\text{g/ml}$  G418, and then harvested for extraction of total proteins which were subjected to Western blot analysis for AP-1 components, c-Jun and c-Fos, in two different cellular fractions: cytoplasmic and nuclear portions. Note that  $\alpha\text{TN4-1}$  cells expressing  $\alpha\text{A-crystallin}$  display upregulated levels of c-Jun and c-Fos protein.

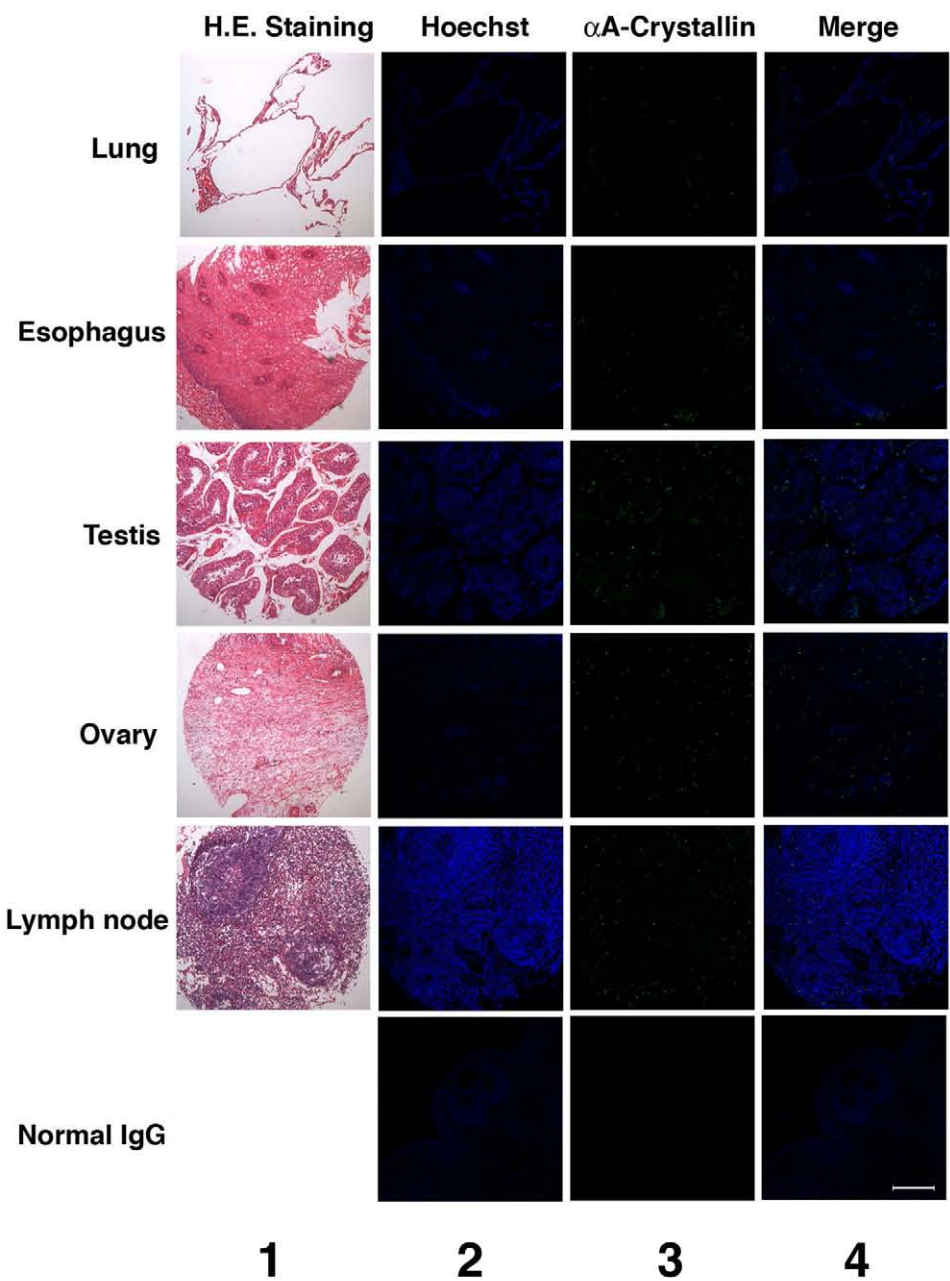
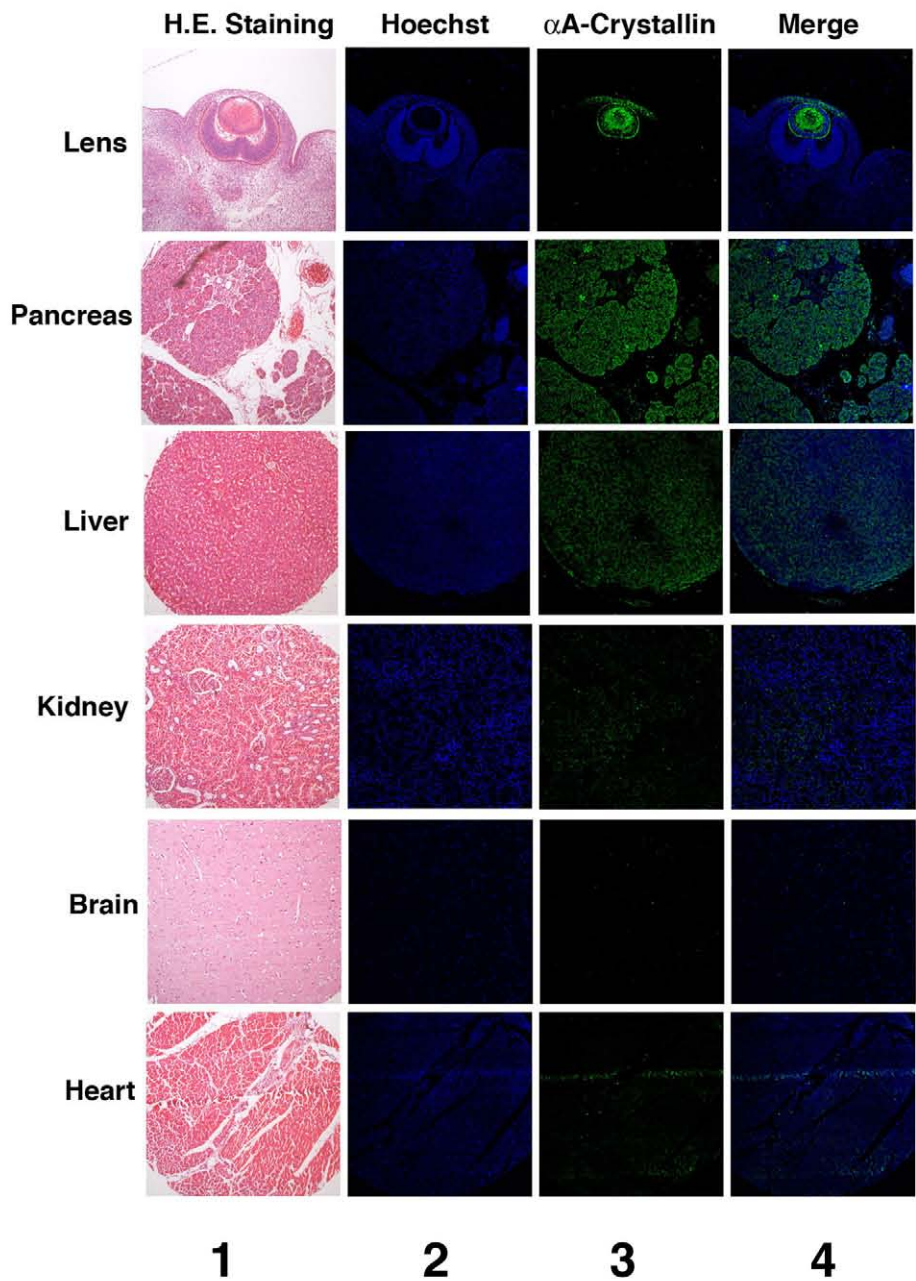
**Supplementary Figure 2.** Expression of  $\alpha\text{A-crystallin}$  in mouse lens epithelial cell line,  $\alpha\text{TN4-1}$  upregulates AP-1 DNA binding activity. Gel mobility shifting assay demonstrates the existence of AP-1 DNA binding activity in pEGFP- $\alpha\text{TN4-1}$  cell nuclear extracts (Lane 2 to 8), which was much lower than that in pEGFP-H $\alpha\text{A}$ - $\alpha\text{TN4-1}$  nuclear extracts (Lane 9). Nuclear extracts prepared from either pEGFP- $\alpha\text{TN4-1}$  cells or pEGFP-H $\alpha\text{A}$ - $\alpha\text{TN4-1}$  cells were incubated with  $\gamma\text{-}^{32}\text{P}$ -ATP-labeled oligos containing wild-type AP-1 binding site (up panel of Fig. 5A left part) under various conditions shown in the figure. Lane 1, gel mobility shifting assay with labeled wild type AP-1 oligo. Lane 2, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP- $\alpha\text{TN4-1}$  nuclear extract. Lane 3, the same assay as in lane 2 except that 50-fold of non-labeled wild-type AP-1 oligos was added into the reaction. Lane 4, the same assay as in lane 3 except that the non-labeled competing oligos contain a mutated AP-1 binding site (bottom panel of Fig. 5A left part). Lane 5, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP- $\alpha\text{TN4-1}$  nuclear extract pre-cleared by anti-c-Jun antibody. Lane 6, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP- $\alpha\text{TN4-1}$  nuclear extract pre-cleared by anti-c-Fos antibody. Lane 7, gel mobility shifting assay with labeled wild type AP-1 oligo and pEGFP- $\alpha\text{TN4-1}$  nuclear extract pre-cleared by anti-c-Jun antibody and anti-c-Fos antibody. Lane 8, the same assay as in lane 2. Lane

9, the same assay as in lane 2 except that the nuclear extracts were from the pEGFP-H $\alpha$ A- $\alpha$ TN4-1 cells.

ACCEPTED MANUSCRIPT

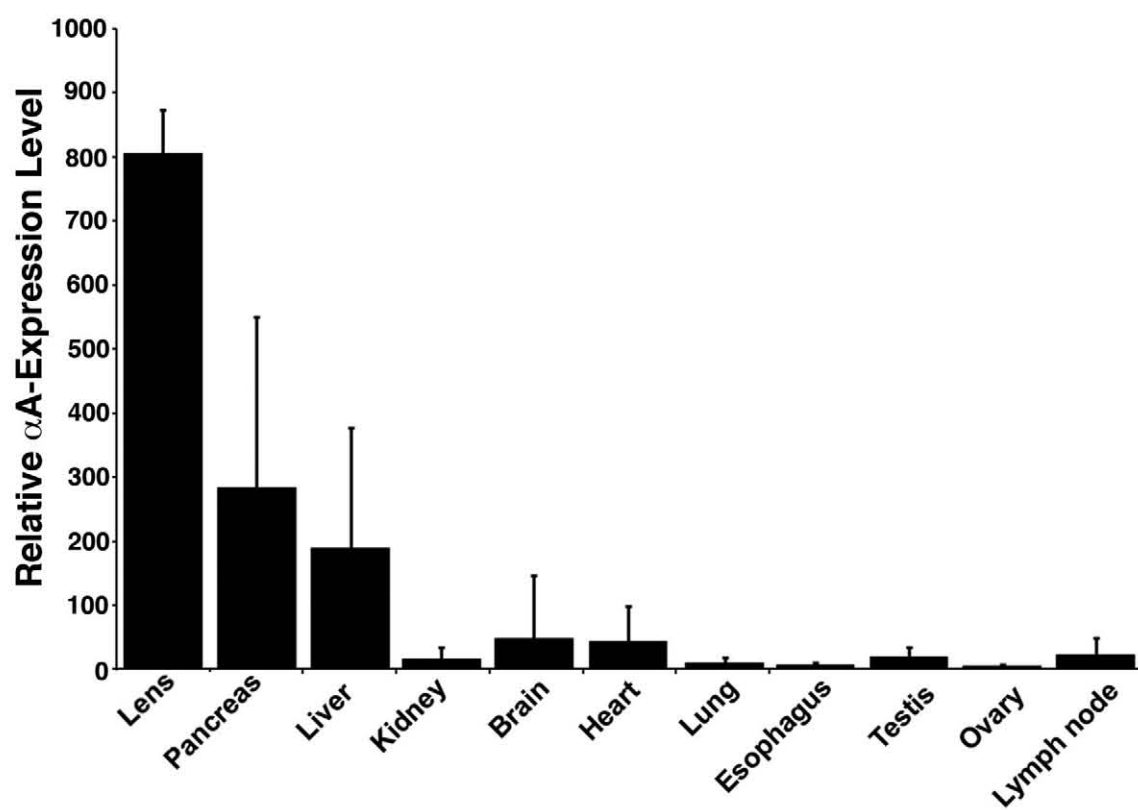
Fig. 1A

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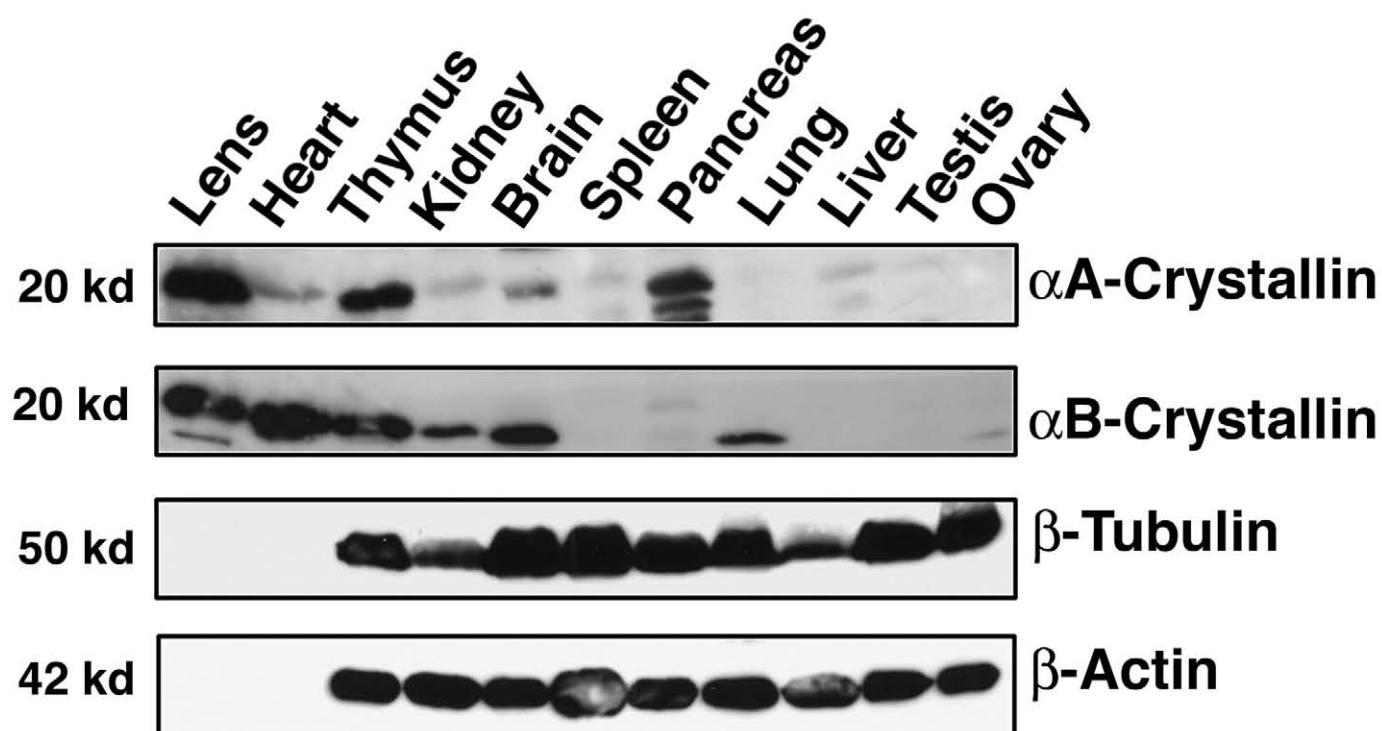


**Fig.1B**

**Deng et al.**



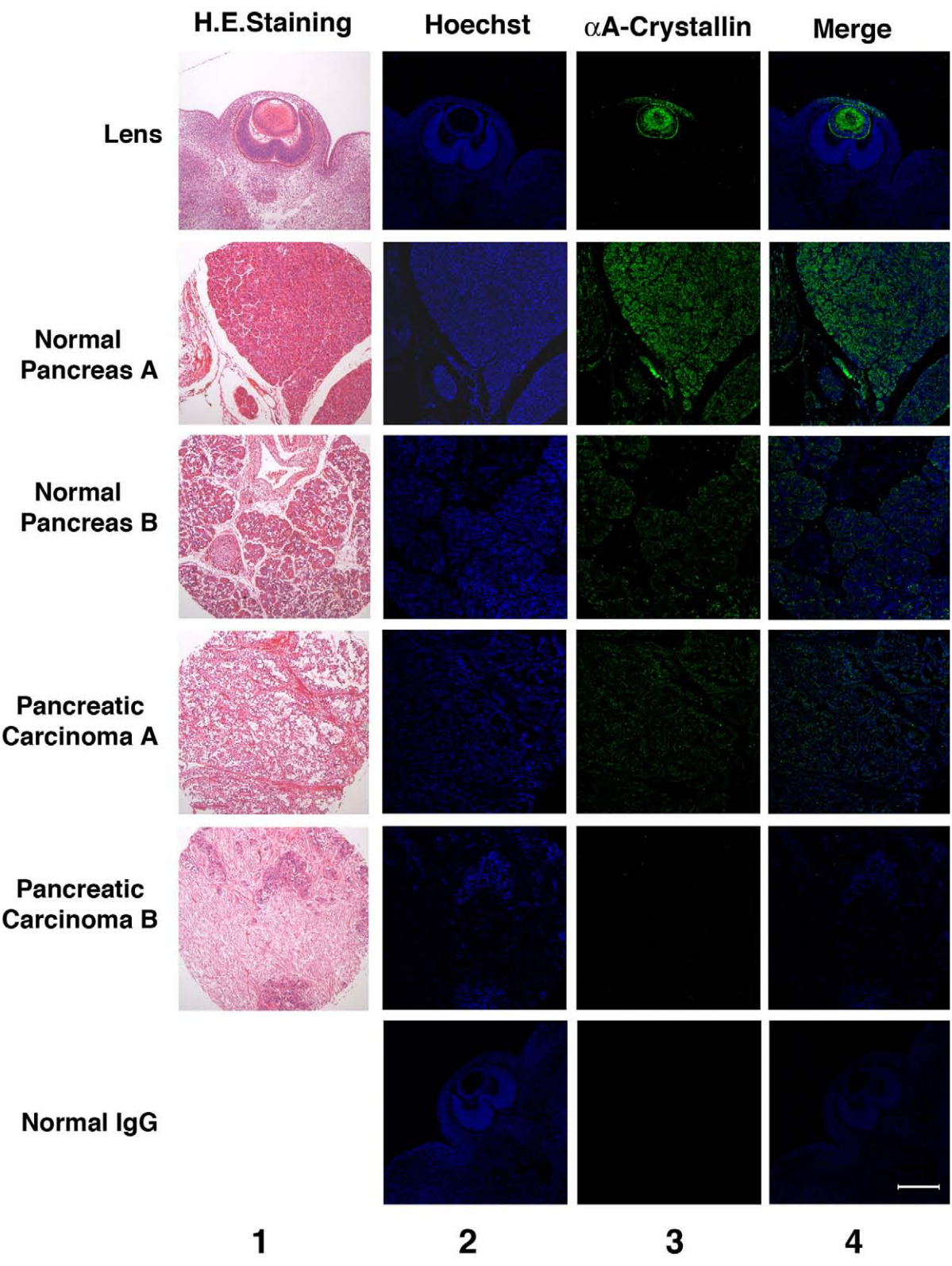
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**Fig. 3A**

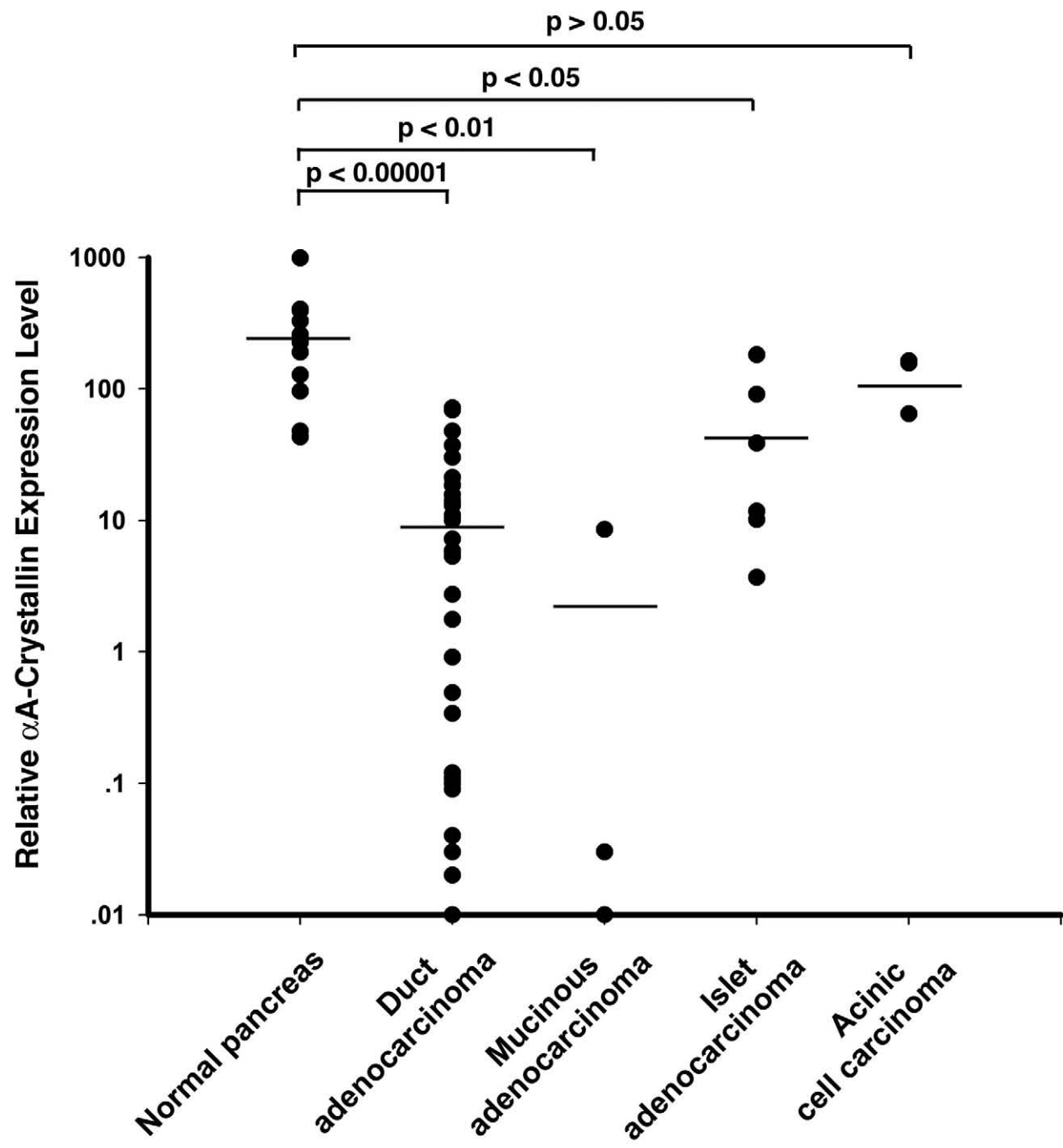
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**Fig. 3B**

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**Fig. 4**

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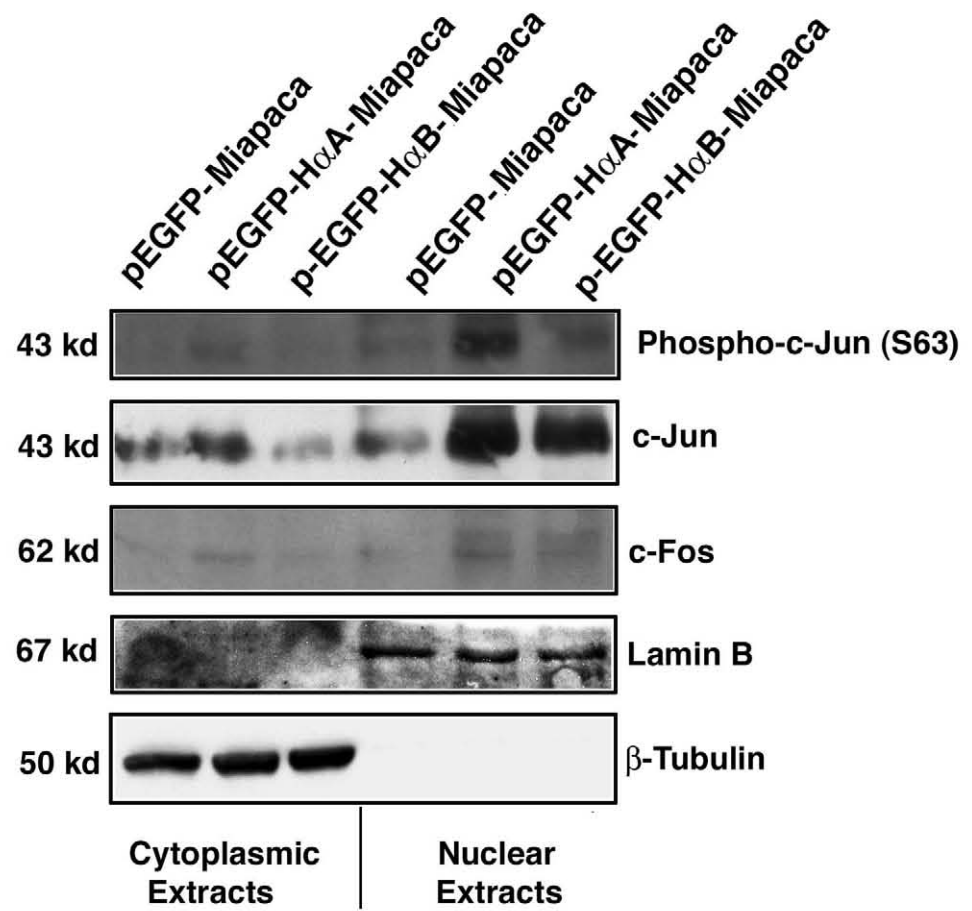


Fig.5

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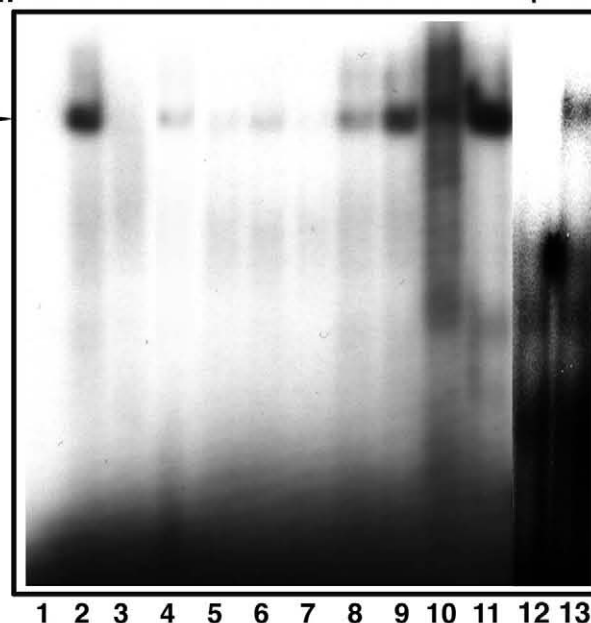
A

Labelled AP-1 Oligo Probe:	+	+	+	+	+	+	+	+	+	+	+	+	+
Nuclear Extract from pEGFP-Miapaca:	-	+	+	+	-	-	-	-	+	-	-	-	-
Nuclear Extract from pEGFP- $\alpha$ A-Miapaca:	-	-	-	-	-	-	-	-	-	+	-	-	-
Nuclear Extract from pEGFP- $\alpha$ B-Miapaca:	-	-	-	-	-	-	-	-	-	+	-	-	-
50 X Wild Type AP-1 Oligo Competition:	-	-	+	-	-	-	-	-	-	-	-	-	-
50 X Mutant AP-1 Oligo Competition:	-	-	-	+	-	-	-	-	-	-	-	-	-
Pre-cleared with Anti-C-Jun Antibody:	-	-	-	-	+	-	-	-	-	-	-	-	-
Pre-cleared with Anti-C-Fos Antibody:	-	-	-	-	-	+	-	-	-	-	-	-	-
Pre-cleared with Anti-C-Jun/c-Fos Antibody:	-	-	-	-	-	-	+	-	-	-	-	-	-
Pre-cleared with Normal IgG Antibody:	-	-	-	-	-	-	-	+	-	-	-	-	-
Nuclear Extract from Miapaca:	-	-	-	-	-	-	-	-	-	-	-	-	+
In Vitro Synthesized p53 Protein:	-	-	-	-	-	-	-	-	-	-	-	+	-

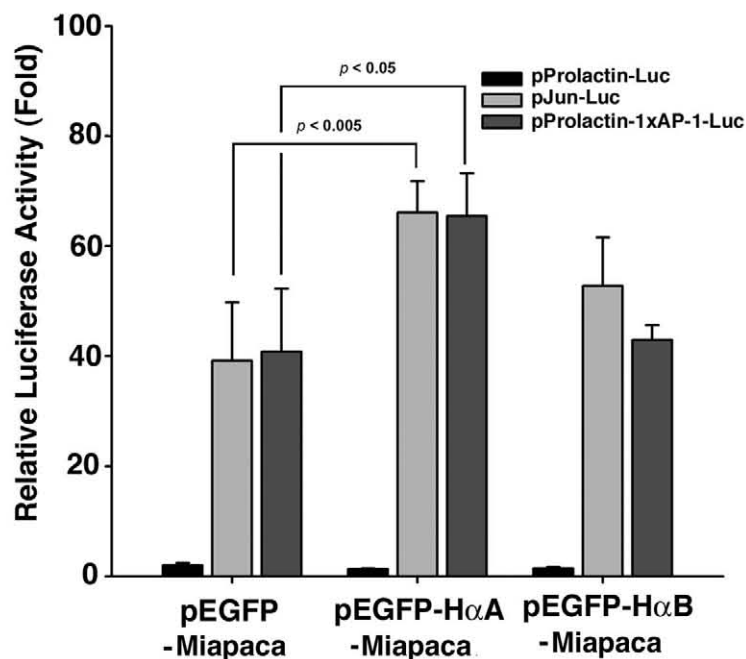
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Oligo with Mutant AP-1  
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AP-1 Complex



B



**Fig.6**

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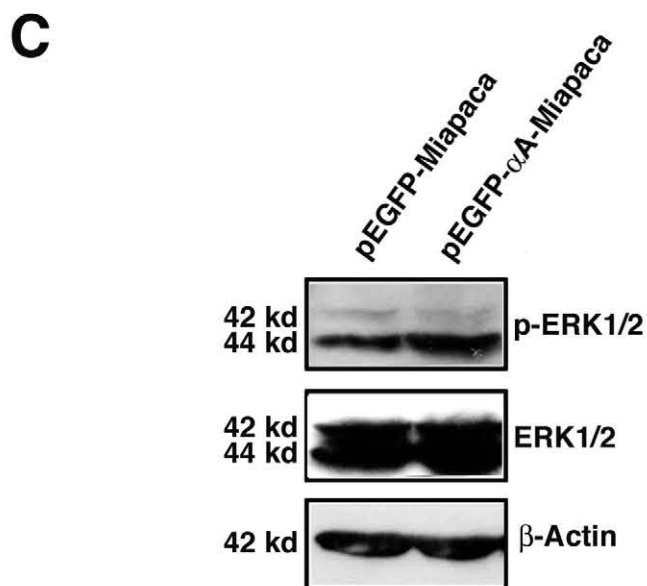
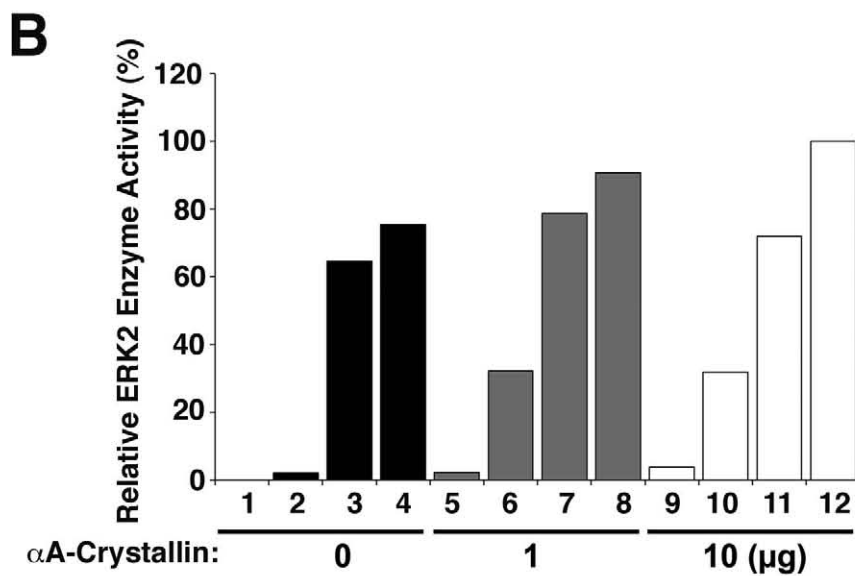
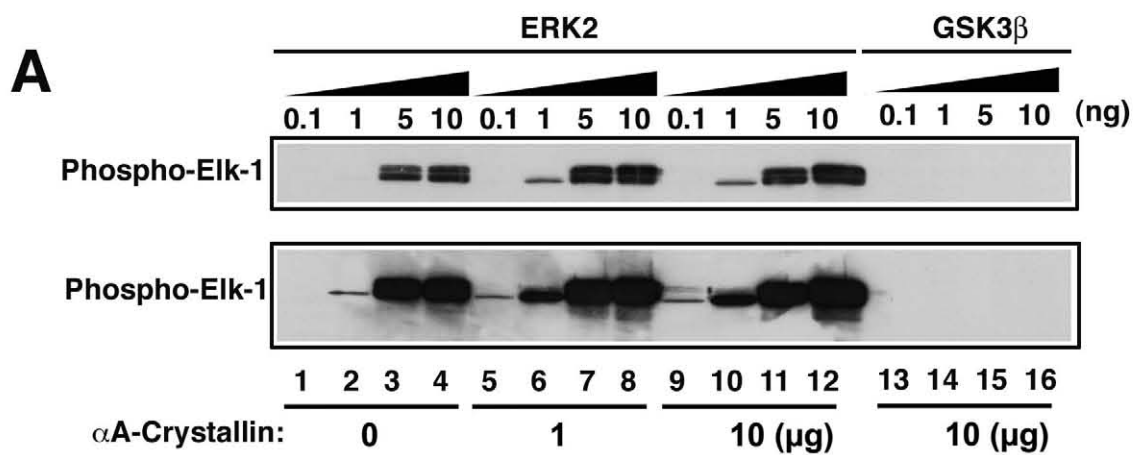
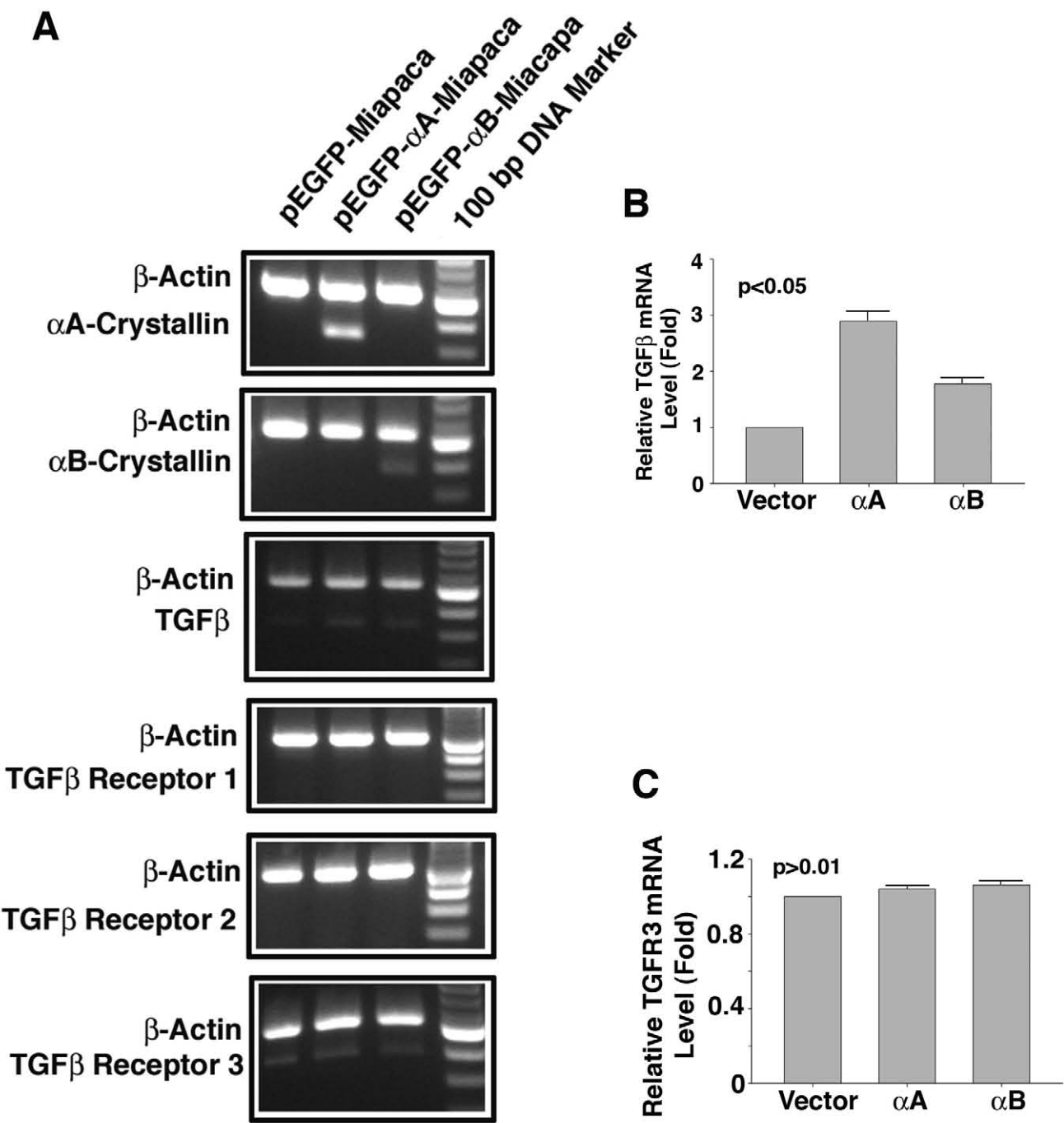


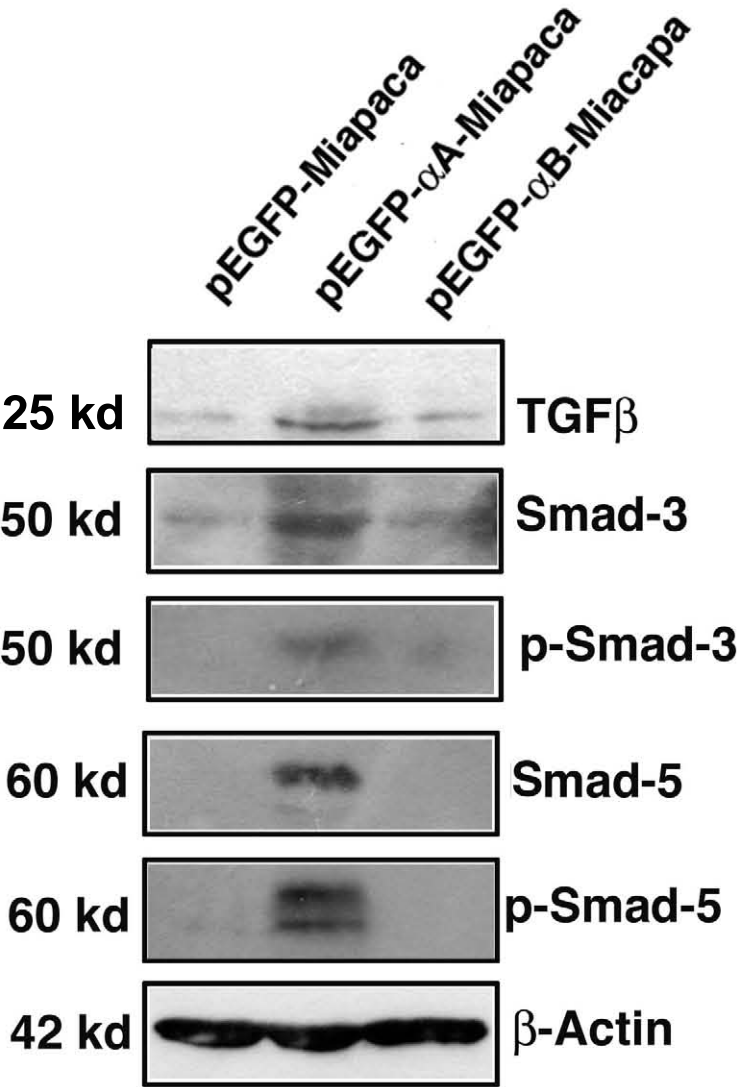
Fig.7

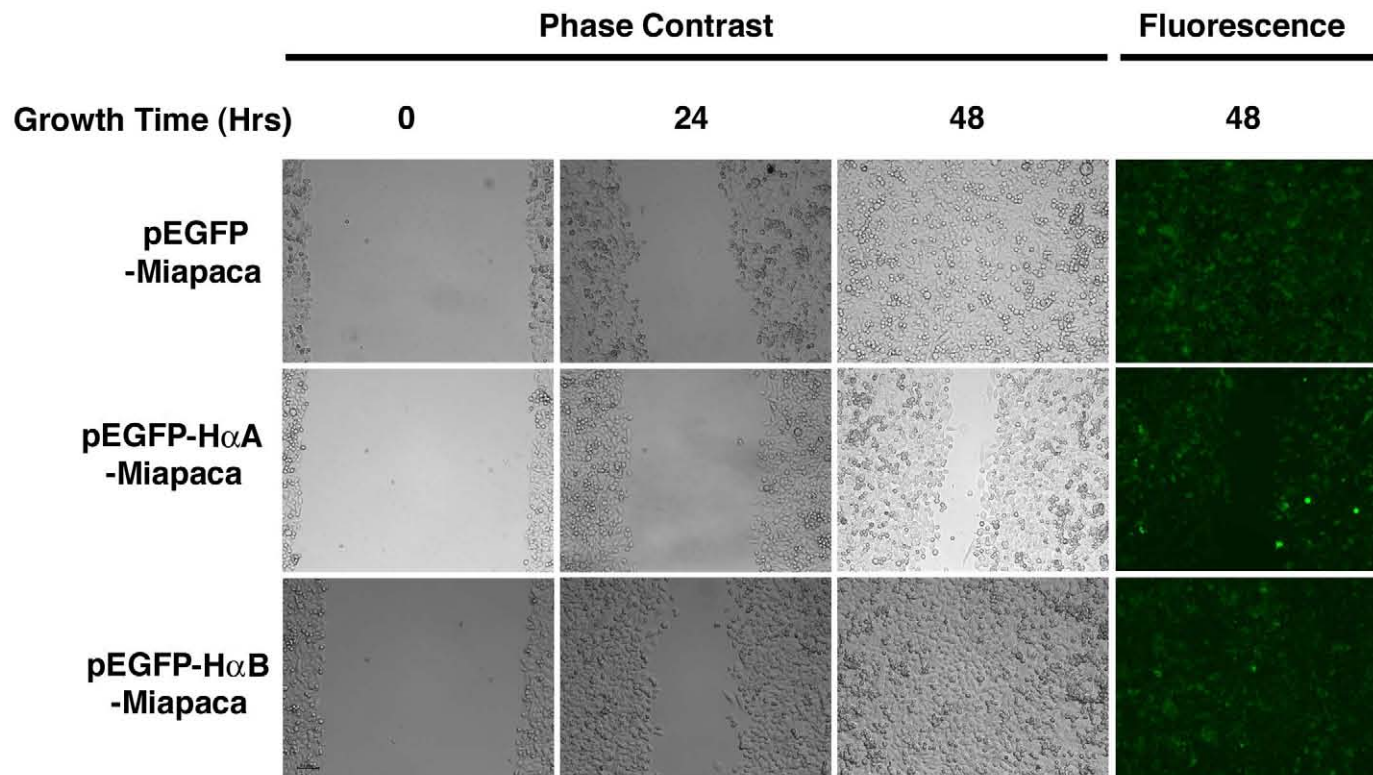
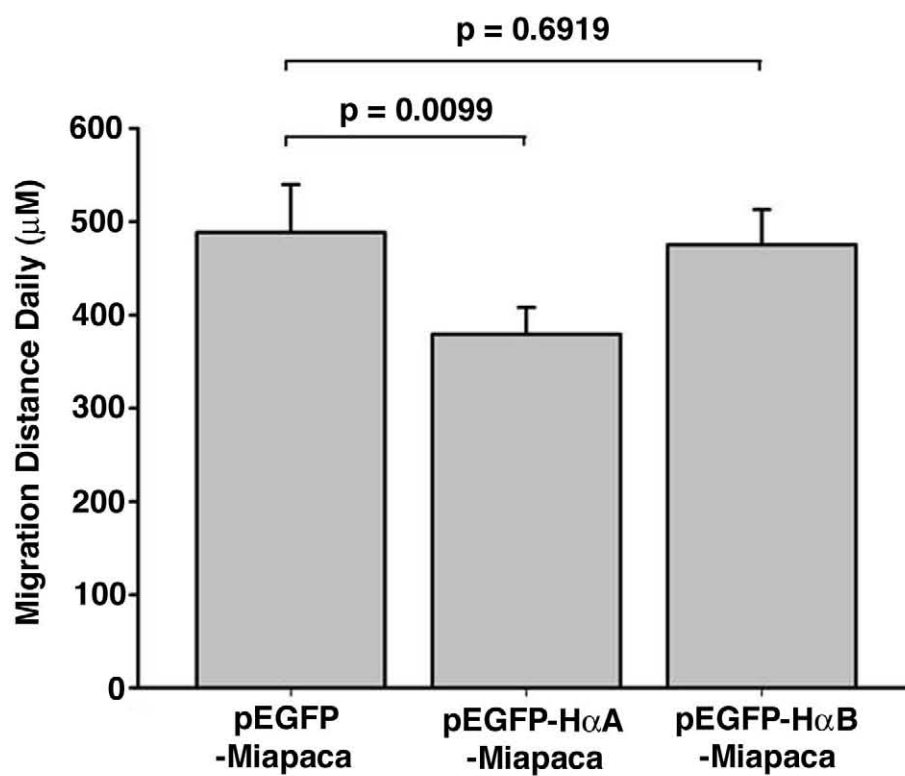
Deng et al.



**Fig.8**

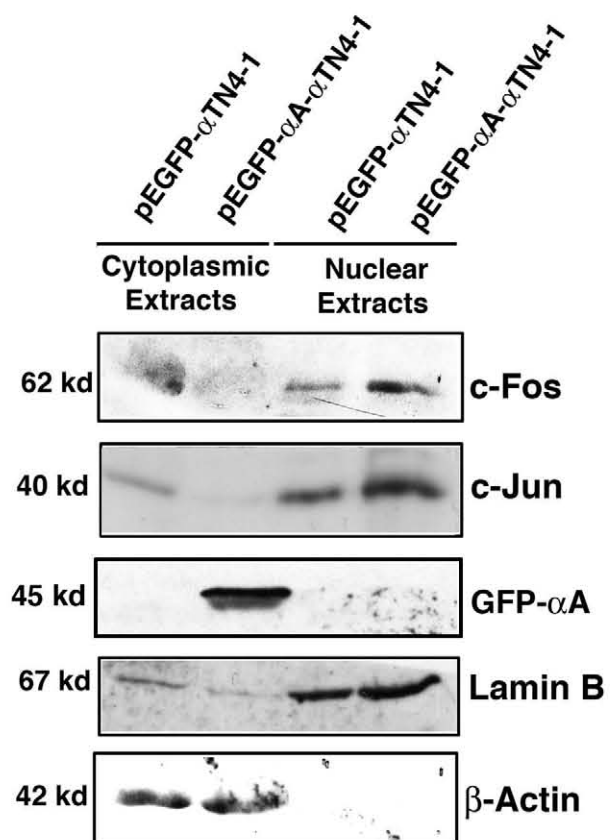
**Deng et al.**



**Fig.9****Deng et al.****A****B**

# Supplementary Fig. 1

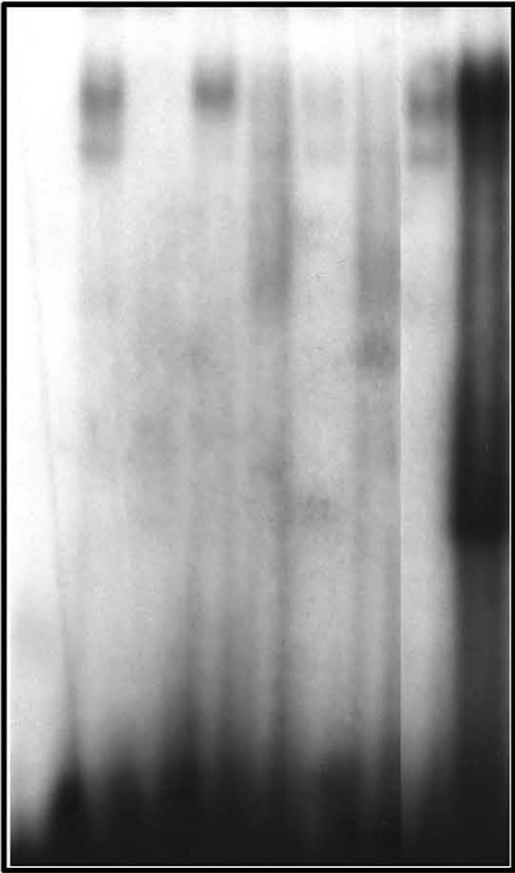
Deng et al.





	1	2	3	4	5	6	7	8	9
Labelled AP-1 Probe:	+	+	+	+	+	+	+	+	+
Nuclear Extract from pEGFP- $\alpha$ TN4-1:	-	+	+	+	-	-	-	+	-
Nuclear Extract from pEGFP- $\alpha$ A- $\alpha$ TN4-1:	-	-	-	-	-	-	-	-	+
50 X Wild Type Oligo Competition:	-	-	+	-	-	-	-	-	-
50X Mutant Oligo Competition:	-	-	-	+	-	-	-	-	-
Pre-cleared with Anti-C-Fos Antibody:	-	-	-	-	+	-	+	-	-
Pre-cleared with Anti-C-Jun Antibody:	-	-	-	-	-	+	+	-	-

AP-1 Complex



1 2 3 4 5 6 7 8 9