Acta Universitatis Tamperensis 2346

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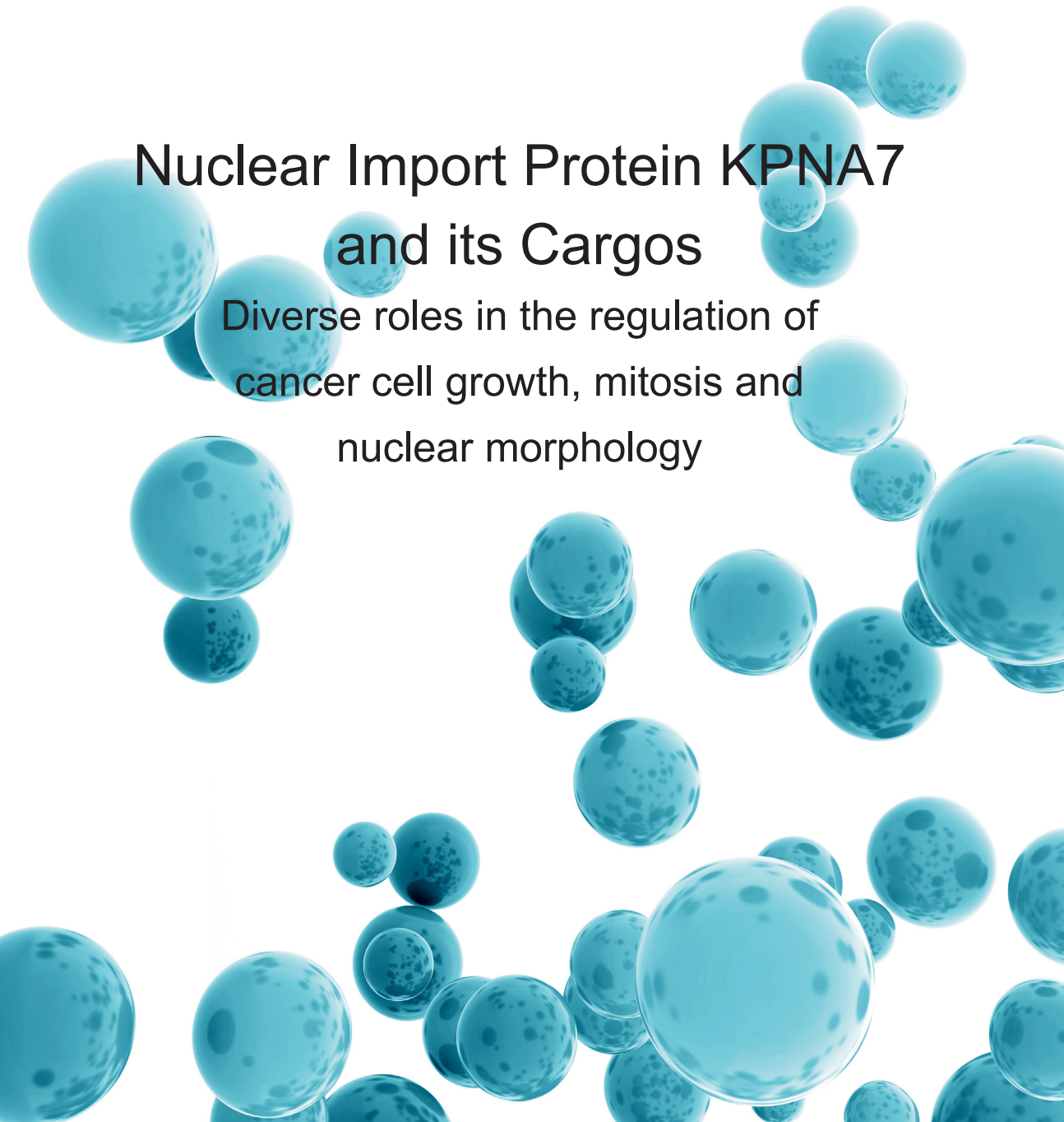
Diverse roles in the regulation of cancer cell growth, mitosis and nuclear morphology



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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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CONTENTS

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This thesis is based on the following communications, which are referred to in the text by the corresponding Roman numerals.

I Laurila EM, **Vuorinen E**, Savinainen K, Rauhala HE, Kallioniemi A. KPNA7, a nuclear transport receptor, promotes malignant properties of pancreatic cancer cells in vitro. (2014). Experimental Cell Research 332:159-167.

II **Vuorinen EM***, Rajala NK*, Ihalainen TO, Kallioniemi A. Depletion of nuclear import protein karyopherin alpha 7 (KPNA7) induces mitotic defects and deformation of nuclei in cancer cells. Submitted for publication.

III **Vuorinen EM***, Rajala NK*, Rauhala HE, Nurminen AT, Hytönen VP, Kallioniemi A. Search for KPNA7 cargo proteins in human cells reveals MVP and ZNF414 as novel regulators of cancer cell growth. (2017). Biochimica et Biophysica Acta – Molecular Basis of Disease 1863:211-219.

*Equal contribution

The publication No. I has also been used in the doctoral thesis of Eeva Laurila.

ABBREVIATIONS

ARH1	Aplasia Ras homolog member 1
BIG3	Brefeldin A-inhibited guanine nucleotide-exchange protein 3
BRCA1	breast cancer type 1 susceptibility protein
CAS	cellular apoptosis susceptibility
CC3	complement component 3
CDC7	cell division cycle 7
CHD4	chromodomain helicase 4
CHK1	checkpoint kinase 1
cNLS	classical nuclear localization signal
co-IP	co-immunoprecipitation
CTNNB1	catenin beta
DAPI	4',6-diamidino-2-phenylindole
DIEXF	digestive organ expansion factor homolog 1
ELAVL1	Elav-like 1
EMT	epithelial-mesenchymal transition
FGF	fibroblast growth factor
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
IF	immunofluorescence
KPNA1-7	karyopherin alpha 1-7
KPNB1	karyopherin beta 1
LUC	luciferase
MDR	multidrug resistance
MS	mass spectrometry
MVP	major vault protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization signal

NPC	nuclear pore complex
NSCLC	non-small cell lung carcinoma
PCR	polymerase chain reaction
PHB1	prohibitin 2
PTEN	phosphatase and tensin homolog
PUM1	Pumilio homolog 1
qRT-PCR	quantitative real-time PCR
RCC1	regulator of chromosome condensation
SAF	spindle assembly factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SSRP1	FACT-complex subunit SSRP1
STAT	signal transducer and activator of transcription
STK35	Serine-threonine kinase 35
SV40	simian virus 40
TAF6	transcription initiation factor TFIID subunit 6
TRF1	telomere repeat factor 1
TSS	transcription start site
ZNF414	zinc finger protein 414

ABSTRACT

Eukaryotic cells are compartmentalized to contain diverse organelles such as the nucleus. The separation of the nucleus from the cytoplasm by the nuclear envelope forms a barrier across which large macromolecules, such as proteins, need to be transported to maintain cellular homeostasis. Disturbances in nuclear import can lead to various diseases, including cancer. Karyopherin alpha 7 (KPNA7) is the newest member of the karyopherin alpha protein family of nuclear importers responsible for the nucleocytoplasmic transport of macromolecules. The aim of this study was to evaluate *KPNA7* expression levels in cancer cells and healthy adult tissues, to characterize the effect of *KPNA7* expression on cancer cell growth, malignant properties, mitosis and nuclear morphology and to identify KPNA7 cargo proteins.

Gene expression studies revealed that *KPNA7* expression is reactivated in cancer cell lines, with highest expression detected in pancreatic cancer cell lines harboring an amplification of the 7q21-22 genomic locus where *KPNA7* is located. In contrast, *KPNA7* expression is almost absent in healthy adult tissues. To probe the consequences of the expression, *KPNA7* was silenced in the cell lines with endogenous expression of the gene. The silencing of *KPNA7* led to decreased proliferation via reduced number of cells in the S-phase of the cell cycle, reduced migration and anchorage-independent growth, and induction of autophagy. By utilizing immunofluorescent assays, it was also noted that *KPNA7*-silencing leads to lobulated nuclei and disturbed mitosis via multipolar mitotic spindles in Hs700T pancreatic and T-47D breast cancer cell lines.

These data led to the hypothesis that the altered subcellular localization of KPNA7 cargo proteins is responsible for the cellular phenotypes observed after *KPNA7* silencing. By utilizing protein pull-down and mass spectrometry, we identified 377 KPNA7 cargo protein candidates, most of which were known or predicted to localize to the nucleus. Two proteins, namely major vault protein (MVP) and zinc finger protein 414 (ZNF414), were shown to bind KPNA7 *in vitro* and their transport to the nucleus was hindered by *KPNA7* silencing. These proteins were also shown to have growth regulatory roles in pancreatic cancer cells.

Together these results suggest that KPNA7, probably via its cargo proteins including MVP and ZNF414, participates in the regulation of phenotypes that are essential for growth and viability of cancer cells. The results also shed light on the contribution of KPNA7 to the regulation of proper mitosis and maintenance of nuclear envelope environment and deepen our understanding on the role of nuclear transfer proteins in cancer pathogenesis.

TIIVISTELMÄ

Aitotumalliset eli eukaryoottiset solut sisältävät useita eri soluelimiä eli organelleja, kuten tuman. Tumakalvo erottaa tuman solulimasta eli sytoplasmasta ja muodostaa esteen makromolekyylien, kuten proteiinien, kulkeutumiselle tuman ja sytoplasman välillä. Solunsisäisen tasapainon ylläpitämiseksi solut tarvitsevat kuljetusmekanismin, jonka avulla suuret makromolekyylit kuljetetaan aktiivisesti osastosta toiseen. Tumakuljetuksen häiriöt voivat johtaa moniin eri sairauksiin, esimerkiksi syöpään. Karyoferiini alfa -proteiiniperheen jäsenet ovat tumakuljetusproteiineja, jotka huolehtivat makromolekyylien kuljetuksesta sytoplasmasta tumaan. Karyoferiini alfa 7 (KPNA7) on kyseisen proteiiniperheen uusin jäsen. Tämän tutkimuksen tarkoituksena oli kartoittaa *KPNA7*:n ilmentymistasoja syöpäsoluissa ja aikuisen terveissä kudoksissa sekä selvittää KPNA7:n merkitystä syöpäsolujen kasvulle, pahanlaatuisille piirteille, tumanjakautumiselle ja tumamorfologian ylläpidolle. Lisäksi pyrittiin tunnistamaan KPNA7:n tumaan kuljettamia proteiineja.

Geenieksprессiotutkimukset osoittivat, että *KPNA7*:n ilmentyminen on uudelleenaktivoitu syöpäsoluissa. Korkeimmat ilmentymistasot havaittiin haimasyöpäsolulinjoissa, joissa esiintyy *KPNA7*:n sisältävän geenialueen 7q21-22 monistuma. Terveissä aikuisen kudoksissa geeniä ei ilmennetä. KPNA7:n merkitystä syöpäsolujen toiminnalle tutkittiin hiljentämällä *KPNA7*-geeni syöpäsolupaneelissa ja tarkastelemalla hiljentämisen aiheuttamia muutoksia kyseisten solujen toiminnassa. *KPNA7*-hiljennys vähensi dramaattisesti solujen kasvua aiheuttaen solusyklin S-vaiheessa olevien solujen määrän vähenemisen. Lisäksi solujen kyky kasvaa pintaan kiinnittymättä väheni ja niissä havaittiin autofagian indusoituminen. Immunofluoresenssimenetelmien avulla havaittiin myös, että KPNA7:n väheneminen häiritsi solujen tumarakennetta ja tumanjakautumista eli mitoosia, aiheuttaen lohkomaisen tuman muodon ja moninapaisen tumasukkulan Hs700T-haimasyöpä- ja T-47D-rintasyöpäsoluissa.

Edellä saadut tulokset johtivat hypoteesiin, että *KPNA7*:n hiljentäminen johtaa sen kuljettamien ns. lastiproteiinien väärään sijoittumiseen solussa ja sitä kautta havaittuihin ilmiasuihin eli fenotyyppeihin. Proteiinien pull-down ja massaspektrometria -menetelmien avulla tunnistettiin yhteensä 377 proteiinia, jotka edustavat todennäköisiä KPNA7:n lastiproteiineja. Suurin osa näistä proteiineista

tiedettiin tai ennustettiin sijoittuvan tumaan. *In vitro* -sitoutuminen KPNA7:n kanssa varmistettiin kahden proteiinin osalta. Nämä proteiinit olivat major vault protein (MVP) ja zinc finger protein 414 (ZNF414). Lisäksi osoitettiin, että KPNA7:n hiljennys todellakin estää näiden proteiinien tumakuljetuksen. MVP:llä ja ZNF414:llä huomattiin olevan kasvunsäätelyrooli haimasyöpäsoluissa, mikä viittaa siihen, että ne voivat hyvinkin olla vastuussa *KPNA7*:n hiljennyksen seurauksena havaitusta syöpäsolujen kasvun vähenemisestä.

Tutkimuksen tulokset osoittavat, että KPNA7 osallistuu syöpäsolujen kasvun ja elinkyvyn säätelyyn. Todennäköisesti säätely tapahtuu KPNA7:n tumaan kuljettamien proteiinien, kuten MVP:n ja ZNF414:n kautta. Tulokset myös tarjoavat uutta tietoa KPNA7:n osuudesta asianmukaisen mitoosin ja tumakalvon rakenteen säätelyssä, ja syventävät tietämystämme tumakuljetusproteiinien merkityksestä syövän synnyssä.

1 INTRODUCTION

Cancer is a major health concern worldwide, affecting millions of people. In 2015, there were 14.5 million new cancer cases, over 90 million people living with cancer and almost 9 million deaths occurred because of it (Vos et al., 2015; Fitzmaurice et al., 2017). This makes it the second leading cause of death in the world, surpassed for now only by cardiovascular diseases (Fitzmaurice et al., 2017; Siegel et al., 2017). The incidence of cancer is rising due to reasons such as population growth and aging and it has already overtaken cardiovascular diseases as a leading cause of death in many European countries, and is predicted to do so also worldwide (European Society of Cardiology, 2016; Fitzmaurice et al., 2017). Despite the improved prognosis of many cancers over the last decades because of better diagnostic tools and improved treatment options, the "War on Cancer" declared in the US in 1971 remains unwon. Hence, further research is still direly needed to conquer the disease.

Cancer is a heterogeneous group of diseases that is plainly defined as uncontrollable growth and proliferation of cells. Despite the heterogeneity, different cancers share common features that distinguishes them from normal, healthy cells. These characteristics, termed hallmarks of cancer, are unlimited replicative potential, self-sufficiency of growth signalling, evasion of growth suppression signals, ability to avoid programmed cell death, induction of angiogenesis and capability to invade surrounding tissues and metastasize (Hanahan and Weinberg, 2011).

As a genetic disease, cancer results from the accumulation of mutations, such as deletions and chromosomal rearrangements, in the genome of the cells. The mutations can be present already at birth (inherited mutations) or occur sporadically during the lifetime of the individual (acquired mutations) (Stratton, 2011). The acquired mutations can be further divided into intrinsic and extrinsic types. Intrinsic mutations result e.g. from errors in DNA replication in cells that are actively dividing; extrinsic mutations are induced by external factors, such as UV radiation of tobacco smoke (Wu et al., 2016). Additional changes not affecting the DNA sequence *per se*, such as epigenetic alterations, also contribute to malignant progression (Stratton et al., 2009; You and Jones, 2012).

Mutations confer the affected cell a growth advantage over its surrounding companions, leading to a process known as transformation. In order for a normal

cell to transform into a malignant cancer cell, a number of genetic changes are needed. The affected genes can be broadly divided into two categories: oncogenes and tumor suppressor genes. Oncogenes are normal growth-promoting genes that are aberrantly activated in cancer cells; tumor suppressors are genes that normally participate in growth-restriction or the maintenance of genomic stability and need to be inactivated for cancer to occur (Lodish et al., 2000; Croce, 2008).

In addition to classical oncogenes, tumor suppressor genes and their protein products, there is a multitude of genes whose incorrect activity may contribute to cancer formation. These genes work in different ways to ensure the proper cellular function and are not necessarily directly involved in the regulation of cell growth or the maintenance of genomic integrity. For example, one component in the progression of cancer is the proper maintenance of cellular homeostasis, including the correct spatiotemporal localization of proteins between the nucleus and the cytoplasm, which are physically separated in eukaryotic cells (Wang and Li, 2014). Because most proteins require an active transport mechanism to enter the nucleus, the disturbance in protein localization and a resulting cellular imbalance can be the consequence of abnormal expression or function of the transport proteins that are responsible for the shuttling.

The seven members of the human karyopherin alpha (KPNA) family of nuclear import proteins are involved in transporting molecules between the cytoplasm and the nucleus (Pumroy and Cingolani, 2015). The dysregulation of nuclear transport resulting from the overexpression of different KPNA proteins has been previously linked to cancer (Stelma et al., 2016). Previous work in our group has identified karyopherin alpha 7 (*KPNA7*), the most recent addition to the KPNA protein family, as a target gene of the chromosomal amplification at locus 7q21-22 in pancreatic cancer (Laurila et al., 2009). The purpose of this study was to examine the role of KPNA7 in cancer pathogenesis and to identify its cargo proteins that might also have relevance for the development and progression of cancer.

2 REVIEW OF THE LITERATURE

2.1 Nuclear import cycle

Eukaryotic cells are divided into distinct nuclear and cytoplasmic compartments by the nuclear envelope. This lipid bilayer presents a barrier to the diffusion of macromolecules above 40 kDa (Faustino et al., 2007; Christie et al., 2016; Stelma et al., 2016). Thus, cells require an active transport machinery to transfer RNA, protein and other molecules to their correct subcellular localizations to maintain homeostasis and normal cellular function (Lott and Cingolani, 2011; Christie et al., 2016). For instance, nuclear proteins such as transcription factors are produced in the cytoplasm but need to be actively transported to their site of function in the nucleus.

Transport into the nucleus takes place through the nuclear pore complexes (NPCs). The NPC is a large, cylindrical macromolecular structure embedded in the nuclear envelope that consists of multiple nucleoporin proteins (Nups) (Hoelz et al., 2011; Knockenhauer and Schwartz, 2016). NPCs fuse the inner and outer nuclear membranes and connect the nuclear and cytoplasmic compartments (Fig. 1) (Grossman et al., 2012; Knockenhauer and Schwartz, 2016). Hence they form a channel that provides a route for the diffusion of small molecules, but they also act as a gateway for receptor-mediated macromolecular transport (Hoelz et al., 2011; Grossman et al., 2012; Knockenhauer and Schwartz, 2016).

Most proteins targeted for transport into the nucleus contain a nuclear localization signal (NLS) motif in their amino acid sequence (Christie et al., 2016). The first NLS to be recognized and also the best characterized is the classical NLS (cNLS) (Lange et al., 2007; Christie et al., 2016). cNLS is typically a lysine-rich stretch of basic amino acids and can be classified as either monopartite or bipartite, containing one basic region (monopartite cNLS) or two basic stretches separated by a linker region (bipartite cNLS) (Lange et al., 2007; Marfori et al., 2011). Monopartite cNLSs can be exemplified by the SV40 large T antigen (PKKKRKV) whereas a bipartite cNLS is represented by nucleoplasmin NLS (KRPAATKKAGQAKKKKL) (Bauer et al., 2015). A second class of NLSs is the PY-type NLS (PY-NLS), characterized by a consensus motif of $RX_{2\text{-}5}PY$ (Lee et al., 2006; Xu et al., 2010).

The NLS is recognized and bound by a transport receptor that carries the NLS-containing cargo into the nucleus. For proteins, the most utilized nuclear import pathway is mediated by karyopherins (Kau et al., 2004). In this system, depicted in Fig. 1, karyopherin alphas (KPNAs, also known as importin alphas) function as adaptors that recognize and bind to cNLSs in their cargo proteins, followed by recruitment of karyopherin beta 1 (KPNB1, importin beta 1) (Lusk et al., 2007; Di Ventura and Kuhlman, 2016). KPNB1 then ferries the ternary protein complex through the NPC (Pemberton and Paschal, 2005). The transport via the NPC depends on transient interactions of KPNB1 with the Nups during its passage through the NPC channel (Pemberton and Paschal, 2005; Hoelz et al., 2011).

Once inside the nucleus, KPNB1 binds the GTP binding nuclear protein Ran (RanGTP) and releases the KPNA and cargo protein (Fig. 1) (Mor et al., 2014). The RanGTP-bound KPNB1 and KPNA are recycled back to the cytoplasm by export receptors (Kau et al., 2004). The directionality of the import is determined by the differential concentration of Ran-GTP between the nucleus and cytoplasm, which is maintained by guanine nucleotide exchange factors (GEFs) in the nucleus and GTPase activating proteins (GAPs) in the cytoplasm (Wente and Rout, 2010). Proteins with non-classical NLSs can also be bound and transported directly by KPNBs, without the need for KPNA adaptors (Mosammaparast and Pemberton, 2004; Lusk et al., 2007; Christie et al., 2016).

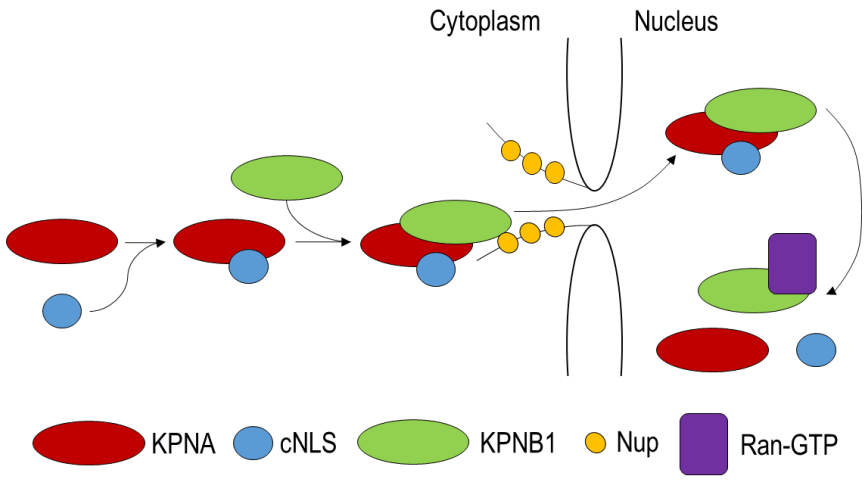


Figure 1. Classical nuclear import pathway. The transport system utilizes karyopherin alphas (KPNA, red) as adaptors and karyopherin betas (KPNB1, green) as import receptors to import cNLS-containing proteins (blue) from the cytoplasm to the nucleus through the nuclear pores via interaction with the Nups (yellow). RanGTP (purple) is responsible for dissociating the transport complex in the nucleus.

2.2 Karyopherin alpha protein family

The human karyopherin alpha protein family contains seven members that have been highly conserved throughout evolution (Goldfarb et al., 2004; Pumroy and Cingolani, 2015). All KPNAs share a common structure consisting of a body of ten helical Armadillo (ARM) repeats, a short C-terminal region of acidic amino acids and an N-terminal importin beta binding domain (IBB) (Fig. 2a) (Conti et al., 1998; Herold et al., 1998; Fontes et al., 2000; Marfori et al., 2011). The general structure of the classical nuclear import complex in illustrated in Fig. 2b.

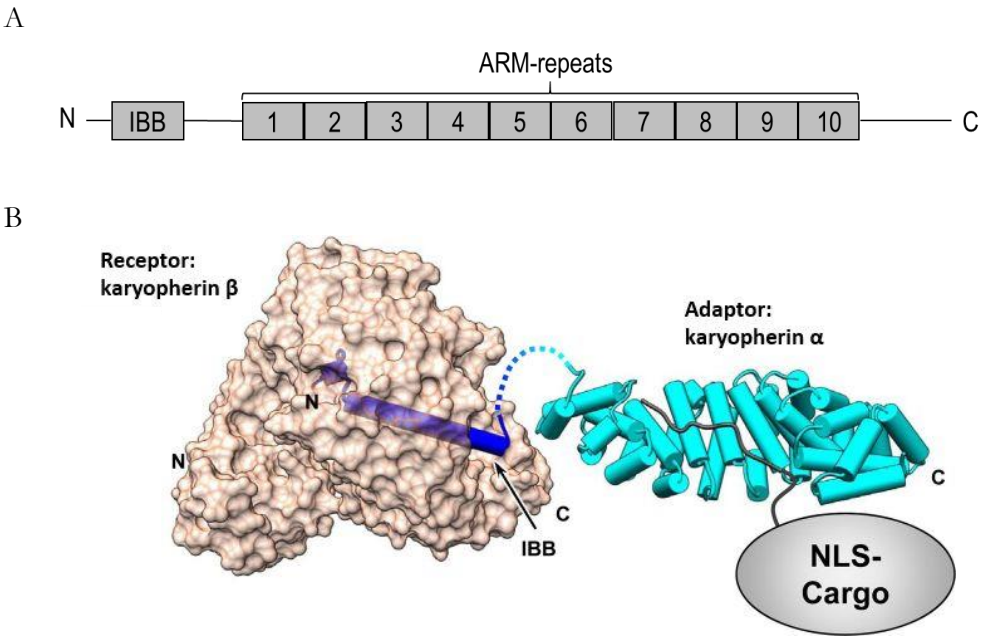


Figure 2. (A) Schematic structure of a generic KPNA. Shown are the importin beta binding domain (IBB) and the body of armadillo (ARM) repeats. (B) Structure of the classical nuclear import complex. Mouse Kpna2 (turquoise) is bound to an NLS-containing cargo protein (grey) and to Kpnb1 (beige) via the IBB domain (dark blue). Panel B adapted from Pumroy and Cingolani, 2015. The N and C termini of each protein are noted with an N or C, respectively in both panels.

The ARM repeats are responsible for cargo NLS recognition and binding as shown in Fig. 2b, whereas the C-terminal region functions as a binding site for a nuclear exporter known as cellular apoptosis susceptibility (CAS) (Herold et al., 1998; Goldfarb et al., 2004; Miyamoto et al., 2016). The N-terminal IBB domain is responsible for the binding to KPNB1 as shown in Fig. 2b, but has also an important

role in the regulation of cargo protein binding to KPNAs: it regulates the accessibility of the NLS binding site via an autoinhibitory mechanism by mimicking the structure of an NLS (Kobe, 1999; Pumroy and Cingolani, 2015). When the KPNAs are not bound to their NLS-containing cargos, the IBB domain folds back to occupy the NLS binding site and only cargos with high affinity can bind to it (Kobe, 1999; Pumroy and Cingolani, 2015). Alternatively, the autoinhibition may be released through formation of KPNA-KPNB1 heterodimer, thus freeing the NLS binding site to become accessible for cargos with lower affinity (Fanara et al., 2000; Pumroy and Cingolani, 2015). The autoinhibitory mechanism mediated by the IBB domain most probably reduces futile import of empty adaptors and hinders cNLS binding to KPNAs when KPNB1 is not available for nuclear translocation (Lott and Cingolani, 2011; Christie et al., 2016).

KPNAs can be divided into three subfamilies according to their primary amino acid sequence similarities (Miyamoto et al., 2016). The alpha 1 family consists of KPNA2 and KPNA7; alpha 2 family contains KPNAs 3 and 4; the third family, alpha 3, includes KPNAs 1, 5 and 6 (Fig. 3) (Pumroy and Cingolani, 2015). There is evidence that despite their structural similarities, the KPNA family members exhibit tissue-specific expression patterns and have distinctive cargo specificities that are dependent on cellular context (Köhler et al., 1999; Quensel et al., 2004; Friedrich et al., 2006; Miyamoto et al., 2016). For example, the roles of KPNAs during murine embryonic development have been studied using knock-out animals and the results show that different KPNAs function at distinct stages of the developmental process (Yasuhara et al., 2009; Miyamoto et al., 2012). KPNAs have also been shown to have different affinities to KPNB1 (Kelley et al., 2010), suggesting that there are several levels of diversity in karyopherin-mediated nuclear import.

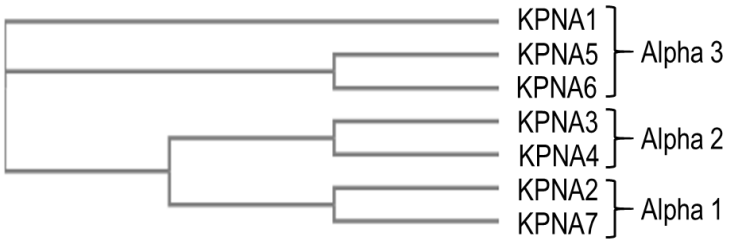


Figure 3. Phylogenetic tree of human KPNAs indicating the three subfamilies. The ClustalW program (Larkin et al., 2007) was used to generate this tree.

2.2.1 KPNA7

Karyopherin alpha 7 (KPNA7) is the most recent addition to the KPNA family. First identified in 2010 (Kelley et al., 2010), it remains the least studied KPNA family member. It is related most closely to KPNA2 with 55% amino acid sequence similarity and hence belongs to alpha 1 subfamily (Fig. 3) (Kelley et al., 2010). It has significant resemblances with the other members as well, as it also consists of the ARM repeat core and an N-terminal IBB domain conserved in all KPNA family members (Kelley et al., 2010).

The IBB domain of human is KPNA7 less similar with the IBBs of other KPNAs and displays a higher affinity to KPNB1 (Kelley et al., 2010; Kimoto et al., 2015), suggesting an inherent difference in cargo specificity and possibly cellular function. *In vitro* assays are inconclusive whether KPNA7 is capable of binding classical NLSs. In one study it was demonstrated that KPNA7 binds retinoblastoma NLS very weakly and has no affinity to SV40 or nucleoplasmin NLSs (Kelley et al., 2010) while another study challenged this result by showing evidence of KPNA7-SV40 interaction (Kimoto et al., 2015). Apart from the study by Kimoto and colleagues (2015) reporting the identification of a few KPNA7 cargos, the proteins that KPNA7 transports to the nucleus in human cells are still mainly unknown.

KPNA7 orthologs have been identified in bovine, porcine, mouse and rainbow trout and these previous animal-based studies report that *KPNA7* is mainly expressed in oocytes and during early embryogenesis and is essential for normal embryonic development and fertility (Tejomurtula et al., 2009; Hu et al., 2010; Park et al., 2012; Wang et al., 2014). In both mouse and cattle, *KPNA7* expression was found predominantly in the ovaries of adult animals (Tejomurtula et al., 2009; Hu et al., 2010). KPNA7 function in human tissues is poorly understood, although germline mutations in the gene have been linked to infantile spasms and cerebellar malformation in two siblings (Paciorkowski et al., 2014). The protein is localized to the nuclei in human cells (Kelley et al., 2010; Kimoto et al., 2015), but other than that, KPNA7 function remains somewhat a mystery, also in terms of whether KPNA7 has a role in human embryogenesis or fertility.

2.3 Roles of karyopherin alphas in cancer

Correct spatiotemporal and subcellular localization of proteins is critical for their proper function and thus the trafficking between the various compartments of the

cell, such as the nucleus and cytoplasm, is essential for cellular homeostasis. Deviations in nuclear import, e.g. because of the abnormal function of members of the nuclear transport machinery, result in incorrect localization of proteins that might subsequently lead to various diseases including cancer (Faustino et al., 2007; Lee et al., 2013; Mor et al., 2014). The abnormally localized proteins can represent key mediators of oncogenesis, like cell cycle regulators, transcription factors or tumor suppressors. For example, inactivation of the tumor suppressor protein p53 has been demonstrated to occur via nuclear exclusion in breast cancer (Moll et al., 1992) and in some cases, this mislocalization takes place due to a truncated form of a KPNA (Kim et al., 2000).

The dysregulation of nuclear transport in cancer may occur at many junctions. Altered expression levels of the transport receptors is the most obvious reason and indeed, increased expression of KPNAs has been observed in various cancer types (Stelma et al., 2016) and correlates with enhanced nuclear import efficiencies of cargo proteins (Kuusisto et al., 2012). The elevated KPNA expression might allow the increased nuclear entry of tumor-promoting factors. The underlying cause of KPNA overexpression has been attributed for example to dysregulated activity of transcription factors of the E2F family that regulate KPNA expression (van der Watt et al., 2011) and decreased expression of micro-RNAs regulating the translation of KPNAs (Lin et al., 2015). Downregulation of KPNA expression in cancer appears to be rare but does occur in the case of KPNA4 (Wang et al., 2015), which will be discussed further under section 2.3.2

The accurate function of karyopherins depends on their ability to interact with their cargos and other members of the transport cycle. In some cases, the NLS of cargo protein might be mutated, disabling the cargo interaction with KPNAs, as has been reported for p53 in head and neck squamous carcinoma (Mandic et al., 2005). Mutations can occur also the transport proteins: a KPNA with a truncating mutation in NLS-binding site has been shown to be retained in the cytoplasm of breast cancer cells despite being able to bind to KPNB1 (Kim et al., 2000). This example suggests that the correct formation of the ternary cargo-KPNA-KPNB1 –complex is essential for the nuclear import cycle and interaction with KPNB1 alone is not sufficient for nuclear translocation.

Overall, mutations in cargo protein NLSs or KPNAs themselves seem to be relatively rare events in cancer and the majority of dysregulation occurs via alterations in KPNA expression levels (Wang and Li, 2014). According to the COSMIC database (http://cancer.sanger.ac.uk/cosmic/), mutations in KPNAs do occur and are mostly single nucleotide substitutions, but there is no evidence on any

causative role in cancer development. In the case of KPNA7, one report describes two amino-acid substitutions predicted to localize in the NLS binding region and to reduce the cargo-binding activity of KPNA7 (Paciorkowski et al., 2014). The consequences of these mutations were congenital neurological defects rather than cancer (Paciorkowski et al., 2014). Nevertheless, this example illustrates that mutations in karyopherins with indications to disease do exist and they might emerge in the future also in cancer.

Healthy cells can also regulate nuclear trafficking by utilizing endogenous transport inhibitors (Stelma et al., 2016). Examples of such inhibitors are Complement Component 3 (CC3/TIP30) and Aplasia Ras Homolog Member 1 (ARH1/NOEY2) (Stelma et al., 2016). CC3 is a central player of the complement system in innate immunity and normally it e.g. promotes phagocytosis and supports inflammatory responses against pathogens (Sahu and Lambris, 2001). ARH1 on the other hand is a GTPase and a member of the Ras superfamily and has been shown to be a tumor suppressor (Yang et al., 2009). Both CC3 and ARH1 interfere with nuclear import via physical interaction with karyopherins and have been found to be downregulated or absent in a range of cancers (King and Shtivelman, 2004; Huang et al., 2009; Stelma et al., 2016). Disruption of the regulation of nuclear transport again leads to the altered localization of essential proteins and may yield advantages to tumor development and progression. The known cancer-related roles of the members of different KPNA subfamilies and their cargos are discussed below.

2.3.1 Alpha 1 subfamily: KPNA2

KPNA2 was the first human alpha karyopherin to be identified over two decades ago (Weis et al., 1995) and has been considered a general importer of NLS-containing proteins since then (Köhler et al., 1999). Cancer-related nuclear transport alterations involving KPNA2 are perhaps the best characterized of all the KPNAs. Elevated levels of KPNA2 have been reported in many cancer types including, but not limited to, breast (Dahl et al., 2006), lung (Wang et al., 2011a), prostate (Mortezavi et al., 2011), and colorectal (Yu et al., 2017) cancers. Importantly, elevated KPNA2 levels have been detected already in early lesions (Christiansen and Dyrskjøt, 2013), suggesting that it actively participates in the carcinogenesis process.

Functional studies support the participation of KPNA2 in cancer pathogenesis, demonstrating that enhanced KPNA2 expression leads to increased proliferation and migration in different cell types (Mortezavi et al., 2011; Wang et al., 2011a;

Noetzel et al., 2012). These effects are probably caused by the increased nuclear transport of KPNA2 cargos, which have been characterized in some detail. Examples of verified KPNA2 cargos are the cell cycle regulator checkpoint kinase 2 (Chk2) (Zannini et al., 2003), DNA repair-associated breast cancer type 1 susceptibility protein (BRCA1) (Chen et al., 1996), androgen receptor (AR) (Cutress et al., 2008; Mortezavi et al., 2011) and tumor suppressor p53 (Wang et al., 2012a). In addition to these aforementioned cancer-associated proteins, KPNA2 has been indicated to transport multiple transcription factors involved in a variety of cancer-contributing processes (Christiansen and Dyrskjøt, 2013). Interestingly, a recent study demonstrated that KPNA2 is localized at the cell surface in some cancer cells, and promotes their proliferation via interaction with the fibroblast growth factor (FGF) signalling pathway (Yamada et al., 2016). The FGF family members are effective regulators of cell differentiation, proliferation, migration and survival (Grose and Dickson, 2005). Thus, they can be classified as oncogenes (Grose and Dickson, 2005). Taken together, the current data highlights KPNA2 as an important player in cancer pathogenesis.

2.3.2 Alpha 2 subfamily: KPNA3 and KPNA4

KPNA4 and KPNA3, members of the karyopherin alpha subfamily 2, also represent KPNAs with a proven association with cancer. They have been shown to mediate the nuclear import of the members of the NF-κB transcription factor family as well as the Notch intracellular domain after its activation and release from plasma membrane, causing the activation of these pathways (Fagerlund et al., 2005; Sachan et al., 2013). Both pathways control cell survival and proliferation as well as differentiation and are hence relevant to cancer (Thomas et al., 2015; Yuan et al., 2015; Nowell and Radtke, 2017). Furthermore, KPNA4 is a transporter of the tumor suppressor p53 (Marchenko et al., 2010) and the signal transducer and activator of transcription 3 and 6 (STAT3/6) proteins (Chen and Reich, 2010; Liu et al., 2005). STAT3 and STAT6, like the other STAT family members, are transcription factors that are phosphorylated by Janus kinases as a result of cytokine signalling and translocate to the nucleus to activate genes involved in e.g. cell growth and differentiation (Villarino et al., 2015). The participation of KPNA3 and KPNA4 in the regulation of these pathways underline their importance in cancer.

In glioblastoma, downregulation of KPNA4 expression by the micro-RNA miR-181b leads to reduced malignancy via suppression of epithelial-to-mesenchymal

transition (EMT) (Wang et al., 2015). Wang and colleagues hypothesize that the reduced transport of NF-κB after KPNA4 inhibition is the mechanism behind the EMT suppression, as NF-κB is known to be essential for the EMT process (Huber et al., 2004; Wang et al., 2015). However, no experimental evidence is provided to corroborate this speculation.

More recently, KPNA4 expression was found to be positively correlated with prostate cancer progression and to promote prostate cancer cell migration *in vitro* as well as invasion and metastasis *in vivo* (Yang et al., 2017). The cause of these effects was again traced to the NF-κB pathway. KPNA4 depletion was shown to lead to reduced nuclear import of NF-κB and hence reduced activity of NF-κB –controlled signalling cascades, including cytokine signalling (Yang et al., 2017). The report showed that in KPNA4-depleted cells, the cytokines tumor necrosis factor alpha and beta (TNF-α and –β, respectively) were downregulated and mediated the observed cellular effects (Yang et al., 2017).

2.3.3 Alpha 3 subfamily: KPNA1, KPNA5 and KPNA6

Cancer-related roles of the members of the karyopherin alpha 3 subfamily have been less extensively studied thus far and reports describing their abnormal expression in cancer are scarce. One paper identified KPNA6 overexpression in chronic myeloid leukaemia (Mascarenhas et al., 2014). However, there are reports linking the subfamily alpha 3 members with cancer-associated cargo proteins. KPNA1 and KPNA6 are transporters of STAT1 and STAT3 (Ma and Cao, 2006), members of the already-mentioned STAT family. Recently all three karyopherins of the alpha 3 subfamily were reported to interact with prohibitin 2 (PHB2), which acts as a tumor suppressor in breast cancer, and these KPNAs are responsible for PHB2's nuclear translocation (Kim et al., 2015). KPNA1 has also been implicated in the nuclear import of FGF1 (Zhen et al., 2012). Another known cargo of KPNA1 is phosphorylated E47, a regulator of E-cadherin expression and suppressor of EMT in colon cancer (Zhu et al., 2016).

2.3.4 KPNAs as biomarkers and prognostic tools in cancer

Late cancer diagnosis at a point when the cancer has already progressed and metastasized is an important factor in poor patient outcomes. This emphasizes the need for new and effective biomarkers for cancer detection as well as tools to predict

the cancer progression, i.e. prognostic markers. Karyopherin alphas, when dysregulated in cancer, may represent a promising target for the development of such markers.

For example, KPNA2 overexpression has been studied extensively in relation to patient outcome in many cancer types (Stelma et al., 2016; Zhou et al., 2016b). High tumor tissue KPNA2 levels are associated with increased degree of malignancy, recurrence, tumor spread and poor patient outcome irrespective of the cancer type (Christiansen and Dyrskjøt, 2013; Zhou et al., 2016b), suggesting that KPNA2 is a useful prognostic tool in multiple cancers. The fact that elevated KPNA2 levels can be found at an early state of tumor progression makes it an attractive biomarker (Christiansen and Dyrskjøt, 2013). Furthermore, elevated KPNA2 expression can in some cases be detected in the serum of cancer patients (Wang et al., 2011b), fulfilling a requirement for easily obtainable patient sample that is prerequisite for a usable biomarker.

As discussed above, KPNA4 overexpression has also been associated with disease progression in prostate cancer and strongly correlates with pathological stage (Yang et al., 2017). In glioblastoma, patients with elevated miR-181b expression had a better prognosis because of KPNA4 inhibition and the reversal of EMT (Wang et al., 2015). Yang et al. also found a link between another miRNA, miR-708 and downregulation of KPNA4 expression levels, which led to the inhibition of tumor cell invasion (Yang et al., 2016). Based on these results KPNA4 and its miRNA regulators might represent potential prognostic markers in prostate cancer and glioblastoma.

2.4 Functions of karyopherins beyond nuclear transport

The functional role of the KPNAs in the cell is not limited to nuclear transport but is more complex. For example, when the nuclear envelope is disassembled and reassembled during cell division to allow the segregation of chromosomes to the nuclei of daughter cells, the transport receptors take on new roles. These and the other non-transport functions of KPNAs are discussed below.

2.4.1 Formation of the mitotic spindle

During mitosis, karyopherins participate in the assembly of the mitotic spindle, where the duplicated chromosomes are aligned, and thereby ensure the fidelity of cell division (Mosammaparast and Pemberton, 2004; Forbes et al., 2015). KPNB1, with the aid of KPNAs, binds proteins known as the spindle assembly factors (SAFs) and keeps them inactive by spatially isolating them from the chromatin to the periphery of the cell (Forbes et al., 2015). The SAFs are released at an appropriate location near the mitotic chromosomes, thus ensuring proper localization of the mitotic spindle (Forbes et al., 2015). This process, like the release of KPNA cargos in the nucleus, is regulated by the differential concentration of Ran-GTP between the nucleus and cytoplasm (Clarke and Zhang, 2008; Forbes et al., 2015). The chromatin-bound Ran-GEF regulator of chromatin condensation 1 (RCC1) maintains a cloud of Ran-GTP around the chromatin when the nuclear envelope dissociates during mitosis (Clarke and Zhang, 2008; Forbes et al., 2015). In addition, Ran-GAPs in the cytoplasmic regions catalyse the Ran-GTP-to-Ran-GDP conversion of any Ran-GTP diffused away from the vicinity of chromosomes (Kalab and Heald, 2008). In this way, the KPNA/KPNB1 heterodimer ensures the correct spatiotemporal release of the SAFs only in close proximity to mitotic chromosomes and the mitotic spindle forms exclusively in that locale.

2.4.2 Reassembly of the nuclear envelope

Subsequent to chromosome separation, KPNAs, again regulated by RanGTP, are also involved in the reassembly of the nuclear envelope that consists of the inner and outer nuclear membranes and associated proteins, mainly lamins and the NPC proteins (Mosammaparast and Pemberton, 2004; Hachet et al., 2004; Forbes et al., 2015). One way KPNAs participate in this process is naturally through the interaction with NLS-containing proteins. For example, lamins contain a cNLS that can be bound by KPNAs. The KPNA-lamin interaction prevents the lamins from prematurely assembling into filaments outside of the nuclear lamina, suggesting a regulatory role for KPNAs in the formation of the nuclear envelope (Adam et al., 2008). However, in the vicinity of Ran-GTP around the chromosomes, the lamins are released from KPNAs and are free to polymerize (Adam et al., 2008). The other function of KPNAs in the assembly of the nuclear envelope is more unconventional and occurs via the association of dephosphorylated KPNAs with the membranes that are in the process of forming the nuclear envelope (Hachet et al., 2004). This

membrane association of KPNAs possibly regulates the fusion of the nuclear envelope that is required for its reformation (Hachet et al., 2004).

2.4.3 Regulation of gene expression

Karyopherin alphas, especially KPNA2, have been indicated to participate in the regulation of gene expression in a way that is not dependent on the transport of transcription factors into the nucleus. A study by Huang and co-workers suggests that KPNA2 participates in the regulation of p21 gene transcription by promoting the anchorage of transcription factors on the p21 promoter (Huang et al., 2007). A later study exhibited a similar result, with KPNA2 localizing to *Serine/threonine kinase 35* (*STK35*) promoter region and accelerating its transcription (Yasuda et al., 2012). However, it remained undetermined whether KPNA2 associated with the DNA directly or via an NLS-containing TFs.

2.4.4 Cytoplasmic retention of cargo proteins

Certain studies have demonstrated that KPNAs are also capable of negatively regulating the nuclear import of select cargos through cytoplasmic retention. For example, telomere repeat factor 1 (TRF1), cell division cycle 7 (Cdc7) and transcription regulator Snail are all transported to the nucleus by KPNB1 alone, and KPNA inhibits their nuclear import (Forwood and Jans, 2002; Kim and Lee, 2006; Sekimoto et al., 2011). TRF1 controls telomere length (Chong et al., 1995; Walker and Zhu, 2012), Cdc7 regulates the G1/S transition of the cell cycle and DNA replication (Jiang and Hunter, 1997; Yamada et al., 2014) and Snail is involved in regulating EMT (Nieto, 2002; Wang et al., 2013). Cdc7 and Snail interact directly with KPNA whereas the transport inhibition of TRF1 occurs via association of TRF1-KPNB1 complex with KPNA (Forwood and Jans, 2002; Kim and Lee, 2006; Sekimoto et al., 2011). Furthermore, in mouse embryonic stem cells, transcription factors Oct6 and Brn2 are retained in the cytoplasm in a transport-incompetent state by Kpna2 until differentiation occurs (Yasuhara et al., 2013). Upon differentiation, Kpna2 levels are diminished and Oct6 and Brn2 are released to fulfil their duties as transcription factors (Yasuhara et al., 2013). These examples illustrate that the proteins bound by KPNAs are not necessarily targets of the KPNA/KPNB1 transport pathway but may participate in other functions in the cell, accentuating the multifaceted roles of karyopherins.

3 AIMS OF THE STUDY

Alterations in cellular processes that sustain appropriate transport between the cytoplasm and the nucleus and maintain correct nuclear morphology are known to have an important role in cancer pathogenesis. In addition, nuclear transport proteins have been recently implicated as prognostic markers and therapeutic strategies. In our previous studies we have identified the nuclear import receptor karyopherin alpha 7 (KPNA7) as a target gene of the 7q21-22 chromosomal amplicon in pancreatic cancer (Laurila et al., 2009). The aim of this study was to obtain new knowledge on KPNA7 in cancer in order to shed more light on the role of nuclear import and its aberrations in cancer. The specific aims were as follows:

1. To functionally characterize KPNA7 in pancreatic cancer cells with high endogenous expression levels.
2. To unveil the role of KPNA7 in universal regulation of cancer cell growth and maintenance of proper mitosis and nuclear morphology
3. To identify KPNA7 cargo proteins in pancreatic cancer cells.

4 MATERIALS AND METHODS

4.1 Cell lines (I, II, III) and RNA samples (I, II)

Twenty-one established pancreatic and breast cancer cell lines were used in this study. Seventeen of these (AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, Hs700T, Hs766T, MCF-7, MDA-MB-231, MDA-MB-453, MIA PaCa-2, PANC-1, Su.86.86, SW 1990, and T-47D) as well as the normal pancreatic cell line hTERT-HPNE, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and three (DanG, Hup-T3, and Hup-T4) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell lines were authenticated by genotyping and were grown under recommended culture conditions. The cells were regularly tested for Mycoplasma infection.

Four normal pancreatic RNA samples were obtained from commercial providers (Ambion, Austin, TX, USA; Biochain, Hayward, CA, USA; and Clontech, Mountain View, CA, USA) and the panel of normal RNA samples was purchased from Ambion. A cDNA panel representing various fetal tissues (human fetal MTC panel) was obtained from Clontech.

4.2 qRT-PCR (I, II, III)

Quantitative real-time PCR was performed using the Lightcycler 2.0 instrument (Roche, Mannheim, Germany) with LightCycler® TaqMan® Master reaction mix (Roche). Universal probe library (UPL) probes (Roche) and associated primers (Sigma-Aldrich, St. Luis, MO, USA) for *KPNA7*, *MVP* and *ZNF414* genes were used and Roche's Reference Gene Assay for HPRT was used for normalization.

4.3 Transfections

4.3.1 Transfection of small interfering RNAs (siRNAs) (I, II, III)

Transfections were performed on 24-well plates with 25,000 (I) or 35,000 (II) cells per well or on 6-well plates with 150,000 cells per well. Twenty-four hours after seeding the cells were transfected using 10nM siRNA and Interferin reagent (Polyplus Transfection, SanMarcos, CA, USA) as instructed by the manufacturer. Briefly, siRNAs were mixed with Interferin reagent in the cell line-specific medium base without supplements and added to cells cultured in complete media after 10 min incubation. The following siRNAs were used: for *KPNA7*, four specific small interfering RNAs (siRNAs) against the gene were designed using the siRNA Selection Program of the Whitehead Institute, Cambridge, MA, USA and the siRNAs were obtained from Dharmacon (Lafayette, CO, USA). A pool containing an equal amount of each of the four siRNAs was prepared. *MVP* and *ZNF414* siRNAs were obtained from the Dharmacon siRNA library (siGENOME SMARTpool siRNAs). A siRNA targeting the firefly luciferase (*LUC*) gene was used as a control in all experiments. Efficient silencing of the target gene was confirmed in each experiment using qRT-PCR. The sequences of the siRNAs used in this study are listed in Table 1.

Table 1. Sequences of siRNAs used in gene silencing experiments.

Gene	siRNA sequences in the pool
KPNA7	GAACAGACCUUAAAGAGAA, GAAGAAGAUGAGAGCCAAA, GAGCCAAGUCAUAGACCAA, CAACAAGCGCAUCGGCCAA
MVP	UAAAGGCGCUGCUUGAUUU, GAACUCAGCCCGCAUCAUU, GGAUAAAGAUGGAGACAAG, GAGGAGCAGUUCACAGUGU
ZNF414	ACGCACCGCUCGCUCUUCA, CUUGAACCCUGCGCCCUUU, GGCCUGACCAGCAUAGUCU, ACUACAAACCCAAUCGCUA

4.3.2 Transfection of plasmids (III)

Plasmid transfections were performed on Millicell® EZ chamber slides (Millipore, Tullagreen Carrigtwohill Cork, Ireland), 6-well plates or 100 mm cell culture dishes with 50,000; 170,000 or $2.2x10^6$ cells per well, respectively, using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, plasmid DNA and Lipofectamine were diluted in the cell line-specific medium base separately and then combined. The transfection mixture was added to cells cultured in complete media after 10 min incubation.

4.4 Immunofluorescence (IF) assays (I, II, III)

The IF stainings in original communication I were performed as previously described in (Kallio et al., 2011) and in original communications II and III as described in (Ihalainen et al., 2015). For LC3B IF, a methanol permeabilization step performed at -20°C was added as recommended by the antibody manufacturer. The primary antibodies used are summarized in Table 2. Alexa Fluor secondary antibodies 1:200 (Molecular Probes, Eugene, OR, USA) were used. Samples were mounted in ProLong Antifade Gold reagent with DAPI (Molecular probes). The fluorescently labeled cells were analyzed and photographed using either the Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan), Zeiss LSM 780 laser scanning confocal microscope (Zeiss, Oberkoche, Germany) or the Zeiss Apotome (Zeiss).

4.5 Functional assays

4.5.1 Cell proliferation (I, II, III)

Cell proliferation assays were performed on 24-well plates and the cells were counted 72 or 96 hours after siRNA transfections using a Coulter Z2 Coulter Counter (Beckman Coulter, San Diego, CA, USA). The assays were done in six replicates and repeated at least twice.

4.5.2 Cell cycle and apoptosis assays (I, II)

For cell cycle and apoptosis analyses, 150,000 cells per well on a 6-well plate were transfected with siRNAs and collected 48 or 72h after transfection. For cell cycle analyses, cells were suspended in 500 µl of hypotonic propidium iodide staining buffer (0.1 mg/mL sodium citrate tribasic dehydrate, 0.03% Triton X-100, 50 µg/µL propidium iodide, 2 µg/mL RNase A). For the apoptosis assay the Annexin V FITC Apoptosis Detection Kit was used (Calbiochem, Nottingham, UK). The cell cycle distributions and the number of apoptotic cells were analyzed using the Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA) and the ModFit LT software (Verity Software House Inc, Topsham, ME, USA). All experiments were performed in six replicates and repeated at least twice.

4.5.3 Colony formation (I)

Potential for anchorage independent growth was assayed by growing siRNA transfected cells on 0.35% agarose on six-well plates. After 14 days, twelve images per well were captured with the Olympus IX71 microscope (Olympus Corporation) using the Capture Pro 6.0 program. The number, size and total area of colonies were quantified using the ImageJ software (Abràmoff et al., 2004).

4.5.4 Autophagy (I)

Autophagy levels were assessed in siRNA-treated cells 96 h after transfection. Analysis was performed by labeling lysosomes by adding 50 nM Lysotracker (Life Technologies) to cell culture medium followed by incubation for 1 h at 37°C or by immunofluorescent staining of autophagy marker LC3B.

4.6 Western blotting (I, II, III)

4.6.1 Protein extraction (I, II)

Total protein from the cell lines was collected by first washing the cell monolayer twice with PBS and then lysing the cells into RIPA buffer (1% PBS, 1% non-idet P-

40, 0.5% sodium deoxycholate, and 0.1% SDS) containing Complete mini protease inhibitor cocktail (Roche).

For co-immunoprecipitation, cells were lysed as recommended by the Dynabeads® Co-Immunoprecipitation Kit manual (Life Technologies, Carlsbad, CA, USA). Shortly, cells were lysed in Extraction Buffer A (150 mM NaCl, 0,01% Tween) containing Complete mini protease inhibitor cocktail (Roche) in 1:9 (w/v) ratio and cleared lysates were used for co-IP.

4.6.2 Nuclear-cytoplasmic fractionation (I, III)

Nuclear and cytoplasmic fractions were extracted using NE-PER nuclear and cytoplasmic fractionation kit (Thermo Fisher Scientific, Bremen, Germany) according to the manufacturer's instructions. Briefly, the cytoplasmic proteins were first extracted with cytoplasmic extraction reagents I and II. After this, the nuclei were pelleted and lysed with nuclear extraction reagent.

4.6.3 Gel electrophoresis and protein detection (I, II, III)

30-50 μg of total protein extract was separated in a 10% SDS-PAGE gel. The proteins were transferred onto a polyvinylidene fluoride membrane (Roche) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with Blocking Reagent (Roche) in tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1h at RT. After blocking, the membrane was probed with a primary antibody diluted in 3% BSA in 0.05% TBS-Tween-20 (TBST) overnight at 4^0C and subsequently with HRP-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA) 1:8000 in 0.05% TBST for 1h at RT. The protein bands were detected with BM Chemiluminescence Western Blotting Substrate (Roche). All antibodies and dilutions used in Western blot analyses are summarized in Table 2.

Table 2. Primary antibodies and dilutions used in Western blots and immunofluorescence analyses.

Antibody	Dilution used in		Manufacturer	Cat. number
	Western	IF		
CDK2	1:200	-	Santa Cruz Biotechnology	sc-6248
CDK6	1:200	-	Santa Cruz Biotechnology	sc-7180
Cleaved Caspase-3	-	1:500	Cell Signaling Technology	#9664
Cyclin A	1:200	-	Santa Cruz Biotechnology	sc-751
Cyclin E	1:200	-	Santa Cruz Biotechnology	sc-247
E-Cadherin	-	1:500	Abcam	ab40772
Fibrillarin	1:1000	-	Cell Signaling Technology	C13C3
KPNB1	1:500	-	Abcam	ab2811
Lamin A/C	1:500	1:200	Abcam	ab8984
Lamin B1	1:1000	1:500	Abcam	ab16048
LC3B	-	1:200	Cell Signaling Technology	#3868
MVP	1:1000	1:500	Thermo Fisher	PA5-22296
NUP153	1:1000	1:1000		ab24700
p21	1:100	-	Santa Cruz Biotechnology	sc-397
p27	1:500	-	Santa Cruz Biotechnology	sc-528
Phalloidin 568	-	1:200	Molecular probes	A12380
p-Myosin light chain II	-	1:200	Cell Signaling Technology	#3674S
Streptag	1:1000	-	Abcam	ab184224
Streptag	-	1:500	Abcam	ab184526
β-Tubulin	1:20,000	-	Sigma-Aldrich	T7816
γ-Tubulin	-	1:500	Abcam	ab179503
V5	-	1:2000	Abcam	ab27671
Vimentin	-	1:500	Sigma-Aldrich	V6630
ZNF414	-	1:100	Santa Cruz Biotechnology	sc-249554

4.7 Generation of stable KPNA7-expressing cell lines (III)

Stable, inducible KPNA7-overexpressing Hs700T and MIA PaCa-2 cell lines were generated using the Lenti-X™ Tet-On® Advanced Inducible Gene Expression System (Clontech) according to manufacturer's instructions. In brief, stable Tet-On

cell lines were first generated via lentiviral transduction of pLVX-Tet-On Advanced plasmid and selection with geneticin. Then, KPNA7-Twin-Strep-tag® pLVX-Tight-Puro plasmid was transduced into these cells and positive cells selected with puromycin. Induction of KPNA7 expression was achieved with doxycycline treatment. The expression of KPNA7 mRNA was verified with qRT-PCR and the presence of the KPNA7 protein with Western blotting using anti-Streptag antibody. Control cell lines expressing only green fluorescent protein (GFP) were also generated.

Clones were isolated from the heteropopulation using cloning rings and characterized with qRT-PCR for *KPNA7* expression in both induced and uninduced states. The highest *KPNA7*-expressing clones with minimal uninduced expression from both cell lines were chosen for protein studies.

4.8 Affinity chromatography (III)

To isolate KPNA7 interaction partners, affinity chromatography was performed. To this end, 1.5 x 10^6 cells were seeded to 145 mm cell culture plates. The next day, induction of KPNA7 expression was achieved using doxycycline. Cells were collected 48 h after induction and lysates used for chromatography by the Strep-Tactin based method. Corresponding GFP cell lines were used as controls and subjected to similar treatments. Eluted proteins were pooled and concentrated and the isolation of the recombinant KPNA7 protein was confirmed with Western blotting with anti-Streptag antibody.

4.9 Mass spectrometry (III)

Proteins from pooled elution fractions were identified with SDS-PAGE and subsequent mass spectrometry compatible silver staining (protocol available at http://www.btk.fi/proteomics/services/protocols/). In brief, the gels were fixed for 1 h (30% ethanol, 10% acetic acid), then rinsed for 15 min 20% ethanol and distilled water. The proteins were shortly sensitized with sodium thiosulfate and stained with silver nitrate for 30 min. The bands were developed with potassium carbonate until the bands reached a desired intensity and stopped with Tris-base stop solution. Protein bands observed in KPNA7 lanes but not present in GFP lanes were analyzed with liquid chromatography-electrospray ionization-tandem mass

spectrometry (LC-ESI-MS/MS) at the Proteomics Facility, BTK, University of Turku. The corresponding areas from GFP lanes were also analyzed as negative controls.

The data obtained from mass spectrometry analysis was subsequently further filtered according to the following parameters: a) protein size must match with the size of the band in the gel (±30 kDa for large proteins ≥100 kDa, ±15 kDa for medium-sized 50-90 kDa proteins, and ±10 kDa for small proteins ≤50 kDa), b) at least 2 unique peptides must match the candidate protein sequence, and c) the proteins must exhibit 2-fold increase in the number of peptides in KPNA7-fraction compared to control GFP fraction. Common false positives, such as keratins, keratin-associated proteins and serum albumin were excluded from the analysis. The CRAPome database (Mellacheruvu et al., 2013) was also utilized to exclude most likely false positive hits.

4.9.1 Web-based analysis of MS results (III)

The MS results were subjected to a variety of web-based analyses. The NLS prediction algorithm NLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi; Kosugi et al., 2009) was used to analyze the entire candidate protein sequences for possible nuclear localization signals. Literature and protein database searches were also utilized to assess possible known nuclear localization and/ or function. A functional analysis using ToppGene Suite software (https://toppgene.cchmc.org/; Chen et al., 2009) was used to reveal enriched biological process pathways. The gene ontology categories yielded by ToppGene were further analyzed with Revigo software (http://revigo.irb.hr/; Supek et al., 2011) to reduce redundancy and to increase the legibility of the list. The subcellular localization of the proteins was retrieved from the COMPARTMENTS database (http://compartments.jensenlab.org/). A domain analysis was performed using Pfam database (http://pfam.xfam.org/; Binder et al., 2014) to identify protein domains that are present in the identified proteins with higher frequency than would be expected for a random sampling of the human proteome.

4.10 Validation of MS results (III)

4.10.1 Co-immunoprecipitation (III)

Nine putative KPNA7 cargos were chosen for validation of binding to KPNA7. V5-tagged pcDNA6.2/EmGFP-Bsd/V5-DEST constructs were generated using the Genome Biology Unit cloning service (Biocenter Finland, University of Helsinki). Briefly, entry clones from the human ORFeome collaboration library were transferred into the pcDNA6.2/EmGFP-Bsd/V5-DEST destination vector using the standard LR reaction protocol. Each plasmid construct was individually transfected into stable KPNA7-expressing cell lines. Induction of KPNA7 expression with doxycycline was performed 3 h after transfection.

Interactions were confirmed with co-immunoprecipitation (co-IP) using V5-antibody and Dynabeads® Co-Immunoprecipitation Kit (Life Technologies, Carlsbad, CA, USA) as instructed by the manufacturer. Co-immunoprecipitated protein complexes were analyzed with Western blotting using anti-V5 and anti-Streptag antibodies. Interaction with KPNB1 was included as a positive control. To this end, endogenous KPNB1 was co-immunoprecipitated from KPNA7-expressing cell lines using KPNB1-specific antibody. Protein complexes were analyzed with Western blotting using anti-KPNB1 and anti-Streptag antibodies.

4.10.2 Functional validation of KPNA7 cargos (III)

To confirm the nuclear import of MVP and ZNF414 by KPNA7, parental Hs700T cells were transfected with V5-tagged ZNF414 plasmid 24 h after plating and subsequently 24 h later with *KPNA7* or *LUC* siRNAs. For MVP analysis, cells were transfected with only *KPNA7* siRNA 24 h after plating. For Western blotting, nuclear and cytoplasmic fractions were extracted and analyzed with anti-MVP or anti-V5 antibodies. IF was performed using anti-MVP and anti-ZNF414 antibodies. To assess the functional roles of validated KPNA7 cargos their expression was silenced in Hs700T cells using siRNAs followed by assays for cell proliferation.

4.11 Statistical analyses (I, II, III)

The Mann-Whitney test was used to compare the medians of the test and control groups in all functional studies.

5 SUMMARY OF THE RESULTS

5.1 *KPNA7* expression is reactivated in cancer cells (I, II)

The *KPNA7* gene is located in the 7q21-22 chromosomal locus that is frequently amplified in pancreatic cancer cells (Laurila et al., 2009). The amplification leads to overexpression of *KPNA7* in these cells, whereas its expression in normal pancreas is almost absent. Due to this expression pattern that suggests a cancer-specific reactivation of the *KPNA7* gene, a qRT-PCR analysis was performed in a panel of 20 normal adult tissues, 16 pancreatic cancer cell lines, four breast cancer cell lines, four normal pancreatic tissues and one normal pancreatic epithelial cell line to map *KPNA7* expression levels. The expression levels were found to be very low in all adult tissues with the exception of ovary and trachea whereas being markedly elevated in a subset of pancreatic cancer cell lines (I, Fig. 1A). In normal pancreatic tissue the expression levels were negligible and normal pancreatic epithelial cell line exhibited no *KPNA7* expression (I, Fig. 1A; II, Supplementary Fig. S1). Three out of four breast cancer cell lines also exhibited *KPNA7* expression, albeit on lower levels than most of the pancreatic cancer cell lines (II, Supplementary Fig. S1). One breast cancer cell line (MDA-MB-453) with no *KPNA7* expression was also found (II, Supplementary Fig. S1). Based on these results, it seems that *KPNA7* expression is reactivated in a subset of cancer cells. The reactivation leads to different expression levels depending on whether the *KPNA7* gene locus is amplified or not.

5.2 KPNA7 is a key regulator of cancer cell growth (I, II)

To study the functional consequences of *KPNA7* overexpression, the gene was knocked down with a pool of siRNAs in a variety of cell lines. To obtain a comprehensive view on the functional role of KPNA7, cell lines with different *KPNA7* expression levels were chosen for these analyses. AsPC-1 and Hs700T pancreatic cancer cells harbour amplification of the *KPNA7* gene and as a result exhibit high endogenous levels of *KPNA7*. The other pancreatic (MIA PaCa-2, SU.86.86) and breast (MCF-7, MDA-MB-231, T-47D) cancer cell lines have no

KPNA7 gene amplification and exhibit varying but lower levels of *KPNA7* expression than AsPC-1 and Hs700T. Two cell lines (hTERT-HPNE normal pancreatic epithelial cell line and MDA-MB-453 breast cancer cell line) without endogenous *KPNA7* expression were utilized as negative controls.

Depletion of *KPNA7* resulted in a dramatic reduction (in the range of 20-54%) of cell growth in all cell lines with endogenous *KPNA7* expression (Table 3; I, Fig. 2A; II, Fig. 1A,B). The growth defects could be seen equally in pancreatic and breast cancer cells. As expected, the most dramatic effect was seen in the AsPC-1 and Hs700T cell lines where the endogenous expression level is the highest. Cancerous (MDA-MB-453) or normal (hTERT-HPNE) cell lines without endogenous *KPNA7* were not affected (Table 3; II, Fig. 1C).

Table 3. Summary of the growth defects and cell cycle alterations caused by *KPNA7* silencing in different cell lines (I, II)

Cell line	Type	KPNA7 expression status	Growth reduction[a]	Fraction of S-phase cells	
				siLUC	siKPNA7
AsPC-1	Pancreatic cancer	High	37%**	35%	20%**
Hs700T	Pancreatic cancer	High	54%**	42%	23%**
MIA PaCa-2	Pancreatic cancer	Low	18%**	11%	7%**
SU.86.86	Pancreatic cancer	Low	31%**	N/A	N/A
MCF-7	Breast cancer	Low	28%**	N/A	N/A
MDA-MB-231	Breast cancer	Low	20%*	N/A	N/A
T-47D	Breast cancer	Low	20%**	16%	4%**
MDA-MB-453	Breast cancer	None	Not altered	N/A	N/A
hTERT-HPNE	Normal pancreatic epithelium	None	Not altered	N/A	N/A

[a] Compared to siLUC control; N/A, not analysed; *p<0.05, **p<0.005

To determine whether the cause of the growth arrest phenotype was increased cell death or decreased proliferation, cell cycle analyses and apoptosis assays were performed. There were no differences in the number of apoptotic cells, but an obvious accumulation of cells in G1 phase and a decrease in S phase of the cell cycle could be detected 72 h after *KPNA7* knock-down in AsPC-1 and Hs700T cell lines (Table 3; I, Fig. 2C). In the other cell lines included in the cell cycle assays with a lower *KPNA7* levels, namely T-47D breast and MIA PaCa-2 pancreatic cancer cells, a similar S-phase reduction was observed although in this case it was not accompanied by a distinct G1 accumulation (Table 3; II, Fig. 2).

In exploration of the mechanism of the cell cycle alterations, the protein levels of six well-known cell cycle regulators (CDK2, CDK6, Cyclin A, Cyclin E, p21 and p27) were assessed in both nuclear and cytoplasmic fractions after *KPNA7* silencing in AsPC-1 and Hs700T cells. A clear induction in the protein levels of p21 was observed in both cell lines (I, Fig. 3A). The increment could be seen in both nuclear and cytoplasmic fractions (I, Fig. 3A), suggesting that the decreased nuclear transport of p21 due to KPNA7 depletion was not the mechanism of function in this case. To verify this, qRT-PCR analysis of *CDKN1A*/p21 mRNA levels was utilized. The analysis showed that *CDKN1A*/p21 mRNA levels were increased already at 48 h after *KPNA7* silencing (I, Fig. 3B), demonstrating that *CDKN1A*/p21 expression is induced at transcriptional level in AsPC-1 and Hs700T cell lines.

In conclusion, these results indicate that *KPNA7* reactivation confers a growth advantage to cancer cells. Any level of endogenous *KPNA7* expression is sufficient for growth promotion.

5.3 High *KPNA7* expression contributes to cancer-associated phenotypes (I)

In addition to increased growth rates, cancer cells exhibit a number of malignant properties. To assess whether *KPNA7* expression affects these characteristics, anchorage-independent growth potential, EMT, apoptosis and autophagy were analysed in *KPNA7* knock-down cancer cells.

The capability for anchorage-independent growth, a hallmark of cancer cells, was studied in KPNA7-depleted cells by culturing AsPC-1 and Hs700T cells in soft agar for 14 days. AsPC-1 total colony area was reduced 29% in siKPNA7 treated cells as compared to controls, due to reduced colony size (Fig. 4; I, Fig. 2). For Hs700T cells,

both the colony size and number of colonies was dramatically decreased with 79% reduction in total colony area (Fig. 4; I, Fig. 2).

Furthermore, *KPNA7*-silencing altered the morphology of Hs700T cells. Five days after siKPNA7 transfection, the cells acquired an elongated, fibroblast-like shape instead of their normal raft-like appearance (I, Fig. 4A). Immunostaining of E-cadherin and vimentin demonstrated that EMT did not contribute to this phenotype. Apoptosis was also not the cause as cleaved caspase-3 was not detected. However, an increase in lysosomal compartment volume in *KPNA7*-silenced cells was observed 72 h post-transfection (I, Fig. 4B), indicating induction of autophagy. This finding was substantiated by immunofluorescent staining of autophagy marker protein LC3B (Fig. 5; I, Fig 4C). Quantitation of the LC3B levels with ImageJ revealed that the amount of LC3B was increased 400% as compared to controls (I, Fig. 4C), confirming the autophagy induction.

These data suggest that in addition to cell growth, *KPNA7* expression promotes other cancer-associated phenotypes, like anchorage-independent growth and suppression of autophagy in pancreatic cancer cells.

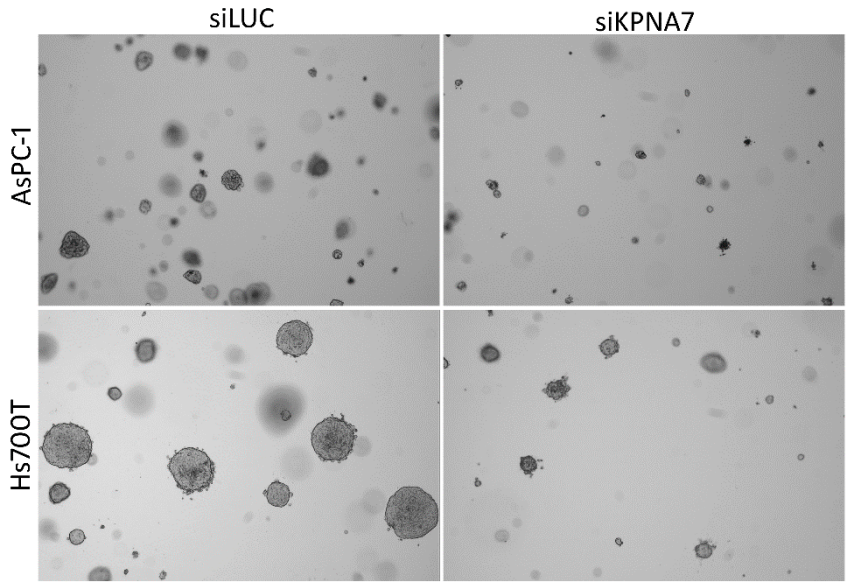


Figure 4. *KPNA7* depletion decreases the potential for anchorage-independent growth. AsPC-1 and Hs700T pancreatic cancer cells were treated with *LUC* or *KPNA7* siRNAs and grown on soft agar for 14 days.

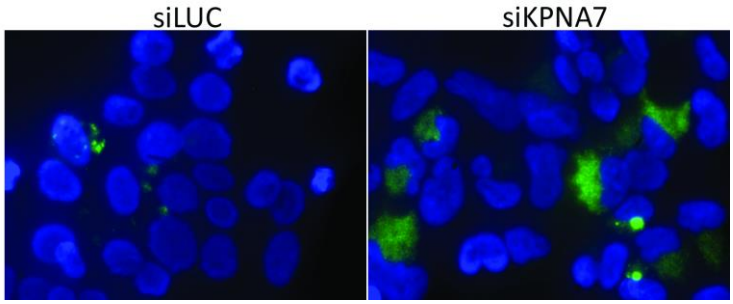


Figure 5. *KPNA7* knock-down induces autophagy is Hs700T cells. The cells were transfected with *LUC* or *KPNA7* siRNAs and autophagy marker LC3B (green) was immunofluorescently stained 96 h later with a concomitant counterstaining of nuclei with DAPI (blue).

5.4 Correct mitotic spindle formation is affected by *KPNA7* depletion (II)

The growth defects and cell cycle distribution changes in the siKPNA7-treated cells led to the theory that the cells undergo disturbances in mitosis. To study this, the organization of the mitotic spindle and centrosomes was explored by staining of γ-tubulin, their major structural component, in Hs700T and T-47D cells 96 h after siRNA transfections. The stainings revealed that the control cells had two centrosomes at the opposite ends of the cell, as normal cells should. The structure of the mitotic spindle was also ordinary and chromatin was localized correctly in the metaphase plate. In contrast after *KPNA7* silencing, a noteworthy fraction (20%) of mitotic Hs700T and T-47D cells had an anomalous number of centrosomes and mitotic spindles that originate from them. Typically, three or even four centrosomes were detected, leading to subsequent aberrant alignment of the chromatin (Fig. 6; II, Fig. 3).

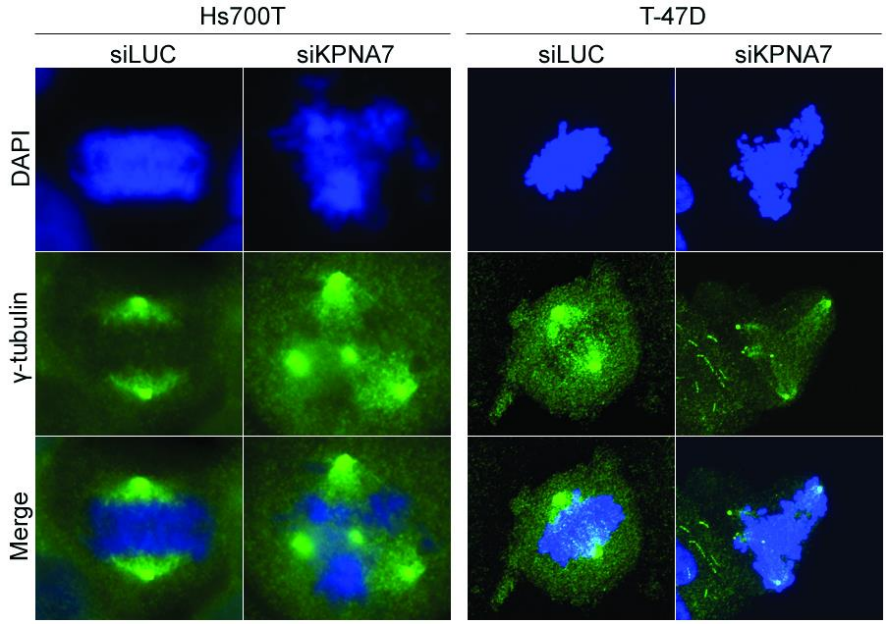


Figure 6. *KPNA7*-silenced cells undergoing mitosis have anomalous number of centrosomes and aberrant organization of the mitotic spindle. Hs700T pancreatic and T-47D breast cancer cells were transfected with siRNAs targeting *KPNA7* or *LUC* control and γ-tubulin (green) was immunofluorescently labelled 96h post-transfection. Nuclei were counterstained with DAPI (blue). Adapted from II, Fig. 3.

5.5 *KPNA7* silencing leads to abnormal nuclear morphology (II)

Based on the DAPI staining in the above-described experiments, it was noted that the *KPNA7*-silenced interphase cells had aberrant nuclear shape (Fig. 7; II, Fig. 4). To study this effect, the KPNA7-depleted cells were immunofluorescently stained for different lamin proteins, which are the major components of the nuclear lamina. The stainings revealed that KPNA7-depleted Hs700T and T-47D cells had a dramatically increased number of aberrant nuclei when compared to control cells (87% vs. 17% and 72% vs. 16%, respectively, Fig. 8a; II, Figs. 4B,D).

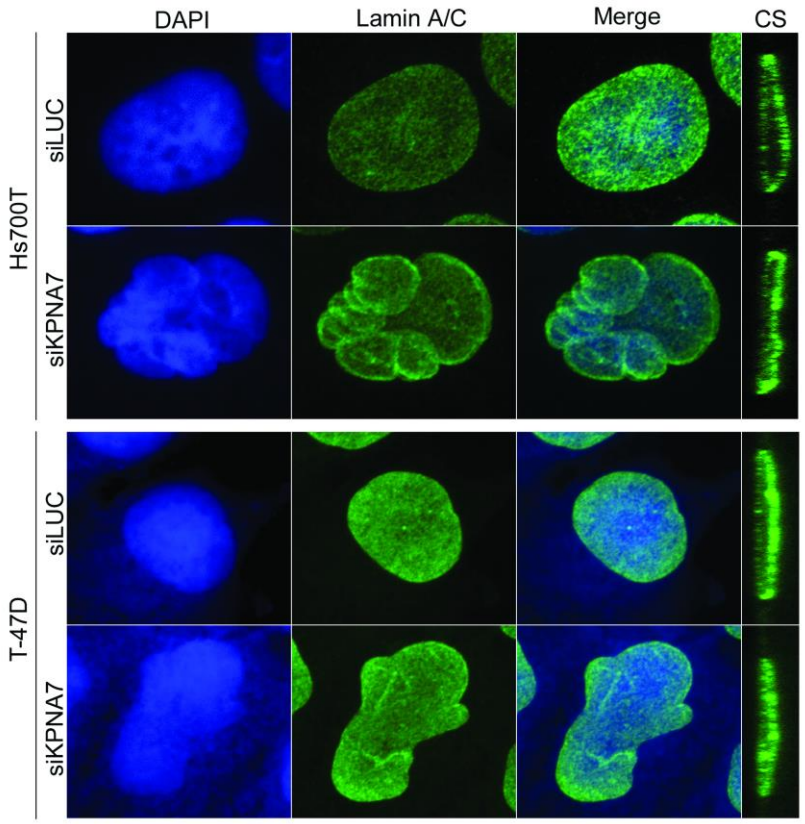
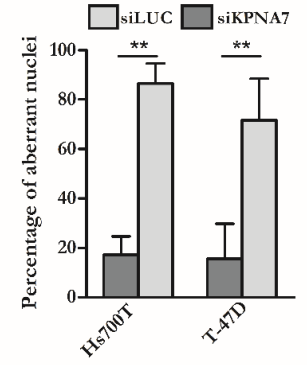


Figure 7. Example of morphological alterations induced by *KPNA7* knock-down. Hs700T pancreatic cancer cells and T-47D breast cancer cells were treated with control (*LUC*) or *KPNA7* siRNA and stained with lamin A/C antibody (green) 96h post-transfection. The nuclei were counterstained with DAPI (blue). CS=cross section of the nucleus. Adapted from II, Fig. 4.

Analysis of nuclear size and shape showed that the siKPNA7-treated Hs700T cells had significantly larger nuclei than control cells and exhibited a flattening of the nuclei, as evidenced by an increase in the YZ-directional aspect ratio of the nuclei (Fig. 8b; II, Fig. 4E) and their cross-sectional view (Fig. 7; II, Fig. 4A). In T-47D cells, the *KPNA7* depletion resulted in extended, distorted nuclei as shown by an increased XY-directional aspect ratio, but no flattening was observed (Figs. 7, 8b; II, Figs. 4C,F). Western blot analysis of the amount of the lamin proteins (Fig. 8b; II, Fig. 5) showed no major changes in lamin A/C amount in Hs700T cells but a noticeable decrease in T-47D cells after *KPNA7* silencing. In contrast, the amount of lamin B1 protein was clearly increased in siKPNA7-treated Hs700T cells compared to controls whereas no alterations were seen in T-47D cells.

To assess whether the morphological changes and flattening of the nuclei result from the activation of stress fibers, phosphorylated myosin light chain II (pMLCII) was immunologically stained in siRNA-treated Hs700T and T-47D cells. No radical alterations in the organization or amount of stress fibers was seen (II, Supplementary Fig. S4).

A



B

	Hs700T	T-47D
Nuclear area[a]	37% increase***	NC
XY aspect ratio[a]	NC	19% increase***
YZ aspect ratio[a]	63% increase***	NC
Lamin A/C amount[a]	NC	Increased
Lamin B1 amount[a]	Increased	NC

[a]Compared to siLUC control; NC, no change

Figure 8. (A) Quantitation of aberrant nuclei. Percentage of aberrant nuclei (mean and SD) in siKPNA7-treated Hs700T and T-47D cells compared to siLUC controls were calculated from at least six microscopic images. (B) Summary of the alterations in nuclear morphology induced by KPNA7 depletion. **p<0.005, ***p<0.0005. Data from II, Figs. 4 and 5.

5.6 Identification of KPNA7 cargo proteins (III)

The phenotypes caused by *KPNA7* silencing made us hypothesize that the aberrant subcellular localization of KPNA7 cargo proteins is the basis of the cellular changes. To pinpoint proteins transported to the nucleus by KPNA7 and hence responsible for the phenotypes, we created Hs700T and MIA PaCa-2 pancreatic cancer cell lines that stably overexpressed recombinant strep-tagged KPNA7 and performed Streptactin-based affinity chromatography followed by mass spectrometry (MS).

5.6.1 Putative KPNA7 cargo proteins contribute to a wide variety of biological processes

MS analysis revealed a total of 377 putative KPNA7 cargos, including essential members of the nuclear transport machinery and proteins previously shown to represent KPNA7 binding partners. However, a majority of the proteins identified

in this study were unique KPNA7 interaction partner candidates that have not been described in the existing literature.

To examine the specific biological processes the KPNA7 cargo candidates participate in, a gene ontology (GO) analysis was performed using ToppGene software. Many of the identified proteins had functions in RNA-related processes, like mRNA metabolism and RNA processing (III, Supplementary Table S3). Pathways associated with translation and protein localization were also represented among the cargo candidates (III, Supplementary Table S3). The enriched GO categories obtained with ToppGene were then evaluated with Revigo software in order to reduce redundancy of the GO terms. The results showed that in addition to the RNA-related pathways mentioned above, the putative cargos participated in many other biological processes, including those linked with e.g. cell cycle regulation (III, Fig. 1). The majority (49%) of the identified proteins were predicted to localize to the nucleus (Fig. 9a; III, Fig. 2A) and almost all (97%) contained a medium-strength to strong NLS (Fig. 9b; III, Fig. 2B).

Together, these results unveiled a number of novel KPNA7 cargo candidates with diverse roles in relevant biological pathways. The putative cargos were mainly localized to the nucleus and/or were predicted to contain an NLS in their amino acid sequence.

A

Subcellular localization	% of proteins
Nucleus	49
Cytosol	19
Mitochondrion	7
Cytoskeleton	7
Unknown	6
Endoplasmic reticulum	5
Extracellular	3
Plasma membrane	2
Other	2

B

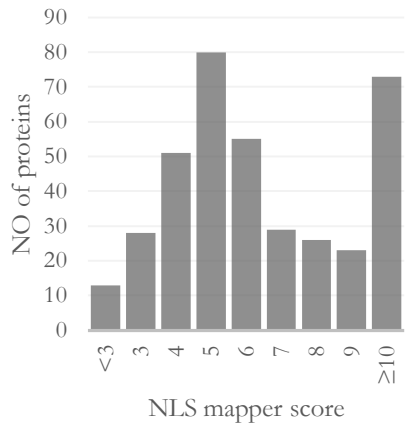


Figure 9. (A) Subcellular localizations of putative KPNA7 cargo proteins identified in proteomic screen. The localization data was obtained from Genecards database. (B) Distribution of NLS Mapper scores among the KPNA7 interaction partner candidates. Proteins with NLS score ≥8 are exclusively nuclear, those with scores 7 or 6 partially localize to the nucleus, those with a score of 3, 4, or 5 are localized to both the nucleus and the cytoplasm whereas proteins with a score of 1 or 2 localize to the cytoplasm. Re-illustrated data from III, Fig. 2.

5.6.2 MVP and ZNF414 represent novel KPNA7 cargo proteins that regulate pancreatic cancer cell growth

Nine putative KPNA7 cargos identified in the proteomic screen (CHD4, CTNNB1, DIEXF, ELAVL1, MVP, PUM1, SSRP1, TAF6, and ZNF414) were then selected for *in vitro* validation. The criteria used to choose the proteins for validation included, but was not limited to, known localization to the nucleus, and/or known function with possible cancer association. Binding to KPNA7 was confirmed with co-IP for two of the proteins, MVP and ZNF414 (III, Fig. 3).

To determine the basal subcellular localizations of MVP and ZNF414 and to illustrate that they are indeed transported to the nucleus by KPNA7, *KPNA7* was silenced in Hs700T cells and subsequent nuclear-cytoplasmic fractionation and Western blotting was performed. The analysis demonstrated that in control cells, MVP is localized almost exclusively to the nucleus, whereas ZNF414 has also a minor cytoplasmic protein pool (III, Fig. 4). The cytoplasmic pools of MVP and ZNF414 were prominently increased as a result of the *KPNA7* silencing, accompanied by a simultaneous decrease in nuclear ZNF414 levels (III, Fig. 4), indicating a disrupted nuclear import pathway. The nuclear levels of MVP were not changed, perhaps reflecting the longer half-life of MVP. The elevated amount of cytoplasmic MVP was also confirmed with immunofluorescence (III, Supplementary Fig. S4).

To assess the contribution of MVP and ZNF414 to the cellular phenotypes seen after *KPNA7* silencing, a siRNA-mediated silencing of the genes was performed in Hs700T cells followed by a cell proliferation assay. Silencing caused a dramatic reduction in the proliferation of Hs700T cells when compared to control cells (III, Fig. 5). For MVP, the silencing decreased growth by roughly 30%, while the knock-down of ZNF414 led to an even more drastic effect with 40% decrease in cell number at 96 h post-transfection (III, Fig. 5).

In conclusion, two putative KPNA7 interaction partners, MVP and ZNF414, identified in the proteomic screen were verified as KPNA7 cargos. These two proteins were shown to possess growth regulatory roles in pancreatic cancer cells.

6 DISCUSSION

6.1 KPNA7 is an important regulator of growth and malignant properties of cancer cells

Previous work in our research group has pinpointed *KPNA7* as one of the target genes of the 7q22 amplicon in pancreatic cancer (Laurila et al., 2009). In **Study I**, strong overexpression of *KPNA7* was demonstrated in a subset of pancreatic cancer cell lines that harbour the amplification. In **Studies I** and **II**, *KPNA7* expression was also detected at lower levels in pancreatic and breast cancer cell lines that do not contain the amplification, whereas the expression in normal adult tissues, was very low or absent. This result is supported by a large-scale RNA-sequencing study in normal human tissues, which revealed extremely low expression levels in adult tissues (The Human Protein Atlas https://www.proteinatlas.org/ENSG00000185467-KPNA7/tissue; Uhlén et al., 2015).

Studies in several animals have shown that KPNA7 is expressed in oocytes, during embryogenesis and later silenced in adult, terminally differentiated cells (Tejomurtula et al., 2009; Hu et al., 2010; Wang et al., 2012b; Wang et al., 2014). The mode of *KPNA7* silencing that occurs is not completely clear. It is possible that it takes place via epigenetic mechanisms; some data in cattle identified differentially methylated CpG sites in *KPNA7* promoter proximal regions in embryos vs. somatic tissue (Wang et al. 2013). The same study also identified a binding site for transcription factor Sp1 near KPNA7 transcription start site (TSS) (Wang et al. 2013). Sp1 is known to control tissue-specific expression of other genes and is highly conserved in eukaryotes (O'Connor et al., 2016). Human *KPNA7* has multiple transcription factor binding sites in a 6,000 bp region upstream of its TSS that overlap with open chromatin, including human SP1 (ENCODE Project Consortium, 2012). This region is likely to contain the promoter region. These results indicate that the expression of *KPNA7* might be regulated by both epigenetic and transcription factors also in human tissues. Taken together, the expression pattern of *KPNA7* suggests that it is abnormally reactivated in cancer cells. Such

cancer-related reactivation has been previously described for multiple genes with established roles during development (Kelleher et al., 2006; Ben-Porath et al., 2008).

To examine the functional consequences of *KPNA7* reactivation to cancer cells, its expression was inhibited with siRNAs and a series of functional assays was performed. *KPNA7* knock-down consistently decreased cell growth in all cell lines with endogenous *KPNA7* expression, even in those where the endogenous level was minimal. However, the most drastic effect was seen in AsPC-1 and Hs700T cells with the highest endogenous expression. This suggests that even low amounts of KPNA7 confer growth advantage to cancer cells, but higher expression levels boost the growth even more. The phenotypes were detected equally in pancreatic and breast cancer cell lines, indicating that the role of KPNA7 is not limited to pancreatic cancer. Importantly, cell lines without endogenous *KPNA7* expression exhibited no changes in proliferation, confirming the specificity of the phenotype to KPNA7 depletion. In Hs700T and AsPC-1 cells, the potential for anchorage-independent growth, another key feature of cancer cells (Hanahan and Weinberg, 2011), was also diminished in siKPNA7-treated cells when compared to control cells. In previous studies, the proliferative effect of KPNAs has been established also for the closest relative of KPNA7, KPNA2, which was shown to promote cell proliferation in many malignancies (Umegaki-Arao et al., 2013; Ikenberg et al., 2014; Ma and Zhao, 2014; Zhou et al., 2016a). This result supports the observation that KPNAs are essential for cell propagation.

The growth decrease observed in **Studies I** and **II** was accompanied by alterations in the cell cycle. All the studied cell lines exhibited a decrease in the fraction of S-phase cells that readily explains the growth reduction phenotype. In Hs700T and AsPC-1 cells, a G1 accumulation of cells was also detected, possibly induced by increased amounts of p21. The p21 protein is a well-known inhibitor of cyclin-dependent kinases (Xiong et al., 1993), mainly those that regulate G1/S transition of the cell cycle (Harper et al., 1993; Dutta and Abbas, 2009). The induction of p21 was shown to occur at mRNA level in both nuclear and cytoplasmic fractions, indicating that its nuclear import was not affected. It is possible that KPNA7 inhibition leads to a decreased nuclear trafficking of a negative regulator of p21 expression, resulting in the observed p21 transcriptional induction. However, this is purely speculation and no experimental evidence exists to confirm this.

In T-47D and MIA PaCa-2 cells no clear G1 arrest could be detected. These cell line specific phenotypes probably result from different genetic and phenotypic characteristics of the cell lines (Moore et al., 2001; Kenny et al., 2007; Deer et al., 2010), or are perhaps due to different KPNA7 cargos in these cells. Nevertheless,

the decreased fraction in proliferating S-phase cells detected in all cell lines assayed suggests some common mechanism behind the phenotype

In Hs700T cells, *KPNA7* knock-down dramatically altered the cell morphology, causing the cells to acquire a fibroblast-like, elongated shape instead of their normal raft-like appearance. The phenomenon was not caused by EMT. However, an increase in lysosomal compartment volume as well as LC3B staining was observed, indicating an induction of autophagy. Autophagy is a catabolic process that leads to the lysosomal degradation of damaged or unnecessary macromolecules and organelles and can be induced in response to various cellular stresses, like starvation (Kondo et al., 2005; Mathew et al., 2007; Fulda, 2017). In cancer, autophagy has a dual role: it functions as a tumor-suppressing mechanism by constraining tumor initiation while on the other hand promoting tumor cell survival by providing nutrients for sustained growth (Kimmelman and White, 2017). In the case of Hs700T cells, the induction of autophagy might reflect secondary effects of cellular stress and not be directly linked with *KPNA7* silencing, but is nonetheless an interesting phenomenon.

All the above-described effects of *KPNA* knock-down on cell behaviour are likely mediated by the decreased nuclear import of key KPNA7 cargo proteins that control cell proliferation and cell cycle. For example, in the case of KPNA2, it has been suggested that its cancer-associated cargos, like p53, are likely to play a role in KPNA2-mediated carcinogenesis and enhanced cancer cell proliferation (Ikenberg et al., 2014). Especially in the cell lines where KPNA7 expression is high, it is feasible that the cells have developed a dependency for the enhanced nuclear import KPNA7 cargos. However, it is also possible that the growth defects associated with KPNA7 depletion are related to other, non-transport functions of the protein. In order to clarify the reason behind the phenotypes resulting from KPNA7-depletion, knowledge of KPNA7 cargos is direly needed.

6.2 KPNA7 depletion induces mitotic defects and deformation of nuclei in cancer cells

The cell cycle alterations detected in KPNA7-depleted cells led to the question whether mitosis is somehow disturbed in these cells. To address this issue, γ-tubulin staining was performed in **Study II** and revealed an abnormal number of centrosomes and spindle poles in a fifth of mitotic siKPNA7-treated Hs700T and

T-47D cells, with chromatin being pulled by three or more mitotic spindles towards as many centrosomes.

Centrosome multiplication and spindle misorientation have been previously discovered in human tumors (Pease and Tirnauer, 2011) and associate with genomic instability (Maiato and Logarinho, 2014). However, the absence of such aberrant spindles in the control cells indicates that this phenotype is indeed connected to KPNA7 depletion and not an artefact of the two cancer cell lines used in the study. It has been shown that mislocalization of Ran-GEF RCC1, the protein responsible for the maintenance of the higher Ran-GTP concentration in the nucleus compared to the cytoplasm, results in a multipolar spindle resembling the ones seen in our cells (Clarke and Zhang, 2008). RCC1 has an NLS and is transported into the nucleus by a heterodimer of KPNA3 and KPNB1 (Clarke and Zhang, 2008). Based on this, it is feasible that KPNA7 transports either RCC1 or another GEF into the nucleus and *KPNA7* knock-down disrupts this transport, resulting in mislocalization of the GEF and aberrant spindle formation during mitosis. In support of this, **Study III** revealed the GEF Ran-binding protein 10 as a putative KPNA7 cargo.

However, KPNA7 itself has been shown to localize to the spindle structures in murine cells (Hu et al., 2010), suggesting an involvement in spindle formation. Moreover, KPNB1 has been demonstrated to regulate the formation of the spindle via its importin alpha binding domain, further supporting the possible role of karyopherins in the regulation of spindle formation (Roscioli et al., 2012). These results indicate that KPNA7, possibly via its binding partners, plays a role in the proper organization of the spindle and its depletion causes aberrations in spindle formation.

In **Study II** it was demonstrated that in addition to effects on cell behavior, KPNA7 depletion induced distinct changes in the nuclear morphology in both pancreatic (Hs700T) and breast cancer (T-47D) cell lines used. Altered nuclear morphology has been associated with dysregulation of several important cellular functions, such as cell migration, intracellular signalling, DNA repair, cell division and gene expression (Chow, Factor et al. 2012), highlighting the relevance of our findings.

Changes in nuclear shape are usually attributed to different lamin proteins, which are intermediate filaments that form the nuclear lamina in the inner nuclear membrane (Gruenbaum and Foisner, 2015). Lamins A and C make nuclei stiffer, stabilizing them against mechanical stress, whereas B-type lamins lend elasticity (Swift et al., 2013; Osmanagic-Myers et al., 2015). Mutations in lamin proteins have been linked to many diseases known as laminopathies that are often associated with

altered nuclear structure and shape (Schreiber and Kennedy, 2013). For example, nuclear alterations similar to those demonstrated in our results are seen in Hutchinson–Gilford progeria syndrome (HGPS) where mutations in lamin A leads to lobular nuclear shape and to reduction in total amount of lamin B1 (Taimen et al., 2009).

Despite the similar appearance of the nuclei to those in HGPS, an increase in the amount of lamin B1 protein in Hs700T cell line was detected after KPNA7 depletion. The changed YZ directional aspect ratio of the nuclei in Hs700T cells suggests that the nuclei are flattened after siKPNA7 treatment and the cross-sectional views of the lamin protein stainings verified this observation. The increased amount of lamin B1 in Hs700T cells is likely to render the nuclear lamina more elastic (Osmanagic-Myers et al., 2015), thus allowing the nuclei to become flatter. The flattening of the nuclei is most likely responsible also for the increased nuclear area detected in Hs700T cells. In T-47D, no increase in lamin B1 was detected but lamin A/C amount was decreased. This might have a similar effect to nuclear rigidity as lamin B1 increase, as the A/C lamins are known to regulate nuclear rigidity and lamin A/C-depleted cells have been shown to have reduced nuclear stiffness (Lammerding et al., 2006). The nuclei in T-47D cells have initially high YZ aspect ratios, meaning that they are initially relatively flat. This may explain why the YZ aspect ratio and hence the nuclear area was not significantly affected in T-47D cells, as it is challenging to flatten nuclei with such high aspect ratios. The altered XY-directional aspect ratio in T-47D suggests that the nuclei in siKPNA7 treated cells adopt an elongated, more elliptical form.

Loss of lamin B1 levels have been linked with cellular senescence, which is a powerful tumor suppressive mechanism that leads to an irreversible cell cycle exit, and the lamin B1 loss has also been suggested as a senescence-associated biomarker (Freund et al., 2012; Chojnowski et al., 2015). For example, lamin B1 silencing in WI-38 human lung embryonic fibroblast cells induced untimely senescence (Shimi et al., 2011). However, upregulation of lamin B1 has been linked with induction of senescence in other studies (Shimi et al., 2011). Thus, it seems that the change in lamin B1 levels is not fully responsible for the senescence phenotype (Hutchison, 2014; Chojnowski et al., 2015). This notion is in concert with data in **Study I**, which demonstrates that the siKPNA7-treated Hs700T cells do not exhibit senescence-like characteristics despite the altered lamin B1 amount observed in **Study II**.

Based on the observation that a majority (80%) of KPNA7-depleted cells had altered nuclear morphology while a fifth of mitotic cells exhibited aberrant spindle structures, it can be concluded that the main impact of KPNA7 knock-down is

targeted to lamins and hence nuclear morphology. Lamins contain a NLS in their amino acid sequence (Loewinger and McKeon, 1988; Dechat et al., 2010) and thus can be assumed to directly interact with KPNA7. The depletion of KPNA7 then disrupts this interaction, perhaps leading to the reorganization of the lamins and the observed nuclear morphology. It is also possible that KPNA7 transports transcription factors that are essential for the expression of the lamins and regulates the lamin levels this way. Nuclear lamins have also been shown to participate in the formation of the mitotic spindle (Dechat et al., 2010). The observed mitotic defects might thus be a result of the dysfunction of the lamins that results from KPNA7 knock-down, leading to improper spindle formation. More studies are needed to accurately evaluate the chronological sequence of the aberrant mitosis and nuclear morphology, but perhaps based on these knowledge one could speculate that the nuclear morphology might be the first event of the sequence that then leads to abnormal mitosis.

6.3 KPNA7 cargo proteins and their relevance in cancer

The phenotypes observed in **Studies I** and **II** led to the straightforward hypothesis that the altered subcellular localization of proteins transported into the nucleus by KPNA7 is the cause of these changes. In **Study III**, this aspect was addressed with a proteomic approach aiming to identify these cargo proteins. At the time of Study III, only one report had focused on the investigation of KPNA7 interaction partners in human cells (Kimoto et al., 2015) and Study III was the first that aimed at identifying KPNA7 cargo proteins in the context of malignancy.

The protein pull-down of recombinant KPNA7 followed my mass spectrometry demonstrated KPNA7 binding to known members of the nuclear import pathway, like KPNB1 and Nups, thus confirming the functionality of the experimental approach. Known KPNA7 cargos, like p53 (Miyamoto and Oka, 2016), were also identified in the screen. However, the majority of the putative cargos revealed in the screen had not been shown to interact with KPNA7 in earlier studies. The identified KPNA7 cargos were mostly found to localize to the nucleus and/or to contain an NLS, supporting the nuclear transporter activity of KPNA7.

Analysis of the biological function of the putative KPNA7 cargos yielded enriched gene ontologies, like RNA processing and mRNA metabolic process, that had also been implicated in earlier data (Kimoto et al., 2015). Furthermore, pathways that are extremely relevant when considering the KPNA7 depletion phenotypes were

found to be enriched amongst the putative KPNA7 cargos. For example, KPNA7 binding partner candidates participate in cell cycle regulation, a phenotype that was demonstrated to be altered after KPNA7 silencing in **Studies I** and **II**.

In addition to previously demonstrated KPNA7 binding partners, many novel KPNA7 cargo candidates were successfully identified. Of these, major vault protein (MVP) and zinc finger protein 414 (ZNF414) were shown to bind KPNA7 *in vitro* as evidenced by co-IP experiments. Nuclear import by KPNA7 was also proven for MVP and ZNF414 as their cytoplasmic pools were noticeably increased in KPNA7-depleted cells, indicating their nuclear import interruption. In functional studies, the knock-down of both MVP and ZNF414 produced a growth arrest phenotype that distinctly matched the one seen after KPNA7 depletion. This result supports the view that the altered subcellular localization of MVP and ZNF414 resulting from KPNA7 inhibition is indeed responsible for the phenotypic effects seen in *KPNA7*-silenced cells in **Studies I and II**.

However, it is unlikely that the two proteins that were chosen for further studies were the only true KPNA7 cargos in the proteomic screen. Especially in the light of the protein localization data for the cargo candidates (demonstrating nuclear presence for most of the proteins) and NLS Mapper scores obtained in **Study III**, the hundreds of putative cargos probably contain multiple relevant KPNA7 cargos. Particularly those proteins that belong to the enriched gene ontologies, like cell cycle regulation, represent interesting targets for future validation and characterization.

6.3.1 Major vault protein

Major vault protein (MVP) is the main constituent in the structure of vaults, which are large ribonucleoprotein complexes conserved in eukaryotes and widely expressed in many cell types but whose exact function remains undetermined (Kedersha and Rome, 1986; Lara et al., 2011). MVP has been shown to be expressed widely in human normal and tumor tissues, thus suggesting an important role (Izquierdo et al., 1996b). Interestingly however, $MVP^{-/-}$ mice models did not exhibit any remarkable phenotypic effects and were completely viable and healthy (Mossink et al., 2002), leading the authors to speculate that other members of the vault complex might be able to fulfil the functional role of vaults in the absence of MVP.

MVP was originally identified as a protein overexpressed in multidrug resistant (MDR) lung cancer cell lines (Scheper et al., 1993) and thus named lung resistance protein (LRP). MVP expression has been demonstrated in almost 80% of human

cancer cell lines (Izquierdo et al., 1996a). Its expression levels correlate with drug resistance (Izquierdo et al., 1996a; Laurencot et al., 1997) and it has been proposed that MVP contributes to MDR by transporting cytotoxic drugs out of the nucleus (Kitazono et al., 1999; Han et al., 2012).

MVP is upregulated in multiple cancer cell lines upon anticancer treatment (Kitazono et al., 1999; Berger et al., 2000). Recently, frequent amplification of MVP was reported in glioblastoma and was associated with poor chemotherapy response (Navarro et al., 2015). In addition to its role in drug resistance, MVP and vaults have been indicated to function in a variety of cellular tasks, including as scaffolds or regulators of signalling cascades like Mek/Erk and PI3-kinase/Akt pathways (Yu et al., 2002; Kolli et al., 2004; Kim et al., 2006) that are closely involved in cancer. For example, MVP was shown to support glioblastoma survival and migration via upregulation of EGFR/PI3K signalling pathway (Lötsch et al., 2013). Also, vaults and MVP have been suggested as participants in DNA damage repair (reviewed in Lara et al., 2011) and resistance to apoptosis by modulation of Bcl-2 expression (Ryu et al., 2008). The research done on MVP and its involvement in many cancer-related processes highlights the role of MVP in cancer.

6.3.2 Zinc finger protein 414

Zinc finger protein 414 (ZNF414) is a zinc finger domain-containing protein identified in large-scale proteomic screens (Rolland et al., 2014; Rual et al., 2005). The function of ZNF414 both in normal and cancerous tissues is quite unknown for the time being, but other proteins with zinc finger domains have been well characterized and are known to participate in a variety of cellular activities, such as nucleic acid binding, protein-protein interactions and membrane association (reviewed in Laity et al., 2001). ZNF414 contains a classical C2H2 zinc finger domain and thus belongs to the krüppel C2H2-type zinc-finger protein family (Uniprot database http://www.uniprot.org/uniprot/Q96IQ9). C2H2-type zinc-finger proteins generally act as transcription factors recognizing specific DNA sequences via their zinc finger motifs and contribute to processes like development, differentiation, and suppression of malignant cell transformation (Razin et al., 2012).

Members of the C2H2-type zinc-finger protein family are abundant in the mammalian genome and indeed, an RNA-sequencing study in adult tissues revealed wide expression of *ZNF414* (NCBI Gene Database https://www.ncbi.nlm.nih.gov/gene/84330; Fagerberg et al., 2014). There is also

evidence of ZNF414 expression on protein level in many tissues (The Human Protein Atlas https://www.proteinatlas.org/ENSG00000133250-ZNF414/tissue; Uhlén et al., 2015). Interestingly, based on The Cancer Genome Atlas (TCGA) RNA sequencing data, *ZNF414* expression seems to be a favourable prognostic factor in head and neck, endometrial, pancreatic and urothelial cancers (data visualization at The Human Protein Atlas https://www.proteinatlas.org/ENSG00000133250-ZNF414/pathology; data generated by TCGA https://cancergenome.nih.gov/). This is also the case in KM plotter database for breast cancer (http://kmplot.com/analysis; Győrffy et al., 2010). This data is in disagreement with the results of **Study III**, where silencing of *ZNF414* led to severe inhibition of cancer cell growth suggesting a growth-promoting role for ZNF414. On the other hand, KM plotter predicts a favourable outcome for lung and gastric cancers exhibiting low *ZNF414* expression (http://kmplot.com/analysis/; Győrffy et al., 2013; Szász et al., 2014), supporting the results of **Study III**. In light of these conflicting results, and considering the lack of knowledge in terms of the function of ZNF414, its cancer association highlights it as an interesting subject for future studies.

7 CONCLUSIONS

Nuclear import is an essential process for all eukaryotic cells and its disturbances can lead to dysregulation of cellular homeostasis and ultimately to variety of diseases, including cancer. Karyopherin alpha (KPNA) family of nuclear import proteins have been previously shown to be overexpressed in different cancers. The aim of this study was to decipher the role of Karyopherin alpha 7 (KPNA7), the newest KPNA family member, to the function of cancer cells and to identify the transport cargo proteins of KPNA7.

KPNA7 expression was found to be very low in healthy adult tissues whereas it was highly overexpressed in certain pancreatic cancer cell lines that have chromosomal amplification of the *KPNA7* gene locus. Lower level expression was also detected in pancreatic and breast cancer cell lines without the amplification. This expression pattern suggests a cancer-specific reactivation of the gene. KPNA7 was demonstrated to promote cancer cell growth and malignant properties in cancer cell lines expressing *KPNA7* at any level. The inhibition of *KPNA7* expression via siRNA-based silencing attenuated cell proliferation, cell cycle alterations and anchorage-independent growth and led to induction of autophagy. These data indicate that KPNA7, even at low levels and most probably through its cargo proteins, is involved in the regulation of phenotypes that are essential for sustained growth and viability of cancer cells. Its effects are not limited to pancreatic cancer where it is overexpressed but can be detected in breast cancer cell lines as well. This suggests a broader relevance for KPNA7 in cancer. KPNA7 depletion also disturbed the proper mitosis via multipolar spindle structures and caused alterations in nuclear morphology, clarifying the contribution of KPNA7 to the maintenance of nuclear envelope environment and corroborating the alternative functions of karyopherins in cell division.

The altered subcellular localization of KPNA7 cargos due to KPNA7 depletion is most probably responsible for the cellular phenotypes observed after KPNA7 silencing. In this study, multiple putative KPNA7 cargos with functions relevant to the observed phenotypes were identified. The two proteins validated in vitro as true KPNA7 cargos, major vault protein (MVP) and zinc finger protein 414 (ZNF414), were shown to have growth regulatory roles in cancer cells as well, suggesting that

they might indeed be responsible for the growth arrest seen after *KPNA7* knock-down. These results provided new information on the growth regulation exerted by KPNA7 on cancer cells and opened interesting new avenues for future research.

In summary, this study provided information on the role of KPNA7 on human cancer. The results obtained advance our knowledge on how abnormal nuclear import protein expression interferes with cellular homeostasis. The data also shed light on how KPNA7 and its cargo proteins mediate cancer cell growth and the maintenance of nuclear envelope environment, thus enhancing our knowledge on the role of nuclear import and its aberrations in cancer pathogenesis. The expression pattern of *KPNA7* provides interesting possibilities regarding its use as a prognostic tool or therapy target, both desperately needed especially in pancreatic cancer.

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Research Article

KPNA7, a nuclear transport receptor, promotes malignant properties of pancreatic cancer cells *in vitro*

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ABSTRACT

Pancreatic cancer is an aggressive malignancy and one of the leading causes of cancer deaths. The high mortality rate is mostly due to the lack of appropriate tools for early detection of the disease and a shortage of effective therapies. We have previously shown that karyopherin alpha 7 (*KPNA7*), the newest member of the alpha karyopherin family of nuclear import receptors, is frequently amplified and overexpressed in pancreatic cancer. Here, we report that *KPNA7* expression is absent in practically all normal human adult tissues but elevated in several pancreatic cancer cell lines. Inhibition of *KPNA7* expression in AsPC-1 and Hs700T pancreatic cancer cells led to a reduction in cell growth and decreased anchorage independent growth, as well as increased autophagy. The cell growth effects were accompanied by an induction of the cell cycle regulator p21 and a G1 arrest of the cell cycle. Interestingly, the p21 induction was caused by increased mRNA synthesis and not defective nuclear transport. These data strongly demonstrate that *KPNA7* silencing inhibits the malignant properties of pancreatic cancer cells *in vitro* and thereby provide the first evidence on the functional role for KPNA7 in human cancer.

Introduction

Pancreatic cancer is among the ten most common cancer types and is one of the most aggressive malignancies with an extremely high mortality rate. Despite extensive research efforts, there has been very little improvement in the prognosis of pancreatic cancer and the 5-year survival rate is only 6% [1]. The main clinical problem is the late diagnosis at a stage when the tumor has already invaded and metastasized into surrounding tissues and there is no efficient treatment available [2]. Furthermore, therapy resistance is another common feature of pancreatic cancer and contributes to the poor outcome [3].

Complex transport machinery is required to ensure the correct localization of proteins within various cellular compartments. The karyopherin (also known as importin) alpha protein family consists of seven highly conserved members (KPNA1–7) that function in the transportation of proteins from the cytoplasm to the nucleus [4,5]. The family members share a common structure containing Armadillo (ARM) repeats and an N-terminal importin beta binding domain (IBB) [4–7]. The alpha karyopherins recognize and bind the nuclear localization signal (NLS) of cargo proteins via the ARM motifs and subsequently recruit a beta karyopherin (importin beta) that mediates the transport of the entire protein complex into the nucleus [6,8]. In the nucleus,

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the cargo protein is released and is available for its designated function. The beta karyopherins are also able to import cargoes directly and thus the alpha karyopherins typically act as adapters that expand the selection of cellular cargoes transported to the nucleus [7]. However, there is evidence suggesting that alpha karyopherins can also import cargoes without the aid of beta karyopherins [9] thereby emphasizing the impact of these proteins in nuclear transport.

KPNA7 (karyopherin alpha 7/importin alpha 8) is the newest and most divergent member of the karyopherin alpha family, being structurally most closely related to KPNA2 [5]. Beyond the structural similarity, there is hardly any information on the function of KPNA7 either in normal or diseased tissues. The IBB domain of KPNA7 is less similar to those of the other importin alpha family members but shows stronger binding to karyopherin beta [5]. *In vitro* assays demonstrated that KPNA7 binds weakly to the NLS of the retinoblastoma protein but failed to bind to those of SV40 or nucleoplasmin [5]. Yet, the actual proteins transported to the nucleus by KPNA7 are currently unknown. Additional data on the possible function of KPNA7 has been obtained from studies of other animal species. *KPNA7* was strongly expressed in cattle, porcine and mouse oocytes and early embryos, and was required for normal fertility and embryonic development [10–12]. Interestingly, the bovine KPNA7 was able to bind to nucleoplasmin 2 [10], thus suggesting that there is some variation in the binding affinities between different species. In addition, the porcine KPNA7 was recently shown to bind several nuclear or NLS containing proteins, such as ubiquitin B [13].

Previously, we identified *KPNA7* as one of the putative target genes of the 7q22 amplicon in pancreatic cancer [14]. The *KPNA7* locus was commonly amplified in about 25% of pancreatic cancer cell lines and primary tumors, and in cell lines the amplification resulted in consistent *KPNA7* overexpression [14]. Abnormal expression of other members of the karyopherin family and resulting mislocalization of proteins has been previously shown to be involved in various diseases, including cancer [15]. Here, we explored the functional consequences of *KPNA7* expression in pancreatic cancer cells to establish its possible contribution to altered nuclear transport and thereby to cancer pathogenesis.

Results

KPNA7 is highly expressed in a subset of pancreatic cancer cell lines

In a previous study we demonstrated a connection between *KPNA7* amplification and elevated mRNA expression [14]. Here, we extend these data and show that *KPNA7* was expressed at very low levels, if at all, in four normal pancreatic RNA samples and in the hTERT-HPNE normal pancreatic ductal epithelial cell line (Fig. 1A). As shown before, the *KPNA7* expression levels varied considerably in the pancreatic cancer cell lines with highest expression in the AsPC-1 and Hs700T cells, which also carry the *KPNA7* amplification [14]. Here, elevated *KPNA7* expression in comparison to the majority of the normal pancreas samples was detected in five additional pancreatic cancer cells lines (HPAC, HPAF-II, HupT3, Capan-1, and SU.86.86; Fig. 1A). To examine *KPNA7* protein expression levels, three different commercially available antibodies as well as one custom-made antibody were tested but failed to produce a specific band corresponding to the known size of the protein in western analysis. Moreover, the Human Protein Atlas database (www.proteinatlas.org) also indicates that their KPNA7 antibody produces data not supported by the predicted size of the protein.

To assess *KPNA7* expression in normal human tissues, mRNA levels were measured in a panel of 21 tissues and revealed expression only in ovary and trachea (Fig. 1B). However, the level of expression in these two tissues was very low, corresponding to that seen in the Panc-1 cells, one of the pancreatic cancer cell lines with minimal expression (Fig. 1A and B). Since *KPNA7* was previously implicated in embryonic development in cattle, porcine and mouse [10–12], *KPNA7* expression was also examined in a panel of human fetal tissue samples but revealed only marginal expression in lung (ages 20–30 weeks, data not shown). Finally, the GeneSapiens (www.genesapiens.org), Oncomine (www.oncomine.org), and Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) databases were queried to gather information on *KPNA7* expression in primary pancreatic tumors, but no datasets were available.

Silencing of *KPNA7* inhibits cell growth and colony formation in the AsPC-1 and Hs700T pancreatic cancer cells

To investigate the functional consequences of elevated *KPNA7* expression, we silenced the gene in the AsPC-1 and Hs700T cells having the amplification and strong overexpression. Four different *KPNA7* specific siRNAs were first individually transfected to AsPC-1 cells and resulted in efficient silencing of *KPNA7* expression. For all subsequent experiments, the four siRNAs were pooled and showed an average of 80% reduction in *KPNA7* mRNA level as compared to non-silencing *LUC* control siRNA (Fig. S1). The silencing was observed already at 24 h after transfection and persisted for up to 96 h (the maximum time range for all experiments except the soft agar assay). A 50% reduction in mRNA level was still evident eight days after transfection. We also ascertained that the *KPNA7* silencing did not reduce the mRNA expression levels of the other alpha karyopherins (data not shown).

Silencing of *KPNA7* resulted in a reduction in cell number in both the AsPC-1 and Hs700T cell lines (Fig. 2A). For the AsPC-1 cells, a trend for slower cell growth was seen at 72 h after transfection and at 96 h the growth reduction was 37% (compared to the *LUC* siRNA transfected cells; $p<0.005$). For Hs700T, a statistically significant 23% growth reduction was observed already at 72 h ($p<0.005$) and an even more striking 54% growth reduction was evident at 96 h ($p<0.005$). In fact, both cell lines showed no change in absolute cell number between the 72 and 96 h time points after *KPNA7* silencing, indicating a halt in cell division, whereas the control cells continued their growth. Panc-1 cells were used to demonstrate that the *KPNA7* siRNAs had no effect on the growth of pancreatic cancer cells with very low endogenous *KPNA7* expression, indicating that the phenotype is not caused by an off-target effect (Fig. 2A).

Anchorage independent growth is one of the established hallmarks of cancer cells. To test whether *KPNA7* silencing has any effect on this phenotype, we allowed *KPNA7* and *LUC* siRNA transfected cells to grow on soft agar for 14 d. Efficient *KPNA7* silencing was verified at the time of seeding of the cells. Overall, the AsPC-1 cells formed less and smaller colonies on soft agar

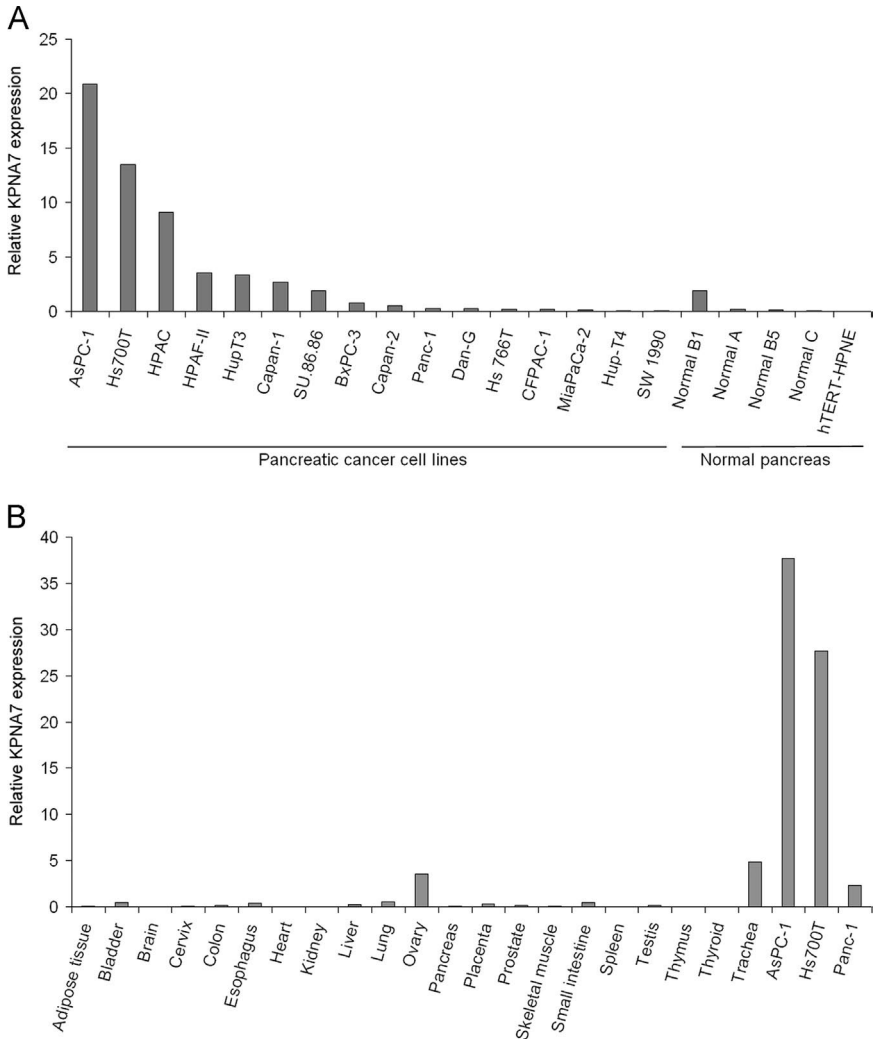

Fig. 1 – Relative *KPNA7* mRNA expression in pancreatic cancer cell lines and in normal human tissues. The expression levels were quantified using qRT-PCR and normalized against the housekeeping gene *GUSB*. (A) *KPNA7* expression in pancreatic cancer cell lines and normal pancreatic RNA samples. Samples were arranged according to their *KPNA7* expression levels. (B) *KPNA7* expression in various normal human tissue samples. Expression of *KPNA7* in AsPC-1, Hs700T and Panc-1 were measured at the same experiment and are shown for reference.

than the Hs700T cells. The number of AsPC-1 colonies was not noticeably affected by *KPNA7* silencing but a decrease in colony size (36% reduction compared to *LUC* siRNA transfected cells) was seen, leading to a statistically significant 29% reduction in total colony area ($p<0.05$; Fig. 2B). For the Hs700T cells, *KPNA7* silencing greatly decreased both the size and the number of colonies (65% reduction, $p<0.0005$ and 32% reduction, $p<0.05$, respectively, compared to *LUC* siRNA transfected cells), resulting in a dramatic almost 80% decrease in the total area of the colonies (Fig. 2B).

KPNA7 silencing leads to a G1 arrest of the cell cycle via induction of p21

We speculated that the reduced cell growth characteristics observed after *KPNA7* silencing were likely to be caused by either increased apoptosis or decreased cell proliferation rate. Through measurements of Annexin V levels, no difference in the number of apoptotic cells was detected between the *KPNA7* and *LUC* siRNA treated cells at 48 h after transfection (6.6% *vs.* 7.4% respectively). In contrast, there was an evident change in the cell cycle distribution with a significant increase of cells in G1-phase and a concomitant decrease in S-phase in both cell lines after *KPNA7* siRNA treatment (Fig. 2C, $p<0.005$). The fraction of cells in the G1 phase of the cell cycle was 42% and 66% for the *LUC* and *KPNA7* siRNA treated Hs700T cells, respectively, at 72 h after transfection. The corresponding figures for the AsPC-1 cells were 51% and 62% (Fig. 2C).

To study the possible mechanisms of the *KPNA7* silencing induced cell cycle alterations, we examined the protein expression levels of six well-known cell cycle regulators (CDK2, CDK6, Cyclin A, Cyclin E, p21, and p27) in *KPNA7* and *LUC* siRNA treated AsPC-1 and Hs700T cells. Due to the role of *KPNA7* in nuclear transport, we considered the possibility that *KPNA7* silencing might not alter the expression level but the cellular localization of the cell cycle regulators, and thus studied the nuclear and

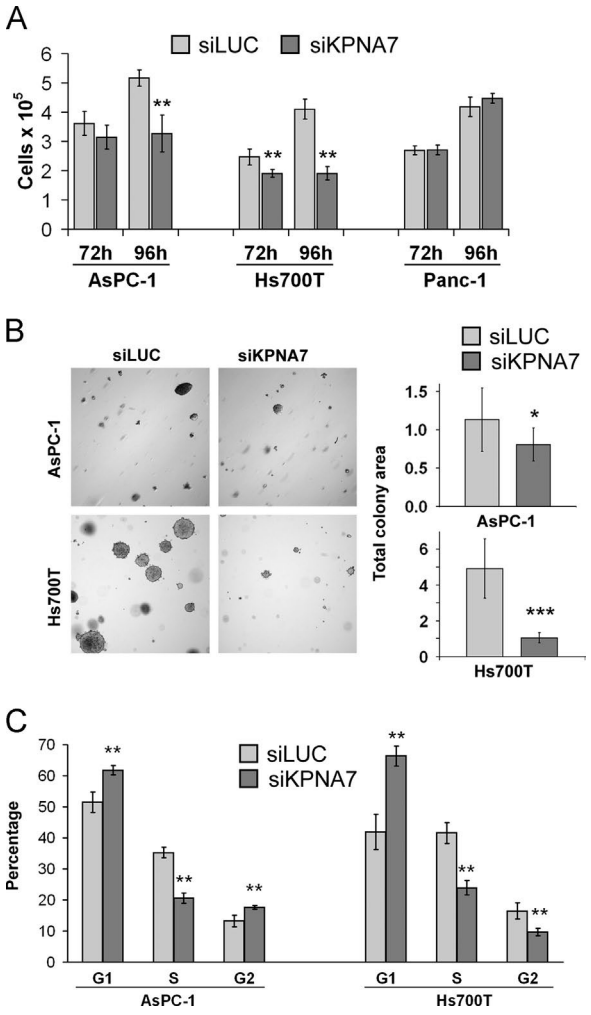

Fig. 2 – Silencing of *KPNA7* decreases the growth of pancreatic cancer cells. (A) AsPC-1, Hs700T, and Panc-1 cells were collected and counted 72 and 96 h after *siKPNA7* or *siLUC* transfections. Mean and SD of six replicates are shown. (B) AsPC-1 and Hs700T cells transfected with *siKPNA7* or *siLUC* were grown on soft agar for 14 d and total colony area was measured using the ImageJ software. Representative images and quantification of the total colony area are shown. (C) The cell cycle distributions were analyzed 72 h after *siKPNA7* or *siLUC* transfections in AsPC-1 and Hs700T cells using flow cytometry. Mean and SD from six replicate experiments are shown. $^{*}p<0.05$, $^{**}p<0.005$, and $^{***}p<0.0005$.

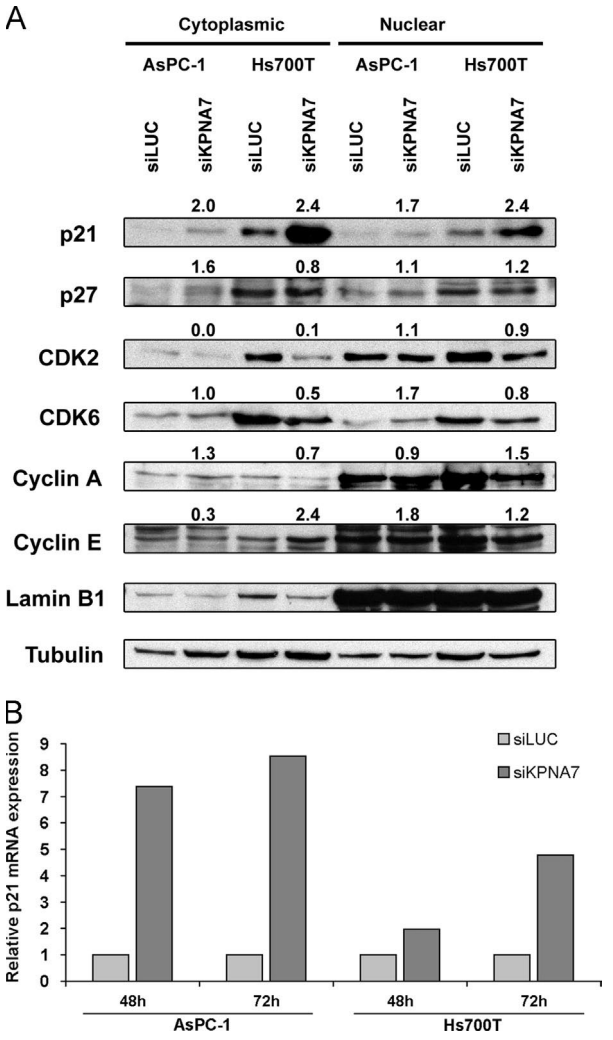

Fig. 3 – *KPNA7* inhibition induces p21 expression both in mRNA and protein level. (A) Nuclear and cytoplasmic protein fractions were collected from AsPC-1 and Hs700T cells 72 h after transfection with *siKPNA7* or *siLUC* and the expression levels of six cell cycle regulator proteins were determined by western blotting. Lamin B1 was used to verify successful fractionation. The expression levels were quantitated using ImageJ software and were normalized against the tubulin loading control. The figures indicate relative expression levels for the *siKPNA7* transfected cells as compared to the corresponding *siLUC* controls. (B) p21 mRNA levels were measured 48 and 72 h after *siKPNA7* transfection in AsPC-1 and Hs700T cells. Bars indicate relative expression levels as compared to *siLUC* transfected control cells.

cytoplasmic protein fractions separately. The p21 protein levels were clearly induced in both cell lines after *KPNA7* silencing but the change was especially prominent in Hs700T cells (Fig. 3A). The induction was seen in both the cytoplasmic and nuclear fractions indicating no major defects in the nuclear transport of p21. In addition, the cytoplasmic but not the nuclear levels of CDK2 were decreased after *KPNA7* silencing (Fig. 3A). Finally, to assess whether the increased p21 protein levels were caused by an induction of mRNA synthesis, p21 mRNA levels were measured. A marked increase in p21 mRNA expression was observed at 48 h after *siKPNA7* transfection in both AsPC-1 and Hs700T cells (Fig. 3B), thus indicating that *KPNA7* silencing does lead to induction of p21 expression at transcriptional level.

Inhibition of *KPNA7* induces autophagy

In addition to the effects on cell growth, we also noted that *KPNA7* silencing altered the morphology of Hs700T but not AsPC-1 cells. Five days after siRNA transfection the Hs700T cells that normally grow as raft-like structures acquired a very different fibroblast-like shape (Fig. 4A). Both the *KPNA7* and *LUC* siRNA treated Hs700T cells readily expressed E-cadherin with small clusters of cells showing vimentin expression (data not shown) suggesting that epithelial–mesenchymal transition (EMT) did not contribute

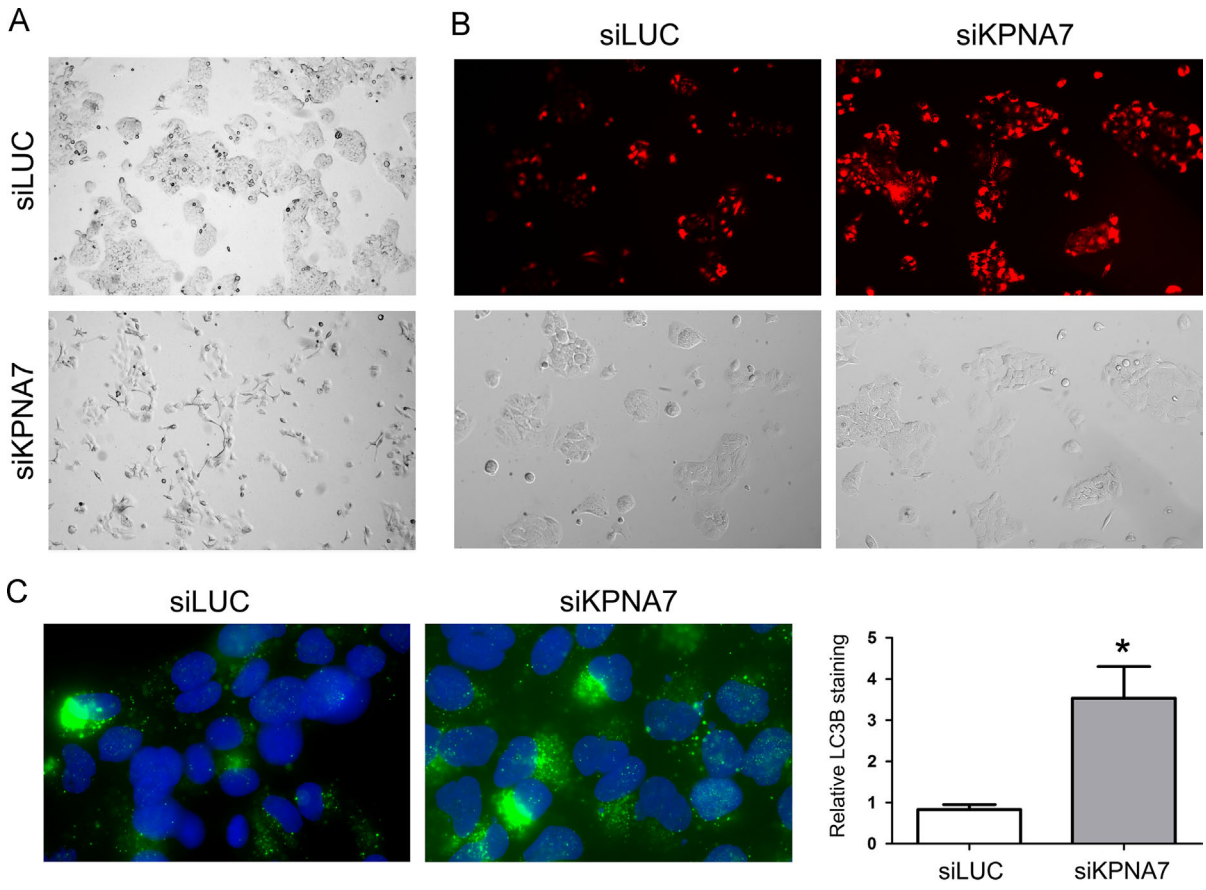

Fig. 4 – *KPNA7* inhibition induces autophagy in Hs700T cells. (A) Hs700T cells transfected with *siKPNA7* or *siLUC* were grown on cell culture plates for five days and photographed using an Olympus IX71 microscope, 100 × magnification. (B) The volume of lysosomal compartment is increased *siKPNA7* transfected cells. The *siKPNA7* or *siLUC* transfected cells were grown for 72 h and the amount of lysosomes was assayed. Top row: lysotracker images (red), bottom row: brightfield images of the corresponding area showing cell density, 100 × magnification. (C) The *siKPNA7* or *siLUC* transfected cells were grown for 72 h and the LC3B levels (green) were measured using immunofluorescence. Nuclei were stained with DAPI (blue). Images were quantified using the ImageJ program and the amount of LC3B staining relative to the cell area was determined (on right). *$p<0.05$.

to the observed change in cell morphology. As mentioned above, we did not observe any increase in the number of apoptotic cell at 72 h after *KPNA7* siRNA transfection. In line with these data, there were no cleaved caspase-3 positive cells at this time point either (data not shown). However, we did observe an about 3-fold increase in lysosomal compartment in the *KPNA7* silenced cells (Fig. 4B) indicating possible induction of autophagy. To substantiate this finding, LC3B (marker for autophagic membranes) levels were quantitated 72 h after transfection and showed a similar 3-fold increase in the *KPNA7* silenced Hs700T cells as compared to *siLUC* treated cells (Fig. 4C, $p<0.005$).

Discussion

Nuclear transport machinery is a critical player in the maintenance of cellular homeostasis. This very complex network of proteins is responsible for transporting various types of proteins, such as transcription factors and histones, as well as RNA molecules, to their correct locations, either in or out of the nucleus [6]. Defects in nuclear import, for instance due to abnormal function of members of the nuclear transport machinery, result in incorrect localization of proteins that might subsequently lead to various diseases including cancer [15]. For example, the tumor suppressor protein p53 has been shown to be inactivated through mislocalization in cancer [16]. In some cases, this mislocalization was proven to be caused by a truncated form of alpha karyopherin that is incapable of transporting p53 into the nucleus, its proper location of action [17]. In addition to such mutations, members of the karyopherin alpha family, especially KPNA2, have been often shown to be aberrantly expressed in cancer [18–26].

We initially found the nuclear importin karyopherin alpha 7 (*KPNA7*) gene to be a putative target for the 7q22 amplification in pancreatic cancer [14]. The same chromosomal region has been found to be amplified in several other malignancies, including gastric and hepatocellular cancers [27–30], suggesting that activation of one or more genes within this region may be involved in the pathogenesis of different tumor types. Here, we show strong overexpression of *KPNA7* in a subset of pancreatic cancer cell lines, with very low to no expression in normal pancreas. Unfortunately there is no data available on *KPNA7* expression in primary pancreatic cancers or practically in any other tumor type. However, the gene was originally isolated from the LNCaP

prostate cancer cells and was shown to be expressed in the HeLa cervical cancer cells [5]. The scarcity of microarray data on *KPNA7* expression in different tissues and tumor types might be partly explained by the fact that it was until very recently merely a hypothetical predicted protein [5], thus not present in most array formats. Nevertheless, our results, which demonstrate very low levels of expression across different normal adult tissues, argue that the expression profile of *KPNA7* is quite limited.

In several animals, *KPNA7* has been shown to be expressed during embryogenesis [10–12] suggesting that the gene might normally act during development and is then silenced in adult differentiated cells. A recent study illustrated the role of *KPNA7* also in human development by reporting autosomal recessive mutations in two individuals with severe neurodevelopmental disease [31]. The mutations resulted in amino acid substitutions in the NLS-binding site of *KPNA7* and thereby were predicted to affect the nuclear import of yet unidentified cargo proteins [31]. Multiple genetic pathways involved in normal embryonic development or stem cell homeostasis are often altered in cancer [32,33]. Moreover, aggressive tumors are known to overexpress a variety of genes generally enriched in embryonic stem cells [34]. Taken together, the information from previous studies and our expression data imply that KPNA7 mainly functions during embryonic development and is then aberrantly activated in a subset of cancer cells.

To examine the functional consequences of *KPNA7* activation *in vitro*, we inhibited its expression in two pancreatic cancer cell lines, Hs700T and AsPC-1, which harbor high-level amplification and subsequent overexpression of *KPNA7*. Silencing of *KPNA7* resulted in a significant decrease in cell growth as well as a reduction in the ability of the cells to grow in soft agar, both of which are key features of cancer cells [35]. The growth reduction was shown to be caused by a G1 arrest of the cell cycle and was accompanied by an increase in p21 expression. p21 is a cyclin-dependent kinase inhibitor and one of the key regulators of the cell cycle [36–38]. It inhibits the G1/S cyclin-dependent kinases, mainly the activity of CDK2–cyclin-E complexes, and is frequently silenced in human tumors [36]. Interestingly, the p21 induction in *KPNA7* silenced cells was not caused by deficient nuclear transport, since both the cytoplasmic and nuclear protein levels were elevated in a similar manner. Although we were able to demonstrate increased mRNA synthesis, the direct mechanism that leads to the high p21 levels after *KPNA7* silencing remains to be established. Besides the well-established role in nuclear transport, alpha karyopherins have also been suggested to have other cellular functions, for example those involved in mitosis [4]. Karyopherins control the activity of proteins, such as NUMA and TPX2, that regulate the formation of the mitotic spindle. These proteins are bound to alpha karyopherin and thereby kept inactive, and then subsequently released during mitosis [4,39,40]. It is thus possible that the cell proliferation associated effects of *KPNA7* silencing are not explained by changes in nuclear transport, but are related to some other functions of the protein.

In addition to the universal changes in cell growth, inhibition of *KPNA7* expression also induced prominent changes in the morphology of the Hs700T cells. Despite their fibroblast-like appearance, no induction of EMT could be demonstrated in *KPNA7*-silenced Hs700T cells. However, a clear increase both in the lysosomal compartment and LC3B staining was observed indicating possible induction of autophagy. Autophagy is an important physiological process where proteins and cytoplasmic organelles are degraded and recycled in response to various cellular stresses, such as starvation [41]. In terms of tumor cells, current evidence supports a role for autophagy in maintaining cell viability during metabolic stress, especially at later stages of tumor progression [42,43]. Consequently, the induction of autophagy in Hs700T cells might not be directly linked with *KPNA7* silencing but rather reflects secondary effects caused by cellular stress.

KPNA2 is the nearest relative of *KPNA7* [5] and has been proposed to play an important role in various malignancies. It is overexpressed and suggested to act as a diagnostic, prognostic or predictive marker for bladder, brain, breast, esophageal, gastric, lung, ovarian, and prostate cancers [18–26]. Recently in non-small cell lung cancer, KPNA2 was shown to mediate the nuclear transport of Oct4, a major transcription factor involved in the maintenance of pluripotency as well as the determination of malignant potential of tumor cells [44]. In terms of functional data, KPNA2 overexpression induced colony formation and increased the migration of MCF7 breast cancer cells whereas silencing led to opposite phenotypic effects [45]. Similar results were obtained in lung and prostate cancer cells where KPNA2 silencing led to reduced cell migration, cell viability, and cell proliferation [23,24]. Together with our results these data demonstrate that abnormal expression of nuclear transporters in cancer cells interferes with cellular homeostasis and thereby contributes to tumor pathogenesis.

To conclude, this study provides the first evidence on the role of *KPNA7* in pancreatic cancer, and in fact, in any human cancer. Our data clearly demonstrates that *KPNA7* silencing leads to growth inhibition in pancreatic cancer cells *in vitro* and that this effect is accompanied by induction of p21 expression and a G1 cell cycle arrest. Although alterations in *KPNA7* expression are not that common in cancer, the basic idea of defective nuclear transport is extremely interesting as a mechanism for dysregulation of key cellular functions in cancer cells. The cargo protein(s) actually transported to the nucleus by human KPNA7 are currently unknown. These cargo proteins may participate in the regulation of cancer cell growth also in cases without KPNA7 alterations, and thus their identification is of outmost importance.

Materials and methods

Cell lines and RNA samples

Sixteen established pancreatic cancer cell lines were used in this study. Thirteen of these (AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, Hs700T, Hs766T, MIA PaCa-2, PANC-1, Su.86.86, and SW 1990) as well as the normal pancreatic cell line hTERT-HPNE, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and three (DanG, Hup-T3, and Hup-T4) were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All cell lines were authenticated by genotyping and cells were grown under recommended culture conditions. Four normal pancreatic RNA samples were obtained from commercial providers (Ambion, Austin, TX; Biochain, Hayward, CA; and Clontech, Mountain View, CA) and the panel of normal RNA samples was purchased from Ambion. A cDNA panel representing various fetal tissues (human fetal MTC panel) was obtained from Clontech.

Gene expression analyses

Quantitative real-time PCR (qRT-PCR) was performed using the Roche LightCycler 2.0 instrument (Roche, Mannheim, Germany). Universal Probe Library (Roche) probes and associated primers (Sigma, St Louis, MO) were used for the *KPNA7* and p21 (*CDKN1A*) genes and Roche's Reference Gene Assay for *HPRT* or *GUSB* were used for normalization. All primer and probe sequences are listed in Table S1.

KPNA7 silencing

Four specific small interfering RNAs (siRNAs) against the *KPNA7* gene were designed using the siRNA Selection Program of the Whitehead Institute, Cambridge, MA [46] and the siRNAs were obtained from Dharmacon (Lafayette, CO). A pool containing an equal concentration of each of the four siRNAs was prepared. A siRNA targeting the firefly luciferase (*LUC*) gene was used as a control. Sequences for all siRNAs are listed in Table S2. Transfections were performed either on 24-well or 6-well plates, with 25,000 or 150,000 cells per well, respectively, using 10 nM siRNA and Interferin reagent (Polyplus-Transfection, San Marcos, CA) as described [14]. The efficacy of the *KPNA7* silencing was verified in each experiment using qRT-PCR.

Cell growth, cell cycle and apoptosis assays

In cell proliferation assays, 25,000 cells per well were seeded on a 24-well plate and transfected with *KPNA7* or *LUC* siRNAs as described above. Cells were counted at 72 and 96 h after transfection using a Coulter Z2 Coulter Counter (Beckman Coulter, San Diego, CA). In cell cycle and apoptosis studies, 150,000 cells per well were seeded on 6-well plates, transfected with *KPNA7* or *LUC* siRNAs and analyzed 48 or 72 h after transfection. In the cell cycle analyses, standard propidium iodide staining was performed as described [47] and for the apoptosis assay the Annexin V FITC Apoptosis Detection Kit was used (Calbiochem, Nottingham, UK). The cell cycle distributions and the number of apoptotic cells were analyzed using Accuri flow cytometer (BD Accuri Cytometers, Ann Arbor, MI) and the ModFit LT software (Verity Software House Inc, Topsham, ME). All experiments were performed in six replicates and repeated at least twice.

Colony formation assay

Potential for anchorage independent growth was assayed by growing *KPNA7* or *LUC* siRNA transfected cells on 0.35% agarose on six-well plates (5000 cells per well for Hs700T, 7000 cells per well for AsPC-1) for 14 d. Images (12 images per well) were captured with Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan) using Capture Pro 6.0 program and the number, size and the total area of colonies (as percentage of total image area) were quantified using the ImageJ software [48].

Western blot

For the western analyses, the *LUC* or *KPNA7* siRNA transfected cells were collected 72 h after transfection. Nuclear and cytoplasmic fractions were extracted using the NE-PER nuclear and cytoplasmic extraction kit as instructed (Thermo Scientific, Rockford, IL). Protein extraction, SDS-PAGE gel electrophoresis, blotting and visualization using the BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Roche) were done as described [49]. The following antibodies (Santa Cruz Biotechnology, CA) and dilutions were used: CDK2 (1:200), CDK6 (1:200), Cyclin A (1:200), Cyclin E (1:200), p21 (1:100), and p27 (1:500). One *KPNA7* primary antibody was custom-made by GenWayBiotech (San Diego, CA) and three commercial *KPNA7* antibodies were purchased from LifeSpan Biosciences (Seattle, WA), Sigma, and Aviva Systems Biology (San Diego, CA) (1:500–1:1000 dilution for all three). Antibodies against Tubulin (1:20,000, Sigma) and Lamin B1 (1:1000, Abcam) were used as controls for loading and successful fractionation.

Immunofluorescence assays

Immunofluorescent stainings and preceding fixation were performed for cells (72 h after transfection with *KPNA7* or *LUC* siRNAs) as described [49]. For LC3B immunofluorescence, a methanol permeabilization step was added as recommended by the antibody manufacturer: after cell fixation, cells were incubated for 10 min in ice-cold methanol at −20 °C and subsequently rinsed for 5 min in PBS. The following antibody dilutions were used: cleaved Caspase-3 (1:500, Cell Signaling, Danvers, MA), E-cadherin (1:500, Abcam, Cambridge, UK), LC3B (1:200, Cell Signaling), Vimentin (1:500, Sigma), and Alexa Fluor secondary antibodies (1:200 Molecular Probes, Eugene, OR). To label lysosomes, Lysotracker (Life Technologies) staining was performed according to manufacturer's instructions. Shortly, 50 nM Lysotracker was added to cell culture medium and incubated for 1 h at +37 °C. The fluorescently labeled cells were analyzed and photographed using the Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan).

Statistical analyses

The Mann–Whitney test was used to statistically compare the medians of the test and control groups.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2013.11.014.

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Search for KPNA7 cargo proteins in human cells reveals MVP and ZNF414 as novel regulators of cancer cell growth

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ABSTRACT

Karyopherin alpha 7 (KPNA7) belongs to a family of nuclear import proteins that recognize and bind nuclear localization signals (NLSs) in proteins to be transported to the nucleus. Previously we found that KPNA7 is overexpressed in a subset of pancreatic cancer cell lines and acts as a critical regulator of growth in these cells. This characteristic of KPNA7 is likely to be mediated by its cargo proteins that are still mainly unknown. Here, we used protein affinity chromatography in Hs700T and MIA PaCa-2 pancreatic cancer cell lines and identified 377 putative KPNA7 cargo proteins, most of which were known or predicted to localize to the nucleus. The interaction was confirmed for two of the candidates, MVP and ZNF414, using co-immunoprecipitation, and their transport to the nucleus was hindered by siRNA based KPNA7 silencing. Most importantly, silencing of MVP and ZNF414 resulted in marked reduction in Hs700T cell growth. In conclusion, these data uncover two previously unknown human KPNA7 cargo proteins with distinct roles as novel regulators of pancreatic cancer cell growth, thus deepening our understanding on the contribution of nuclear transport in cancer pathogenesis.

1. Introduction

Nucleocytoplasmic transport is central to the function of eukaryotic cells, allowing the differential subcellular localization of macromolecules important for the maintenance of cellular homeostasis. In the case of proteins, the most common nuclear import pathway is the karyopherin-mediated transport via nuclear pore complexes [1]. In this system, karyopherin alphas (KPNAs, also known as importin alphas) function as adaptors that recognize and bind to classical nuclear localization signals (NLS) in their cargo proteins, followed by recruitment of karyopherin beta 1 (KPNB1, importin beta 1) [1,2]. KPNB1 then ferries the trimeric protein complex through the nuclear pores into the nucleus where the complex dissociates, thus releasing the cargo protein [1,2]. The karyopherin alpha family consists of seven conserved members (KPNA1-7) that share a common structure containing Armadillo (ARM) repeats and an N-terminal importin beta binding domain (IBB) [2–5]. The ARM motifs are responsible for the recognition and binding of NLSs on the cargo proteins [4,5]. The accessibility of the NLS binding site is regulated by an autoinhibitory mechanism. When the KPNAs are not bound to their NLS-containing cargos, the IBB domain occupies the NLS binding site and only cargos with high affinity can bind to it [6,7]. Alternatively, the autoinhibition may be released through binding of KPNA to KPNB1 and thereby the NLS binding site becomes accessible for lower affinity cargos [7,8].

KPNA7 (karyopherin alpha 7/importin alpha 8) is the newest and most divergent member of the KPNA protein family [3]. Its closest structural relative is KPNA2 (importin alpha 1) with 55% amino acid sequence similarity [3]. KPNA7 function in human tissues is poorly understood, although germline *KPNA7* mutations have been associated with infantile spasms and cerebellar malformation in two individuals [9]. Previous studies in other mammals report that *KPNA7* is mainly expressed during early embryogenesis and in oocytes and is required for normal embryonic development [10–12]. The IBB domain of KPNA7 shares less similarity with the IBBs of other karyopherin alphas and exhibits higher affinity to importin beta [3,13]. In vitro assays are inconclusive as to the capability of KPNA7 to bind classical NLSs. One study showed that KPNA7 exhibits very weak binding to retinoblastoma (Rb) NLS and no affinity to SV40 or nucleoplasmin NLSs [3] while another contradicted this result by demonstrating that KPNA7-SV40 interaction does occur [13]. Apart from the study by Kimoto and colleagues (2015) [13] that reported the identification of KPNA7 cargos, the proteins transported to the nucleus by KPNA7 in different human cell types and contexts are still largely unknown.

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We have previously identified *KPNA7* as one of the putative target genes of the 7q21-22 amplicon in pancreatic cancer [14] and shown that the gene is overexpressed in a subset of pancreatic cancer cell lines [15]. Silencing of *KPNA7* in these cell lines resulted in a G1 arrest of the cell cycle via transcriptional induction of CDKN1A/p21, leading to a distinct reduction in cell proliferation and anchorage independent growth, as well as induction of autophagy [15]. These data suggest that KPNA7, most probably through its cargo proteins, is involved in the regulation of phenotypes that are essential for sustained growth and viability of cancer cells. Identification of such cargo proteins is thus essential and is expected to shed more light on the maintenance of cancer cell homeostasis. Here, we applied protein affinity chromatography to search for KPNA7 cargos. For the study, we used pancreatic cancer cell lines with endogenous KPNA7 expression, as these cells are likely to express the relevant cargo proteins.

2. Materials and methods

2.1. Cell lines

Hs700T and MIA PaCa-2 pancreatic cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were authenticated by genotyping and were grown under recommended culture conditions. The cells were regularly tested for Mycoplasma infection.

2.2. Generation of stable KPNA7 expressing cell lines

Stable, inducible KPNA7-overexpressing Hs700T and MIA PaCa-2 cell lines were generated using the Lenti-X™ Tet-On® Advanced Inducible Gene Expression System (Clontech, Mountain View, CA, USA) according to manufacturer's instructions. In brief, stable Tet-On cell lines were first generated via lentiviral transduction of pLVX-Tet-On Advanced plasmid and selection with geneticin. Then, KPNA7-Twin-*Strep*-tag pLVX-Tight-Puro plasmid was transduced into these cells and positive cells selected with puromycin. Induction of KPNA7 expression was achieved with doxycycline treatment. The expression of *KPNA7* mRNA was verified with qRT-PCR and the presence of KPNA7 protein with Western blotting using *anti*-Streptag antibody (Abcam, Cambridge, UK). Control cell lines expressing only GFP were also generated.

To isolate clones from the heteropopulation, cells were plated sparsely to 100 mm plates to obtain colonies originating from a single cell. Plates were incubated for 4 to 15 days until the colonies reached an 8–10 cell state, after which they were trypsinized using cloning rings with 5 mm diameter and transferred to 96-well plates. Clones were characterized with qRT-PCR for *KPNA7* expression in both induced and uninduced states and compared to parental cell line KPNA7 expression levels (Fig. S1). The highest *KPNA7*-expressing clones with minimal uninduced expression from both cell lines were chosen for protein studies.

2.3. qRT-PCR

Quantitative real-time PCR was performed using the Lightcycler 2.0 instrument (Roche, Mannheim, Germany) with LightCycler® TaqMan® Master reaction mix (Roche). Universal probe library (UPL) probes (Roche) and associated primers (Sigma-Aldrich, St. Luis, MO, USA) for KPNA7, MVP and ZNF414 genes were used and Roche's Reference Gene Assay for HPRT was used for normalization. Primer sequences and probe information are given in Table S1.

2.4. Western blotting

Cell lysates were separated in a 10% SDS-PAGE gel. The proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Roche) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with Blocking Reagent (Roche) in tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h at RT. After blocking, the membrane was probed with primary antibody diluted in 3% BSA in 0.05% TBS-Tween-20 (TBST) overnight at 4 °C and subsequently with HRP-conjugated horse *anti*-mouse IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) 1:8000 in 0.05% TBST for 1 h at RT. The protein bands were detected with BM Chemiluminescence Western Blotting Substrate (Roche). The following antibodies and dilutions were used: anti-Streptag (ab184224, Abcam) 1:1000, anti-KPNB1 (ab2811, Abcam) 1:500, anti-V5 tag (ab27671, Abcam) 1:2000, anti-Fibrillarin (C13C3, Cell Signaling Technology, Boston, MA, USA) 1:1000 and anti-β-Tubulin (T7816, Sigma-Aldrich) 1:20,000.

2.5. Affinity chromatography

1.5×10^6 cells were seeded to 145 mm cell culture plates and cultured overnight. The next day, induction of KPNA7 expression was achieved using 500 ng mL^{-1} (Hs700T) or 100 ng mL^{-1} (MIA PaCa-2) doxycycline. Cells were collected 48 h after induction by washing the plates in ice-cold PBS, scraping the cells into PBS over ice followed by centrifugation at 500 × *g* for 5 min. Cells were lysed with mammalian lysis buffer (IBA, Göttingen, Germany) containing 50 mM Tris/HCl pH 8.0, 7.5% glycerol, 150 mM NaCl, 1 mM EDTA and 1% Triton-X and 1x Complete Mini EDTA free Protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 14,000 × *g* for 30 min at 4 °C.

The strep-tag based method was used for chromatography. Lysates were allowed to flow into Strep-Tactin Macroprep columns with 1 mL column bed volume (IBA). Corresponding GFP cell lines were used as controls and subjected to similar treatments. Protein complex purification was then performed according to manufacturer's instructions. Elution fractions 2–4 from the purifications were pooled and concentrated using Amicon Ultra 4 mL Centrifugal Filters for Protein Purification and Concentration (Millipore, Tullagreen Carrigtwohill Cork, Ireland) at 4000 × *g* for 10 min at 4 °C. The isolation of the recombinant KPNA7 protein was confirmed with Western blotting from column eluates using *anti*-Streptag antibody (Abcam).

2.6. KPNA7 cargo identification by mass spectrometry

Proteins from pooled elution fractions were identified with SDS-PAGE using Criterion TGX precast gels (Bio-Rad) or self-made 20 × 20 cm gels and subsequent mass spectrometry compatible silver staining (protocol available at http://www.btk.fi/proteomics/services/protocols/). Briefly, the gels were fixed for 1 h (30% ethanol, 10% acetic acid), rinsed for 15 min (20% ethanol) and washed with distilled water. After short sensitization (300 mg L^{-1} $Na_2S_2O_3 \cdot 5H_2O$) and washing, the gels were stained with silver for 30 min (2 g L^{-1} $AgNO_3$). After washing, the gels were developed for 2–5 min (30 g L^{-1} K_2CO_3, 15 mg L^{-1} $Na_2S_2O_3 \cdot 5H_2O$ and 700 μL L^{-1} 37% formaldehyde) until the bands reached a desired intensity. The reactions were stopped with stop solution (50 g L^{-1} Tris base in 2.5% acetic acid).

Protein bands observed in KPNA7 lanes but not present in GFP lanes were excised from the gels. The corresponding areas from GFP lanes were also excised as negative controls. The bands were cut into approximately 1 × 1 mm pieces, washed twice with deionized water and finally dried with 200 μL of acetonitrile for 10 min at RT. Bands were delivered in a dried form and further processed for mass spectrometry by Proteomics Facility, BTK, University of Turku. In brief, the proteins were in-gel digested with trypsin and analyzed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS) using a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Database searches were performed by Mascot against the SwissProt protein sequence database. The data was filtered according to the following criteria: a) Mascot Significance

Threshold: 0.01, b) Peptide Minimum confidence: High, c) Minimal number of peptides per protein: 1.

2.7. Processing of mass spectrometry data

The data obtained from mass spectrometry analysis was subsequently further filtered according to the following parameters: a) protein size must match with the size of the band in the gel (±30 kDa for large proteins ≥100 kDa, ±15 kDa for medium-sized 50–90 kDa proteins, and ±10 kDa for small proteins ≤50 kDa), b) at least 2 unique peptides must match the candidate protein sequence, and c) the proteins must exhibit 2-fold increase in the number of peptides in KPNA7-fraction compared to control GFP fraction. Common false positives, such as keratins, keratin-associated proteins and serum albumin were excluded from the analysis. Furhermore, the CRAPome database [http://www.crapome.org, [16]] was employed to exclude most likely false positive hits.

2.8. Web-based analyses

After filtering, the NLS prediction algorithm NLS mapper [http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi, [17]] was used to analyze the entire candidate protein sequences for possible nuclear localization signals using NLS mapper score 3 as cut-off. Literature and protein database searches were also utilized to assess possible known nuclear localization and/or function.

The proteins were subjected to Web-based functional analysis using ToppGene Suite software [https://toppgene.cchmc.org/, [18]] to reveal enriched biological process pathways. The GO categories yielded by ToppGene were further analyzed with Revigo software [http://revigo.irb.hr/, [19]] to reduce redundancy and to increase the legibility of the list.

The subcellular localization of the proteins was retrieved from the COMPARTMENTS database (http://compartments.jensenlab.org/). COMPARTMENTS localization data is integrated from literature manual curation, high-throughput microscopy-based screens, predictions from primary sequence with algorithms like PSORT and YLOC, and automatic text mining [20]. NLS mapper is not stated to be used by the COMPARTMENTS database. In cases where the COMPARTMENTS localization data contained multiple possible subcellular localizations, only the organelle with the highest confidence score was used in the analysis.

A domain analysis was performed using Pfam database [http://pfam.xfam.org/, [21]] to identify protein domains that are present in the identified proteins with higher frequency than would be expected for a random sampling of the human proteome.

2.9. Validation of KPNA7 cargo protein binding

Nine putative KPNA7 cargos were chosen for validation of binding to KPNA7. For validation experiments, V5-tagged pcDNA6.2/EmGFP-Bsd/V5-DEST constructs were generated using the Genome Biology Unit cloning service (Biocenter Finland, University of Helsinki). Briefly, entry clones from the human ORFeome collaboration library were transferred into the pcDNA6.2/EmGFP-Bsd/V5-DEST destination vector using the standard LR reaction protocol.

Each constructs was individually transfected into stable KPNA7-expressing cell lines using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. Induction of KPNA7 expression with doxycycline was performed 3 h after transfection. Interactions were confirmed with co-immunoprecipitation using V5-antibody (Abcam) and Dynabeads® Co-Immunoprecipitation Kit (Life Technologies, Carlsbad, CA) as instructed by the manufacturer, with 7 μg antibody coupled to mg of Dynabeads. Co-immunoprecipitated protein complexes were analyzed with Western blotting using *anti*-V5 and anti-Streptag antibodies (Abcam). Interaction with KPNB1 was included as a positive control. To this end, endogenous KPNB1 was co-immunoprecipitated from KPNA7-expressing cell lines using KPNB1-specific antibody (Abcam). Protein complexes were analyzed with Western blotting using anti-KPNB1 and anti-Streptag antibodies (Abcam).

2.10. siRNA based gene silencing

Transfections were performed using 10 nM siRNA and Interferin reagent (Polyplus Transfection, SanMarcos, CA, USA) as instructed by the manufacturer. The following siRNAs were used: for KPNA7, four specific small interfering RNAs (siRNAs) against the gene were designed using the siRNA Selection Program of the Whitehead Institute, Cambridge, MA, USA and the siRNAs were obtained from Dharmacon (Lafayette, CO, USA). A pool containing an equal amount of each of the four siRNAs was prepared. MVP and ZNF414 siRNAs were obtained from the Dharmacon siRNA library (siGENOME SMARTpool siRNAs). A siRNA targeting the firefly luciferase (LUC) gene was used as a control. Efficient silencing of the target gene was confirmed in each experiment with qRT-PCR.

2.11. Immunofluorescence (IF) assays

IF was used to assess the subcellular localization of recombinant KPNA7 (Fig. S2), MVP and ZNF414 proteins in Hs700T cells. The IF stainings were performed as previously described [22]. The following antibodies and dilutions were used: *anti*-Streptag 1:500 (ab184526, Abcam), anti-MVP 1:500 (PA5-22296, Thermo Fisher Scientific), anti-ZNF414 1:100 (C-15, Santa Cruz Biotechnology, Paso Robles, CA, USA) and Alexa Fluor secondary antibodies 1:200 (Molecular Probes, Eugene, OR, USA). Samples were mounted in ProLong Antifade Gold reagent with DAPI (Molecular probes). The fluorescently labeled cells were analyzed and photographed using the Zeiss Apotome (Zeiss, Oberkoche, Germany).

2.12. Confirmation of KPNA7-mediated nuclear import of MVP and ZNF414

Parental Hs700T cells were plated for IF on Millicell® EZ chamber slides (Millipore, Tullagreen Carrigtwohill Cork, Ireland) with 50,000 cells per well or for Western blotting on 6-well plates with 170,000 cells per well. For ZNF414 analysis, cells were transfected with V5-tagged ZNF414 24 h after plating as described in Section 2.9. and subsequently 24 h later with KPNA7 or LUC siRNASs. For MVP analysis, cells were transfected with KPNA7 siRNA 24 h after plating.

For Western blotting, the cells were collected 72 h after siRNA transfection. Nuclear and cytoplasmic fractions were extracted using NE-PER nuclear and cytoplasmic fractionation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Western blotting was performed as described in Section 2.4. IF was performed 72 h after siRNA transfections using *anti*-MVP or anti-ZNF414 antibodies as described above.

2.13. Functional characterization of KPNA7 cargo proteins

To assess the functional roles of validated KPNA7 cargos their expression was silenced in Hs700T cells using siRNAs followed by assays for cell proliferation. Transfections were performed on 24-well plates in six replicates with 35,000 cells per well as described in Section 2.10. Cells were counted 96 h after transfection using a Coulter Z2 Coulter Counter (Beckman Coulter, San Diego, CA, USA). The knock-down of target genes was confirmed 24 h after transfection with qRT-PCR. Each experiment was repeated at least twice. The Mann–Whitney test was used to statistically compare the means of the test and control groups.

3. Results

3.1. Proteomic analysis reveals a number of novel KPNA7 cargo proteins

To pinpoint proteins transported to the nucleus by KPNA7, we stably overexpressed recombinant strep-tagged KPNA7 in Hs700T and MIA PaCa-2 pancreatic cancer cell lines and performed streptactin affinity chromatography followed by mass spectrometry (MS). After filtering of the MS data to remove commonly occurring false positive hits, a total of 377 proteins with at least 2-fold increase in the number of peptides in KPNA7-expressing cells compared to the GFP controls were found, representing the most promising candidates for KPNA7 cargo proteins (Table S2). These proteins included essential components of the nuclear transport machinery, like importin beta 1 (KPNB1) and several nuclear pore complex proteins (e.g. nuclear pore complex protein Nup214 and nuclear envelope pore membrane protein POM 121C). In addition, proteins such as p53, zinc finger CCCH-type antiviral protein 1 (ZC3HAV1), heterogeneous nuclear ribonucleoprotein F (HNRNPF), and DnaJ homolog subfamily B member 1 (DNAJB1), previously shown or suggested to represent KPNA7 binding partners [13, 23] were discovered. However, most of the proteins identified in this study represented novel KPNA7 cargos that have not been described in the existing literature.

3.2. The majority of KPNA7 cargos are nuclear proteins that contribute to a wide variety of biological processes

To evaluate whether the putative KPNA7 cargo proteins participate in specific biological processes, a pathway analysis was performed with ToppGene software [18]. A notable fraction of the identified proteins functioned in RNA-related processes, such as mRNA metabolism and RNA processing, as well as those associated with translation and protein localization (Table S3). To reduce redundancy and to increase the legibility of the list, the enriched Gene Ontology (GO) categories obtained with ToppGene were further evaluated with Revigo software [19]. With redundant terms removed, the results highlighted that, in addition to the functions mentioned above, the putative cargo proteins were involved in a variety of other biological processes, including those linked with multi-organism metabolism and cell cycle regulation (Table S4, Fig. 1). Analysis of the protein domains present in the cargo candidates indicated that they represented those commonly encountered in the human proteome and no enrichments in any specific domain families was observed (data not shown).

The known or anticipated subcellular localization of the cargos was retrieved from the COMPARTMENTS database [20] (Table S5), which integrates evidence on subcellular protein localization from manually curated literature, high-throughput screens, automatic text mining, and sequence-based prediction methods. According to the COMPARTMENTS data, 49% of the identified cargo candidates localized to the nucleus while the second largest group (19%) consisted of cytosolic proteins (Fig. 2A). An NLS prediction algorithm, NLS mapper [17], was used to examine the candidate protein sequences for possible nuclear localization signals. The analysis showed that almost all proteins identified (97%) were predicted to contain medium-strength to strong NLS (score greater than 3), with only 3% having a score below 3 indicating cytoplasmic localization (Fig. 2B). When the localization data obtained from the COMPARTMENTS database was combined with the NLS mapper predictions, we observed that a fraction (30%) of proteins with NLS mapper score 8 or higher, indicating exclusively nuclear localization, were classified as non-nuclear according to the COMPARTMENTS data. Conversely, some proteins (25%) with NLS mapper score below 3 did

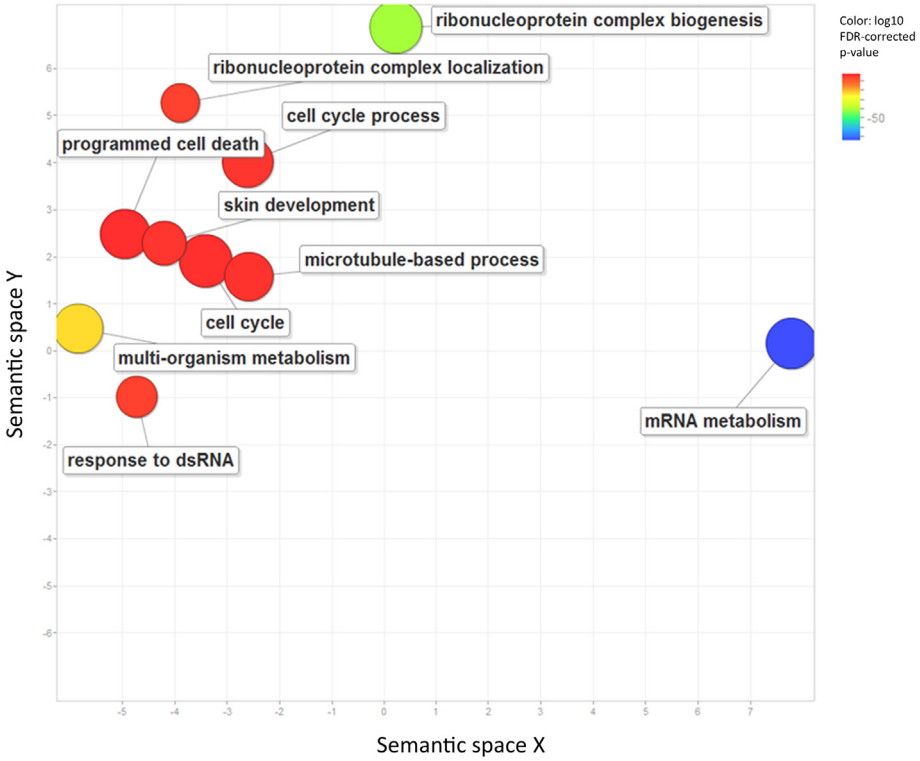

Fig. 1. Evaluation of the biological functions of the putative KPNA7 binding partners. KPNA7 cargo candidates were subjected to pathway analysis with ToppGene suite and the results obtained were further refined according to their semantic similarities with Revigo software to reduce redundancy. The scatterplot shows the cluster representatives (terms remaining after redundancy reduction) in a two-dimensional space by applying multidimensional scaling to a matrix of the GO terms semantic similarities. Each GO term is assigned a dispensability value that depicts the terms semantic closeness to other terms and is based both on the semantic distance and p-values from the ToppGene pathway analysis. Ten of the most indispensable pathways are shown. The bubble color depicts the log10 p-value for each pathway enrichment, with most significant terms shown in blue.

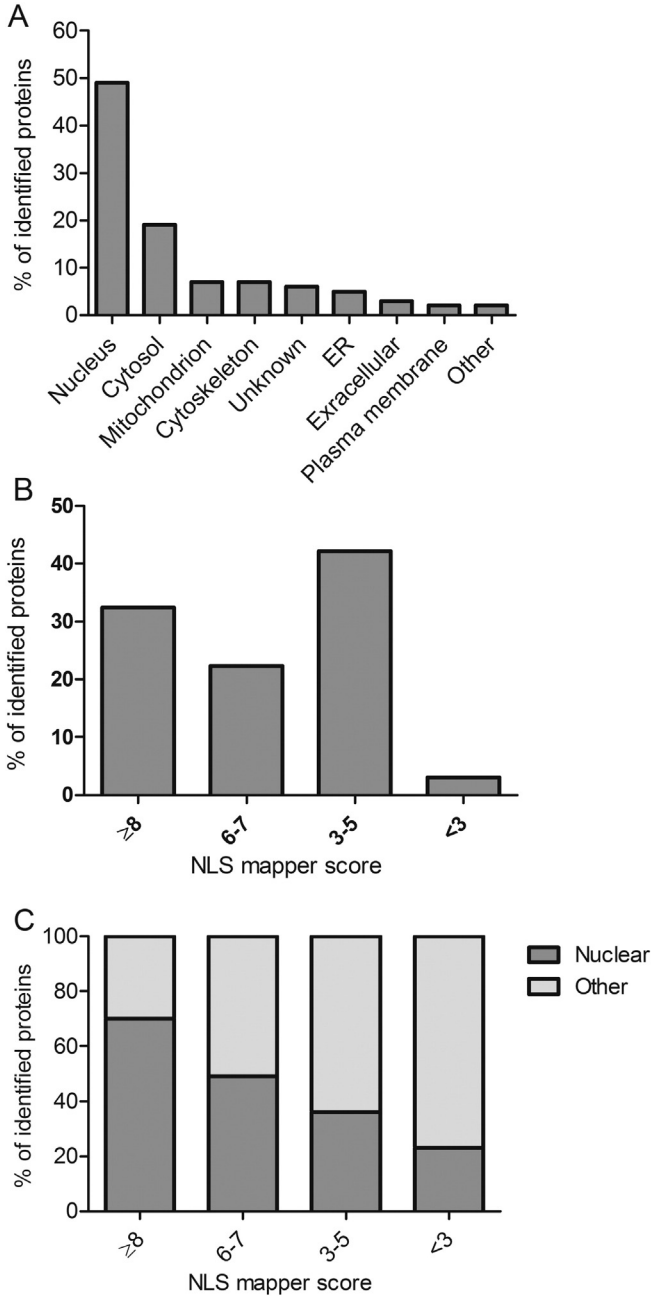

Fig. 2. Predicted subcellular localization of putative KPNA7 cargo proteins. A localization prediction was performed for the KPNA7 cargo candidates using (A) the COMPARTMENTS database and (B) the NLS mapper. Proteins with an NLS score of eight and above are predicted to be exclusively nuclear. Scores from seven to six indicate partial localization to the nucleus. Scores from five to three indicate localization to both the nucleus and the cytoplasm. Proteins with scores below three are predicted to be localized to the cytoplasm. (C) Comparison of NLS mapper scores and the COMPARTMENTS localization data (from panels A and B) for KPNA7 binding partners.

localize to the nucleus in the COMPARTMENTS database. Despite of these differences, the majority of the proteins identified in this study are expected to be nuclear.

3.3. MVP and ZNF414 represent novel KPNA7 cargo proteins

As expected, KPNB1, which binds to cargo-bound KPNAs [1,2], was one of the proteins identified. We used this known interaction to ascertain that the co-immunoprecipitation (co-IP) method is able to validate KPNA7 cargos. Indeed, co-IP with KPNB1-specific antibody confirmed the interaction between KPNA7 and KPNB1 (Fig. 3). In a previous study of HeLa cells [13], DDB2 was the strongest binging partner of KPNA7 and thus we decided to test this interaction in our pancreatic cancer cell lines, even though this protein was not detected in our own MS analysis. However, DDB2 did not interact with KPNA7 in the Hs700T and Mia PACA-2 cells (Fig. 3), most likely due to cell type dependent factors.

Nine putative KPNA7 cargo candidates identified in MS (CHD4, CTNNB1, DIEXF, ELAVL1, MVP, PUM1, SSRP1, TAF6, and ZNF414) were then selected for validation with co-IP based on a combination of criteria including: (1) the higher number of identified peptides in KPNA7-expressing cells versus GFP controls, (2) a low CRAPome score, (3) known nuclear localization, and/or (4) known function with possible cancer association. The KPNA7-overexpressing cell lines were individually transfected with each of the V5-tagged candidate cargo proteins and whole cell lysates were used for co-IP. Interaction with KPNA7 was confirmed for two of the chosen proteins, MVP and ZNF414 (Fig. 3). The other cargo candidates did not exhibit binding to KPNA7 in these experimental conditions (Fig. 3 or data not shown).

According to the COMPARTMENTS database, MVP is localized uniformly across the cell, whereas ZNF414 is predicted to be nuclear. The NLS mapper predicts both MVP and ZNF414 to contain multiple classical NLSs spread across the protein sequences (Table S6 and Fig. S3). The strongest NLS mapper scores for MVP and ZNF414 are 3.9 and 4.5, respectively. To experimentally evaluate the subcellular localization of these proteins in pancreatic cancer cells, we performed nuclear-cytoplasmic fractionation followed by Western blotting. This analysis showed that MVP is almost exclusively localized to the nucleus of Hs700T cells, as seen from the siLUC-treated control cells, and that the majority of the ZNF414 protein is nuclear with a smaller proportion in the cytoplasm (Fig. 4). An immunofluorescent (IF) assay confirmed the nuclear localization of MVP (Fig. S4), but unfortunately the only *anti*-ZNF414 antibody for IF applications did not show specific staining in Hs700T cells.

To illustrate that MVP and ZNF414 are indeed transported to the nucleus by KPNA7, we silenced *KPNA7* in Hs700T cells and subsequently performed nuclear-cytoplasmic fractionation to determine the subcellular localizations of the two cargos after KPNA7 depletion. The analysis demonstrated that the amounts of cytoplasmic MVP and ZNF414 were markedly increased as a result of the *KPNA7* silencing, together with a concomitant decrease in nuclear ZNF414 levels (Fig. 4). Interestingly, there were no apparent changes in the nuclear MVP levels. Nevertheless, we were able to confirm the cytoplasmic retention of MVP after KPNA7 silencing by immunofluorescence (Fig. S4).

3.4. Silencing of MVP and ZNF414 impairs pancreatic cancer cell growth

As we have previously shown, silencing of KPNA7 in pancreatic cancer cells with high endogenous KPNA7 expression leads to G1 arrest and thereby to decreased proliferation and anchorage-independent growth [15]. To characterize whether MVP and ZNF414 contribute to this phenotype, the genes were silenced in Hs700T cells using siRNAs followed by an assay for cell proliferation. Silencing (leading to more than 80% reduction in mRNA levels, Fig. S5) resulted in a statistically significant decrease in the proliferation of Hs700T cells compared to control cells ($p = 0.002$; Fig. 5). For MVP silencing, the growth decreased approximately 30%, whereas the silencing of ZNF414 led to an even more dramatic effect with 40% decrease in cell number at 96 h after transfection.

4. Discussion

In eukaryotic cells, bidirectional nucleocytoplasmic transport of proteins is an essential part of many processes such as control of gene expression and cell cycle. Aberrations in this pathway can lead to a variety of cellular dysfunctions, for example cancer [24], with alterations involving KPNA2 being perhaps the most well characterized. Elevated levels of KPNA2 have been detected in many cancer types

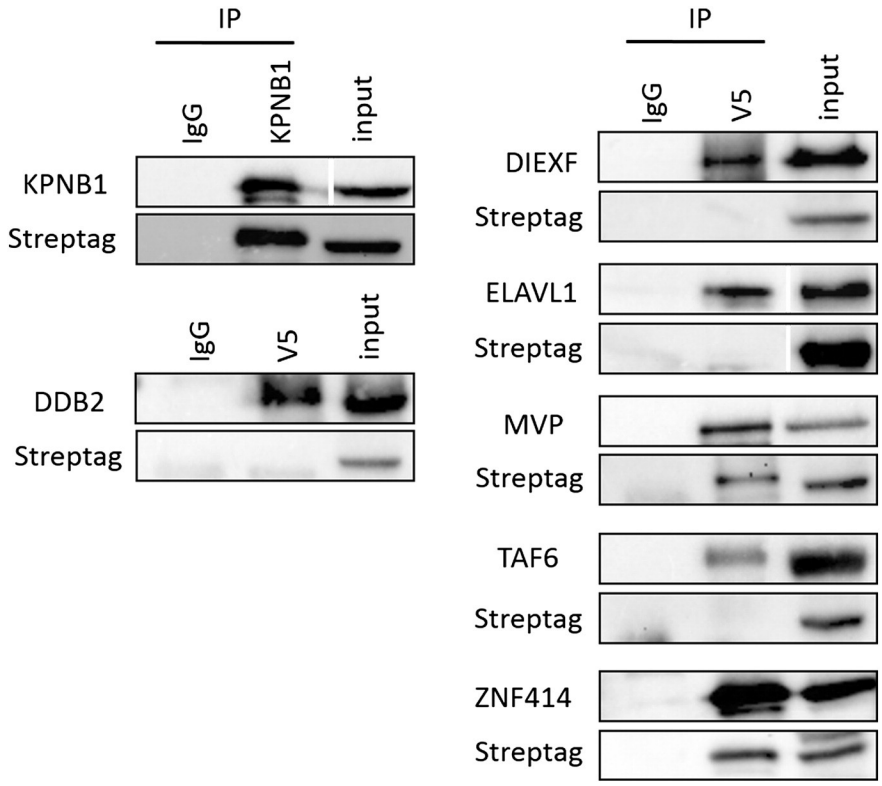

Fig. 3. Validation of KPNA7 binding partners using co-immunoprecipitation assays. Endogenous KPNB1 was co-immunoprecipitated from cell lysates with KPNB1-specific antibody. Each V5-tagged cargo candidate construct was individually transfected into Hs700T and MIA PaCa-2 cells and whole cell lysates used for co-IP with anti-V5 antibody. The bound protein complexes were analyzed by Western blotting using the indicated antibodies. Non-essential or empty lanes have been cropped where indicated with white vertical lines.

including, but not limited to, breast cancer [25], melanoma [26], lung cancer [27] and prostate cancer [28]. KPNA2 overexpression has been established as an independent marker of poor prognosis in several studies and cancer types, (reviewed in [29]). However, KPNA2 expression levels are not elevated in pancreatic cancer [30]. We have previously shown that the newest karyopherin family member and the closest homolog of KPNA2, KPNA7, is overexpressed in a subset of pancreatic cancer cell lines. More importantly, its expression is absent in normal adult tissues with the exception of ovary and trachea [15], indicating reactivation of gene expression in cancer cells. Furthermore, our data implicated KPNA7 as a key regulator of pancreatic cancer cell growth [15].

Since our preceding study showed that depletion of KPNA7 in pancreatic cancer cells leads to growth arrest [15], we hypothesized that the decreased nuclear import of KPNA7 cargo proteins is the cause of the observed phenotype. Here, protein affinity chromatography was utilized to search for KPNA7 cargo proteins in pancreatic cancer cells. The present study is the first aiming to identify KPNA7 cargo proteins in the context of malignancy. Moreover, only one study has investigated KPNA7 interaction partners in human cells [13], adding to the prior reports in porcine [31] and rainbow trout [32] tissues. The proteomic

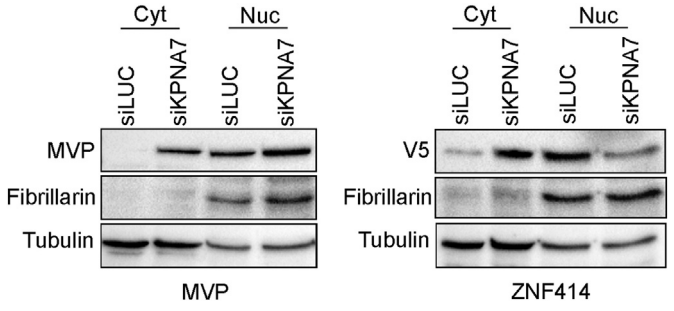

Fig. 4. Inhibition of KPNA7-mediated nuclear import of MVP and ZNF414. For MVP Western Hs700T cells were transfected with KPNA7 siRNA. For ZNF414, Hs700T cells were first transfected with V5-tagged ZNF414, then 24h later with siKPNA7. Cells were harvested 72h after siRNA transfections and nuclear and cytoplasmic fractions were separated and analyzed by Western blotting using the indicated antibodies. Fibrillarin was used as a control for successful fractionation and tubulin as a loading control.

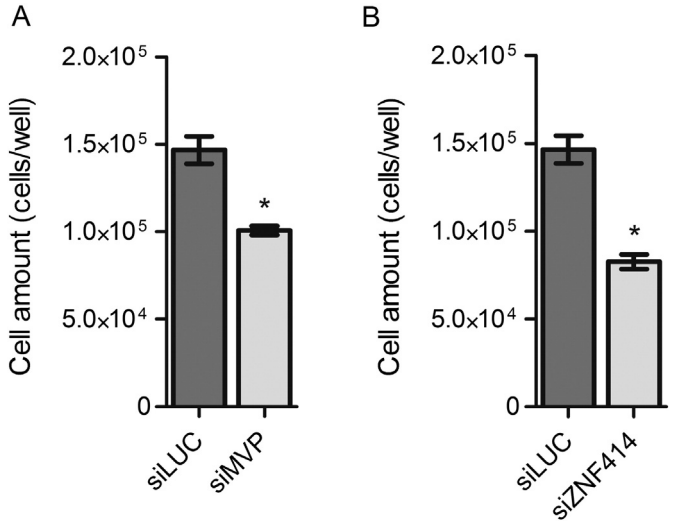

Fig. 5. Functional characterization of validated KPNA7 binding partners. MVP (A) and ZNF414 (B) were silenced in Hs700T cells using siRNA knock-down and cell numbers were counted 96h after transfection. siLUC = control siRNA targeting the firefly luciferase gene. Mean and SD from six replicates are shown. The experiments were repeated at least twice. *P=0.002 (the Mann–Whitney test).

analysis revealed altogether 377 putative KPNA7 cargos. The majority of these were predicted to be localized to the nucleus and/or contained an NLS, thereby supporting the function of KPNA7 as a nuclear importer.

Consistent with previous reports [3,10,13,23,31], the proteomics screen demonstrated binding of KPNA7 to essential components of the nuclear import pathway, including KPNB1 and nuclear pore complex proteins, as well as to other proven or suggested KPNA7 cargos, such as p53, zinc finger CCCH-type antiviral protein 1 (ZC3HAV1), heterogeneous nuclear ribonucleoprotein F (HNRNPF), and DnaJ homolog subfamily B member 1 (DNAJB1). Yet, the majority of the proteins detected at the present study have not been previously shown to interact with KPNA7. Global evaluation of the biological functions of the putative KPNA7 cargos revealed multiple biological pathways, like RNA processing and mRNA metabolic process, in agreement with earlier data [13]. It also disclosed new functional categories, most importantly the cell cycle regulation, that are highly relevant when considering the growth inhibitory phenotype in KPNA7-silenced cells [15]. Overall, these results imply that the set of KPNA7 cargo proteins varies depending on the cell and tissue type in a context-dependent manner. This phenomenon has been previously demonstrated for KPNA2, where divergent interaction partners have been observed in experiments carried out using different cell lines [33].

There is evidence that different KPNA family members show tissue specific expression patterns and possess distinct context-dependent cargo specificities, although some cargo proteins may be imported into the nucleus via multiple KPNAs [34–36]. As an example of the latter, a recent report demonstrated that the prototype foamy virus transactivator Bel1 can be transported into the nucleus individually by KPNA1, KPNA6 and KPNA7 [37]. In line with the data in [34,37] suggesting that KPNAs have common cargos, our proteomic screen uncovered proteins, like minichromosome maintenance complex component 5 (MCM5) and histone deacetylase 2 (HDAC2), that have been previously indicated to bind to KPNA2 [13,33,38]. Considering the homology between KPNA2 and KPNA7, it is perhaps not unexpected that they should share some cargos. Interestingly, we also found KPNA7 to have noticeable similarities in interaction partners with a more distant relative, KPNA6 (importin alpha 7) [39]. For example, chromodomain helicase DNA binding protein 4 (CHD4), SWI/SNF complex subunit SMARCC2 (BAF170) and nucleolar RNA helicase 2 (DDX21) were discovered in both studies. These results indicate that our knowledge on KPNA cargo specificity is still incomplete and that different KPNA homologs are likely to share common cargos, either in tissue or cell type dependent or independent manner. However, one must keep in mind that the current understanding on cargo binding is primarily based on in vitro analyses and that the in vivo binding affinities may also vary, bringing another level of complexity into the picture. In addition to known binding partners of KPNA7, we succeeded in defining novel KPNA7 cargos not described elsewhere, most importantly the major vault protein (MVP) and zinc finger protein 414 (ZNF414). These two cargos were successfully demonstrated to bind KPNA7 in vitro. MVP and ZNF414 were also shown to be imported into the nucleus by KPNA7 as their cytoplasmic amounts were markedly increased after KPNA7 depletion, indicating an interruption in their nuclear import.

Furthermore, we demonstrated that the silencing of *MVP* and *ZNF414* induced quite dramatic growth-attenuating effects in Hs700T pancreatic cancer cells with 30% and 40% reduction in cell number, respectively. This growth arrest phenotype matches distinctly with the one seen after the silencing of *KPNA7* itself [15]. These data indicate that the incorrect subcellular localization of MVP and ZNF414 due to KPNA7 depletion may indeed be responsible for the phenotypic effects seen in *KPNA7*-silenced cells. However, one must bear in mind that we cannot not rule out the possibility that MVP and ZNF414 are transported into the nucleus also by other KPNA family members, and KPNA7 is not necessarily their only transporter. Nevertheless, the growth arrest phenotype caused by *MVP* and *ZNF414* silencing is very intriguing and invites further studies.

MVP is the major structural component of vaults, large ribonucleoprotein complexes that are highly conserved among eukaryotes and abundantly expressed in a variety of cell types but whose precise function is unclear [40,41]. MVP expression levels are correlated with drug resistance and induced upon anticancer treatment [42–46]. Recently, frequent amplification of *MVP* was reported in glioblastoma and was associated with poor chemotherapy response [47]. In addition to its role in drug resistance, MVP and vaults have been proposed to function in a variety of cellular tasks, including as regulators of signaling cascades like the Mek/Erk and PI3-kinase/Akt pathways [48–50] and as participants in DNA damage repair (reviewed in [41]). Furthermore, based on the localization of vaults adjacent to nuclear pore complexes and their hollow barrel-like structure, they have been suggested to be involved in nucleocytoplasmic transport [51–53]. However, to our knowledge there is no direct evidence to support this notion.

In prior studies, MVP was mainly localized to the cytoplasm or the cytoplasmic surface of the nuclear envelope [51,54], with others describing equal localization between cytoplasm and nucleus [55]. Our results clearly indicate that MVP is almost exclusively found in the nuclei of Hs700T pancreatic cancer cells with minimal cytoplasmic staining. Similar predominantly nuclear localization of MVP was also recently seen in 253J bladder carcinoma cells [47]. These observations imply that in some cellular contexts MVP is indeed transported to the nucleus and that the interaction with KPNA7 is not merely caused by the fact that MVP as a building block of vaults is a component of the nucleocytoplasmic transport machinery.

Another KPNA7 cargo protein discovered in our study was ZNF414, a zinc finger-containing protein identified through large-scale proteomic screens [56,57]. Presently, there are no reports on the function of ZNF414 either in normal or cancerous tissues, but zinc finger proteins are in general known to be involved in a variety of cellular activities, such as nucleic acid binding, protein-protein interactions and membrane association (reviewed in [58]). ZNF414 belongs to the krüppel C2H2-type zinc-finger protein family and thus contains a classical C2H2 zinc finger domain (http://www.uniprot.org/uniprot/Q96IQ9). Members of this family are abundant in the mammalian genome and many of them operate as transcription factors that recognize specific DNA sequences and participate in processes like development, differentiation, and suppression of malignant cell transformation [59]. Despite of the lack of knowledge in terms of its function, the fact that silencing of *ZNF414* led to severe inhibition of cell growth highlights it as an interesting subject for future studies.

In the present study, we aimed to identify the cargo proteins of KPNA7, the newest member of the karyopherin alpha family that was previously shown to be re-expressed in pancreatic cancer cell lines and to contribute to their malignant properties. The study revealed a set of novel human KPNA7 cargos that participated in a wide variety of biological processes including cell cycle regulation and were mainly localized to the nucleus and/or contained an NLS, thus supporting the predicted function of KPNA7 as a nuclear import protein. Two of the identified cargos, MVP and ZNF414 were successfully confirmed to be transported into the nucleus by KPNA7 and shown to regulate the growth of pancreatic cancer cells. These results provide new information on the role of KPNA7-mediated regulation of pancreatic cancer cell growth and advance our current knowledge on nuclear import and its aberrations in cancer pathogenesis.

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Author contributions

E.M.V., N.K.R., H.E.R., V.P.H. and A.K. designed the research; E.M.V., N.K.R., H.E.R. and A.T.N. performed research and analyzed data; E.M.V. wrote the paper; N.K.R. and A.K. provided critical revisions to the intellectual content.

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Competing interests

No competing interests declared.

Transparency document

The Transparency document associated with this article can be found, in online version.

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