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THE FUNCTION AND MECHANISM OF CHMP1A IN TUMOR DEVELOPMENT

Thesis submitted to
the Graduate College of
Marshall University

In partial fulfillment of
the requirements for the degree of
Master of Science
in Biological Sciences
by

Jing Li

Committee Members:

Dr. Simon Collier, Committee Chairperson
Dr. Maiyon Park
Dr. Guozhang Zhu

Marshall University
May 2008

ABSTRACT

THE FUNCTION AND MECHANISM OF CHMP1A IN TUMOR DEVELOPMENT

By Jing Li

Chmp1A (Chromatin modifying protein 1A/Charged multivesicular protein 1A) is a member of the ESCRT-III (Endosomal Sorting Complex Required for Transport) family. ESCRT complexes play central roles in endosome mediated trafficking via MVB (multivesicular body) formation and sorting. Chmp1A is a potential tumor suppressor, especially in the pancreas. Knockdown of Chmp1A resulted in an increase of anchorage-independent growth of HEK 293T cells. Chmp1A shRNA expressing HEK 293T cells transformed these non-tumorigeneic cells to form tumors in xenograft assays. Doxycycline inducible over-expression of Chmp1A in human pancreatic ductal tumor cells (PanC-1) induced growth inhibition *in vitro* and *in vivo* xenograft assays. Knockdown of Chmp1 via short hairpin RNA (shRNA) in PanC-1 cells promoted cell growth. Over-expression of Chmp1A strongly increased the protein level of pan-P53 and phospho-P53.

All-trans retinoic acid (ATRA) and its derivatives play an important role in regulating proliferation. Cellular retinol-binding protein (CRBP-1) is a key regulator of ATRA through controlling ATRA metabolism and nuclear localization. Chmp1A positively regulated CRBP-1 at mRNA level. To investigate the specific role of Chmp1A in ATRA signaling, ATRA responsive and non-responsive pancreatic tumor cells were treated with ATRA *in vitro*. Growth assays were performed and confirmed the previously reported growth inhibitory activity of

ATRA. In the ATRA responsive cell line, ATRA treatment apparently increased the expression of Chmp1A, CRBP-1, phospho-P53 and pan-P53. ATRA also facilitated translocation of Chmp1A into the nucleus. The knockdown of Chmp1A via shRNA abolished the growth inhibition of ATRA on pancreatic cancer cell lines. Taken together, our data indicates that Chmp1A is a potential tumor suppressor and Chmp1A is indispensable for anti-proliferative action of ATRA in pancreatic cancer cell lines; Chmp1A may mediate ATRA signaling by regulating the expression of CRBP-1, and P53.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS / NOMENCLATURE

ATRA	all-trans retinoic acid
AMP	ampicillin
bp	base pair
Chmp1A	chromatin modifying protein 1A charged multivesicular protein 1A
CRBP-1	cellular retinol binding protein-1
CRBP-1	cellular retinol binding protein-2
CRABP-1	cellular retinoic acid binding protein-1
CRABP-1	cellular retinoic acid binding protein-2
ddH2O	double distilled H2O
dH2O	distilled H2O
DEPC	diethylpyrocarbonate
DMEM	dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
dNTP	deoxyribonucleotide triphosphate
DNA	deoxyribonucleic acid
Dox	doxycline
E2Fs	elongation factor-2s
EDTA	ethylenediamine tetraacetic acid
ESCRT	endosomal sorting complex required for transport
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
hTERT	telomerase reverse transcriptase
kb	kilobase
LT	SV40 large antigen
MDM2	murine double minute
MVBs	multivesicular bodies
MCS	multicloning site
O.D.	optical density
PBS	phosphate buffered saline

pRb	phospho-retinoblastoma protein
RT-PCR	reverse transcriptase-polymerase chain reaction
RAR	retinoid alpha receptor
RNA	ribonucleic acid
RXR	retinoid X receptor
ST	SV40 small antigen
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAE	tris-acetate-EDTA
TE	tris-EDTA
TET	tetracycline
TGN	*trans* Golgi network
TBS	tris-buffered saline
VPS	vacuolar protein-sorting

Chapter1

Chmp1A Is Involved in Anti-proliferative Effects of All-trans Retinoic Acid *in vitro* in Pancreatic Cancer cells

Abstract: All-trans retinoic acid (ATRA) and its derivatives play an important role in regulating proliferation. Cellular retinol-binding protein 1 (CRBP-1) is a key regulator of ATRA through its ability to control ATRA metabolism and nuclear localization. ATRA inhibits tumor growth by regulating genes such as p53, a known tumor suppressor that is mutated in many human cancers. Microarray studies using HEK 293T cells showed that Chmp1A (Chromatin modifying protein/Charged multivesicular protein) positively regulated CRBP-1 at the mRNA level, indicating a potential involvement of Chmp1A in ATRA signaling. To test this hypothesis, we treated ATRA responsive and non-responsive pancreatic tumor cells with ATRA *in vitro*. Growth assays were performed and they confirmed the previously reported growth inhibitory activity of ATRA. In the ATRA responsive cell line, ATRA treatment significantly increased the protein expression of Chmp1A, CRBP-1, P53 and phospho-P53 at serine 15 and 37 position. We found that knockdown of Chmp1A via shRNA abolished the growth inhibitory effect of ATRA and promoted the growth of PanC-1 cells. Furthermore, Chmp1A shRNA treatment diminished the increase of Chmp1A, CRBP-1, P53 and phospho-P53 protein expression induced by ATRA treatment. In the ATRA non-responsive cells, however, ATRA treatment did not have any effect on the protein level of Chmp1A, CRBP-1, P53 and phospho-P53. Interestingly, ATRA treatment facilitated translocation of Chmp1A into the nucleus in ATRA responsive cells. In the ATRA-non-responsive cells, Chmp1A

expression appears to be localized mainly at the plasma membrane in the presence or absence of ATRA. Collectively our data provide evidence that Chmp1A mediates the growth inhibitory activity of ATRA in pancreatic cancer cells through positively regulating CRBP-1 expression and P53 activity. Our results also suggest that nuclear localization of Chmp1A is important in mediating ATRA signaling.

Review of the literature

Retinoic Acid

Retinoids are natural or synthetic derivatives of vitamin A. Retinoids exert their biological effects by both retinoid receptors and retinoid-binding proteins. On the one hand, the active form of retinoids (ATRA or 9-*cis*-RA) in nucleus interacts with their nuclear receptors, retinoic acid receptors (RARα, β,and γ), and retinoic X receptors (RXRα, β, and γ), and each receptor has several isoforms [1, 2]. There has been major progress in understanding the interactions between retinoids and their receptors. RARs preferentially bind RXRs to form a heterodimer [3-5]. However, RXRs bind other receptors including a number of orphan receptors [6]. Binding of retinoid to RAR, not RXR in the heterodimer allows RXR to interact with its ligand, which results in synergistic increase in target gene transcription [7, 8]. All-trans retinoic acid (ATRA) is one of the most active and physiological members of the retinoid family. By binding to its receptor RAR, ATRA effects broad spectrum of biological processes such as proliferation and differentiation [9]. Because of anti-proliferative effects of ATRA, it has been used as therapeutic and/or preventive agent in certain cancers such as leukemia [10].

Cellular retinol-binding protein-I

On the other hand, the retinoid-binding proteins are composed of two cellular retinol-binding proteins (CRBP- I and II) and two cellular retinoic acid-binding protein isoforms (CRABP- I and II) [11]. Each has a different expression pattern and function (Fig.1). Cellular retinol-binding protein-I (CRBP-I) is a key regulator of ATRA through its ability

to control ATRA metabolism and nuclear localization [11-13]. The regulation of tumor growth associated with CRBP-I have been proven to be RARs-dependant [13, 14].

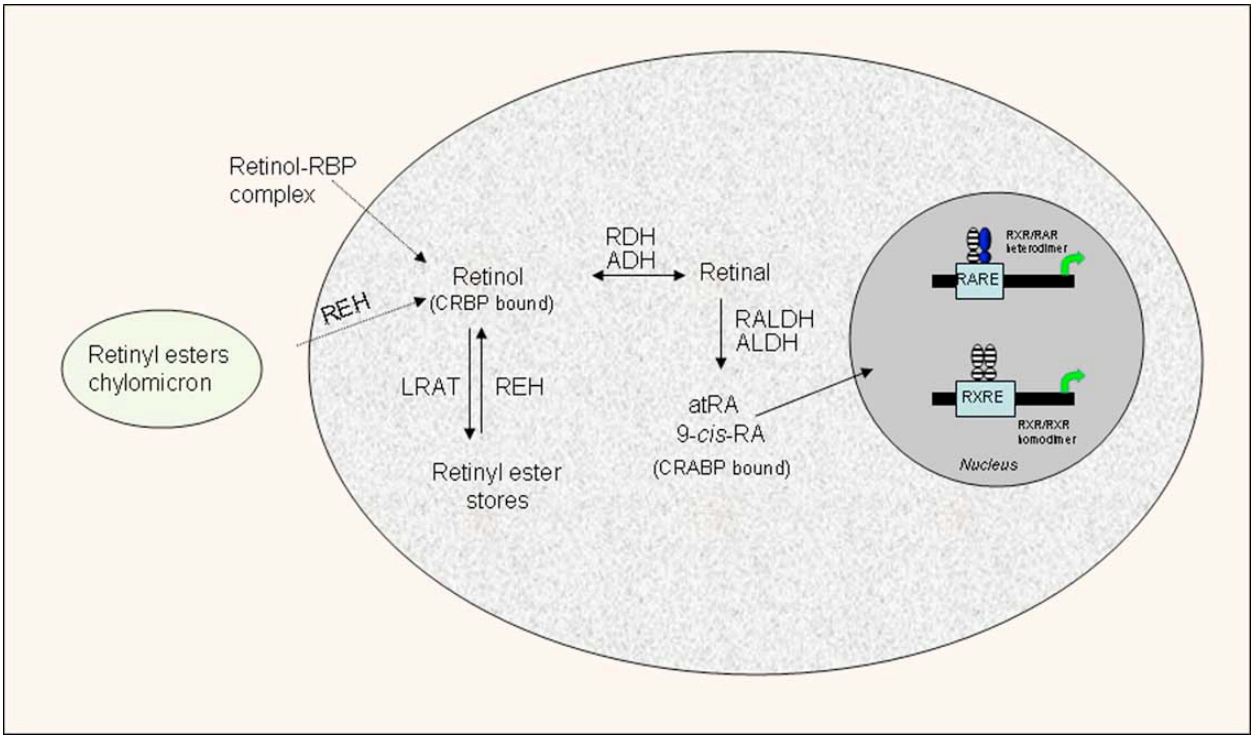

Figure 1. Metabolism of vitamin A in target cells to biologically active retinoic acid. Once in the nucleus the retinoid signal is transduced by means of gene expression by two families of nuclear receptors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). –Dr. Kenneth J. Soprano's Lab [15]

Pancreatic Cancer

Despite recent insights into the molecular basis of pancreatic cancer, the prognosis for this disease remains extremely poor [16]. In 2006, the American Cancer Society reported that the survival rate of pancreatic cancer patients is 20% for 1-year and only 4% for 5-year for all stages combined. Cancer Statistics, 2007 [17] predicted that pancreatic cancer will be the 4th causes of cancer death, following lung and bronchus,

colon and rectum, and breast, based on American Cancer Society estimation. However, the survival rate of pancreatic cancer patients is the lowest among all the cancer types combined [17] . Therefore, development of new efficient anticancer therapy is imminently needed to increase survival rate of pancreatic cancer patients.

Chmp1A

Chmp1A/Vps46p belongs to class E family of vacuolar protein sorting (Vps) and has different names such as Chromatin Modifying Protein1 (Chmp1), Pcoln3 (Procollagen (type III) N-endopeptidase), and Sal1 (Supernumerary Aleurone Layers 1) [19-21]. For simplicity we will refer this protein “Chmp1A” in this report. Chmp1A has been reported to silence gene activation by interacting with a transcriptional repressor Polycomb-group (PcG) protein, BMI1 [18-21]. Chmp1A has also been shown to physically associate with the multivesicular sorting protein, SKD1/VPS4 (Vacuolar Protein-Sorting 4) [19]. Chmp1A localizes at the endosomes, where it functions in vesicle sorting and MVB (multivesicular body) formation [19]. In maize, a mutation in Chmp1A homolog sal1, results in more aleurone cell layers, suggesting that Sal1 might play a role in cell growth [22]. Collectively these studies indicate that Chmp1A may play critical roles in development by controlling cell growth and signaling activity via MVB formation/transcription repression [23]. However, the functions and signaling activities of Chmp1A in vertebrates have not been investigated.

INTRODUCTION

Retinoids are natural or synthetic derivatives of vitamin A. Nuclear receptors and cellular binding proteins are involved in mediating the biological effects of retinoids [4, 15, 24, 25]. The active form of retinoids (ATRA or 9-*cis*-RA) interacts with their nuclear receptors, retinoic acid receptors (RAR α, β, and γ), and retinoic X receptors (RXR α, β, and γ). All-trans retinoic acid (ATRA) is one of the most physiologically active members of the retinoid family. By binding to its receptor RAR, ATRA exercises a broad spectrum of biological effects such as proliferation and differentiation [9]. Because of anti-proliferative effects of ATRA, it has been used as a therapeutic and/or preventive agent in certain cancers such as promyelocytic leukemia [10].

There are two cellular retinol-binding proteins; CRBP- I and II [11]. Cellular retinol-binding 1 controls ATRA activity by presenting the retinol to various enzymes for retinoic acid synthesis [11-13]. CRBP-I protein expression was previously shown to correlate with tumor growth [13, 14]. P53, a known tumor suppressor, is mutated in most tumors including pancreatic cancer. The mutation of P53 causes further mutation of the P53 gene itself, an increase in an ubiquitin-dependant degradation of P53 mediated by MDM2 [26], and an inactivation of P53 [27]. p53 gene located at chromosome 17; P53 has a DNA binding domain and acts as a “genome gatekeeper” [28]. The inherited loss of one allele of p53 [29, 30] usually results in several independent tumors in early adulthood (Li-Faumeni syndrome) [31].

Chmp1A (Chromatin Modifying Protein 1A/Charged multivesicular protein 1A) belongs to the class E family of Vps and is also called Vps 46p [19, 23, 32-35]. Chmp1A was shown to physically associate with the multivesicular sorting protein, SKD1/VPS4

(Vacuolar Protein-Sorting 4), with AMSH, an endosome-associated ubiquitin isopeptidase, and with VPS4 ATPases [19]. Chmp1A localizes at the endosomes, where it functions in vesicle sorting and multivesicular body (MVB) formation [19]. Chmp1A has also been reported to silence gene activation by interacting with a transcriptional repressor Polycomb-group (PcG) protein, BMI1 [18, 20, 21, 36]. In maize, mutation in the Chmp1A homolog sal1, results in more aleurone cell layers, suggesting that Sal1 might play a role in cell growth [22]. Collectively these studies indicate that Chmp1A may play critical roles in vertebrate development by controlling cell growth and signaling activity via MVB formation/transcription repression [23]. However, the functions and signaling activities of Chmp1A in vertebrates have not been investigated.

Pancreatic cancer has the worst prognosis of all cancers with a dismal 5-year survival rate. ATRA alone or in combination with other chemotherapeutic reagent has been successful in treating tumors [37-39]. Preclinical studies using ATRA for treating human pancreatic cancer suggest this compound might useful for treatment. The molecular mechanism by which ATRA inhibits growth of pancreatic cancer cells is not clear. This paper focuses on the role of Chmp1A in ATRA mediated growth inhibition. The objective of our study was to investigate whether Chmp1A expression and/or localization is essential for ATRA induced growth inhibition of human pancreatic tumor cells.

Materials and methods:

Antibodies and chemicals:

Rabbit polyclonal antibody against Chmp1A was generated in our laboratory by using recombinant Chmp1A protein (Belogortseva and Park, unpublished). Other antibodies were purchased from commercial sources: rabbit polyclonal antibodies against P53 (Cell Signaling) and Phospho-P53 (Cell Signaling); rabbit polyclonal antibodies against P53 phosphorylated at Ser37 (Cell Signaling), Ser15 (Cell Signaling); mouse monoclonal antibodies against Gapdh (Cell Signaling), and rabbit polyclonal antibody against CRBP-1 (abcam). Goat anti-rabbit/mouse HRP conjugated secondary antibody was purchased from Chemicon. All Trans Retinoic Acid was purchased from Sigma. Puromycin was from Invitrogen. All other chemical reagents were purchased from Sigma, unless otherwise described.

Cell Culture

All cell lines were obtained from American Type Culture Collection (Manassas, VA). PanC-1 (human pancreatic ductal tumor cells, poorly differentiated), CRL-2151 (mouse acinar tumor cells), and HEK 293T (human embryonic kidney, CRL-11268) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS, Gibco). Capan-2 (human pancreatic ductal tumor, well differentiated) cells were cultured in McCoy with 10% fetal bovine serum. All cell culture assays were performed at 37°C under 5% CO2.

RT-PCR

HEK 293Tcells (CRL-11268) were transiently transfected with Chimp1A-Cs2+ or control CS2+ plasmid using Lipofectamine and Plus reagent (Invitrogen). Total RNA was isolated 16 hours post-transfection using Trizol reagent (Invitrogen). Quality and quantity of isolated RNA was analysed using Bioanalyzer (Agilent). RT-PCR (reverse-transcriptase-PCR) was performed using Titan one tube RT-PCR system that was purchased from Roche. For each reaction, 500 ng of total RNA was reverse transcribed and used to amplify Chmp1A and CRBP-1. PCR products were separated on 1.5 % agarose gel containing ethidium bromide. The primer used for Chmp1A was: 5'–GAGACAGCGGGTCCGTAAC-3', and 5'–ACCTGGGCCATATTCTTGGT-3' for forward and backward primer respectively. CRBP-1 primer used in this experiment was described in Arapshian, A., et al., [40]. The cycling parameter for amplifying PCR products was: 94℃ for 2 min, 10 cycles of (94℃ for 30 second, 48℃ for 30 second, 68 ℃ for 1 min), followed by 15, or 18 additional cycles of (94℃for 30 second, 48℃ for 30 second, 68℃ for 1 min), and last step is 68℃ for 7min.

ATRA treatment:

PanC-1 and Capan-2 cells were seeded at 350,000 and 300,000 cells per 10 cm plate respectively. The next day, cells were replaced with fresh media containing either vehicle (DMSO) or ATRA (20 μM final concentration). We used 20 μM of ATRA as a working concentration since that concentration showed obvious growth inhibition. ATRA treated cells were kept in the dark since ATRA is light sensitive. Every other day, cells were given fresh media containing either DMSO or ATRA. The cells were cultured for

up to 5 to 6 days for the following experiments. For PanC-1 and CRL-2151 cells, the media was supplemented with 10% FBS containing antibiotics during ATRA treatment. For Capan-2, serum free media supplemented with antibiotics was used for ATRA treatment [41].

Generation of stable Chmp1A knockdown cell lines

PanC-1 cells were cultured in DMEM media supplied with 10% FBS. RNAintro^{TM} pSM2 retroviral vector (Open Biosystems) was used to subclone control and Chmp1A shRNAs. The shRNA sequence was designed by online software from Open Biosystems. This vector contains a puromycin-resistant marker site for positive colony selection. The specificity of Chmp1A shRNA was verified by transient transfection using Arrest-In transfect reagent (open system) followed by Western blotting. To generate stable cell lines, shRNAs targeted to Chmp1A or non-silencing control were transfected into PanC1 cells. Stable transfectants were selected in the presence of 2 ug/ml puromycin (Invitrogen), which was determined by kill curve. Cells derived from these transfectants were used for Western blotting to confirm the decrease of Chmp1A protein expression. Chmp1A knockdown stable Panc-1 cells were maintained in DMEM media supplied with 10% FBS containing 1ug/ml puromycin.

Western blot analysis

The cell lysates were prepared from cells using RIPA buffer plus complete mini protein inhibitor cocktail (Roche). Protein concentration of cell lysates was measured by BCA assay kit (Pierce). The cell lysates were subjected to 10% SDS-PAGE, and the proteins

were electroblotted to nitrocellulose membranes. After blotting the membranes were incubated with appropriate primary antibody followed by peroxidase-conjugated secondary antibody and was developed using the enhanced chemiluminescence kit (Amersham).

Confocal microscopic analysis

PanC1 and CRL2151 cells were seeded onto sterile glass coverslips in twelve-well plates at an approximate density of 0.5×105 cells/well in DMEM (Gibco) containing 10% FBS (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). These cells were incubated at 37° C with 5% CO2 (Gibco). The following day cells were treated with ATRA dissolved in DMSO (20 umol, final concentration). Following 24, 48 and 72 hours of incubation, cells were washed with 1 x cold PBS, and fixed with 4% formaldehyde in 1X PBS at room temperature for 30 min. Fixed cells were then washed with PBS before being permeabilized in PBS/0.1% Triton X-100 (Sigma) for 5-10 min at room temperature. Following permeabilization cells were washed three times in cold PBS and incubated in blocking solution (PBS, 10% heat inactivated FBS) for 30 min. Next, the cells were incubated with a primary antibody (Chmp1A antibody) for 3 hours. After being washed three times with 1 x PBS cells were incubated with anti-rabbit Alexa Flour 488 secondary antibody (Molecular Probes). In each case secondary antisera were used at a dilution of 1/300 in blocking buffer. Cells were washed in PBS and mounted on the slide using Vectashield (Vector Laboratories, Inc. Burlingame). Confocal images were taken by using a confocal laser scanning microscope (Carl Zeiss LSM510) at Marshall University Imaging Core.

Statistical Analysis

Statistical analysis was performed with Sigma Stat software using paired student test analysis. All numerical data are reported as the Mean ± SEM. P values less than 0.05 were considered statistically significant, and all P values are one-sided.

Results:

Overexpression of Chmp1A positively regulates CRBP-I expression

A microarray screen identified Chmp1A and cellular retinol binding protein-1 (CRBP-I) as up-regulated genes in HEK 293T cells overexpressing Chmp1A. Chmp1A (NM_002768) and CRBP-1 (NM_002899) showed a 9.44 and a 3.46 fold change respectively upon Chmp1A overexpression compared with a control. Reverse transcriptase PCR (RT-PCR) was carried out to verify the microarray data. As shown in Fig. 2, Chmp1A mRNA was increased by 2.2 fold at 25 cycles and 1.7 fold at 31 cycles compared to the control. CRBP-1 mRNA level was also increased by 1.3 fold at 25 cycle and 1.2 fold at 31 cycle compared to control. Consistent with the microarray data, the increase of Chmp1A mRNA was greater that the increase of CRBP-1 mRNA. Since CRBP-1 is involved in retinoid metabolism and function we developed a hypothesis that Chmp1A might be involved in ATRA signaling.

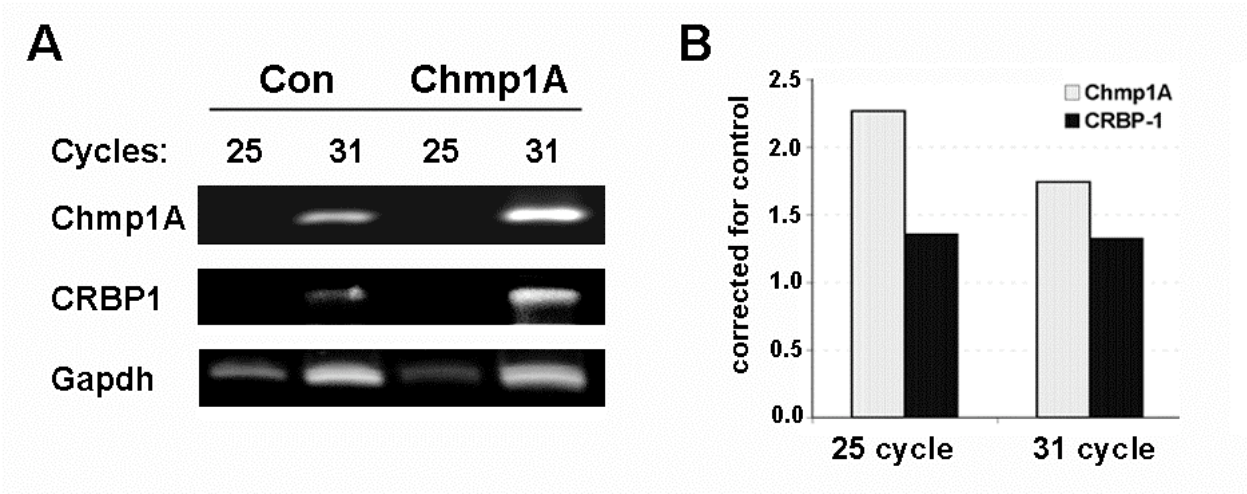

Figure 2. Chmp1A over-expression positively regulates CRBP-1 transcription. (A) Reverse transcriptase PCR (RT-PCR) indicates that CRBP-1 transcripts were increased at 25 and 31 cycles respectively. RT-PCR control Gapdh was amplified similarly in control and Chmp1A over-expression respectively. (B) Densitometric analysis reveals that CRBP-1 are up regulated at both 25 and 31 cycle by Chmp1A over-expression, compared with control that was set as 1 (not shown in the graph).

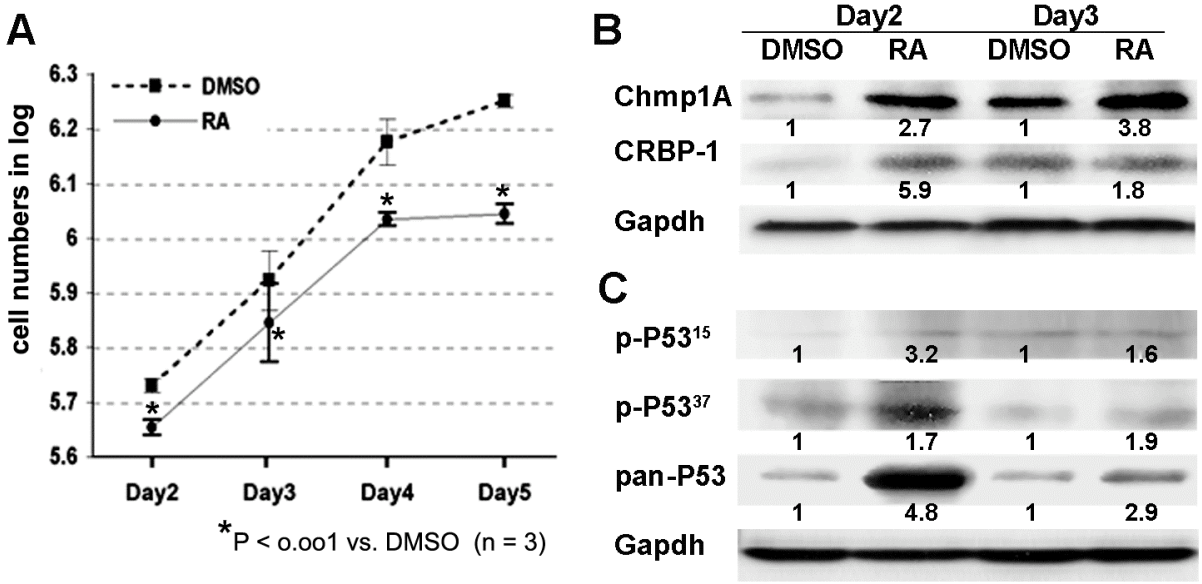

Figure 3. The growth inhibition of Capan-2 cells by ATRA was accompanied by an increase in the protein level of Chmp1A, CRBP-1, P53, and phospho-P53. (A) Growth

curve demonstrates that ATRA significantly decreases the growth of Capan-2 cells. Black line with square represents DMSO treated and gray line with circle represents ATRA treated. *t*-test was used to obtain P-value. (B) Upon ATRA treatment Chmp1A protein level was increased on day 2 and 3. CRBP-1 protein was up regulated highly on day 2 only and moderately on day 3. Gapdh protein indicates equal loading of protein samples. (C) P53 protein showed a strong 4.8 fold increase on day 2. The protein levels of phospho-P53 at serine 15 and 37 position were increased on both days. The fold difference was obtained by setting control DMSO as 1 on each day, as shown below the blots.

ATRA induced growth inhibition and increase of Chmp1A, CRBP-1, P53 and phospho-P53 protein levels in human pancreatic ductal tumor cells

Human pancreatic ductal tumor cells, Capan-2 and PanC-1 were treated with ATRA (20 μM) [42]. Capan-2 is poorly differentiated and PanC-1 is a highly metastatic ductal adenocarcinoma cell line. Cell number was counted on a daily basis for 5 days using a hemocytometer. ATRA treatment induced growth inhibition of Capan-2 and PanC-1 cells (Fig. 3A and 4A respectively) compared with vehicle DMSO treatment. This data is consistent with the results reported from other researchers [43-46]. To determine if Chmp1A is involved in the growth inhibition of ATRA, we performed Western blot analysis. As shown in Fig. 3B and 4B, the expression level of Chmp1A protein was increased upon ATRA treatment in both cell lines compared to control. Furthermore, CRBP-1 protein expression was increased in both cell lines upon ATRA treatment compared to control (Fig. 3B and 4B). ATRA is shown to regulate tumor growth by controlling a known tumor suppressor P53 [47-49]. Thus we investigated whether ATRA controls the expression of P53 and phospho-P53 in both ATRA responsive cell lines. Western blot analyses (Fig. 3C and 4C) demonstrated that ATRA increased the protein

expression of P53 and phospho-P53 (at Serine 15 and Serine 37, respectively) in ATRA responsive Capan-2 and PanC-1 cells. The fold increases of Chmp1A, CRBP-1, P53 and phospho-P53 (at serine 15/37) protein level compared with control was reported below each blot.

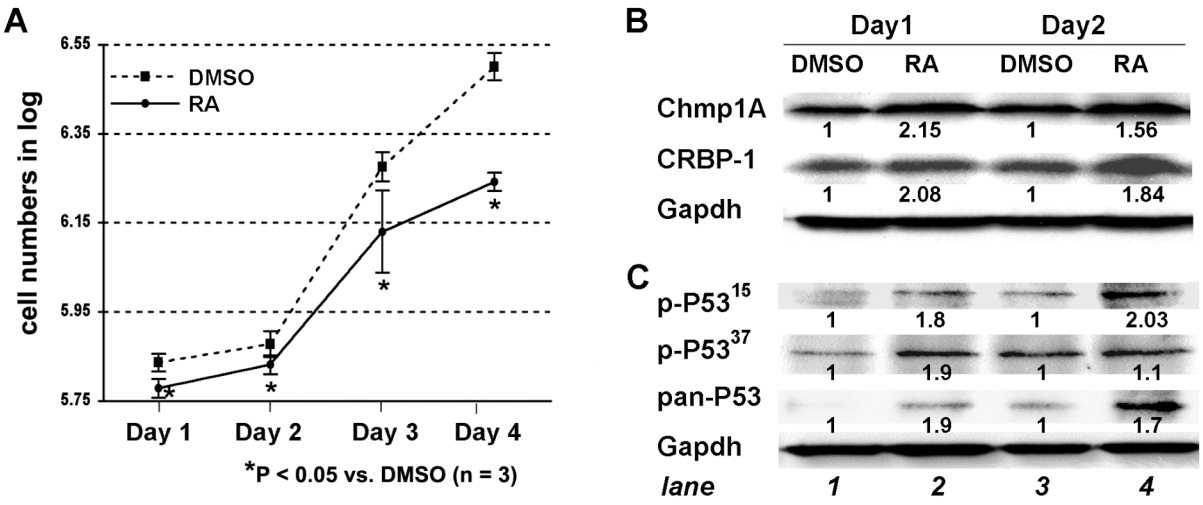

Figure.4. The growth inhibition of PanC-1 cells by ATRA was accompanied by an increase of Chmp1A, CRBP-1, P53 and phospho-P53 protein expression. (A) Growth curve demonstrates that ATRA induces growth inhibition of PanC-1 cells compared to DMSO vehicle treated cells. P value was obtained using *t*-test. (B) Chmp1A protein level was increased greater on day 1 than on day 2 by ATRA treatment. CRBP-1 also showed similar increase on day one and two upon ATRA treatment. (C) The protein levels of P53 and phospho-P53 at serine 15 and 37 position were increased both on day 1 and 2. The fold difference was obtained by setting control DMSO as 1 on each day, as shown below the blots.

Chmp1A, CRBP-1 and P53 protein level was not changed in ATRA non-responsive cells

To determine whether the increase of Chmp1A protein is linked with ATRA function we treated CRL 2151 cells with ATRA. This cell line was previously shown to be resistant to ATRA-mediated growth inhibition [50]. Consistent with previous reports ATRA did not inhibit growth of CRL 2151 cells (Fig. 4A). In ATRA-responsive cells Chmp1A protein expression was increased upon ATRA treatment (Fig. 3B and 4B). However, as shown in Fig. 5B, the Chmp1A protein level did not change upon ATRA treatment in this cell line. In addition, as shown in Fig. 5B, the expression level of P53 remained similar in ATRA treated cells compared to DMSO treated cells.

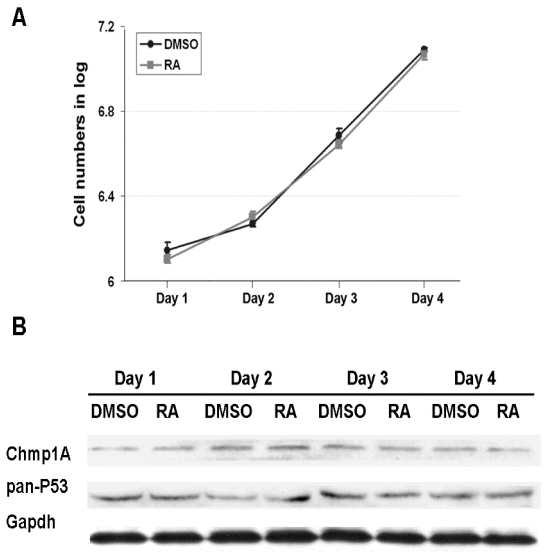

Figure 5. CRL-2151 cells are resistant to ATRA treatment in growth inhibition and did not exhibit the increase of proteins in Chmp1A and P53. (A) CRL-2151 cells did not show any difference in growth in the presence or absence of ATRA. Notice the similar growth pattern between DMSO treated and ATRA treated cells. (B) Western blot analysis reveals similar levels of Chmp1A and P53 protein expression in vehicle DMSO treated or ATRA treated cells

Chmp1A is required for ATRA mediated growth inhibition

Next we investigated whether Chmp1A is necessary for the growth inhibition of ATRA. We generated stable cell lines that express shRNA to knockdown Chmp1A in PanC-1 cells. Before we used the stable knockdown cells for growth assays, we determined the knockdown efficiency by Western blot analysis. Knockdown stable colony 1 and 2 (KD 1

and KD 2) showed a significant decrease of Chmp1A protein (Fig. 6A) by 72% and 85%, respectively compared to a control colony that stably expressed non-silencing shRNA. Since stable colony 2 showed greater knockdown efficiency we used this colony for the following growth analysis.

We treated cells, which stably express Chmp1A shRNA or control shRNA, with ATRA (or DMSO as control) and tested whether Chmp1A was required for growth inhibition of ATRA signaling. Similar to non-treated cells, the non-silencing shRNA expressing cells exhibited growth inhibition upon ATRA treatment (black line), compared with DMSO treatment (black dashed line) (Fig. 6B). On the other hand, the cells that stably express Chmp1A shRNA promoted cell growth in the presence of ATRA or DMSO. The growth promotion by Chmp1A knockdown was obvious in the DMSO treated cells (gray dashed line) as well as in the ATRA treated cells (gray line) compared with control. Importantly, ATRA treated PanC-1 cells that express Chmp1A shRNA revealed the same growth pattern as DMSO treated cells demonstrating that ATRA signaling is not translated to growth inhibition in the absence of Chmp1A. In addition, the growth was promoted in both ATRA and vehicle treated PanC-1 cells that express Chmp1A shRNA compared to the cells that express non-silencing shRNA. Our data indicates that Chmp1A knockdown mediates growth promotion, and that Chmp1A is indispensable for the growth inhibition of ATRA signaling.

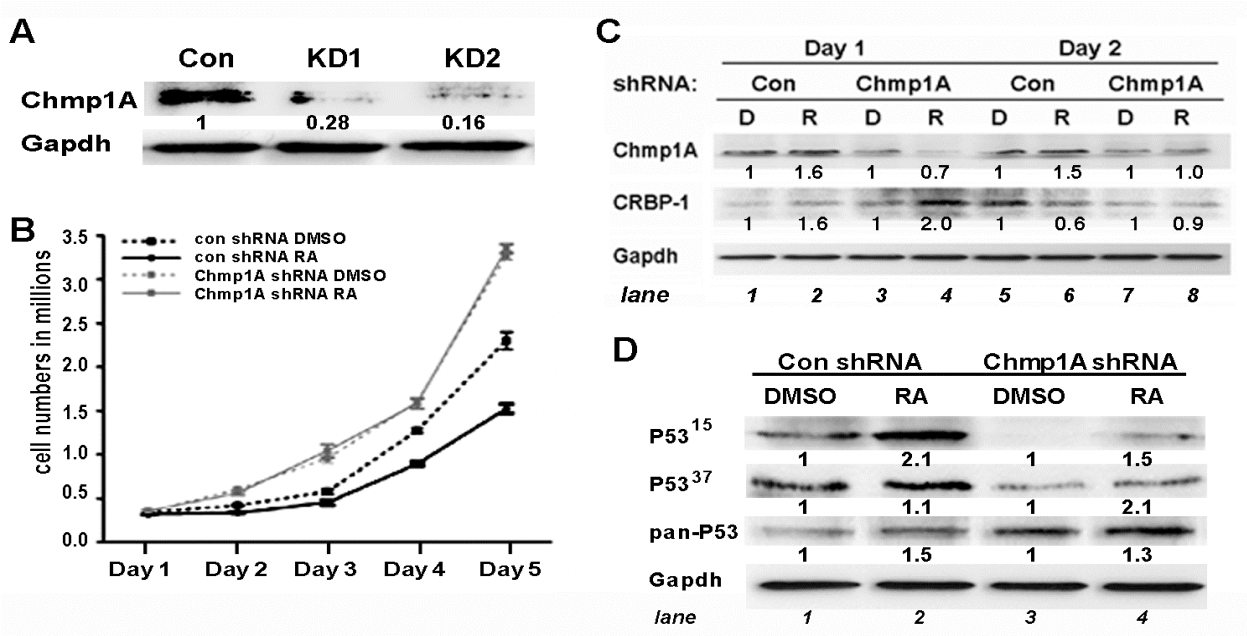

Figure 6. Chmp1A knockdown abolished ATRA mediated growth inhibition and the increase of protein levels of Chmp1A, CRBP-1, P53 and phospho-P53. (A) Two Chmp1A knockdown colonies (KD1 and KD2) of PanC-1 cells demonstrated the decrease of Chmp1A protein by 72% and 84% respectively compared to control. Non-silencing shRNA was used as control. Densitometric analysis was shown below the blots. (B) Control shRNA expressing cells showed growth inhibition upon ATRA treatment that is similar to non-transfected PanC-1 cells, as shown in Fig. 3A. However, Chmp1A knockdown cells did not exhibit any growth inhibition upon ATRA treatment. In addition, both ATRA and DMSO treated Chmp1A knockdown cells showed significant growth promotion compared to control cells. (C) Compared to control Chmp1A shRNA expressing cells showed robust reduction of Chmp1A protein in both DMSO and ATRA treated on both days. Compare Chmp1A expression in lane 3, 4 and 7, 8 to control lane 1, 2 and 5, 6. CRBP-1 expression was deceased only on day 2 in Chmp1A depleted cells upon ATRA treatment (compare the lane 8 to 7). Compared with control Chmp1A depleted cells showed the reduction of CRBP-1 in the presence of ATRA and DMSO (compare the lane 7, 8 to control 5, 6). (D) The protein expression of P53 was increased slightly by ATRA treatment in both control and Chmp1A shRNA expressing cells. However, Phospho-P53 expression at serine 15/37 in control cells were greatly reduced

in Chmp1A depleted cells (compare lane 3 and 4 to 1 and 2). ATRA treatment did not abolish the increase of Phospho-P53 expression at serine 15/37 in Chmp1A depleted cells (compare lane 4 to 3). The fold difference was obtained by setting control DMSO as 1 on each day and in each group (control shRNA and Chmp1A shRNA), as shown below the blots.

The knockdown of Chmp1A diminished the ATRA mediated increase in protein expression level of Chmp1A, CRBP-1, P53 and phospho-P53

We have shown that Chmp1A expression is increased upon ATRA treatment. Thus we tested if Chmp1A expression was changed upon ATRA treatment in Chmp1A depleted cells. In the control shRNA-expressing cells, Chmp1A expression was increased on day one and day two compared with control upon ATRA treatment (compare lane 2 and 6 with 1 and 5 respectively in Fig. 6C). However, in the Chmp1A shRNA-expressing cells, Chmp1A expression was either decreased (compare lane 4 to 3 in Fig. 6C) or remained the same (compare lane 8 to 7 in Fig. 6C) upon ATRA treatment. Chmp1A protein level was decreased upon both ATRA and DMSO treatment in Chmp1A depleted cells in comparison with that in control, verifying the knockdown of Chmp1A in these cells (compare lane 3, 4 to lane 1, 2 and lane 7, 8 to lane 5, 6 respectively).

Our data suggest that Chmp1A mediates ATRA signaling through the transcriptional activation of CRBP-1 (Fig. 2A and B). Thus we investigated the CRBP-1 protein expression in Chmp1A knocked-down stable cells. On day one CRBP-1 protein was increased in control as well as Chmp1A shRNA expressing cells upon ATRA treatment compared with DMSO treatment (compare lane 4 and 2 to lane 3 and 1 respectively). The fold increase of CRBP-1 in these cells was similar to that was shown in non-treated PanC-1 cells (2 fold increase in Fig. 3B compared to 1. 6 and 2 fold

increase in Fig. 6C). On day two, however, the up-regulation of CRBP-1 by ATRA was abolished in the Chmp1A depleted cells compared with control, from 1.6 and 2.0 fold increase to 0.6 and 0.9 fold increase in control and Chmp1A-shRNA expressing cells that were treated with ATRA (compare lane 6 and 8 to lane 2 and 4 in Fig. 6C). In addition, in Chmp1A depleted cells, CRBP-1 expression was not increased upon ATRA treatment compared with DMSO treatment on day 2 (compare lane 8 to 7 in Fig. 6C).

Next we tested whether Chmp1A knockdown affected P53 activity by determining the level of total P53 and phospho-P53 at serine 15/37. Since we observed the significant changes in the expression of these proteins on day one we showed the results obtained on day one. In control shRNA expressing cells, ATRA treatment elevated the P53 and phospho-P53 expression (6D). In control expressing cells, the fold increases in P53 and phospho-P53 at serine 15 was similar to that we obtained from non-treated PanC-1 cells (compare lane 2 in Fig. 6D to lane 2 in Fig. 4C). Both DMSO and ATRA treatment decreased phospho-P53 expression at serine 15/37 in Chmp1A shRNA expressing cells compared with non-silencing shRNA expressing cells (compare P53 in lane 4 to lane 2 in Fig. 6D). However, it appears that the knockdown of Chmp1A did not abolish the ATRA mediated increase in total P53 expression (compare lane 4 to lane 2 in Fig. 6D).

Nuclear expression of Chmp1A is important for ATRA mediated growth inhibition

ATRA is known to exert its effect via interacting with its nuclear receptors [4, 51]. Chmp1A protein is shown to be distributed in the cytoplasm and nucleus. It is possible that ATRA regulates cellular growth by enhancing the nuclear localization of Chmp1A.

We tested this hypothesis by determining the subcellular distribution of Chmp1A in the presence of ATRA or vehicle DMSO. Confocal microscopic analysis demonstrated that Chmp1A protein is ubiquitous with stronger staining in the nucleus in ATRA responsive PanC-1 cells. When cells were treated with ATRA, Chmp1A staining was increased especially in the nucleus compared to control (Fig. 7. A b,d compare with A a, c). In ATRA non-responsive cells Chmp1A protein was also ubiquitous initially, and up to day 2 with or without ATRA treatment (Fig. 7. B a, b). By day three, Chmp1A was recruited into the plasma membrane and remained at the membrane in both ATRA and DMSO treated cells (Fig.7. B c, d).

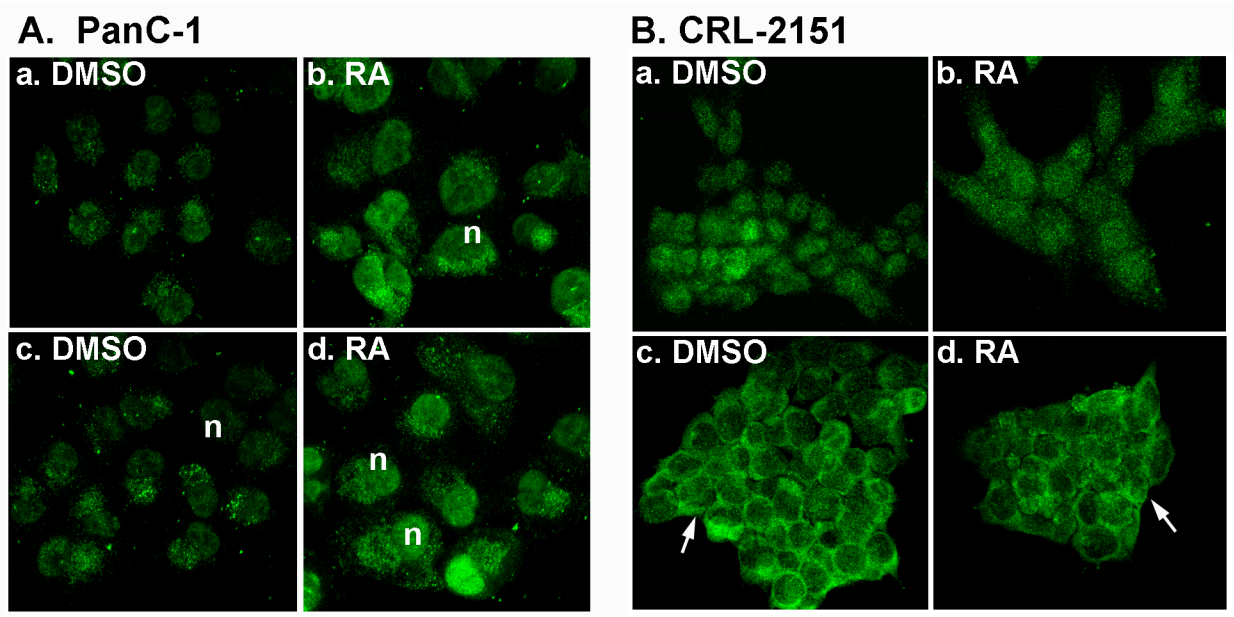

Figure 7. Chmp1A protein was translocated to the nucleus in PanC-1 cells upon ATRA treatment, but recruited to the membrane in CRL-2151 cells in the presence or absence of ATRA. (A) In the presence of DMSO, Chmp1A protein was modestly detected in the nucleus and cytoplasm in PanC-1 cells (a, c). However, Chmp1A protein expression became robust in the presence of ATRA, especially in the nucleus (n in c, d). (a, b) and (c, d) is one and two days after vehicle or ATRA treatment respectively. (B) Chmp1A was initially distributed ubiquitously in CRL-2151 cells in the presence of ATRA or

DMSO (a, b). From day 3 on, however Chmp1A protein was mainly detected and remained at the membrane in both DMSO and ATRA treated cells (arrows in c, d). (a, b) is for day two and (c, d) is for day three after DMSO and ATRA treatment respectively.

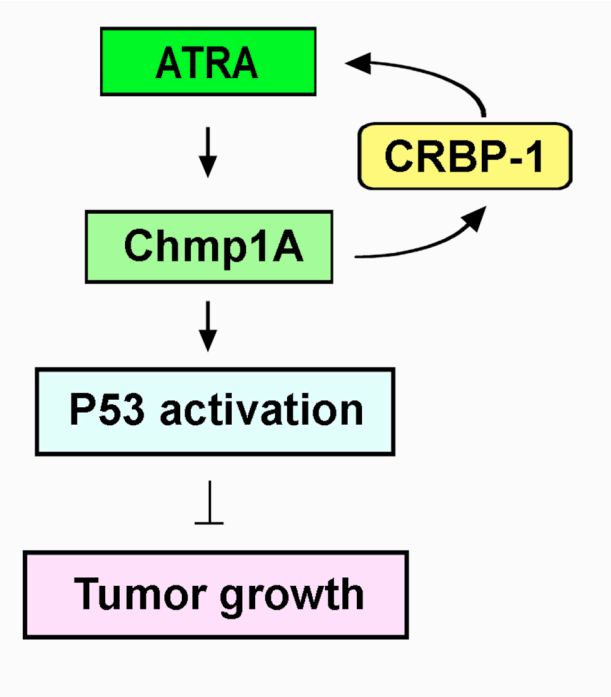

Figure 8. Model: Chmp1A mediated ATRA signaling amplification. Chmp1A increases the expression of CRBP-1. In turn, CRBP-1 controls the activity of ATRA via regulating the storage and metabolism of retinol A. ATRA increases the expression level of Chmp1A, which increases total and 'active' P53 resulting in a decrease in cell proliferation.

DISCUSSION

In this study we provide a new insight into the function and mechanism of the ESCRT family by studying Chmp1A, a member of ESCRT-III family. As shown in our model in Fig. 8, Chmp1A regulates CRBP-1 expression. In turn, CRBP-1 regulates the availability of ATRA via controlling the storage and metabolism of retinol A [11, 52]. ATRA then increases the protein level of Chmp1A, which activates its own expression as shown in our RT-PCR and Western blot analyses. This model proposes a positive amplification of ATRA signaling resulting in inhibition of tumor cell proliferation mediated at least in part by Chmp1A.

CRBP- I binds retinol and is thought to carry this retinoid to various enzymes for its metabolism into retinoic acid [11]. The expression of CRBP-1 is reduced in various tumors including breast [53], prostate [54], ovarian [55], and endometrial carcinomas [56]. The decrease of cytoplasmic immunoreactivity of CRBP-1 is associated with the increase of tumor grade in endometrioid carcinomas [56]. In our study, we have shown that Chmp1A positively regulated the expression of CRBP-1. This protein localizes to lipid rafts, specialized membrane domains, where retinol is stored [13]. We have identified SPFH domain containing proteins as Chmp1A binding partners (Belogortseva and Park, unpublished). SPFH domain-containing proteins are also found in the lipid rafts of various membrane including plasma membrane and endosomes [57]. Thus it is possible that Chmp1A, by interacting with SPFH domain containing proteins at lipid rafts of various membranes, could have effects on CRBP-1 and retinol storage.

We examined the involvement of Chmp1A in ATRA signaling by determining the effect of ATRA on Chmp1A expression in pancreatic tumor cell lines. We chose two of ATRA responsive cell lines and one of ATRA non-responsive cell line for our experiments. Capan-2 and PanC-1 cells are poorly differentiated and highly metastatic human pancreatic ductal tumor cells respectively whose growth was inhibited by ATRA treatment. CRL-2151 is mouse pancreatic acinar tumor cell line that did not show growth inhibition upon ATRA treatment. Our results on ATRA-mediated growth inhibition of pancreatic tumor cells are consistent with the already published reports [41, 43, 50, 58]. However, the increase of Chmp1A upon ATRA treatment in two ATRA responsive cell lines is a novel finding. Our observation that ATRA increased iP53 and phospho-

P53 expression also agrees with several reports demonstrating that ATRA inhibits tumor growth in part by regulating P53 expression and activity [49, 59].

We tested whether Chmp1A is required for ATRA induced growth inhibition by using shRNA mediated stable knockdown of Chmp1A. Clones of PanC-1 cells that heavily reduced Chmp1A protein levels were resistant to ATRA-induced growth inhibition. In addition, Chmp1A knockdown resulted in increased growth of pancreatic ductal tumor cells relative to cells expressing control shRNA. These results suggest that Chmp1A mediates the effect of ATRA on cell proliferation, at least in this human pancreatic cancer cell lines. We are currently testing whether Chmp1A might inhibit tumor growth in ATRA-independent manner. Knockdown of Chmp1A also resulted in the decrease of protein levels of CRBP-1, which is consistent with our model (Fig. 8) suggesting the positive effect of Chmp1A on CRBP-1. In addition, the decrease of P53 and phospho-P53 (at serine 15/37) by Chmp1A knockdown is consistent with model that proposes regulation of P53 expression and activation by Chmp1A.

This model is further supported by our experiments with ATRA resistant cells. Since human pancreatic acinar tumor cell lines are not available we used mouse acinar tumor cells for this assay. We hypothesized that ATRA non-responsive cells should not show any difference in Chmp1A or CRBP-1 protein level. Our data indeed demonstrated that the expression of Chmp1A or CRBP-1 did not change upon ATRA treatment. In addition, we did not observe any change in the protein expression of P53 suggesting that ATRA regulation of P53 expression and activity is important for inhibition of cell proliferation.

We investigated whether ATRA had any effect on the nuclear localization of Chmp1A. In the absence of ATRA, Chmp1A was expressed ubiquitously in the cytoplasm and the nucleus (Fig. 7) in both ATRA responsive and non-responsive cells. However, Chmp1A protein had stronger nuclear localization upon ATRA treatment in the responsive cells. In the ATRA non-responsive cells, addition of ATRA resulted in translocation of Chmp1A to the plasma membrane. These results indicate that nuclear localization of Chmp1A might be important for its ability to regulate cell proliferation ATRA signaling activity.

In summary we have novel findings that support a role for Chmp1A in mediating ATRA signaling induced growth inhibition of human pancreatic ductal tumor cells.

Chapter2

IN VIVO AND *IN VITRO* INVESTIGATION OF CHMP1A IN TUMOR DEVELOPMENT

Abstract: Chmp1A (Chromatin modifying protein 1A/Charged multivesicular protein 1A) is a member of the ESCRT-III (Endosomal Sorting Complex Required for Transport) family. ESCRT complexes (0, I, II, and III) play central roles in endosome mediated trafficking via MVB (multivesicular body) formation and sorting. An increasing amount of data suggests that ESCRT complexes are also involved in broader cell signaling events such as cell cycle progression and tumor development. Using *in vitro* and *in vivo* model systems, we provide evidence that Chmp1A is a potential tumor suppressor, especially in the pancreas. The *in vitro* soft-agar assay demonstrated that shRNA mediated knockdown of Chmp1A resulted in an increase of anchorage-independent growth of HEK 293T cells. In addition, Chmp1A shRNA expressing HEK 293T cells transformed these non-tumorigeneic cells to form tumors in xenograft assays. To determine the role of Chmp1A in human tumor development we screened human cancer profiling arrays and human pancreatic tissue arrays. We found out that Chmp1A mRNA and protein is reduced in various human pancreatic tumors. Furthermore, we discovered that Chmp1A protein is either reduced or mis-localized in human pancreatic ductal tumors. To substantiate the data we obtained from cancer profiling arrays, we either over-express or knockdown the expression of Chmp1A and study its effect on PanC-1 cell and tumor growth *in vitro* and *in vivo* respectively. Doxycycline inducible over-expression of Chmp1A in human pancreatic ductal tumor cells (PanC-1) induced growth inhibition *in vitro* and *in vivo* xenograft assays. On the other hand, knockdown of

Chmp1 via short hairpin RNA (shRNA) in PanC-1 cells resulted in the elevation of cell growth *in vitro*. Mechanically, over-expression of Chmp1A strongly increased the protein level of pan-P53 and phospho-P53. Taken together, our data indicates that Chmp1A is a potential tumor suppressor, especially in pancreas and that Chmp1A regulates tumor growth in part through activation of P53.

Review of the literature

Tumor development

Cancer involves uncontrolled cell growth, invasion, and sometimes metastasis. Hyperplasia usually is benign proliferation, and self-limited, whereas neoplasm is a malignant hyperplasia, which is much more dangerous than hyperplasia for people. According to the origin, neoplasm is classified as two groups, one originated from epithelial is defined as carcinoma, and another one originated from mesenchymal is called stroma. Lymphoma and leukemia are derived from hematopoietic cells.

As we know, tumor formation is a multi-step process [60]. Multiple genetic alterations might be involved in tumor formation. According to Robert Weinberg, tumor must acquire six kinds of capabilities acquired, which are self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis [61].

At present, metastasis and angiogenesis of tumors is the hot spot of investigation in cancer biology field.

Cell transformation

Cell transformation is defined by changes of a cell that affects its morphology and physiology through natural or artificial mechanism or sources. In the cancer biology field, a transformed cell is able to survive without growth factors, and they do not require the anchorage to a solid support [62, 63]. The extent of cell transformation is usually measured by soft-agar assay [64, 65].

Definition of Tumor suppressor

In contrast with oncogenes, tumor suppressor genes are defined as the counterpart of oncogene that is able to slow down cell division and repair DNA mistakes. The tumor suppressor gene usually has a low expression level or is mutated in cancer. The known tumor suppressors include P53, RB, pTEN, PP2A, BRCA1, BRCA2, and APC.

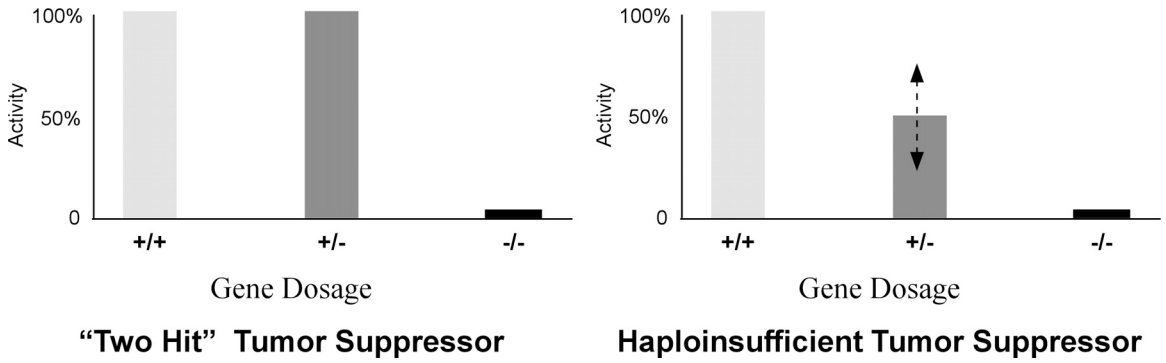

Fig.1. Tumor suppressor activitiy versus gene dosage. Wild type (+/+) activity represents 100% of diploid gene function and true null (–/–) represents complete loss of functionality. Haploinsufficient tumor suppressors exhibit a continuum of activity based on gene dosage with even 50% reduction sufficient for phenotypic manifestation, i.e. accelerated tumorigenesis. While some genes may be less dosage-sensitive than others (in which a true threshold of close to 0% of normal gene product is required in order to detect a phenotype), Christopher J. Kemp predict that most genes will be sensitive to dosage with some threshold that varies on a continuum between 0 and 100%. By Shannon R. Payne and Christopher J. Kemp [66]

Tumor suppressor genetics

There are two kinds of tumor suppressor [66]. The first one is "two hit" tumor suppressor (Fig.1), for example, RB, which involves dominant oncogene and recessive tumor suppressor. The later one is haploinsufficient tumor suppressor (Fig.1), where loss of a single allele can show a strong phenotype. This kind of tumor suppression by tumor

suppressor is gene-dose dependent. The example of this kind of genes includes P53 [67, 68], P27 [69].

P53

P53, a known tumor suppressor, mutated in most tumors including pancreatic cancer. The mis-regulation of P53 includes mutation of P53 gene itself, increased ubiquity-dependant degradation of P53 protein products mediated by murine double minute 2 (MDM2) [26], and some other situations which abolished the activity of P53. P53 might be the clearest protein which got investigated currently [27]. p53 gene is located on chromosome17. P53 protein has a DNA binding domain and act as “genome gatekeeper” [28]. The inherited loss of one copy of allele of p53 [29, 30] usually results in the several independent tumors in early adulthood, which is named as Li-Faumeni syndrome [31].

RB

The full name of RB is retinoblastoma protein-susceptibility gene, which is a prototypical tumor suppressor, which means it inhibits cell growth. Some reports indicated that RB also has important implications in apoptosis, and cell cycle by repression of elongation factor-2s (E2Fs) [70-72]. Hereditary retinoblastoma results from bi-allele loss of RB gene in embryonic retinoblasts. There is also inactivation of genetic mutations in the sporadic cancers.

The phosphorelated form of RB is usually considered as the inactive form of RB, degradation of RB also is one of inactivation mechanism of RB. In one word, this

inactivation mechanism of RB mainly comprised of phosphorylation, degradation, genetic mutation and viral inactivation [71, 73].

Rules for making tumor cells either in rodent or human cells

According to Robert Weinberg, Human embryonic kidney 293 cells expressing SV40 large antigen (LT), telomerase reverse transcriptase (hTERT), H-RAS, and small antigen (ST) have the characteristics of tumorigenesis, whereas cells expressing only LT, hTERT, and H-RAS are immortal but not tumorigenic [62, 74]. HEK293T/17 cells (CRL-11268) constitutively express the SV40 large antigen (LT) only, it is not tumorigenic [75, 76]. Mechanically, previous reports showed that SV40 large antigen is able to inactivate RB and P53.

Transformation of cultured cells is itself a multi-step process, for rodent cells at least two ectopic genetic alterations are required before they acquire tumorigenic competence [61]. However, it is more difficult to repeat this experiment in human cells and species-specific difference between mice and human do exist [77]. Until 1999, Hahn and Weinberg reported that Ras in combination with telomerase apparently were able to transform both human embryonic kidney cells and fibroblast cells into tumor cells by cell transformation assay *in vitro* and xenograft experiment *in vivo* [75]. Consequently, they also published one review which talked about rules of making human normal cells to tumor cells [78].

Introduction

The ESCRT complexes (Endosomal Sorting Complex Required for Transport, 0, I, II, III) mediate the lysosomal degradation of transmembrane proteins and are critical for receptor down regulation, and other normal and pathological cell processes [32, 79, 80]. Proteins, such as receptors at the membrane are internalized by endocytosis [81]. Some membrane receptor proteins (e.g. transferring receptors and low density lipoprotein) are recycled to the membrane, and others (e.g. mannose 6-phosphate receptors) enter into the *trans* Golgi network (TGN). In contrast, misfolded proteins and activated growth factor receptors are transported into the multivesicular bodies (MVBs), which will fuse with late endosome or lysosomes for protein degradation [82, 83]. Chmp1A is a member of the ESCRT-III complex. Mammalian orthologues of ESCRT-III components and their related proteins are collectively called Chmps. All Chmps reported to date have common features: they contain an approximately 200 amino acid long open reading frame (ORF), a coiled-coil region and charged residues, basic at the N terminus and acidic at the C terminus [80]. Chmp1A is a member of these structurally-related Chmp family proteins.

Chmp1A localizes at the endosomes, where it functions in vesicle sorting and MVB formation [19]. Chmp1A is peripherally associated with the membrane of both early and late endosomes and over-expression of Chmp1A alters endosomal structure. Chmp1A was shown to physically associate with the multivesicular sorting protein, SKD1/VPS4 (Vacuolar Protein-Sorting 4) [19]. The binding of Chmp1A to SKD1/VPS4 is shown to mediate ATP-dependent disassembly of the ESCRT-III complex, which leads to restructuring and/or dissociating from the membrane [19]. Recent studies

demonstrate that in addition to their endosomal function in protein sorting, ESCRT complexes have non-endosomal functions. In *Drosophila melanogaster*, genetic defects in Vps25 (Tsg101, ESCRT-I) are shown to cause loss of cell polarity in epithelial tissue, followed by cell autonomous and non-autonomous over-proliferation [84, 85]. Knockdown of mammalian homologue Vps25 (Tsg101) induces cell transformation and forms tumors in mice [86]. HCRP1 (Hepatocellular Carcinoma Related Protein 1, ESCRT- I [87]), a human homologue of Vps37P is frequently deleted in hepatocellular carcinoma (HCC) [88, 89]. In addition, it was shown that Chmp1A (ESCRT-III) functions in gene silencing by interacting with a transcriptional repressor Polycomb-group (PcG) protein, BMI1 [18-21, 36]. Over-expression studies in cultured cells demonstrated that the gene silencing of Chmp1A was due to its effect on chromatin structure [36]. A maize homolog of Chmp1A named SAL1 (supernumerary aleurone layers 1) has been shown to regulate the formation of the aleurone cell layer [22]. Mutation in *sal1* results in more aleurone cell layers, suggesting that Sal1 might play a role in cell growth.

These studies indicate that Chmp1A, a member of ESCRT, may play critical roles in tumor development by controlling cell growth and by regulating signaling activity via MVB formation [19, 36, 90]. However, its functions and signaling activities in tumor development have not been explored. In this paper we provide evidence that Chmp1A functions as a tumor suppressor, especially in pancreas.

MATERIALS AND METHODS

Cell lines and culture

All cell lines were obtained from American Type Culture Collection (Manassas, VA). HEK293T (CRL-11268) and PanC-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS, Gibco). All cell culture was performed at 37°C under 5% CO2. Stable cell lines were cultured in conditional medium as indicated in the following.

Antibodies and chemicals

Rabbit polyclonal antibody against Chmp1A was generated in the lab by using recombinant Chmp1A protein (Belogortseva and Park: unpublished). Other antibodies were purchased from commercial sources: rabbit polyclonal antibodies against pan-P53 (Cell Signaling) and rabbit polyclonal antibodies against phospho-P53, which detect phosphorylated form of P53 at Serine 37 and serine 15 (Cell Signaling), and mouse monoclonal antibodies against Gapdh (Cell Signaling). Goat anti-rabbit/mouse HRP conjugated secondary antibody was purchased from Chemicon. All Trans Retinoic Acid were purchased from sigma. Puromycin was purchased from Invitrogen. Doxycycline was purchased from Clontech. All other chemical reagents were purchased from Sigma, unless otherwise described. To determine whether Chmp1A is differentially expressed in human tumors, we screened cancer profiling arrays II (Clontech) with Chmp1A probe that was generated from the cDNA plasmid (a gift from Dr. Stauffer [36]). The DIG high prime DNA labeling and detection starter kit II was used following the instructions from

the company (Roche). Clontech Cancer Profiling Array II hybridized with digoxigenin labeled human *Chmp1A* probe. The array was stripped and re-probed with human *Ubiquitin* (Ubi, control) probe.

Immunhistochemical analysis of high density Tissue MicroArrays (TMA)

The high-density Tissue MicroArrays (TMA) for human pancreatic tumors was obtained from BioChain (PACA0909, CA). The immunohistochemical analysis was performed based on the manufacture's direction. Briefly, this process includes: deparaffinization of paraffin-embedded tissues, blocking tissue sections, incubation with primary Chmp1A antibody followed by secondary goat anti-rabbit antibody, and color reaction with HRP substrate.

Generation of stable Chmp1A knockdown cell lines of HEK293T cells

PanC1 cells were cultured in DMEM media supplied with 10% FBS. RNAintro^{TM} pSM2 retroviral vector (Open Biosystems) was used to subclone control and Chmp1A shRNAs. The shRNA sequence was designed by online software from Open Biosystems. This vector contains a puromycin-resistant marker site for positive colony selection. The specificity of Chmp1A shRNA was verified by transient transfection using Arrest-In transfect reagent (open system) followed by Western blotting. To generate stable cell lines, shRNAs targeted to Chmp1A or non-silencing control were transfected into PanC1 cells. Stable transfectants were selected in the presence of 2 ug/ml puromycin (Invitrogen), which was determined by kill curve. Cells derived from these transfectants were used for Western blotting to confirm the decrease of Chmp1A protein expression.

Chmp1A knockdown stable HEK cells were maintained in DMEM media supplied with 10% FBS containing 1ug/ml puromycin.

Establishment of stable PanC1 cells expressing Chmp1A conditionally

Tet-On advanced inducible gene expression system was used to generate conditional stable PanC1 cell lines [91, 92] (Clontech). Transfection was performed with Clontechfectin (Clontech) according to the manufacturer's instructions. Stable transfectants were selected by incubation with Geneticin (700 ng/mL; G418; Invitrogen) for a first selection and hygromicin B(300 ng/mL; Clontech) for a second selection, PanC1 stable transfectants were selected by incubating the cells with Geneticin (500 ng/mL; G418; Invitrogen) as first and hygromicin B(200 ng/mL; Clontech) as second selection. Luciferase assays were used for confirmation of success of first transfection: PanC1 cells after the first transfection was transiently transfected with tet-luc plasmid using Clontechfectin (Clontech) according to the manufacturer's instructions. 24 hours after transfection, 50 ul of total 150 ul cell lysate was used to measure luciferase activity using Dual Luciferase kit (Promega) with Berthold Centro 960.

Western blot analysis

The cell lysates were subjected to 10% SDS-PAGE, and the separated proteins in the gel were electroblotted to nitrocellulose membrane. The membrane was incubated with peroxidase-conjugated secondary antibody and visualized by using an enhanced chemiluminescence kit (Amersham).

Anchorage-independent growth assay

HEK293T cells stably expressing Chmp1A shRNA (short hairpin RNA) were subjected to Western blot analysis to determine the knockdown efficiency of Chmp1A. Control shRNA stable transfectants were used as control. The transfectant with higher knockdown efficiency in Chmp1A expression was used in the assay. For anchorage independent experiments, stably trnasfected cells were counted and seeded into soft agar based on the manufacturer's directions (Cell Biolabs INC). The colonies were photographed and counted for statistical analysis after 7 days incubation.

Xenograft assays in nude/nude mice

Five-week old male specific, pathogen free athymic nude/nude mice were purchased from Jackson Laboratoris. 70% confluent cells, which either over-express or knockdown Chmp1A were replaced with fresh medium 3 to 4 hours before harvesting to remove dead and detached cells. Cells were washed 3 times with 1X PBS, dissociated by trypsin, and counted by cellometor (Nexcelom). Next, cells were pelleted, washed with 1X cold PBS and re-suspended to a concentration of 3×10^6 cells/ 300 μl. We cleaned the inoculation area with ethanol.

Cells were mixed and drawn into 1-cc syringe without a needle to avoid negative pressure which can cause cell damage. Cells were subcutaneously (s.c.) injected into the mid-scapular region of the nude mice using a 23-gauge needle [93, 94]. To induce Chmp1A over-expression fresh doxycycline (Dox, Clontech, 631311) in drinking water was supplied at a concentration of 200 μg/ml every other day. For control mice, fresh water without Dox was supplied. Once a tumor was detected, the tumor volume was

measured every three days with vernier calipers (Fisher Scientific). Tumor volume was calculated using the formula, Volume = length×width^2 × 0.5236 [95, 96]. When the tumors reached 2.0 cm in diameter, the mice were sacrificed and tumors were cut out for further investigation.

RESULTS

Reduction of Chmp1A protein increased anchorage- independent growth in HEK 293T cells

Chmp1A, a member of ESCRT, has been shown to function in cell cycle progression [36] and some ESCRT members have been shown to be involved in tumor suppression (references). Hence we investigated whether Chmp1A also has the ability to function as a tumor suppressor. To test this, we knocked-down Chmp1A stably via short hairpin RNA (shRNA) and examined its effect on the anchorage-

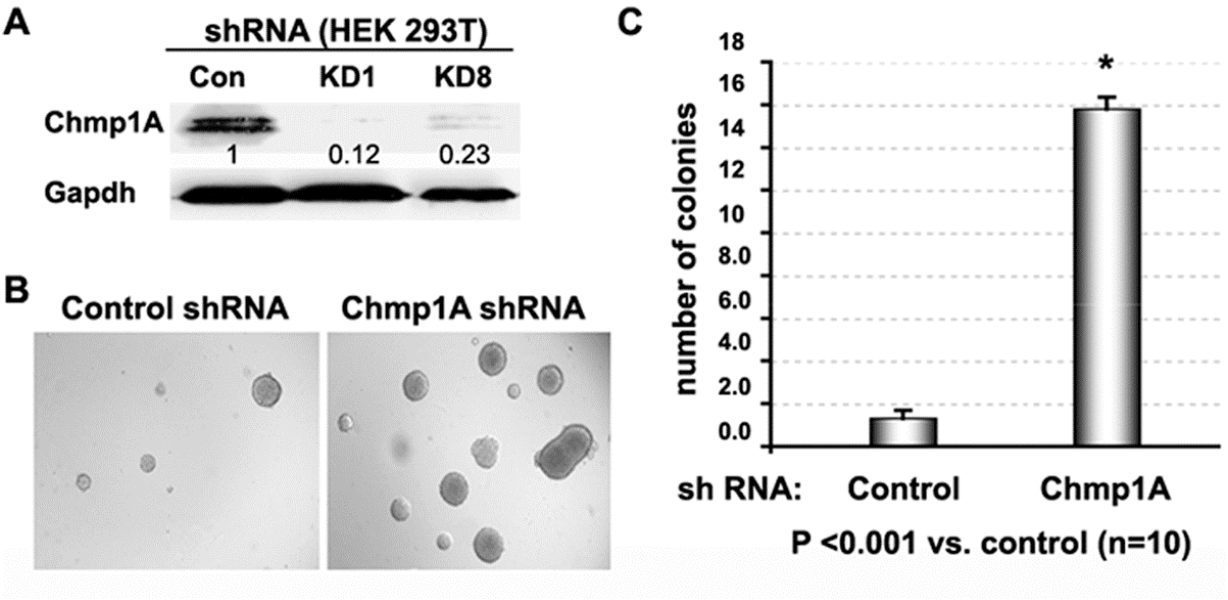

Fig. 2. Knockdown of Chmp1A expression increases anchorage-independent growth of HEK 293T cells. (A) The specificity of short hairpin RNA aimed to knockdown Chmp1A protein is shown. Compare with the Chmp1A protein level in control shRNA transfected. KD 1 and KD 2: knockdown colony 1 and 2. (B, C) The increase of the colony formation by Chmp1A knockdown is shown. The graph in (C) indicates about 30 fold increase of colony formation by Chmp1A knockdown.

independent growth of HEK 293T cells (human embryonic kidney cells). We carried out the assay using a Cytoselect^{TM} 96-well Transformation Assay kit. This kit allowed us to evaluate the anchorage-independent growth in a short period of time since colonies formed faster compared to the conventional method [75]. As shown in Fig. 2A, cells stably expressing Chmp1A shRNA decreased Chmp1A protein level by 70% compared to control cells expressing non-silencing shRNA in two independent colonies. Further, Chmp1A shRNA expressing cells revealed an increase in an anchorage-independent growth compared to control (Fig. 2Bb, compare with control Ba). The graph demonstrates that the knockdown of Chmp1A in HEK 293T cells increased an anchorage independent growth by almost 30 fold (Fig. 2C).

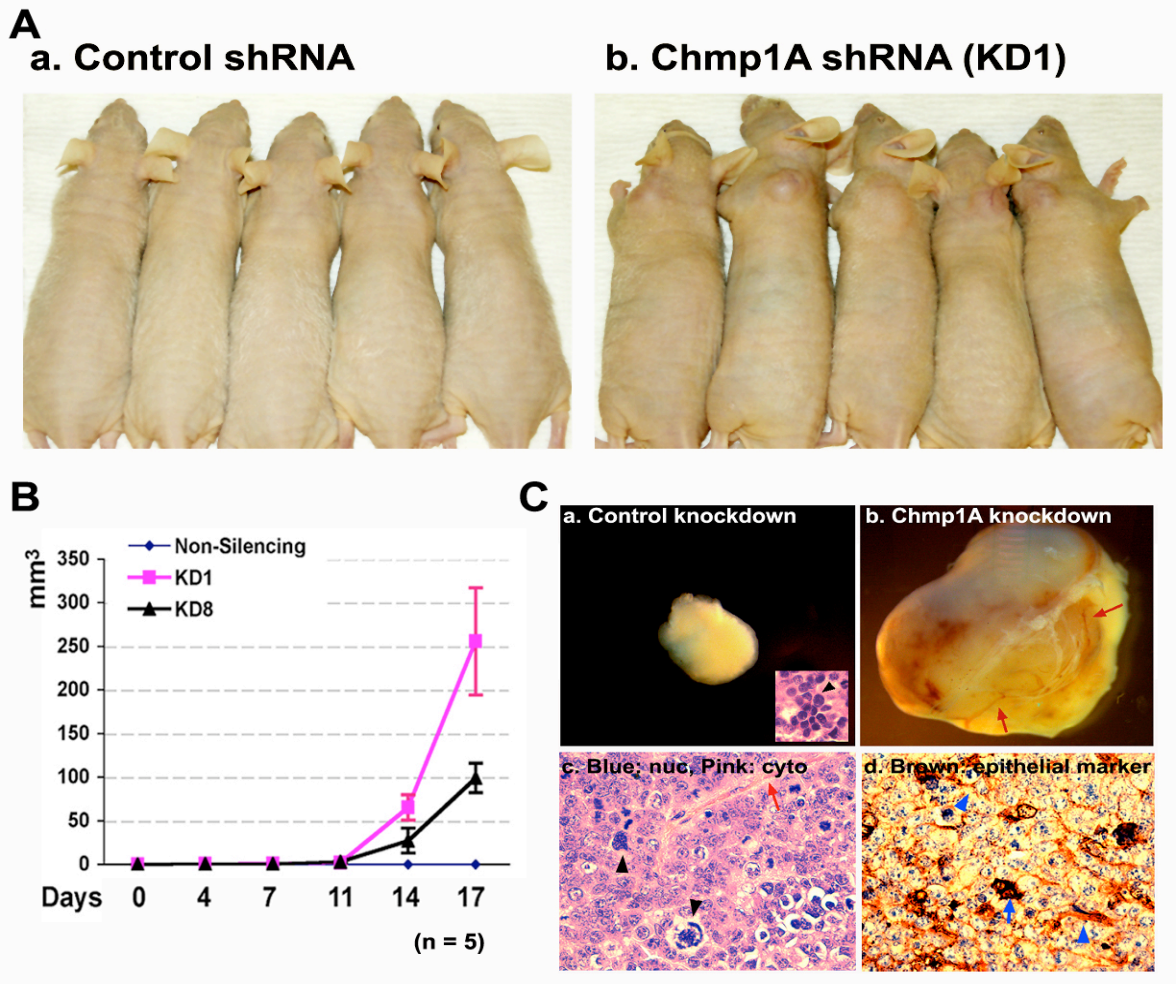

Fig. 3. Chmp1A shRNA expressing HEK 293T cells induce tumors in nude mice. (A) Both Chmp1A knockdown colonies induced tumors in nude mice. (B) The tumor volume induced by the injection of HEK 293T cells expressing knockdown colony 1 and 2 was plotted in a graph. Pink and blue represent KD 1 and KD 8 respectively. No tumor was formed by the injection of HEK 293T cells expressing non-silencing shRNA, (blue at the X-axis). (C) Tumors formed by Chmp1A knockdown developed new blood vessels (red arrows in Cb) compare with control (Ca). The tumor cells exhibit a high nuclear/ cytoplasm ratio, with multinucleated cells and abnormal mitosis (arrow heads in Cc). Blue and pink represent nucleus and cytoplasm respectively. Compare to Chmp1A knocked-down cells, control knocked-down cells remain primitive with no clear nuclear division (arrow head in the inset of Ca). Red arrows indicate blood vessel. The tumor shown appears to be epithelial in origin based on the epithelial marker, pan-Cytokeratin (Invitrogen). Most of the cells in the section express pan-Cytokeratin, either strongly (arrow) or mildly (arrow heads). Tumor was processed for paraffin embedding followed by sectioning for H&E (Hematoxylin and Eosin) and pan-Cytokeratin staining at the Pathology Department of Saint Mary's Hospital at Huntington, WV.

Transformation of HEK 293T cells to tumors by stable knockdown of Chmp1A in xenograft assay

The anchorage-independent growth assay demonstrated that the loss of Chmp1A elevated the colony formation in soft agar, indicating a potential transformation of cells by the Chmp1A knockdown. Thus we tested whether non-tumorigenic HEK 293T cells transformed to tumors when Chmp1A was stably knocked-down via shRNA *in vivo* using a xenograft assay. The nude mice injected subcutaneously with Chmp1A shRNA expressing HEK 293T cells developed tumors compared with the mice injected with HEK 293 cells expressing non-silencing shRNA. Fig. 3 A demonstrates that HEK 293T

cells expressing Chmp1A shRNA developed tumors in nude mice (Fig. 2a), compared to control (Fig. 3 Aa). The graph in Fig. 3B illustrates the average size of tumors formed by the knockdown of Chmp1A (pink and black lines for KD1 and KD8 respectively). Both Chmp1A knockdown colonies (KD1 and KD8) induced tumors although KD1 cells formed tumors with larger volume compared to KD8 cells. None of the mice injected with cells expressing control shRNA exhibited tumor formation (blue line). The tumors induced by Chmp1A knockdown showed evident neo-angiogenesis (red arrow in Fig. 3Cb). Tumor cells exhibited the characteristics of a high grade or poorly differentiated neoplasm such as high nuclear/cytoplasm ratio, multinucleation and abnormal mitosis (arrow heads in Fig. 3Cc). Control cells however remained primitive, showing no clear nuclear division (arrow head in the inset of Fig. 3Ba). In addition, the tumor shown appears to be epithelial in origin based on the epithelial marker, pan-Cytokeratin. Most of the cells in the section express pan-Cytokeratin, either strongly (arrow) or mildly (arrow heads in Fig. 3Bd). Fig.3A is data from 17days nude mice. Section is from 50 days nude mice tumor.

Chmp1A mRNA and protein expression in human pancreatic cancers

Courtesy of Dr.Park (data not shown)

To determine whether Chmp1A is implicated in human cancer development we screened cancer-profiling arrays (designated I and II from Clontech). These arrays contain cDNAs from various tumor tissues and corresponding normal tissues taken from the same patient. When array I (containing one pancreatic tumor sample) was hybridized with *Chmp1A* antisense probe, a significant reduction of *Chmp1A* was noticed in pancreatic tumor compared to the corresponding normal sample. Therefore

we screened cancer- profiling array II, which contains various pancreatic tumor samples. Consistent with the data obtained from array I, pancreatic tumors showed a considerable reduction of *Chmp1A* mRNA expression compared to corresponding normal samples. The reduction of Chmp1A mRNA was robust in adenocarcinoma, which is the most common cancer in human pancreas. Equality of samples was verified by hybridizing the array with human ubiquitin. Densitometric analysis was used to compare the reduction of *Chmp1A* mRNA in pancreatic tumors with corresponding normal samples. Next we examined Chmp1A protein level and localization by immunohistochemical analysis on human pancreatic tumor tissue arrays. In normal ducts of pancreas, Chmp1A is strongly localized to the apical side of ductal cells that face the lumen of the ducts. However the ductal cells of the ductal adenocarcinoma displayed either randomized localization or reduced expression of Chmp1A. Taken together, our results indicated that not only the level of mRNA and protein, but also the localization of Chmp1A protein, is important for proper cell growth.

Over-expression of Chmp1A inhibited the growth of pancreatic cancer cells *in vitro*

To substantiate the results obtained from the arrays we investigated the role of Chmp1A *in vitro* using human pancreatic ductal tumor cells. We chose PanC-1 cells since they are highly metastatic human pancreatic ductal tumor cells. Chmp1A over-expression was carried out via Tet-on Advanced Transgenic System to regulate Chmp1A over-expression in a doxycyclin (Dox) dependent manner. The induction of Chmp1A protein by Dox was determined as shown in Fig. 4A. Two independent colonies revealed the

induction of Chmp1A protein in a doxycyclin dose-dependent manner. We chose 1,000 ng/ml of doxycyclin as a working concentration for *in vitro* growth assay. Western blot analysis demonstrated that at this dose Chmp1A protein was significantly induced without non-specific effect; compare the Chmp1A expression in Dox treated to non-Dox treated samples (Fig. 4B). The same amount of Dox was used to determine the effect of Chmp1A over-expression on PanC-1 cell growth. Chmp1A over-expression inhibited the growth of PanC-1 cells by 35% on day 2 and 30% on day 5 *in vitro* (Fig. 4C). PanC-1 cells are shown to form tumors in xenograft assay.

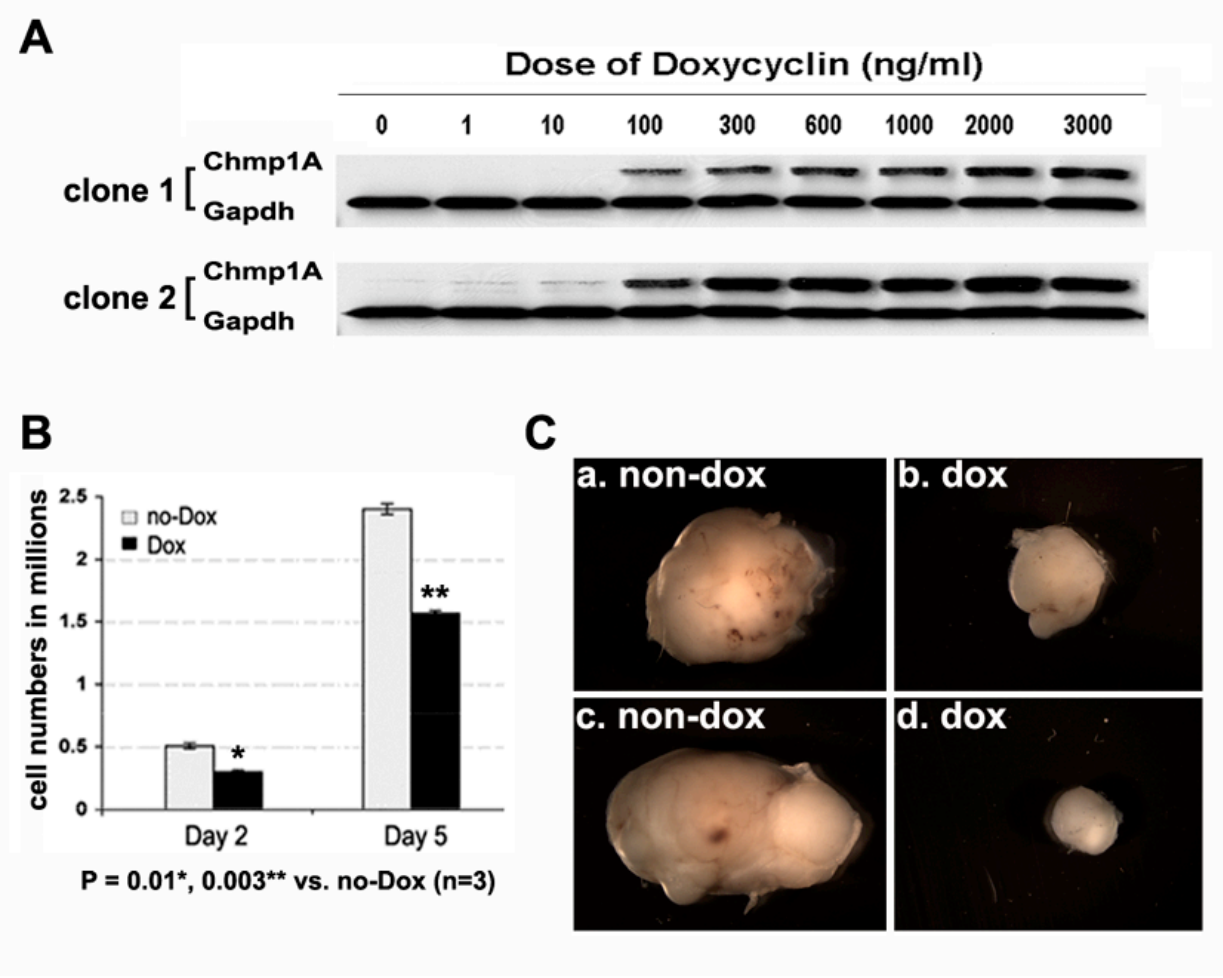

Figure 4. Over-expression of Chmp1A suppresses cell growth and the tumors formed by PanC-1 cells in nude mice. (A) Western blot analysis demonstrates Chmp1A over-

expression upon doxycyclin treatment in two independent colonies. Compare the Chmp1A expression level to non-dox treated control. Gapdh was used as protein loading control. (B) Chmp1A over-expression induced growth inhibition in human pancreatic ductal tumor cells, PanC-1. Over 30% growth inhibition was observed on day 2, and this growth inhibition was maintained through day 5. Cell numbers are in millions. (D) The size of tumors was reduced by a dox-dependent over-expression of Chmp1A (b, d), compared to control (a, c).

Over-expression of Chmp1A inhibits tumor growth in xenograft assay

We have demonstrated that loss of Chmp1A increased an anchorage-independent growth of HEK 293T cells and transformed these non-tumorigenic cells to form tumors in nude mice (Fig. 1 and 2). We also showed that over-expression of Chmp1A induced growth inhibition in human pancreatic ductal tumor cells (Fig. 4. C). Although our findings indicate that Chmp1A is a potential tumor suppressor, it did not illustrate whether Chmp1A could inhibit the growth of tumors, which were already formed. To test this, we subcutaneously injected Chmp1A over-expressing PanC-1 cells into 6-weekold nude mice. The mice were left to develop tumors until the tumors became visible (around two weeks after the injection). The mice with visible tumors were divided into two groups, one for control and the other for Chmp1A over-expression. These two groups of mice were supplied with either regular water for control or with Dox-containing water (200 μg/ml) for Chmp1A over-expression. As shown in Fig. 4D, Dox-mediated Chmp1A over-expression inhibited tumor growth compared to control *in vivo*. Moreover, Chmp1A over-expression appears to inhibit neo-angiogenesis as compared to control since the tumors (Db, d) did not show red blood vessels compared to control (Da, c).

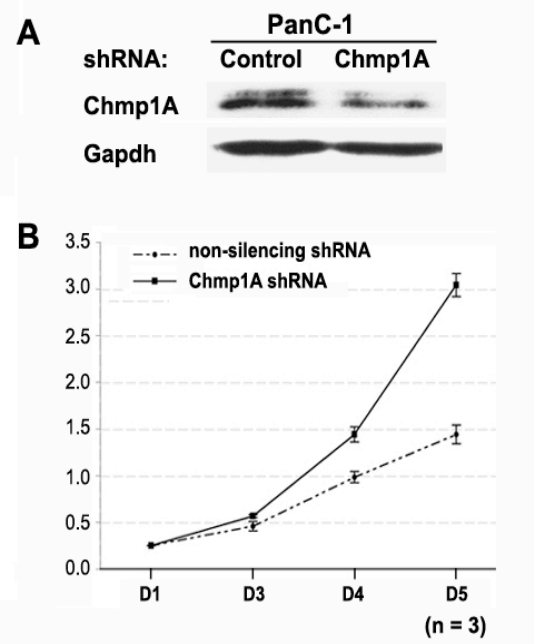

Fig. 5. Decrease of Chmp1A protein expression promotes the growth of PanC-1 cells. (A) Western blot analysis showed the reduction of Chmp1A protein by stable Chmp1A shRNA transfection (KD1 and KD2), compare with control shRNA transfection. (B) PanC-1 cells showed a significant growth promotion by the reduction of Chmp1A expression. More than a two-fold difference in the number of cells was observed by day 5 compared to control.

Knockdown of Chmp1A promoted the growth of pancreatic cancer cells *in vitro*

Next we examined the effect of Chmp1A knockdown on PanC-1 cell growth. Short hairpin RNA technology was used to knockdown Chmp1A in PanC-1 cells. Fig. 5A demonstrated the knockdown efficiency of Chmp1A protein in PanC-1 cells, demonstrating about 65% reduction of Chmp1A protein level compared with non-silencing control shRNA expressing cells. The increase in growth of PanC-1 cells expressing Chmp1A shRNA was apparent. By day five, shRNA expressing PanC-1 cells exhibited more than 1.5 fold increase in growth compared to control. Taken together the results demonstrate that Chmp1A expression inversely regulates the growth of pancreatic tumor cells and provide evidence that Chmp1A is a novel tumor suppressor in pancreas.

Over-expression of Chmp1A accompanies the increase of P53 activation

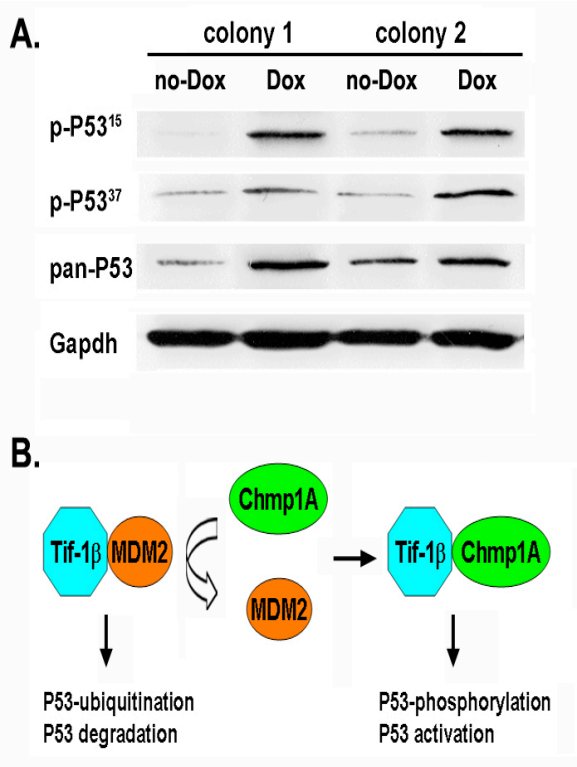

Fig. 6. Over-expression of Chmp1A increased the protein expression of pan-P53 and phospho-P53. (A) Western blot analysis showed the increase of pan-P53 and phospho-P53 at serine 15 and 37 upon Chmp1A over-expression. (B) Potential mechanism of Chmp1A on growth regulation: The binding of Tif-1β with MDM2 induces the ubiquitination of P53 followed by degradation. However, in the presence of Chmp1A, Tif-1β interacts with Chmp1A, which results in the activation of P53.

Chmp1A over-expression induced growth inhibition of PanC-1 cells *in vitro* and *in vivo* (Fig. 4 C, D). To understand the mechanisms of Chmp1A in growth regulation we determined the expression level of a known tumor suppressor, P53. As shown in Fig. 6A, doxycyclin-mediated Chmp1A over-expression led to an increase of pan-P53, and phospho-P53 at serine 15 and 37 compared to control. The activation of P53 by over-expression of Chmp1A is consistent with our preliminary data indicating that Chmp1A interacts with TIF1β /KAP1 (Transcription Intermediary factor 1β/KRAB domain-associated protein 1) (Belogortseva and Park; unpublished). As mentioned in the introduction, Chmp1A has a coiled-coil domain, which potentially mediates its interaction with proteins through the coiled-coiled domain of its binding partners. TIF1β

has previously been shown to interact with MDM2 (ubiquitin E3 ligase) through a coiled-coil domain. The interaction of TIF1β and MDM2 promotes ubiquitination and subsequent degradation of a suppressor P53 [26]. It is possible that the binding of Chmp1A to TIF1β prevents TIF1β from interacting with MDM2. If this is the case, over-expression of Chmp1A should lead to fewer complexes between TIF1β and MDM2, which would lead to an increase of P53 activation (Fig. 6B).

DISCUSSION

There are only two previous publications in the literature on the biologic properties of Chmp1A. It was shown that it affect chromatin structure and cell cycle progression. We used non-tumorigenic human embryonic kidney, HEK 293T cells to determine whether Chmp1A functions as a tumor suppressor. We had reasons to believe that Chmp1A might function as a tumor suppressor. First, Chmp1A belongs to ESCRT family, whose members were reported to be either deleted (HCC) [88, 89] or induce cell transformation and form tumors in nude mice [86]. Second our preliminary data (transient transfection of Chmp1A) indicated that over-expression of Chmp1A induced growth inhibition of HEK 293T cells. We employed short hairpin RNA (shRNA) technology to stably knockdown Chmp1A expression in HEK 293T cells. We have shown that knockdown of Chmp1A expression in HEK 293T cells resulted in an increase in anchorage independent growth *in vitro* and xenograft mediated tumor formation *in vivo*. Chmp1A depleted HEK 293T cells developed tumors with clear neo-angiogenesis. Histological analysis of the tumors indicated that the cells show

abnormal mitosis, high nuclear/cytoplasm ratio and multi-nucleation. Taken together our data demonstrates that Chmp1A is required for normal cell growth and that cells adopt tumorigenic characteristics without proper Chmp1A expression.

We screened human cancer profiling arrays to investigate whether Chmp1A functions as tissues specifically for tumor suppression. Although we focused mainly on the pancreatic tumors in this study Chmp1A mRNA was differentially expressed in many tumors including skin tumors. The reason we determined to study pancreatic tumor is because among many tumors screened Chmp1A mRNA is the most strongly and consistently reduced in various pancreatic tumors compared with corresponding normal tissues. Our finding on profiling arrays thus indicates that Chmp1A might function as tumor suppressor in many different types of tumors.

The results from human pancreatic tissue array supports further our hypothesis on Chmp1A. Chmp1A protein expression was mostly reduced in various pancreatic ductal adenocarcinomas compared with normal ducts, substantiating a role of Chmp1A in tumor suppression. In addition to the expression level of Chmp1A its localization in the ductal cells appears to be important for the maintenance of normal ducts. In the normal ducts Chmp1A is localized towards the lumen of the ducts, which is the apical side of ductal cells. However, in the ductal adenocarcinomas, Chmp1A is localized randomly, at the lateral or basal side of cells. This observation is particularly interesting since we obtained Chmp1A as a binding partner of Strabismus (Park, unpublished) that was shown to function in epithelial cell polarity [97]. It will be a great interest to determine

whether the polarity of Chmp1A expression is directly related to cancer development.

Since Chmp1A mRNA and protein is reduced in pancreatic cancers we chose a PanC-1 cells, pancreatic ductal tumor cells to study the function of Chmp1A further *in vivo* and *in vitro*. As expected, doxycyclin inducible Chmp1A over-expression induced growth inhibition of PanC-1 cells *in vitro*. We had similar effect with transient transfection on the growth of PanC-1 cells (data not shown). In addition, the size of tumors formed by the injection of PanC-1 cells became smaller upon Chmp1A over-expression *in vivo*. Complementary to our over-expression study in PanC-1 cells, knockdown of Chmp1A promoted the growth of cells.

Mechanistically, the increase of pan-P53 and phospho-P53 proteins by Chmp1A over-expression provides new insights supporting our model as shown in Figure 6B. We identified TIF1β as one of Chmp1A binding protein (Belogortseva and Park, unpublished). Since both Chmp1A and TIF1β contains coiled-coil domain, it is possible that these two proteins interact each other through coiled-coil domain. The interaction of TIF1β to MDM2 (ubiquitin E3 ligase) was previously shown to the increase of ubiquitination of a suppressor P53, which results in the degradation of P53 [26]. So we hypothesize that Chmp1A binds with Tif-1β and this interaction leads to the prevention of P53 ubquitination and an increase of phosphorylation and subsequent activation. Although we have shown that Chmp1A regulates tumor growth through P53 activation we speculate it is only part of the mechanisms by which Chmp1A control the tumor growth. Chmp1A contains various domains that could be easily related to signaling

events, which regulates tumor growth. Nonetheless, this study provides *in vitro* and *in vivo* evidences supporting the tumor suppressor function of Chmp1A in pancreatic cancer. The prognosis of pancreatic cancer is extremely poor and there is s need for a broader understanding of the molecular mechanisms underlying pancreatic carcinogenesis. Our present work potentially provides a foundation for developing new treatment and/or early biomarkers to improve the survival rate of pancreatic cancer.

SUMMARY AND CONCLUSIONS

Our data showed that Chmp1A is a potential tumor suppressor and plays a pivotal role in cell growth inhibition and tumorgenesis. Chmp1A participates in the ATRA signaling by regulating CRBP-1 transcription. The main conclusions are:

1. ATRA increases Chmp1A localization in the nucleus in ATRA responsive pancreatic cancer cells.
2. ATRA increases Chmp1A, P53, phospho-P53 and CRBP1 protein expression in ATRA responsive pancreatic cancer cells.
3. Chmp1A knockdown abolishes ATRA cell growth inhibition of PanC-1 cells.
4. Chmp1A knockdown abolishes an increase of Chmp1A, p53, and phospho-p53 expression by ATRA in PanC-1 cells.
5. The partial loss of Chmp1A of HEK293T/17 (non-tumorgenetic cells) cells formed tumor in xenograft nude mice.
6. The dox-inducible ectopic expression of Chmp1A inhibited pancreatic cancer cell growth *in vitro* and tumor growth of xenograft nude mice *in vivo*.
7. The Chmp1A apparently promotes CRBP-1 expression in both transcription and translation levels that in turn facilitate ATRA transportation into nuclear receptor.

In conclusion, the Chmp1A-CRBP-1 loop pathway may be, at least in part, the molecular foundation of growth inhibition of Chmp1A. On the other hand, the expression of Chmp1A also parallel with P53 pathway, which suggests that there is kind of direct link between Chmp1A and P53. One is the functional link; both P53 and Chmp1A act as

tumor suppressor, partial loss of either is able to result in tumor formation. Another link may be related to structure or mechanism which needs more evidence to elucidate. The establishment of association between Chmp1A and P53 might be the molecular mechanism of tumor suppressor of Chmp1A.

Future direction

The tumorgenesis of Chmp1A by xenograft model is strong, but more evidences in transgenic mice model is needed to confirm the role of tumor suppressor of Chmp1A. And the transgenic mice data also is more convincing about mechanism. Thus, transgenic mice work is so attractive for us now. The hypothesized crosstalk between Chmp1A and P53 also needs more data to confirm. The universal expression of Chmp1A do work in both nuclear and cytoplasm. How do they coordinate? This also is an interesting question for next step research. We will Knockdown coiled-coil domain and transfect mutation plasmid into cells to check its cellular distribution.

First of all, flow cytometry analysis will be used to confirm its function in apoptosis. Annexin V-FITC and propidium iodide staining will be good methods for flow cytometry apoptosis analysis. Either western blot or real-time PCR will be used to determine the expression of Bcl-XL, BIM, BAX, or DAPK.

Second, some downstream signals of P53 such as P21, MDM2 will be checked to dissect the Chmp1A-P53 pathway.

Our preliminary data show that over-expression of Chmp1A was able to induce apoptosis of panc-1 cells. The apoptosis may be P53-dependant. To evaluate this hypothesis, knockdown of P53 may be a good tool. Another possibility can be that Chmp1A act as a CDK/Cycline inhibitor. Chmp1A directly regulate cell cycle through inhibiting CDK/Cycline. Western blot will be used to check the effect of Chmp1A on typical CDK/Cycline protein expression. Immunoprecipitation will be employed to confirm the physical interaction derived from proteomics screen.

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