Characterization of hnRNP C, a Potential Telomerase Inhibitor and PinX1 Interacting Partner, on Telomerase Function

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Abstract

Telomere is tandem repeats of DNA found at the ends of eukaryotic linear chromosomes. It serves to protect chromosome ends and maintain genomic stability. Telomerase is the enzyme that adds DNA sequence repeats to telomere. While telomerase expression is absent in most somatic cells, over 85% of immortal cancer cells have overexpressed telomerase. Telomerase activation and telomere maintenance are found to be critical in cellular immortality and tumorigenesis. Therefore, studies on the telomerase regulatory pathway become one of the major targets in cancer research for cancer therapy.

This study focuses on the characterization of hnRNP C on telomerase regulation and its interaction with PinX1, a known telomerase regulator, and telomerase. TRAP assay was used to characterize the effect of hnRNP C and PinX1 on telomerase activity. It is found that hnRNP C can inhibit telomerase activity independent of its interaction with PinX1. Potential telomerase activity inhibitory domain on hnRNP C was identified and the interaction of hnRNP C with telomerase was characterized by co-immunoprecipitation. It was suggested that hnRNP C inhibit the telomerase activity by associating with the telomerase catalytic subunit and it might also related to the binding of hnRNP C with the telomerase RNA. Our study provides further understanding on the telomerase regulation by revealing hnRNP C as a novel PinX1 interacting protein and characterizing the inhibitory effect of hnRNP C on telomerase activity.

摘要

端粒是真核線性染色體端部 DNA。這串聯重複序列的 DNA 旨在保護染色體末端及維持基因組的穩定性。端粒酶是添加端粒 DNA 重複序列的酶。雖然端粒酶在大多數體細胞端粒酶沒有表達，超過 85%的不朽癌細胞卻有過度表達的端粒酶。端粒酶活性和端粒維持對細胞不朽和腫瘤的發生是至關重要的。因此，端粒酶的調控機理成為研究和治療癌病的一個主要目標。

本研究著重於證實端粒酶抑製蛋白 PinX1 與核蛋白 C 之間的相互作用，以及研究它們的互動對端粒酶活性的影響。其中，我們發現核蛋白 C 能獨立地抑制端粒酶活性。因此，研究的第二部分著重於核蛋白 C 對端粒酶的調節。透過免疫共沉澱和 TRAP 法，我們找出核蛋白 C 與端粒酶催化亞基發生相互作用的結合位點，和核蛋白 C 對端粒酶活性的抑制域。我們發現核蛋白 Ç 透過與端粒酶催化亞基的相互作用來抑制端粒酶活性，當中亦涉及到核蛋白 C 與端粒酶 RNA 的結合。我們的研究顯示出核蛋白 C 是一種新型的 PINX1 相互作用蛋白，並揭示了核蛋白 C 對端粒酶活性的抑制效果。這有助我們進一步了解端粒酶的調控機理。

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Chapter 1

General Introduction

1.1 Overview of Telomere and Telomerase in Cancer

Cancer is a leading cause of death worldwide. Most cancers are caused by uncontrolled and unlimited proliferation of the cells. Normal somatic cells have limited life span and cannot divide indefinitely. This phenomenon is due to the telomere-shortening in each cell cycle. When telomeres shorten to a critical length, the cells will eventually undergo cellular senescence or apoptosis (McEachern et al., 2000; de Lange, 2002). This stops the cells from unlimited division and avoids tumor formation. Telomerase is an enzyme that can synthesize telomere repeats at the end of chromosomes, which is absent in most somatic cells. When the expression of telomerase is activated in cells, telomere length can be maintained and therefore, telomere length shortening–induced senescence is bypassed. It has been demonstrated that overexpression of the catalytic subunit of telomerase in primary fibroblasts can turn the cells immortal (Bodnar et al., 1998). This telomerase immortalization phenomenon is also observed in many other human primary cells (Harada et al., 2003; Xiaoxue et al., 2004; Chapman et al., 2006; Li et al., 2007). These findings suggested that the telomere maintenance and the telomerase activation are crucial for cell immortalization and in tumorigenesis.

1.2 Introduction to telomere

1.2.1 General function and structure of telomere

Telomeres are non-coding repeats of G-rich DNA sequence found at the ends of chromosomes in eukaryotic cells. Human telomere consists of DNA repeats of TTAGGG, oriented 5' to 3' towards the end of chromosome, ending essential single-stranded G rich overhang. The length of the single-stranded overhang and the number of tandem repeats varies among different vertebrates (Chakhparonian and Wellinger, 2003). Telomere does not exist as a linear form, but in a looped structure. The 3'single-stranded overhang of the telomere can invade the double-stranded telomeric tracts, displacing the homologous strand of the same telomere. This forms a lasso-like structure called T-loop with a displacement (D)-loop at the invasion site (Griffith et al., 1999; de Lange, 2004). The telomere is bound by six core telomere binding proteins called shelterin complex which involve in the stabilization of the telomere and the regulation of the telomere length (de Lange, 2005; Palm and de Lange, 2008).

The special structure of telomere together with the shelterin complex gives protective caps to chromosome ends. They serve to camouflage the chromosome ends being sensed as DNA double stranded breaks by the DNA repair machinery as well as to protect it from the exonuclease activity inside the cell. In addition, due to the "end-replication problem" caused by DNA polymerase, chromosomes cannot be completely replicated in each cell cycle, the presence of non-coding telomere serve to preserve the coding region of chromosome. Therefore, the main function of telomere is to provide protection to the chromosome ends and maintain the genomic integrity (Blasco, 2005; Verdun and Karlseder, 2007).

1.2.2 Role of shelterin complex in telomere maintenance

As mentioned, shelterin complex is a group of proteins binding at the end of telomere. The six core proteins of the complex include telomeric repeat binding factors 1 and 2 (TRF1 and TRF2), TRF1-interacting protein 2 (TIN2), protection of telomeres 1 (POT1), POT1-TIN2 interacting protein (TPP1) and transcriptional repressor/acticator protein 1 (RAP1). TRF1 and TRF2 are proteins that directly and specifically bind the double-stranded telomeric repeats. TIN2 does not bind telomeric DNA directly; it associates with TRF1 and TRF2. POT1 specially recognizes 5'-TAGGGTTAG-3' and binds to 3'single-stranded overhang of telomere. It also connects with TRF1 and TRF2 via protein interaction involving TIN2 and TPP1. TPP1 interacts and bridges the association between POT1 and TIN2. RAP1 is recruited to telomeres by TRF2 (de Lange, 2005).

1.2.2.1 Telomere protection

A major role of shelterin complex is to provide protection to chromosome ends (de Lange, 2005; Palm and de Lange, 2008; de Lange, 2009). Mouse models with shelterin components deleted experience telomere dysfunction and chromosomal fusions. For example, mouse embryonic fibroblasts (MEFs) with TRF1 depletion show rapidly senescence, increased frequency of chromosome fusion. The TRF1-depleted telomeres are more fragile and prone to breakage (Martinez et al., 2009; Sfeir et al., 2009). Depletion of TRF2 in MEFs leads to similar chromosome fusion as in TRF1 depleted MEFs (Celli and de Lange, 2005). Knockdown of POT1 orthologue in mouse leads to increased DNA damage foci at telomere and induces early senescence (He et al., 2006; Wu et al., 2006). POT1 is also involved in suppressing the ATR-mediated DNA damage response, preventing the chromosome ends mistakenly activate the DNA repair machinery (Denchi and de Lange, 2007; Guo et al., 2007). Furthermore, RAP1 can

protect the telomere from non-homologous end joining activity, which is a double stranded DNA breaks repair pathway (Bae and Baumann, 2007). All these suggest the importance of shelterin in telomere protection.

1.2.2.2 Telomere length maintenance

Shelterin complex also maintains the telomere length through modulating the recruitment and access of the telomerase. TPP1 is implicated in telomerase recruitment through its interaction with POT1 and telomerase. Depletion of TPP1 reduces binding of the telomerase catalytic subunit to telomere and thus telomere shortening (Xin et al., 2007; Abreu et al., 2010; Tejera et al., 2010). On the other hand, most shelterin proteins including TRF1, TRF2, TIN2, RAP1 and POTI, modulate the telomerase activity mainly in negative manner. Over-expression of TRF1 causes telomeres to gradually shorten until a new length setting is achieved (Munoz et al., 2009). Overexpression of TIN2 also inhibits telomere elongation in human cells and the absence of TIN2 enhances the accessibility of telomerase to telomere (Kim et al., 1999). These negative regulations on telomerase in telomerase available cells serve to maintain the telomeres within a relatively narrow size distribution without being over-elongated**.** It is suggested in the protein counting model that longer telomere can bind more shelterin proteins (Loayza and de Lange, 2003; Smogozewska et al., 2000), and this may lead to the suppression of telomerase activity at the end of long telomere while short telomere binds less shelterin proteins and leads to telomerase activation. The presence of shelterin complex helps to balance the telomere length and allows activity of telomerase on short telomere.

1.2.3 Telomere shortening and replicative senescence in human cells

1.2.3.1 End replication problem of DNA polymerase

In human cells, the length of telomere is not remained the same during the lifespan, it undergoes shortening after each cell division. Telomere shortening is due to the "end replication problem" caused by DNA polymerase. DNA polymerase only replicate DNA in 5' to 3' direction initiated by a short RNA primer and in the presence of a complementary strand as a template. During DNA replication, DNA polymerase can continuously and completely synthesize a complementary leading strand towards 5' end of the chromosome. On the other direction, lagging strand is first synthesized as Okazaki fragments, which is initiated by many short RNA primers that bind on the template. Later, the RNA primers are removed and the Okazaki fragments are joined by ligase. However, after removal of the RNA primer that initiates the terminal Okazaki fragment, a single strand deletion remains at the 5' end of the newly replicated strand. This causes the shortening of telomere in successive cell divisions (Lingner et al., 1995; Shore and Bianchi, 2009).

1.2.3.2 Replicative senescence pathway

When the shortened telomere reaches a critical length, the chromosome ends are sensed as double-stranded breaks (Verdun and Karlseder, 2007). This telomeric dysfunction triggers the DNA damage response machinery and activates the phosphorylated ataxia telangiectasia mutated (ATM) pathway or ataxia telangiectasia and Rad3-related (ATR) pathway. Disruption of the ds-telomeric DNA, which can be caused by depletion of TRF2 will activate the ATM pathway. ATR pathway is activated when ss-telomeric DNA is disrupted; for example, in the case of POT1 depletion (Denchi and de Lange, 2007; Guo et al., 2007). Both ATM and ATR pathway converge on p53/p21 or p16/pRB pathway and lead to cellular senescence or apoptosis

(McEachern et al., 2000; de Lange, 2002). This helps to explain why the number of divisions of the normal cells is limited.

1.2.4 Telomere shortening and cancer formation

Under normal circumstance, the fate of cells with telomere shortening stops proliferation and ends up with cellular senescence. However, this can be bypassed if p53 and Rb1 proteins are inactivated in cells carrying mutations (Hara et al., 1991; Shay et al., 1991). In this case, continued proliferation of cells lead to further telomeres shortening, resulting in end-to-end fusion and genomic instability that can lead to further mutations and cell cycle arrest, called crisis (Shay et al., 1991; Wright et al., 1992). Some cells can escape this crisis by activating telomere length maintenance mechanisms, either employing telomerase or alternative lengthening of telomeres (ALT) to become immortal. Over-expression of telomerase reverse transcriptase (TERT) has been shown to prevent senescence and to induce cell immortalization. When cells become immortal with genetic alterations, they can be transformed and give rise to tumours (Counter et al., 1992; Bodnar et al., 1998). This model is consistent with the findings that the length of telomeres in invasive cancers of pancreas, prostate and breast are shorter than those normal tissues (van Heek et al., 2002; Chin et al., 2004; Meeker et al., 2004a). 89% cases of pre-invasive cancers in bladder, cervix, colon, esophagus and oral cavity are also found to have very short telomeres (Meeker et al., 2004b Meeker et al., 2004c). Several studies indicate that shorter telomeres are a risk factor for cancer. Individuals with shorter telomeres seem to have a greater risk for development of lung, bladder, renal cell, gastrointestinal, and head and neck cancers (Wu et al., 2003; McGrath et al., 2007). In conclusion, telomere maintenance is playing a crucial role in maintaining genomic stability; dysfunction of telomere is one of the major routes for cancer formation.

1.3 Introduction to telomerase

1.3.1 Function and organization of telomerase

Telomerase is the enzyme that adds DNA repeats to telomere and maintains telomere length. It is a ribonucleoprotein consists of two core components, the reverse transcriptase (TERT) and the RNA template (TERC/TR). TERT is the catalytic subunit that carries reverse transcriptase activity for adding TTAGGG repeats to the end of telomere (Nakamura et al., 1997). Human TERT (hTERT) carries seven domains that are similar to retroviral and retrotransposon reverse transcriptase while a motif T is telomerase specific (Yang, 2008). TERC/TR is the integral RNA component within the telomerase catalytic core that provides 11-bp template for the addition of telomeric repeats (Autexier and Lue, 2006). The TR from all known species contains two conserved and potentially universal structures that are the template/pseudoknot domain and the CR4/5domain (Chen et al., 2000; Chen et al 2002.) These two domains comprise the regions of the TR required for telomerase activity (Lin et al., 2004). The human TR contains a third domain in the 3'end, the H/ACA domain (Chen et al., 2000). This domain has homology to small nucleolar (sno) and small Cajal body-specific (sca) RNAs, which contain two stem-loops separated by box H and box ACA moieties (Mitchell et al., 1999; Jady et al., 2004) The TR H/ACA domain binds a quartet of proteins, dyskerin, NOP10, NHP2, and GAR1 essential in vivo for telomerase biogenesis and localization (Cohen et al., 2007; Egan and Collin, 2010). This suggested that besides the telomerase core, some other accessory proteins are needed for a functional telomerase inside the cell.

1.3.2 Telomerase expression in normal cells

At the early stages of human development, telomerase activity is ubiquitously present

throughout the embryo (Wright et al., 1996; Ulaner and Giudice 1997). At these stages, telomerase is needed to compensate for the massive number of cell divisions for embryogenesis (Forsyth et al., 2002). At the later stages, hTERT expression is repressed and telomerase activity becomes undetectable in most tissues. At birth, the activity is absent from most human cells with the exception of dividing male germ cell lineages and the rare proliferative cells of the blood, skin and gastrointestinal tract (Wright et al., 1996; Ulaner and Giudice 1997). In tissues that divide rapidly, tissue homeostasis is maintained by the continuous proliferation and differentiation of adult stem cells. It is believed that some telomerase activity is present in the adult stem cells to slow down the rate of telomere loss. However, complete compensation for telomere loss cannot be achieved except in the embryonic stem cells (Forsyth et al., 2002).

1.3.3 Role of telomerase in cancer cells

In contrast to the somatic cells, about 85% of immortal cancer cell lines express high level of telomerase and maintain short telomere length. This suggests the critical role of telomerase in cellular immortalization. As mentioned, telomerase expression and activation are the major steps for cells to maintain telomere length, which help the cells to escape from crisis and become immortal (Kim et al., 1994; Shay and Bacchetti, 1997; Shay, 1998). Ectopic expression of telomerase in human somatic cells can immortalize many of cell types. Some cell types can be immortalized by telomerase without any further genetic modification, for examples, prostate epithelial (Li et al., 2008), urothelium epithelial (Chapman et al., 2006), ovarian surface epithelial (Li et al., 2007). In some cell types, additional genetic alternations or genetic modifications are acquired in order to achieve immortalization. To conclude, telomerase activation and telomere stability are the defining hallmarks for cellular immortalization.

1.3.4 Regulation and recruitment of telomerase

1.3.4.1 Telomerase activation on short telomere and protein counting model

In the telomere studies in mammalian cells and yeast, telomerase is found to be activated when the telomere length is below the threshold (Marcand et al., 1999; Negrini et al., 2007). As suggest in the protein counting model, telomerase activity is suppressed at long telomere by the shelterin complex but it is activated at short telomere. Several studies provide evidences to support this idea. Study in yeast showed that the telomerase activity decreases progressively at the end of an artificially shortened telomere after successive cell cycles as the telomere is elongated. This experiment showed that short telomeres are preferential substrates of telomerase rather than the long one (Marcand et al., 1999). Another study using TERC depleted mice found that, the re-introduction of telomerase activity into TERC lacking mice selectively lengthens the shortest telomere population rather than the longer populations (Hemann et al., 2001; Samper et al., 2001). These suggest that telomerase is preferentially activated at shorter telomere ends.

1.3.4.2 Telomerase regulation by shelterin and its associate factors

Once an active telomerase RNP particle is formed, it must engage the chromosome terminus to facilitate the incorporation of telomere repeats. Crosstalk between telomerase RNP components and telomere capping proteins influences the recruitment of telomerase to the telomere.

Besides the negative regulations mentioned in 1.2.2.2, shelterin complex also plays an important role in governing the structure and folding of telomere and determine the accessibility of telomerase to telomere. In *in vitro* assay, TRF1 can promote the base pairing of duplex telomeric DNA, and may promote the formation of intra-telomeric base pairing and t-loop which can suppress the accession of telomerase. This promotion

effect can be stimulated by TIN2 (Kim et al., 2003b). POT1 has dual roles in telomere length regulation. It can negatively regulate telomere length by blocking the access of telomerase to the telomere 3′-end and inhibit telomere extension (Kelleher et al., 2005 and Lei et al., 2005). In contrast, POT1 binding on internal region of DNA substrate can enhance the activity and processivity of telomerase. The positive effect can be further enhanced by the formation of POT1-TPP1 heterodimer, which has implicated in telomerase recruitment (Zaug et al., 2005; Wang et al., 2007; Wang et al., 2011).

Proteins that are involved in the DNA damage response may also take part in telomerase recruitment. For example, the DNA DSB (double strand break) repair protein Ku, which is required for DSB repair by the non-homologous end-joining pathway, can interact with telomerase in vitro. It can also be recruited to telomere possibly by TRF1, TRF2 and RAP1 and may have a role in the maintenance of telomere length and localization (Chai et al., 2002; Downs and Jackon, 2004; Ting et al., 2005). Besides, recruitment of MRN complex that recognizes DNA damage can activate the ATM-dependent phosphorylation of TRF1 and subsequent dissociation of TRF1 from telomeres. This helps to increase the access of telomerase to telomere (Wu et al., 2007).

1.3.4.3 Cell cycle dependent trafficking of telomerase

Although active telomerase can be detected in the cell extract throughout the cell cycle, telomere synthesis only occurs during S-phase (Wright et al., 1999). A cell-cycle dependent recruitment of telomerase to telomere has been suggested. Throughout most of the cell cycle stages, hTR and hTERT are localized in intranuclear sites that distinct from telomere. During S-phase, both hTERT and hTR are recruited from nucleoli and cajal bodies respectively to subsets of telomeres (Tomlinson et al., 2006; Jady et al., 2006). hTR localization to the Cajal body appears to be involved in telomere elongation

in vivo (Cristofari et al., 2007) and is mediated by hTERT (Tomlinson et al., 2008). Detection of overexpressed hTERT at the telomere has recently been shown to depend on hTR (Cristofari and Lingner, 2006). These data suggest the biogenesis of telomerase and the cell-cycle dependent subcellular localization of its constituent parts are prerequisites for recruitment of telomerase to telomere.

1.4 Introduction to PinX1

1.4.1 Discovery of PinX1 as telomerase inhibitor

PinX1 was first discovered as a TRF1-interacting protein by yeast-2 hybrid screening of human HeLa cell cDNA library in 2001 (Zhou and Lu, 2001). The identified PinX1 gene encodes a 328a.a. protein with only one known structural domain, Glycine-rich patch at its N-terminal. In PinX1 or PinX1-C (a.a. 254-328 of PinX1) stable expressing HT1080 cell lines, the growth rates were reduced after 20-30 population doublings. Besides, PinX1 or PinX1-C overexpression induces crisis in cells (Zhou and Lu, 2001). Further investigation indentified PinX1 as a telomerase inhibitor and suggested that PinX1 and PinX1-C can induce crisis possibly through the inhibition of telomerase activity. PinX1-C possesses even more potent inhibition than the full length PinX1 (Zhou and Lu, 2001). A more recent study showed that PinX1 290-328 a.a. is sufficient for its potent inhibitory effect (Chen et al., 2010). Consistent with the telomerase inhibition property, the telomere length was progressively shortened in stable HT1080 cell line expressing PinX1 or PinX1-C. On the other hand, PinX1 or PinX1-C cannot induce crisis in telomerase negative GM847 cells suggested that the crisis induction in cells is telomerase-dependent (Zhou and Lu, 2001).

1.4.2 Role of PinX1 in telomerase and telomere regulation

1.4.2.1 Interaction between PinX1 and telomerase

PinX1 was shown to directly interact with hTERT and hTR (Zhou and Lu, 2001; Banik et al., 2004). PinX1 consists of two direct hTERT-binding domain separately allocated to its N-terminal (1-142 a.a.) and C-terminal domains (Zhou and Lu, 2001). PinX1 can interact with the N-terminal region of hTERT and overlaps with the RNA-binding domain (hTERT 326-620 a.a.). The C-terminal of PinX1 is responsible

for the binding to hTR (Banik et al., 2004). PinX1 binds to transcribed ^{32}P-labeled hTR *in vitro*. The selectivity of this interaction was demonstrated by the inability of PinX1 to bind to the irrelevant structural RNA U6.

1.4.2.2 PinX1 mediates nucleolar localization of hTERT

hTERT is predominantly distributed in the nucleus, while it appears to shuttle between the nucleoplasm and nucleoli in different stages of cell cycle (Wong et al., 2002; Yang et al., 2002). Ectopic expression of PinX1 forces both the endogenous or ectopic expressed hTERT to concentrate and co-localize with PinX1 within the nucleolar region of cells. This effect is mediated by the middle domain of PinX1 (1 a.a.98-254). Besides, loss of nucleolar localization in PinX1 mutant S254C/C265Y, which is frequently found in hepatocarcinoma patients (Oh et al., 2004), resulted in inability of PinX1 to mediate the nucleolar localization of hTERT (Lin et al., 2007). Finally, it has been demonstrated that sequestration of hTERT within the nucleolus and the inhibition of telomerase activity are two independent action of pinX1 in human cells.

1.4.2.3 Interaction between PinX1 and TRF1

Direct interaction between PinX1 and TRF1 has been demonstrated (Zhou and Lu, 2001). PinX1 interacts with the TRFH domain (68-268a.a.) of TRF1 while TRF1 interacts with the TID (telomerase inhibitory domain, 254-328a.a.) of PinX1 (Soohoo et al., 2010). PinX1 can affect the nucleolar and telomere localization of TRF1. Overexpression of PinX1 forces TRF1 to accumulate in nucleolus in telomerase positive cells and enhance the association between TRF1 and telomere (Yoo et al., 2009). This suggested PinX1 may affect telomerase activity by controlling the amount of TRF1 on telomere. PinX1 was found to localize at the telomere speckles (Zhou and Lu, 2001)

and this is in turn mediated by TRF1. Down-regulation of TRF1 in BEL7404 cells leads to decreased telomere localization of Red Fluorescent Protein fused PinX1 (RFP-PinX1) (Chen et al., 2010). Also, PinX1 L291E mutant, that is unable to interact with TRF1, fails to localize with telomere and resides predominately in nucleolus (Soohoo et al., 2010). This showed that PinX1-TRF1 interaction is essential for the recruitment of PinX1 to telomere.

1.4.2.4 Dual role of PinX1 in telomere maintenance

A recent study has shown that PinX1 does not only negatively regulate the telomerase and telomere; it also plays a positive role in telomere length maintenance. Despite the increase in telomerase activity in the cells extract, silencing of PinX1 causes telomere length shortening. PinX1-silenced cells reduced telomerase association with POT1 telomeric protein component was observed by Zhang and colleagues (Zhang et al., 2009). These suggested that PinX1 may be responsible for telomerase loading to POTI and thus telomere and suggested PinX1 possesses a dual role on telomere maintenance.

1.4.3 Role of PinX1 in cancer cells

1.4.3.1 Genetic analysis of PinX1 in cancers

Due to the potent telomerase inhibitory effect, PinX1 was originally identified as an intrinsic telomerase inhibitor and a potential tumor suppressor. However, the molecular status of the PinX1 gene and its expression patterns showed contradiction between different types of tissues and tumors. Studies on PinX1 mRNA expression and mutational analysis reveal that this gene may not affect the tumorigenesis of gastrointestinal tract carcinoma, hepatocellular carcinoma, medulloblastomas, hereditary prostate cancer (Liao et al., 2003; Oh et al., 2004; Chang et al., 2004; Akiyama et al., 2004; Hawkins et al., 2004). On the other hand, reduced expression of

PinX1 was detected in gastric carcinoma, ovarian carcinoma, breast cancer (Kondo et al., 2005; Cai et al., 2010; Zhou et al., 2011) while PinX1 has enhanced gene expression in B-cell acute lymphoblastic leukemia, T-cell acute lymphoblastic leukemia and chronic myeloid leukaemia (Capraro et al., 2011; Campbell et al., 2006). These reports suggest PinX1 does not solely suppress tumorigenesis ; it may also has positive role in cancer progression, which may be relate to the dual role of PinX1 in telomere maintenance

1.4.3.2 Treating of cancer by targeting PinX1 and its manipulation

Although there are difference in PinX1 expression in various cancer cells, PinX1 can be a target for cancer treatment in some types of cancer. For examples, TAT-fused PinX1-C can treat hepatocellular carcinoma BEL-7404 and hepatoblastoma HepG2 cells by inhibiting their proliferation and tumor formation ability of (Chen et al., 2011). On the other hand, it is found that down regulation of PinX1 can be resulted from the treatment of a class of common cancer therapeutic drugs, Anthracyclines, which can in turn disrupt the association of telomerase to telomere (Zhang et al., 2011). Furthermore, high expression of Pinx1 was found positively correlated with oesophageal squamous cell carcinoma (ESCC)'s resistance to chemoradiotherapy (CRT) and suggested that PinX1 could serve as a novel predictor for a CRT response to ESCC patients. The pathway of PinX1-mediated telomere stability might also be new target to improve the radiotherapy effect of ESCC (Qian et al., 2013). These data provide the basis of anti-cancer drug design targeting PinX1 regulatory pathway.

1.5 Introduction to heterogeneous nuclear ribonucleoprotein C

Because of the dual role of PinX1 regulation on telomere and telomerase, it is believed that other cellular proteins may involve in these regulatory pathways. Efforts have been put in identifying the PinX1 interacting proteins. The identification was done by pull-down assay using PinX1-C (a.a.254-328) against the nuclear fraction of HepG2 lysate. The elution was subjected to 2D gel electrophoresis and mass spectroscopy. Heterogeneous nuclear ribonucleoprotein C (hnRNP C) is one of the potential PinX1 interacting partners identified by our group (Derek Cheung, unpublished data). hnRNP C belongs to the family of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs), a group of RNA binding proteins that form complex with heterogeneous nuclear RNA (hnRNA) and involve in mRNA biogenesis (Pinol-Roma et al., 1988; Dreyfuss et al., 1993).

1.5.1 Functions of hnRNP C as RNA binding protein

1.5.1.1 Functional domains on hnRNP C

hnRNP C is an abundant nuclear protein. There are two C isoforms, hnRNP C1 and C2, with C1 being the more abundant form and C2 being the larger splice variant, with an additional 13 amino acids (Merrill et al., 1989). Each isoform has an RNA recognition motif (RRM), a basic leucine zipper-like motif (bZLM), a nuclear retention signal, and an acidic auxiliary domain that thought to be involved in protein-protein interactions (McAfee et al., 1996a; McAfee et al., 1996b; Nakielny and Dreyfuss, 1996). hnRNP C exists as tetramer ($C1_3C2$) in the 40S hnRNPs particles and the oligomerization domain (CID) has also been identified in hnRNP C1 (Whitson et al., 2005).

1.5.1.2 hnRNP C acts as nascent pre-mRNA transcript chaperone

$C1_3C2$ tetramers are the core members of the 40S hnRNP particle; the major function of the tetramers is to actively package and acts as chaperone for nascent pre-mRNA transcripts. They bind RNA to form an hnRNP C–RNA complex, which is the obligate first event in the assembly of the 40S core particle (Huang et al., 1994). Recently, it was found that hnRNP C selectively binds to the unstructured RNA regions longer than 200 to 300 nucleotides and thus sorts the transcripts into two RNA categories, to be exported as either messenger RNA or uridine-rich small nuclear RNA (U snRNA), depending on whether or not they are longer than the threshold (McCloskey et al., 2012).

1.5.1.3 hnRNP C involves in nuclear-cytoplasmic shuttling of nuclear pre-mRNA

A model has been proposed for the involvement of hnRNP C and other hnRNP proteins in mRNA nuclear export**.** hnRNP C contain nuclear retention signals (NRS) at a.a. 88-165, which can override the nuclear export signals (NES) of other hnRNPs like hnRNP A1. It is proposed that mRNA is retained within the nucleus by the binding of hnRNP A1 and hnRNP C on the mRNA strand. Prior to nuclear export, the removal of hnRNP C allows the mRNA strand being exported out of the nucleus by the NES of hnRNP A1 (Pinol-Roma, 1997).

1.5.1.4 Role of hnRNP C in IRES-dependent translation

During mitosis and apoptosis, normal cap-dependent translation of mRNA was reduced (Pyronnet et al., 2001; Spriggs et al., 2005). Many mRNA necessary for the apoptotic and mitotic progress continue to be translated via IRES (internal ribosome entry site)-dependent mechanisms (Pyronnet et al., 2000). hnRNP C has the ability to increase mRNA translation by binding to the IRES 5'- untranslated region (UTR) of mRNAs. Although hnRNP C is generally localize in the nucleus, its binding to IRESs

occurs in the cytoplasm, which is usually triggered by entry into mitosis. Nuclear export signal (NES) on hnRNP C that is responsible for this cytoplasmic localization was also identified in apoptotic cells (Lee et al., 2004). Translation that can be enhanced by the hnRNP C binding on IRES includes mRNA encoding proto-oncogene c-Myc, Unr (upstream of N-Ras), c-sis and also XIAP (X-chromosome-linked inhibitor of apoptosis) (Sella et al., 1999; Holcik et al., 2003; Kim et al., 2003a; Schepens et al., 2007). hnRNP C also regulates the translation of p53 through the interaction with the cis-element within p53 mRNA (Christian et al., 2008). Most of these proteins regulated by hnRNP C are implicated in cell division and apoptosis.

1.5.2 Role of hnRNP C in cancer cells

One mechanism hnRNPs may use to regulate tumor progression is to control the expression of proteins which has roles in proliferation. One specific example of enhancement of proto-oncogene translation in cancer by hnRNP C is IGF1R. The type I insulin-like growth factor receptor (IGF1R) is a proto-oncogene strongly implicated in human breast cancer and can promote the survival and proliferation of tumor cells as well as metastasis and chemoresistance. hnRNP C was found to bind to the IGF-IR IRES 5'-UTR in mitosis, resulting in a significant increase in activity of the IGF-IR IRES in T47D breast carcinoma cells (Meng et al., 2008).

Besides, hnRNPC can bind to primary miR-21 and promote miR-21 expression in T98G glioblastoma cells. miR-21 is one of the most highly overexpressed miRNAs in glioblastoma multiforme (GBM), and its level of expression correlates with the tumor grade. Silencing of hnRNPC resulted in lowered miR-21 levels, which inhibit migratory and invasive activities of T98G glioblastoma cell (Park et al., 2012).

In contrast, increased expression of hnRNP C in some cases can inhibit the cell growth. Celecoxib is a selective inhibitor of cyclooxy-genase-2 (COX-2) that is a

critical factor in carcinogenesis. It was found that Celecoxib can induce the increase of hnRNP C expression in human oral squamous cell carcinoma (OSCC) YD-10B cells (Lee et al., 2006). hnRNP C is one of factors that bind to 5'-UTR of p27 mRNA, it is believed that increased in hnRNP C can subsequently induce the translation of p27 mRNA, which trigger the inhibition of cell growth via p27-regulated cell cycle arrest (Millard et al., 2000).

The above suggested hnRNP C can regulate cancer progression through its regulation on varies mRNA translation.

1.5.3 Role of hnRNP C in DNA double strand break repair

Multiple links between DNA DSB repair and telomere biology have been elucidated in recent years (Riha et al., 2006). Proteomics identified hnRNP C as a protein that responds to double strand breaks of chromosomal DNA. It binds to chromatin only when the DNA of HeLa cells was damaged by gamma irradiation (Lee et al., 2005). Besides, hnRNP C was found as a component of a nucleoprotein complex containing breast cancer suppressor proteins PALB2, BRCA2 and BRCA1, which is also critical for homologous recombination. Depletion of hnRNP C substantially altered the normal balance of repair mechanisms following DSB induction (Anantha et al., 2013). However, the effect of hnRNP C on DNA repair remains to be resolved.

1.5.4 Role of hnRNPs in telomere and telomerase regulation

1.5.4.1 hnRNP C

hnRNP C was found to associate directly with the integral RNA component of telomerase by binding to a 6-base uridylate tract of the template region in the human telomerase RNA (Ford et al., 2000). Besides, affinity purification and mass spectrometry of telomerase-associated protein identify hnRNP C as an hTERT-binding

protein. The association of hnRNP C with hTR was independent on hTERT as specific hTR coenrichment with hnRNP C was observed in the present and absence of hTERT. Overexpression of hnRNP C results in telomere shortening (Fu and Collins, 2007). In addition, immunofluorence revealed that around 50% of the hnRNP C colocalized with telomere binding proteins TRF1, indicating rising a possibility of hnRNP C/telomere association in vivo.

1.5.4.2 Other hnRNPs

Besides hnRNP C, several researches have shown that other hnRNP members are important for telomere biology. hnRNP A1, hnRNP D and hnRNP U are capable in interacting with the human telomerase holoenzyme (LaBranche et al., 1998; Eversole and Maizels, 2000; Fu and Collins, 2007), while hnRNP A1, A2/B1, hnRNP D, and hnRNP E can associate with the single stranded telomeric repeat sequence in vivo (McKay and Cooke, 1992; Ishikawa et al., 1993; Ding et al., 1999). It is suggested that hnRNPs have a positive role in telomere regulation that bridges the association of telomerase to the telomere (Ford et al., 2002). Out of the above hnRNPs, the role of hnRNP A1 on telomere and telomerase regulations is most characterized. Telomere elongation was observed upon the introduction of exogenous UP1 (LaBranche et al., 1998). Moreover, restoring A1 expression in A1-deficient cells can increase telomere length (Zhang et al., 2006). hnRNP A1 is capable in binding to both single-stranded telomeric DNA and telomeric RNA. hnRNP A1 was found to contribute to the enhancement of the telomerase activity through recruitment and unfolding of the quadruplex of telomeric DNA (Nagata et al., 2008).

1.6 Long term impact and objectives of the study

Cancer is always one of the leading causes of death in human and accounts for 7.6 million deaths which are 13% of all deaths in 2008 according to the WHO. Cancer can begin in nearly every part of the human body, and the pathways that ultimately lead to cancer vary depending on the type. Given that about 85% human tumors have the telomerase expression; it makes the telomerase activity the most universal marker for human cancers. Therefore, the mechanistic study of telomerase regulation and telomere maintenance is an essential direction in cancer therapy. The present study focuses on hnRNP C, which is a potential PinX1 interacting partner and telomerase regulator, and its role in telomerase regulation.

The study is divided into two parts. In the first part, the interaction and biological significance of PinX1/hnRNP C interaction were characterized. The second part of the study is to characterize the interaction of hnRNP C with telomerase and also the inhibitory effect of hnRNP C on telomerase activity. Study on hnRNP C/PinX1 interaction and hnRNP C/hTERT interaction may provide further understanding on regulation of telomerase activity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Plasmids

The original full length clone of PinX1 was kindly provided by Prof. H.F. Kung (The Chinese University of Hong Kong). Plasmid pCI-neo-hTERT was provided by Prof. J.J. Huang (Beijing Institute of Biotechnology). Plasmid pCMV6_XL5 hnRNP C was purchased from OriGene Technologies. Bacterial expression vector pETZ2-1a was provided by European Molecular Biology Laboratory, pGEX-6p-2 was purchased from GEHealthcare. Mammalian expression vectors pCMV-myc, pCMV-TAG2B, pcDNA3.1-mycHisA were gift from Prof. K.F. Lau (The Chinese University of Hong Kong). The clones of pGEX-6p-2 PinX1 and pCMV-myc hTERT and its truncations were work of Dr. Derek Cheung (The Chinese University of Hong Kong).

2.1.2 Bacterial Cells

E.coli strain DH5α was used for cloning of plasmid. *E.coli* strain BL21(DE3)-pLysS was used for protein expression. The *E.coli* cells were cultured in LB medium with appropriate antibiotics.

2.1.3 Mammalian Cells

HEK293T, HeLa (ATCC) cells were cultured in Minimal Essential Medium (Gibco, Invitrogen) with 10% fetal calf serum (Gibco, Invitogen).

2.1.4 Serum and Antibodies

Antibodies used for western blot and immuno-precipitation were: Anti-myc (9B11,

cell signaling), Anti-FLAG (M2, Sigma-Aldrich), Anti-Actin (GenScript), Anti-hTERT (Y182, Abcam), anti-hnRNP C (4F4, Santa Cruz). The secondary antibodies for western blot were: HRP conjugated goat anti-mouse antibody (Biorad), mouse trueblot ultra HRP conjugated rat anti-mouse antibody (eBioscience), HRP conjugated goat anti-rabbit antibody (Invitogen).

2.2 Methods

2.2.1 Molecular Cloning

2.2.1.1 Basic scheme of cloning

The required genes or gene fragments were amplified by corresponding primers using polymerase chain reaction (PCR) with Phusion high-fidelity DNA polymerase (Finnzyme). Site-directed mutagenesis was performed by overlapping PCR. The PCR products were purified by DNA gel extraction kit (Viogene). The purified PCR products together with the targeted vector were digested by corresponding restriction enzymes (New England Biolabs). After subsequent purification of the digested product by DNA gel extraction kit, the digested PCR product and vector were ligated together by T4 DNA Ligase (New England Biolabs). All the processes were preformed according to manufacturers' protocols. Ligation products were transformed to competent DH5α cells by chemical transformation and the transformed cells were then spread on LB plates with appropriate antibiotics. Colonies on the plates were picked and inoculated in 5ml LB medium with appropriate antibiotics in snap cap tube. After overnight shaking in air bath at 37℃, cells were collected and the plasmids were extracted by Mini-Plus Plasmid DNA Extraction System (Viogene). The prepared plasmids were then digested by the corresponding restriction enzymes to check for the presence of the targeted genes in the plasmid. The positive clones were then sent to DNA sequencing (Tech Dragon Limited, Hong Kong, China) to ensure no unwanted mutations were found.

2.2.1.2 Cloning of hnRNP C constructs

The primers and templates used for PCR amplification of hnRNP C gene, truncations and mutants are listed in table 2.1, 2.2 & 2.3. For bacterial expression, wild type hnRNP C was cloned in the pET-28a by NdeI and NotI which expressed with His-Tag. The truncations were cloned in the pETZ2-1a vector by NdeI and NotI which expressed with a His-Ztag2-carrier with size of 10 kDa after TEV protease cleavage. For mammalian

expression, wild type hnRNP C and truncations were cloned into pCMV-myc/pcDNA3.1-mycHisA and pCMV-TAG2B vectors by EcoRI/NotI and BamHI/EcoRI respectively. The site-directed mutation was introduced by over-lapping PCR with primers and templates listed in table 2.3.

2.2.2 Expression of recombinant protein in bacteria

All recombinant proteins were expressed in *E.coli* strain BL21 (DE3)-pLysS. The recombinant plasmid DNA was transformed into BL21(DE3)-pLysS by chemical transformation. Colonies were then picked and inoculated in 100ml LB medium with appropriate antibiotics as starter culture and allowed to grow at 37℃ shaking air bath for 16-18 hours. 20ml starter culture was inoculated to 2L LB medium with appropriate antibiotics and incubated at 37℃ shaking air bath at 250 rpm. After the O.D.600nm reached 0.6-0.8, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture with final concentration 0.4mM for inducing protein expression. After IPTG induction, cells were kept in 30℃ shaking air bath for 5 hours to allow protein expression. Cells were then harvested by centrifugation.

2.2.3 Purification of GST-PinX1

Cell pellets were first resuspended in GST Loading Buffer (PBS, 1.5M NaCl, 5% glycerol, pH 7.4) and then subject to sonication for cell lysis. The cell lysate was centrifuged at 20000 rpm for 60 min at 4℃ to collect the soluble fraction of lysate. The supernatant fraction was loaded onto the column packed with Glutathione Sepharose (GE Healthcare). After sample loading, the beads were washed by 10 bed volumes of loading buffer. Then, the column was further washed by 5 bed volumes of low salt Wash Buffer (PBS, 5% glycerol, pH 7.4). Bound proteins were eluted by Elution Buffer (PBS, 200mM NaCl, 30mM Reduced Glutathione, 5% glycerol, pH 8). Eluted GST-tagged

PinX1 were cut by adding PreScission Protease (GE Healthcare) at 4℃ for overnight. The elution was then subject to heparin column for removal of PreScission Protease and GST. 5ml HiTrap Heparin XL column (GE Healthcare) was equilibrated by Heparin Loading Buffer (PBS, 200mM NaCl, 5% glycerol, pH 8), and the cleaved proteins were then loaded onto the Heparin column. GST and PreScission protease cannot bind on the column and present in flow through of the column. Bound PinX1 or PinX1-N was eluted out by Heparin Elution Buffer (PBS, 800mM NaCl, 5% glycerol, pH 8). PinX1 were further purified by gel filtration column Superdex 75 (GE Healthcare) with PBS as the running buffer.

2.2.4 Purification of hnRNP C and truncations

Cell pellets were resuspended in His Loading Buffer (20mM PB, 1.5M NaCl, 50mM Imidazole, pH 7) and the soluble fractions were prepared as mentioned in 2.2.3. Samples were loaded onto HisTrap HP column (GE Healthcare) equilibrated by His Loading Buffer. After washing with 10 bed volumes of loading buffer, column was further washed by low salt Washing Buffer (20mM PB, 50mM Imidazole, pH7). Bound proteins were eluted by His Elution Buffer (20mM PB, 500mM NaCl, 500mM Imidazole, 5% glycerol, pH 7).

For the full length hnRNP C, the elution was dialyzed against dialysis buffer (20mM PB, 500mM NaCl, 5% glycerol, pH7) for overnight at 4℃. The elution was concentrated by Amicon (Millipore) and then further purified by gel filtration column Superdex 75 (GE Healthcare) with PBS 5% glycerol as the running buffer.

For the truncations cloned into pETZ2-1a, His-tagged TEV-Protease was added to the elution to cleave off the His-Ztag2-carrier and the mixture was dialyzed against dialysis buffer (20mM PB, 500mM NaCl, 5% glycerol, pH7) for overnight at 4℃. The cleaved proteins was re-loaded onto the HisTrap column, His-tagged TEV-Protease and

His-Ztag2-carrier were bound onto the column while hnRNP C truncations were present in the flow through fraction. The flow through collected was concentrated by Amicon (Millipore) and further purified by gel filtration column Superdex 75 (GE Healthcare) with PBS 5% glycerol as the running buffer.

2.2.5 *In-vitro* pull down assay

Direct interaction between PinX1 and hnRNP C and mapping of PinX1 binding site on hnRNP C were performed using pull down assay. 5mg of purified bait protein PinX1 was immobilized onto the 1ml NHS-activated column according to the standard protocol. The column was equilibrated by PBS for 5 column volumes. 1-2mg of ligand protein in 1ml PBS was then injected into the column. The column was closed and incubated at room temperature for 30min to allow protein-protein interaction. Column was extensively washed by PBS for 20 column volumes. Bound protein was eluted out by PBS with 1.5M NaCl. The elution was analyzed by SDS-PAGE and Coomassie Blue staining.

2.2.6 Plasmid transfection into mammalian cells

HEK293T was cultured in T75 flask in MEM with 10% FBS. The cells were seeded at 6cm dishes for 24 hours. Plasmid transfection using Lipofectamine 2000 (Invitrogen) was performed. The amount of lipofectamine 2000 used was 1.5 fold of the amount of plasmid DNA. Lipofectamine/DNA complex was prepared according to the manufacturer's instructions and was then added to the seeded cells. The cells were incubated at 37℃ for 24 hours for protein expression.

2.2.7 Co-immunoprecipitation

After 24 hours transfection, cells were lysed by 500μl ice cold IP buffer (50mM Tris

pH 7.6, 150mM NaCl, 1mM EDTA, 1% triton X-100). Cell lysate was centrifuged at 14000 rpm for 10 min at 4℃. 200μl supernatant was added to 300μl IP Buffer with 1μl anti-myc antibody (9B11, cell signaling), another 200μl supernatant was added to 300μl IP Buffer for antibody negative control. The tubes were incubated at 4℃ with gentle rocking for 16-18 hours. 15μl Protein A beads (50% slurry, Sigma) were added to each tube and incubated at 4℃ with gentle rocking for 1.5 hours. The protein A beads were washed three times by ice cold IP buffer and 30μl 2X protein dye was added. The samples were heated at 95℃ for 10min and subjected to SDS-PAGE electrophoresis, followed by western blot analysis.

2.2.8 TRAP Assay

2.2.8.1 Basic Scheme of TRAP Assay

$2x10^5$ HEK293T cells were counted by hemocytometer and collected per tube. 200μl CHAPS Lysis Buffer was added to resuspend the cell pellet and incubated on ice for 30 min. Cell suspension was centrifuged at 14000 rpm for 20 min at 4℃ and the cell lysate (supernatant) was transferred to a new tube. The TRAP reaction was set up in a 0.1ml PCR tube as follow:

10X TRAP Reaction Buffer	5μl
TS template (0.1μg/μl)	1μl
2.5mM dNTP	1μl
DEPC H_2O	40.4μl
Cell Lysate	1μl
Total	48.4μl

The reaction tube was incubated in 2720 Thermo-Cycler (Applied Biosystem) at 30℃ for 30min to undergo TS extension followed by 95℃ heat inactivation of

telomerase for 5 min. Another reaction mix for the PCR was set up as follow and added to the reaction tube subsequently.

RPa (0.1μg/μl)	1μl
TSK1 (0.1μg/μl)	0.1μl
K1 (0.1μg/μl)	0.1μl
Taq polymerase (5 Units/μl)	0.4 μl
Total	1.6μl

Then the reaction tube was subject to PCR reaction as follow:

1	94℃	5 min
2	94℃	30 s
3	60℃	30 s
4	72℃	30 s
5	72℃	5 min

Steps 2-4 were repeated for 35 cycles

The PCR products were analyzed by 12.5% non-denaturing gel electrophoresis in 0.5X TBE running buffer. The gel was stained by SYBR Green I Nuclei A (Invitrogen) according to the manufacturer's instruction. The components of the buffers and the sequences of primers are listed in table 2.4.

2.2.8.2 TRAP Assay with exogenous purified proteins

Purified proteins were diluted to desire concentration in a new tube. The purified protein were mixed and incubated in room temperature to allow interaction for 30 min before the TRAP assay. The TRAP reaction was set up as follow:

	Single protein	Two proteins
10X TRAP Reaction Buffer	5μl	5μl

TS template (0.1μg/μl)	1μl	1μl
2.5mM dNTP	1μl	1μl
Purified protein(s)	1μl	2μl
DEPC H_2O	39.4μl	38.4μl
Cell Lysate	1μl	1μl
Total	48.4μl	48.4μl

The reaction tubes were incubated on ice for 15 min before subject to incubation at 30℃ for TS extension. The rest of the procedures were the same as mentioned in 2.2.8.1.

2.2.9 Transient knock-down of hnRNP C by siRNA

Transient knock-down experiment was performed in 6-wells plate. Cells were seeded for 24 hours before transfection of siRNA. hnRNP C siRNA and scrambled control siRNA-A were commercially purchased (Santa Cruz) and prepared according to the given protocol. The transfection reagent used was RNAiMAX (Invitrogen). For transfecting in 6-wells plate, 80 pmole of siRNA was added to 7 μl RNAiMAX in 500μl Opti-MEM. Cells were harvested 72 hours post-transfection.

Table 2.1 Primers for hnRNP C cloning in bacterial expression vectors

Clone	Primer	Sequence (5’ → 3’)
pET-28a-hnRNP C	hnRNPC_NdeI_F	CGC ATA CAT ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pETZ2_1a-hnRNP C 1-90	hnRNPC_NdeI_F	CGC ATA CAT ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_90R	CG CAT AGC GGC CGC TTA CAC TTT TGG CTC TGC AGC
pETZ2_1a-hnRNP C 1-130	hnRNPC_NdeI_F	CGC ATA CAT ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_130R	CG CAT AGC GGC CGC TTA TAC ACG TGC TGG GTA ACT G
pETZ2_1a-hnRNP C 1-174	hnRNPC_NdeI_F	CGC ATA CAT ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_174R	CG CAT AGC GGC CGC TTA CTT TCC AGA CTT GGA AGA TC
pETZ2_1a-hnRNP C 95-293	hnRNPC_NdeI_95F	CGC ATA CAT GCA GGT GTG AAA CGA TCT G
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pETZ2_1a-hnRNP C 128-293	hnRNPC_NdeI_128F	CGC ATA CAT GCA CGT GTA CCT CCT CCT C
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pETZ2_1a-hnRNP C 159-293	hnRNPC_NdeI_159F	CGC ATA CAT ATG GGC TTC AAT TCT AAG AGT
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pETZ2_1a-hnRNP C 260-293	hnRNPC_NdeI_260F	CGC ATA CAT GAA GAT CGG GGG GAT GAC
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pETZ2_1a-hnRNP C 95-174	hnRNPC_NdeI_95F	CGC ATA CAT GCA GGT GTG AAA CGA TCT G
	hnRNPC_Not1_174R	CG CAT AGC GGC CGC TTA CTT TCC AGA CTT GGA AGA TC

Table 2.2 Primers for hnRNP C cloning in mammalian expression vectors (pCMV-myc)

Clone	Primer	Sequence (5' → 3')
pCMV-myc-hnRNP C	hnRNPC_EcoRI_F	CGC ATA GAA TTC GG ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pCMV-myc-hnRNP C 1-90	hnRNPC_EcoRI_F	CGC ATA GAA TTC GG ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_90R	CG CAT AGC GGC CGC TTA CAC TTT TGG CTC TGC AGC
pCMV-myc-hnRNP C 1-130	hnRNPC_EcoRI_F	CGC ATA GAA TTC GG ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_130R	CG CAT AGC GGC CGC TTA TAC ACG TGC TGG GTA ACT G
pCMV-myc-hnRNP C 1-174	hnRNPC_EcoRI_F	CGC ATA GAA TTC GG ATG GCC AGC AAC GTT ACC
	hnRNPC_Not1_174R	CG CAT AGC GGC CGC TTA CTT TCC AGA CTT GGA AGA TC
pCMV-myc-hnRNP C 95-293	hnRNPC_EcoRI_95F	CGC ATA GAA TTC GG GCA GGT GTG AAA CGA TCT G
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pCMV-myc-hnRNP C 128-293	hnRNPC_EcoRI_128F	CGC ATA GAA TTC GG GCA CGT GTA CCT CCT CCT C
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pCMV-myc -hnRNP C 159-293	hnRNPC_EcoRI_161F	CGC ATA GAA TTC GG AAT TCT AAG AGT GGA CAG
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pCMV-myc-hnRNP C 260-293	hnRNPC_EcoRI_260F	CGC ATA GAA TTC GG GAA GAT CGG GGG GAT GAC
	hnRNPC_Not1_R	CG CAT AGC GGC CGC TTA AGA GTC ATC CTC GCC
pCMV-myc-hnRNP C 95-174	hnRNPC_EcoRI_95F	CGC ATA GAA TTC GG GCA GGT GTG AAA CGA TCT G
	hnRNPC_Not1_174R	CG CAT AGC GGC CGC TTA CTT TCC AGA CTT GGA AGA TC

Table 2.3 Primers for hnRNP C cloning in mammalian expression vectors (pcDNA3.1-mycHisA and pCMV-TAG2B)

Clone	Primer	Sequence (5’ → 3’)
mycHisA-hnRNP C 95-174	hnRNPC_MycHis_EcoRI_95F	CGC ATA GAA TTC ATG GCA GGT GTG AAA CGA TC
	hnRNPC_MycHis_Not1_174R	C GCA GAG CGG CCG CCA CTT TCC AGA CTT GGA AGA TC
pCMV-TAG2B-hnRNP C	hnRNPC_BamH1_F 5”	CGC GGA TCC ATG GCC AGC AAC GTT ACC
	hnRNPC_EcoRI_R 5”	ATA CGC GAA TTC TTA AGA GTC ATC CTC GCC
pCMV-TAG2B-hnRNP C-F19S	hnRNPC_F19S_F 5”	C TCC CGT GTA TCC ATT GG
	hnRNPC_F19S_R 5”	TAC ACG GGA GTT CAT GGA G
pCMV-TAG2B-hnRNP C-Q56H	hnRNPC_Q56H_F 5”	G TT GCC TTC GTT CAT TAT
	hnRNPC_Q56H_R 5”	AAC GAA GGC AAA GCC CTT ATG

Table 2.4 Buffers and primers list for TRAP Assay

CHAPS Lysis Buffer	CHAPS Lysis Buffer: 10mM Tris-HCl, pH7.5 1mM $MgCl_2$ 1mM EGTA 0.1mM Benzamidine (Can be replaced by PMSF) 5mM b-mercaptoethanol 0.5% CHAPS 10% Glycerol
10X TRAP Reaction Buffer:	100mM Tris-HCl, pH8.3 15mM MgCl2 630mM KCl 0.5% Tween-20 10mM EGTA
TS	5’ AAT CCG TCG AGC AGA GTT 3’
RPa	5" GTG TAA CCC TAA CCC TAA CCC 3’
TSK1	5’ AAT CCG TCG AGC AGA GTT AAA AGG CCG AGA AGC GAT 3’
K1	5’ ATC GCT TCT CGG CCT TTT 3’

Chapter 3

Characterization of PinX1/hnRNP C interaction

3.4 Introduction

After identification of PinX1 interacting partners by pull down assay, hnRNP C was found to be one of the potential PinX1 interacting proteins. Given that hnRNP C has some associations with telomerase, it was chosen for further studies. The following study focuses on the confirmation and characterization of PinX1/hnRNP C interaction and the effect of the interaction on telomerase activity.

3.5 Result

3.5.1 Confirmation of PinX1/hnRNP C interaction

3.5.1.1 Association of PinX1 and hnRNP C inside the cell

To confirm the interaction of PinX1 and hnRNP C inside the cell, co-immunoprecipitation was performed. Myc-tagged PinX1 and FLAG-tagged hnRNP C were transiently co-transfected into HEK293T cells for 24 hours. After cell lysis, anti-myc antibody and protein A beads were added to capture the immunoprecipitant. FLAG-tagged hnRNP C was detected in myc-tagged PinX1 immunoprecipitate, but not in anti-myc negative control, indicated that PinX1 and hnRNP C are associated inside the cell (Figure 3.1). Another control experiment was set by co-transfecting the cells with the empty myc-tagged vector and FLAG-tagged hnRNP C. No FLAG-hnRNP C was present in the myc-immunoprecipitate, indicating that FLAG-hnRNP C did not interact with the myc-tag and the anti-myc antibody.

	1	2	3	4
Anti-myc	+	-	+	-
Myc-PinX1	+	+	-	-
pCMV-myc	-	-	+	+
FLAG-hnRNP C	+	+	+	+

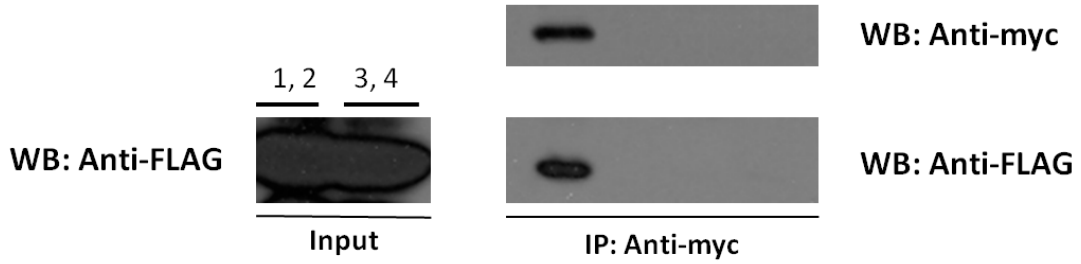


Figure 3.1 Co-immunoprecipitation between PinX1 and hnRNP C. myc-PinX1 and FLAG-hnRNP C were co-transfected into HEK293T. Anti-myc antibody and protein A beads were used to isolate the immunoprecipitant. Empty vector pCMV-myc and FLAG-hnRNP C were co-transfected as a negative control to ensure FLAG-hnRNP C did not interact with anti-myc antibody.

3.5.1.2 Direct interaction between PinX1 and hnRNP C *in vitro*

To prove the direct interaction between PinX1 and hnRNP C, full length PinX1 and hnRNP C were purified from *E. coli* expression (Figure 3.2A). PinX1 was immobilized onto NHS-column as bait and the purified hnRNP C was loaded into the column. After extensive washing, hnRNP C was found in the elution from the PinX1-immobolized column, but not the control NHS column. This showed PinX1 interacts directly with hnRNP C *in vitro* and this interaction is not due to the non-specific interaction between hnRNP C and NHS (Figure 3.2B).

3.5.2 Characterization of PinX1/ hnRNP C interaction

To study how PinX1/hnRNP C interaction is involved in the telomerase regulation, characterization of the interaction between PinX1 and hnRNP C was preformed. Mapping of the PinX1 binding region on hnRNP C was done by *in vitro* pull-down assay. Purified PinX1 was used as bait and immobilized onto the NHS-column. Eight hnRNP C truncations C a.a.1-90, C a.a.95-293, C a.a.128-293, C a.a.260-293, C a.a.1-130, C a.a.1-174 and C a.a.95-174 were designed according to their functional domains (Fig 3.3B). Truncation C a.a.174-293 was also designed; however the expression of this domain was low and the protein was not enough for the pull down assay. The protein truncations purified from *E.coli* expression were used as ligands in the pull down assay. Control column was also prepared and subjected to the same pull-down procedures. No protein was eluted from the control column indicated the hnRNP C truncations did not non-specifically interact with the NHS-column. Truncations C a.a.95-293 and C a.a.128-293, showed interaction with PinX1, while C a.a.1-174 did not interact with PinX1. This indicated that the PinX1 binding site locates on a.a.174-260, which is the CID and acidic domain (Figure 3.3A).

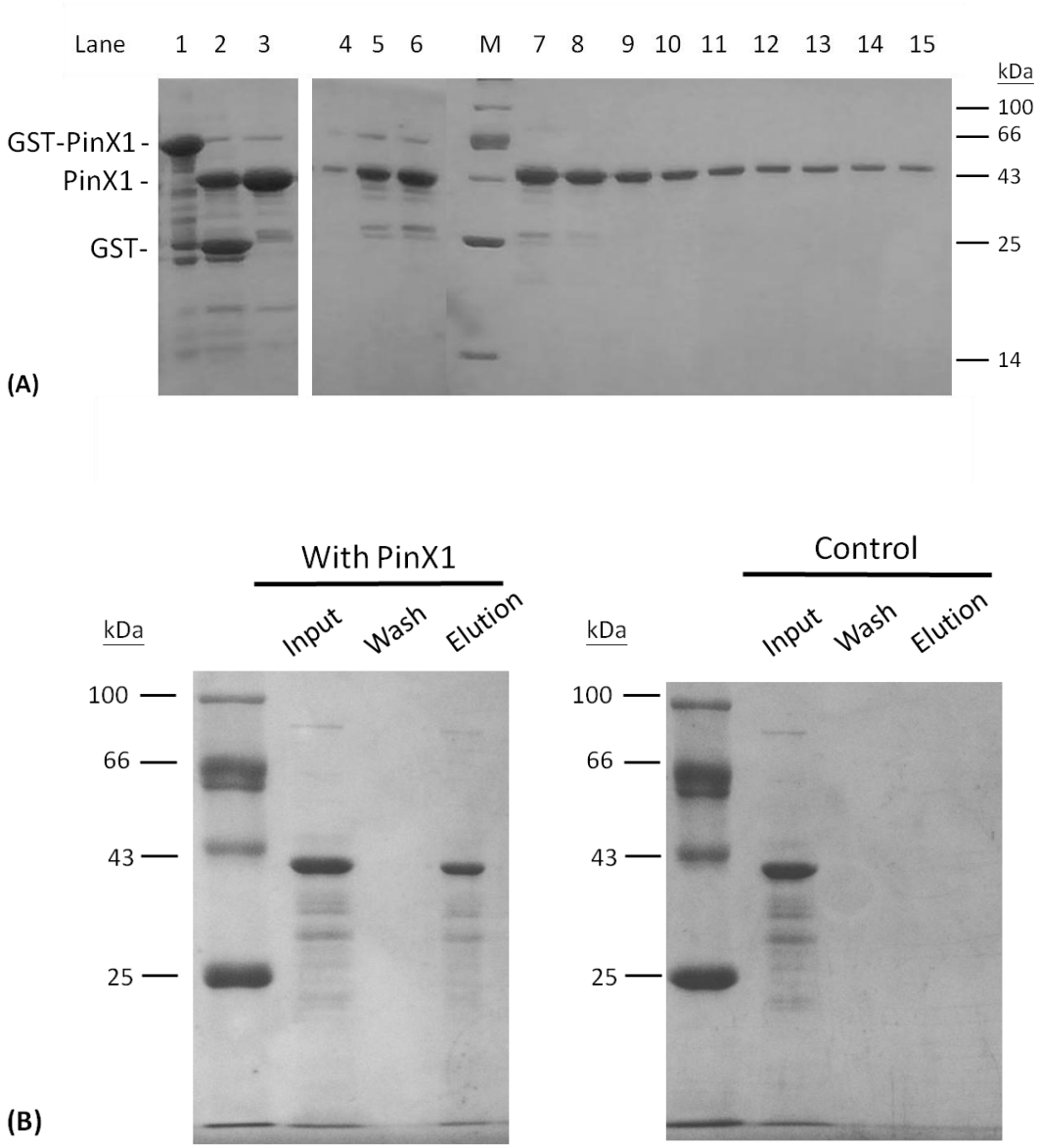
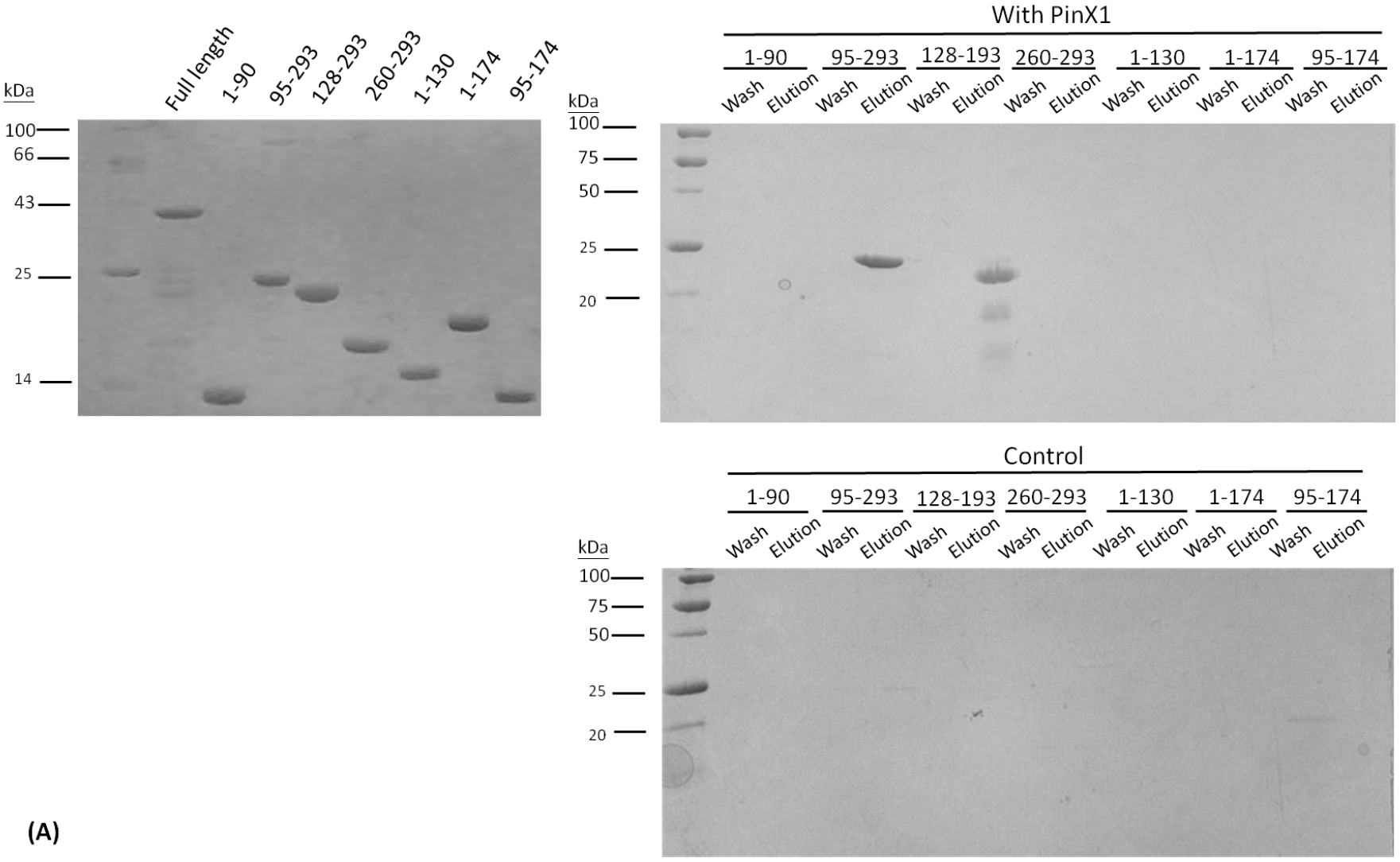


Figure 3.2 Purification of PinX1 and direct interaction between PinX1 and hnRNP C *in vitro.* (A) PinX1 was successfully purified after a three-step purification. Lane 1: GST-tagged PinX1 eluted from GST column. Lane 2: The cleavage product of PinX1 and GST after the addition of PreScission protease. Lane 3: PinX1 eluted from Heparin column. Lane 4-15: Elution fractions after gel filtration. The purity was estimated to be over 90%. **(B)** Purified PinX1 was immobilized on NHS column and purified hnRNP C was loaded and allowed to interact with PinX1 in the column. Control column was prepared by the same immobilization procedures but without PinX1 added.


kDa
Full length
1-90
95-293
128-293
260-293
1-130
1-174
95-174
100
66
43
25
14
With PinX1
1-90
95-293
128-193
260-293
1-130
1-174
95-174
Wash
Elution
kDa
100
75
50
25
20
Control
1-90
95-293
128-193
260-293
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Wash
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(A)

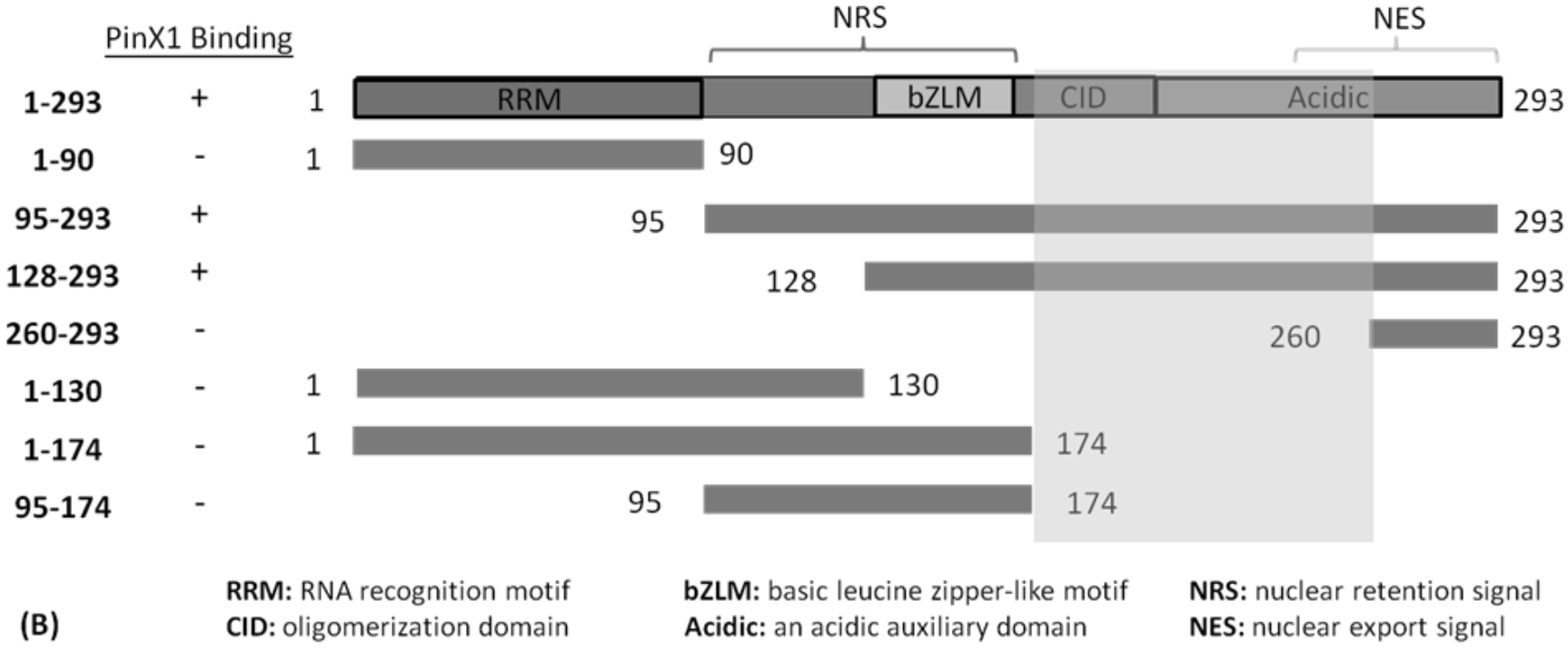


Figure 3.3 PinX1 interacts with the C-terminal region of hnRNPC. (A) The mapping of PinX1 interaction site was carried out by pull down assay in NHS-column by using purified PinX1 as bait. Control column was prepared without PinX1. The input proteins are shown on the left panel. All proteins have their His-Ztag2-carrier removed, except C a.a. 260-290 due to its small size **(B)** Map showing hnRNP C truncations on PinX1 binding, where '-' representing lack of specific binding and '+' indicating specific binding with PinX1. The shaded region indicated the PinX1 interacting region.

3.5.3 Effect of PinX1/hnRNP C interaction on telomerase activity

To examine the effects of hnRNP C and hnRNP C/PinX1 interaction on telomerase activity, TRAP assay was performed. Telomerase-containing cell extracts were incubated with various PinX1 and hnRNP C proteins before the TRAP assay (Figure 3.4). Consistent with the previous finding that PinX1 acts as a potent telomerase inhibitor, PinX1 inhibited telomerase activity in a concentration-dependent manner. Upon the addition of equal amount of hnRNP C together with PinX1, the telomerase activity was lower compared with adding PinX1 alone. It should be noticed that adding hnRNP C alone into the TRAP assay could give an inhibitory pattern similar to that of the PinX1(Figure 3.4). This suggested that hnRNP C can inhibit the telomerase activity and together with PinX1, they give a more intense inhibition on telomerase activity.

There are two possibilities for the increase in telomerase inhibition of PinX1-hnRNP C co-incubation. First, it may because of the binding of hnRNP C on PinX1 can increase the association of hnRNP C to telomerase, which leads to a further decrease in telomerase activity. The second is that hnRNP C and PinX1 can inhibit the telomerase independently. As going to be discussed in the next chapter, hnRNP C truncation, C a.a.1-174 had an inhibitory effect on telomerase activity which was similar to the wild type hnRNP C (Chapter 4.2.2). However, this truncation did not interact with PinX1 as shown by the previous pull down assay. Therefore, another set of TRAP assay was performed by co-incubating PinX1 and C a.a.1-174 with the telomerase extract. The aim of the experiment was to compare the effect on telomerase activity when PinX1 can interact hnRNP C with that when PinX1 cannot interact with hnRNP C. Upon the co-incubation of PinX1 and C a.a.1-174, the inhibitory effect also increased and was similar to that with PinX1-hnRNP C co-incubation. It implied that PinX1 and hnRNP C inhibit the telomerase activity independently without interacting with each other.

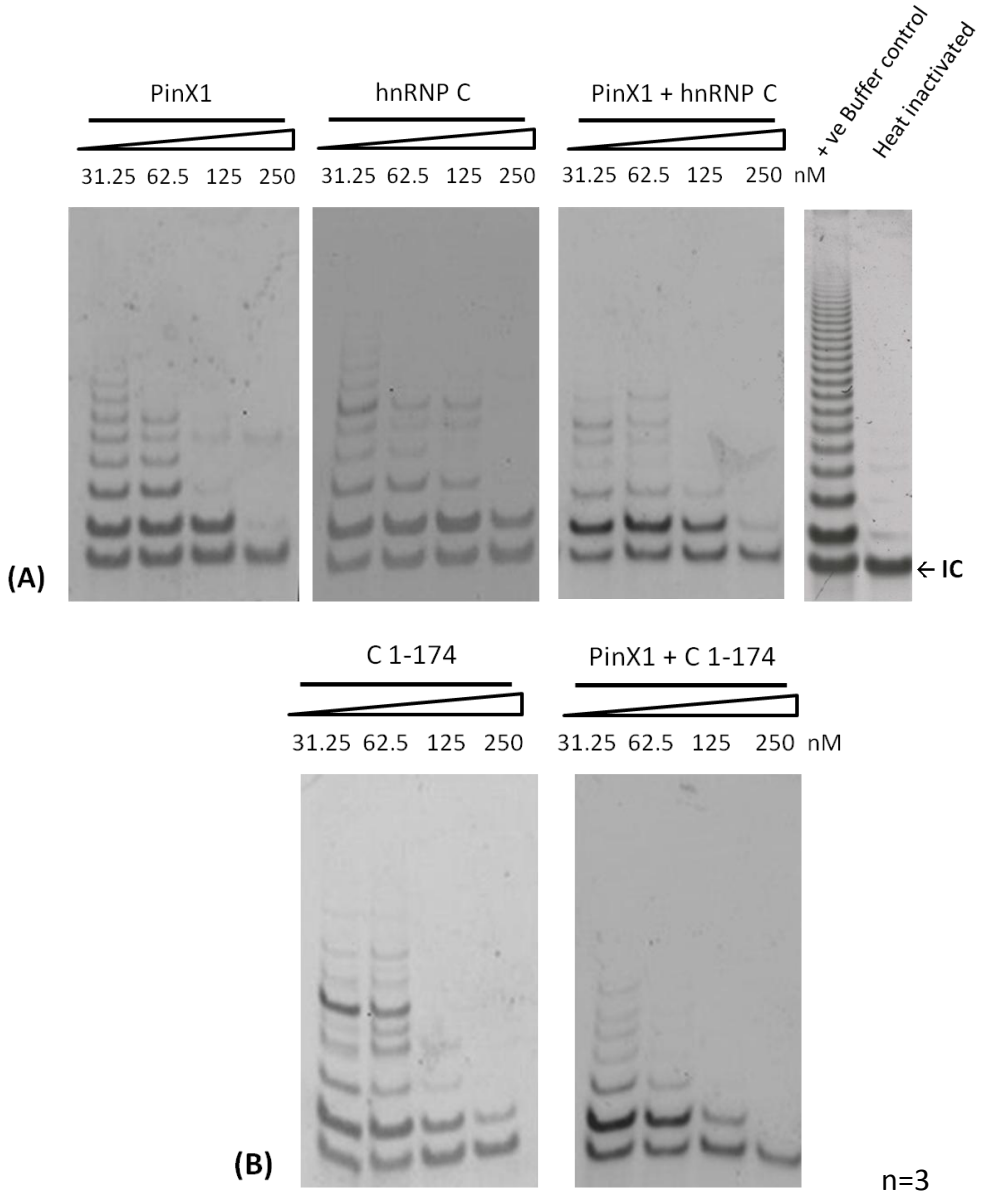


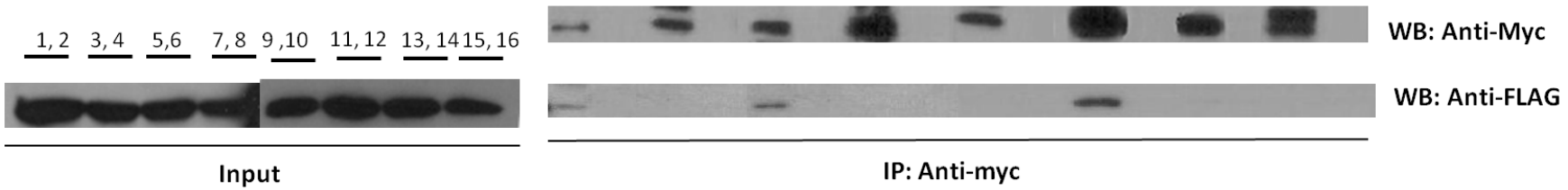
Figure 3.4 hnRNP C inhibits the telomerase activity. Co-incubation of PinX1 with hnRNP C or C a.a.1-174 can increase the telomerase inhibitory effect (A) The telomerase activity upon addition of exogenous purified proteins was measured by conventional TRAP assay. Different concentrations of purified PinX1 and hnRNP C were incubated with telomerase extracts for 10 minutes before TRAP assay. The internal control (IC) indicated the success of PCR and the heat inactivated control was done by heating the extract at 95 ℃ before the assay to ensure no contamination in the assay. A longer DNA ladder indicates a higher telomerase activity. For the co-incubation of proteins, the proteins added were in 1:1 ratio. The concentrations indicated refer to the individual protein concentration in the reaction. **(B)** PinX1 and hnRNP C a.a.1-174 were co- incubated with telomerase extracts.

3.5.4 Binding pattern of hnRNP C on hTERT

It was demonstrated that hnRNP C can inhibit the telomerase activity on its own and PinX1 and hnRNP C have the ability to inhibit the telomerase activity independently. To find out if PinX1 and hnRNP C share the same binding site on hTERT to inhibit the telomerase activity, the binding pattern of hnRNP C on hTERT was compared with that of PinX1.

Different hTERT truncations a.a.1-183, a.a.170-546, a.a.523-924, a.a.915-1132, a.a.1-325, a.a.326-620, and a.a.621-1132 were designed according to Banik et al. (2004). The truncations have been used to map the PinX1 binding on hTERT. In our experiment, myc-tagged hTERT truncations were co-transfected with FLAG-tagged hnRNP C in HEK293T cells for co-immunoprecipitation. FLAG-tagged hnRNP C was detected in the myc-tagged hTERT immunoprecipitate, but not in anti-myc negative control, indicated an association between hnRNP C and hTERT inside the cells. Out of these hTERT truncations, only a.a.1-325 and a.a.170-546 showed interaction with hnRNP C (Figure 3.5). This suggested that hnRNP C may bind to a.a.170-325, which is the overlapping region of these truncations. This binding pattern of hnRNP C on hTERT was compared with that of PinX1 and it was different from PinX1 as PinX1 was found to bind strongly to a.a.326-620 of hTERT.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Anti-myc	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Myc-hTERT	WT		1-183		170-546		523-924		915-1132		1-325		326-620		621-1132	
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FLAG-hnNRP C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+



(A)

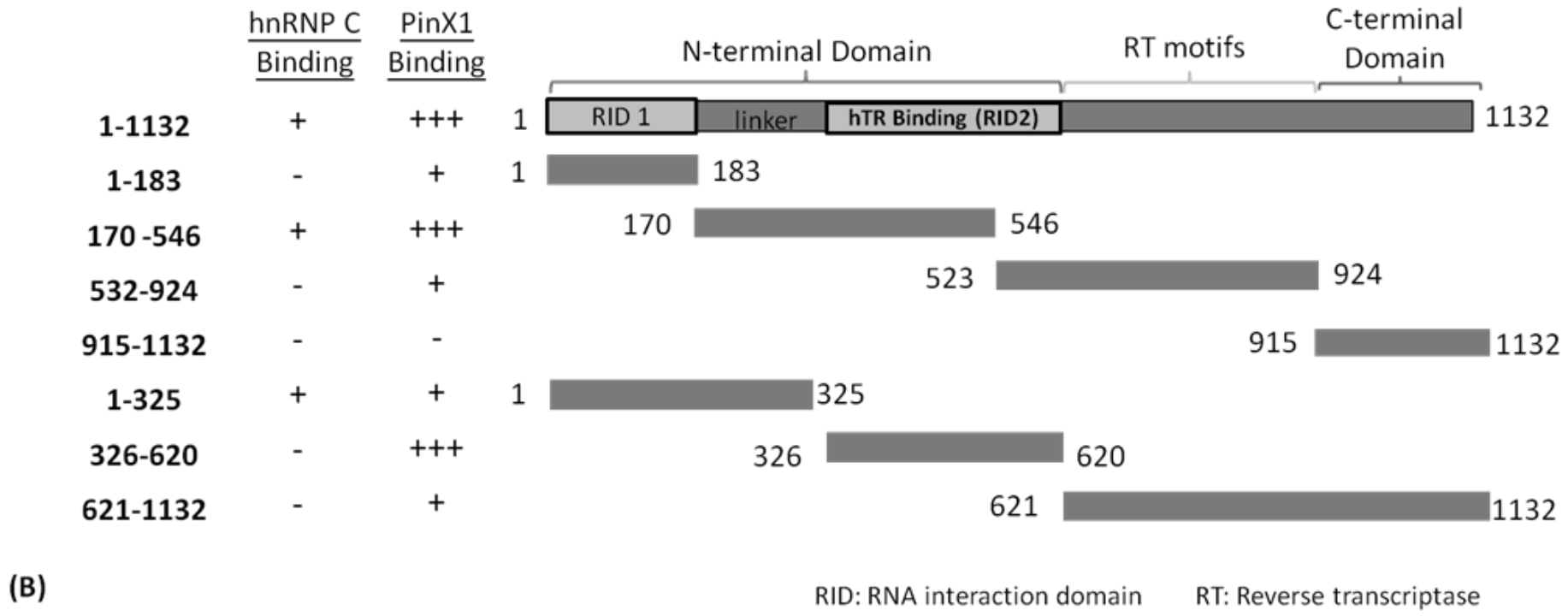


Figure 3.5 PinX1 and hnRNP C showed different binding pattern with hTERT. **(A)** Immunoprecipitation was carried out by co-transfecting the myc-tagged hTERT truncations and FLAG-hnRNP C into HEK293T. The presence of hnRNP C in the myc-immunoprecipitates was analyzed by western blot. Negative control was performed by using the cell lysate expressing full length hTERT without adding anti-myc antibody. **(B)** Map showing hTERT truncations for hnRNP C binding, where '-' representing lack of hTERT binding and the number of '+' indicating the relative strength the binding. The PinX1 binding site was indicated according to the previous work (Banik et al. 2004).

3.6 Discussion

In this study, the interaction between PinX1 and hnRNP C *in vivo* and *in vitro* was confirmed. PinX1 interaction site on hnRNP C was also located at a.a.174-260. This region includes the acidic auxiliary domain and also part of the oligomerization domain. The acidic auxiliary domain was believed to involve in protein-protein interaction, however specific functions of this region has not been determined.

To verify the function of hnRNP C/PinX1 interaction, TRAP assay was performed. PinX1 and hnRNP C alone can show inhibitory effect on telomerase activity. There was an increase in inhibitory effect when these proteins were incubated together in the TRAP assay. However, the increase in inhibition was due to the accumulative effect of these two telomerase inhibitors, rather than the end effect of hnRNP C/PinX1 interaction. It suggested that PinX1 and hnRNP C can inhibit the telomerase activity independently without interacting with each other. Besides, hnRNP C and PinX1 bind different sites on hTERT. This further suggested that hnRNP C inhibits the telomerase activity by a different mechanism than PinX1. However, it should be noticed that although these two proteins were added to the TRAP assay in a one to one ratio, that meant the amount of telomerase inhibitors added was doubled, a double increase in the telomerase inhibition was not observed. It is possible that the binding of one protein on hTERT may hinder the access of the other protein on hTERT; therefore, a more potent telomerase inhibition cannot be achieved.

PinX1 has multiple functions in telomerase regulation, although there is no significant importance of hnRNP C/PinX1 interaction in telomerase activity inhibition, this interaction may be important for other PinX1 functions.

Chapter 4

Characterization of hnRNP C on telomerase regulation

4.4 Introduction

It was demonstrated that hnRNP C alone can inhibit the telomerase activity. Given that overexpression of hnRNP C can cause telomere shortening (Fu and Collins 2007), role of hnRNP C as a telomerase inhibitor was investigated.

4.5 Result

4.5.1 Silencing of hnRNP C on telomerase activity

To further confirm the role of hnRNP C on telomerase activity regulation *in vivo*, hnRNP C was silenced in HKE293T and the telomerase activity of the cells was compared with the mock-transfected control. After 72 hours transfection, $2x10^5$ of hnRNP C siRNA-transfected and scrambled control siRNA-transfected cells were collected. Western blot analysis indicated that hnRNP C was successfully down regulated in the hnRNP C siRNA-transfected cells (Figure 4.1A). The collected cells were lysed by CHAPS lysis buffer and the cell lysate was obtained by centrifugation. The telomerase containing cell lysate was serially diluted to 3 folds and subjected to TRAP assay. The telomerase activity in the hnRNP C silenced-cells was higher than the control (Figure 4.1B). This indicated that presence of hnRNP C attenuates the telomerase activity in cells and hnRNP C regulates the telomerase activity in a negative manner.

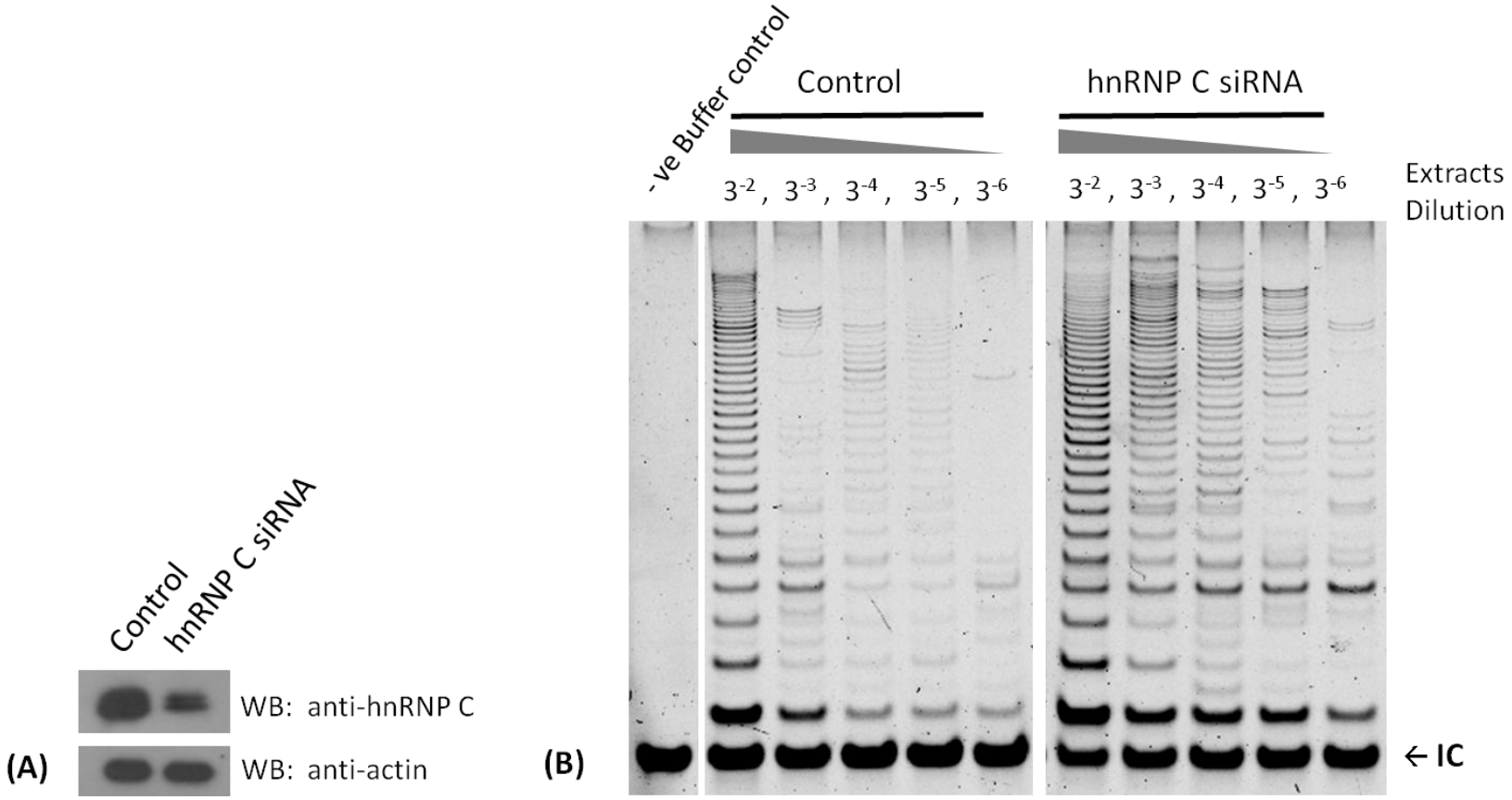
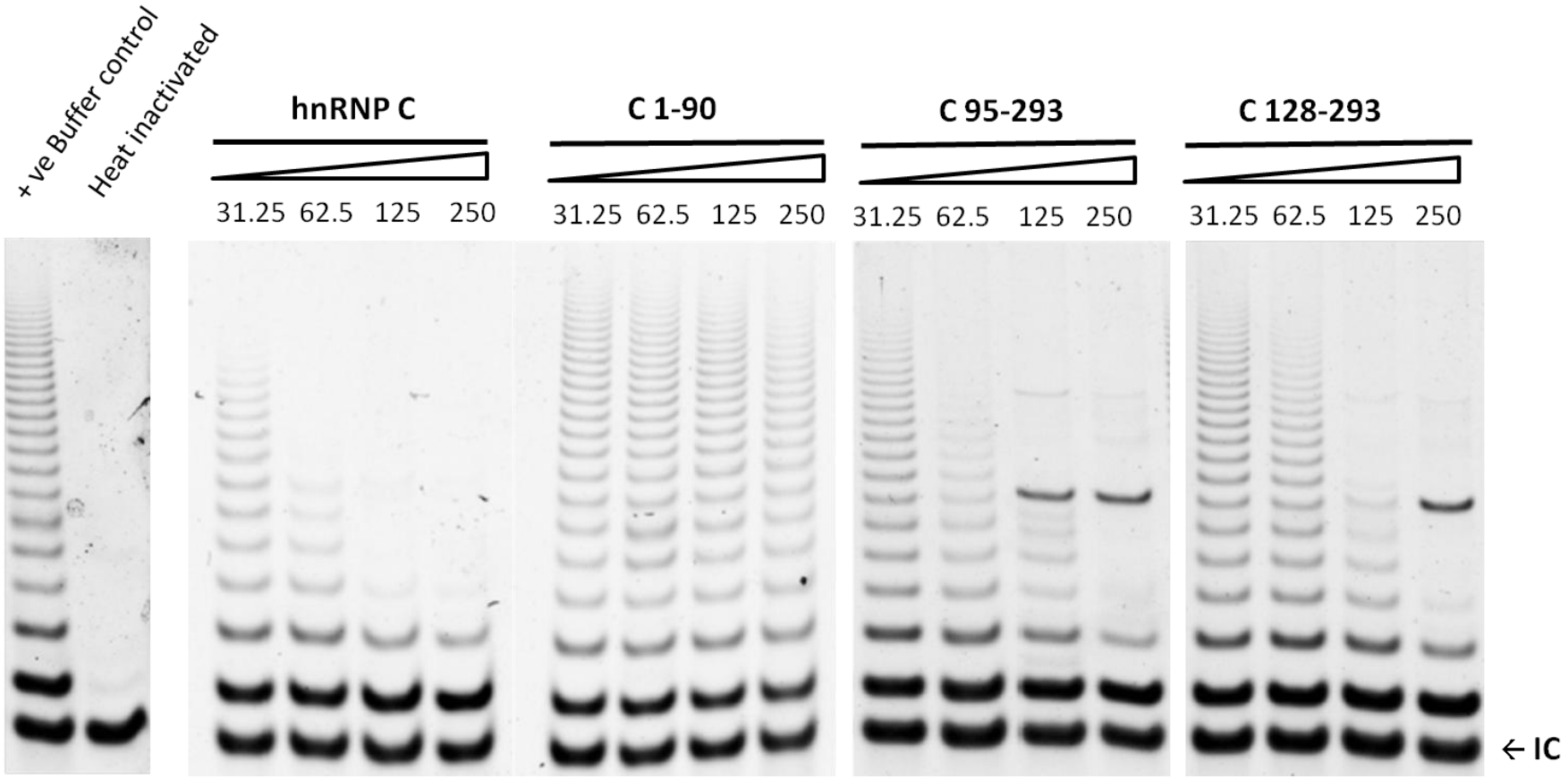


Figure 4.1 Silencing of hnRNP C increases telomerase activity in cells (A) hnRNP C was silenced in HEK293T cell by hnRNP C siRNA transfection. (B) Telomerase extracts from the control and silenced-cells were diluted and subjected to TRAP Assay. Buffer instead of extract was added as the negative control.

4.5.2 Identification of telomerase inhibitory domain on hnRNP C

Role of hnRNP C as a negative telomerase regulator was confirmed. In order to characterize the inhibitory effect of hnRNP C on telomerase activity, domain on hnRNP C that is responsible for inhibiting the telomerase activity was identified. The protein truncations were purified from *E.coli* expression and subjected into the TRAP assay (Figure 4.2, 4.4). Out of the truncations, a.a.1-174 were found to have a similar inhibition on the telomerase activity as the full length hnRNP C. Deletion of a.a.1-174 to a.a.1-130 showed decreased inhibitory effect, while further deletion to a.a.1-90 (the RRM) showed loss of inhibitory effect. This indicated the presence of a.a.130-174 can optimize the inhibition and a.a.95-174 was a critical region for inhibition. The importance of a.a.95-174 was supported by the fact that truncations that contain part of the potential inhibitory domain can show some inhibition on telomerase activity. It has been found that the bZLM (a.a.140-161) is the major determinant for high affinity and salt resistance RNA binding activity of hnRNP C (McAfee etl., 1996). Involvement of bZLM in telomerase inhibition indicated that the inhibition may be related to RNA binding activity. It should be noticed that truncation a.a.95-174, which is the potential inhibitory domain, did not show inhibitory effect as intense as a.a.1-174. Therefore, although the RRM (a.a.1-90) alone did not inhibit the telomerase activity, the presence of this motif together with a.a.95-174 form an important domain for the inhibition. It has been suggested that the canonical RRM together with bZLM forms a single RNA binding domain, this may explain why a.a.95-174 that lack the RRM showed decreased inhibitory effect and suggested an involvement of RNA binding activity in the telomerase inhibition.


+ ve Buffer control
Heat inactivated
hnRNP C
C 1-90
C 95-293
C 128-293
31.25 62.5 125 250
31.25 62.5 125 250
31.25 62.5 125 250
31.25 62.5 125 250
← IC

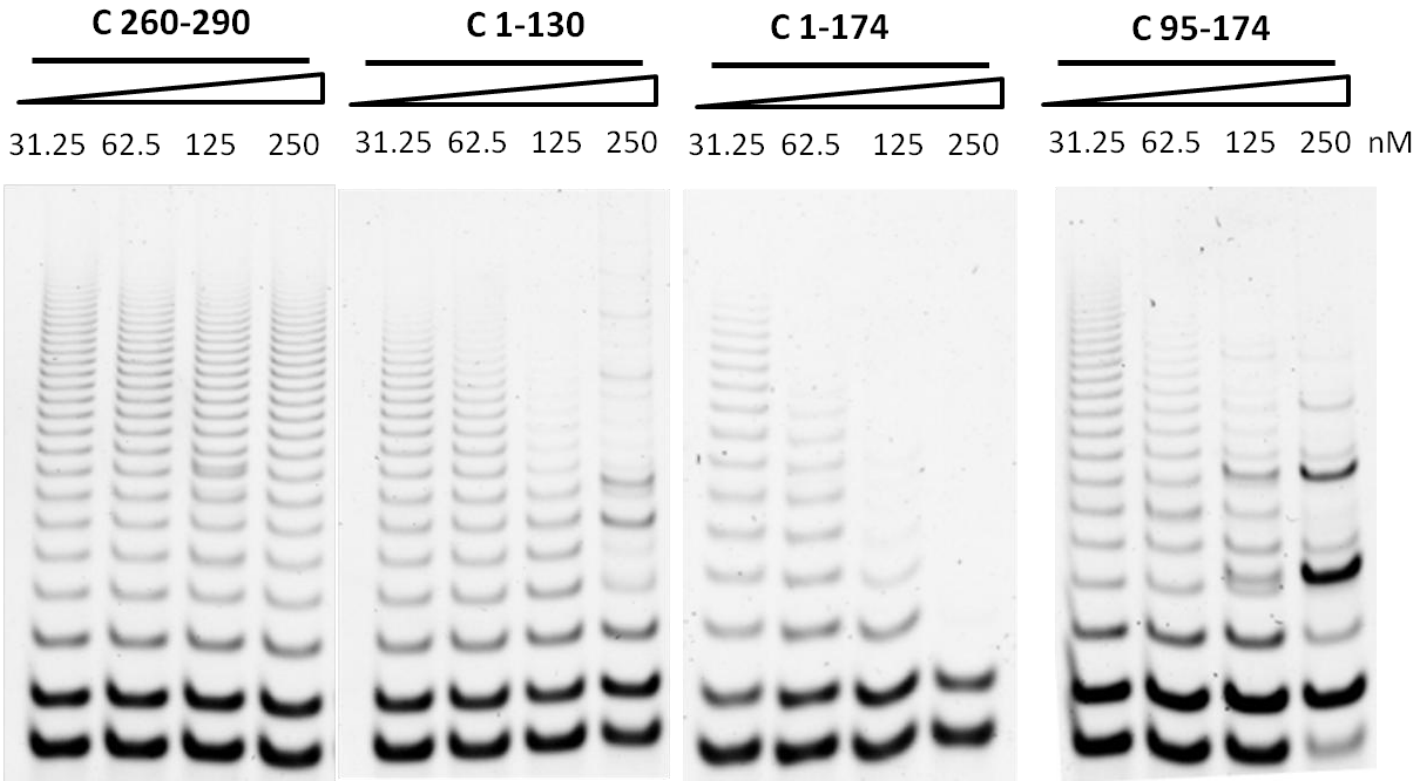
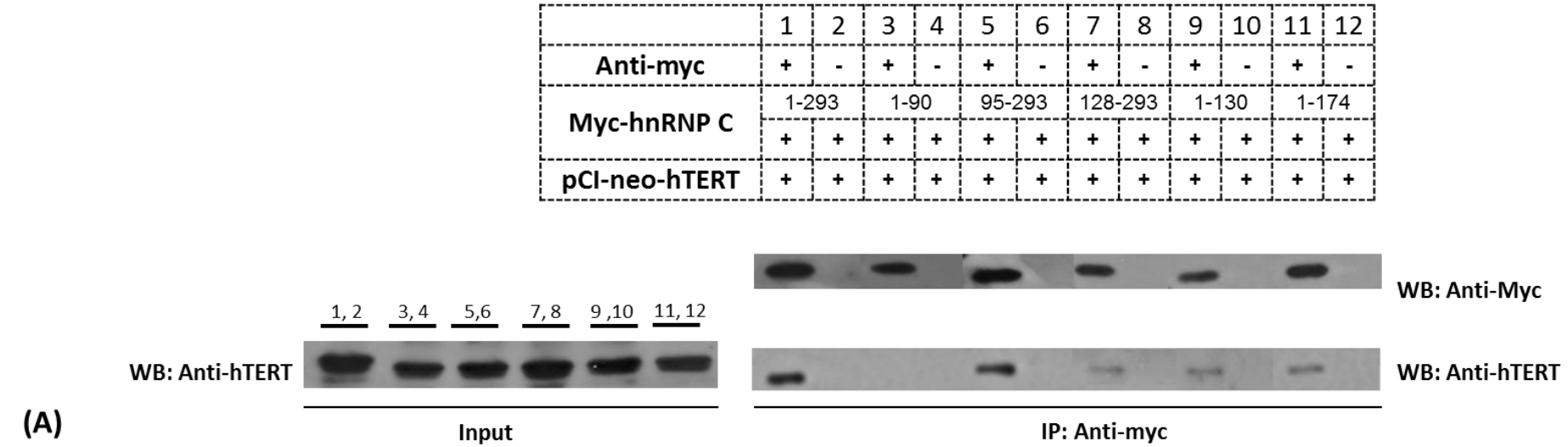


Figure 4.2 Telomerase inhibitory domain is located at a.a. 95-174 of hnRNP C. The telomerase activity upon addition of different hnRNP C truncations again was measured by conventional TRAP assay. Different concentration of the truncations were purified and incubated with telomerase extracts before the TRAP Assay. Some strong bands were found in some samples may due the contamination of proteins.

4.5.3 Identification of hTERT-binding region of hnRNP C

To investigate if the ability of hnRNP C to inhibit the telomerase activity matches the interaction between hnRNP C and the catalytic subunit of telomerase (hTERT), their interaction inside the cells was investigated. Mapping of hTERT binding region on hnRNP C was done by *in vivo* co-immunoprecipitation. HKE293T was transiently transfected with myc-tagged hnRNP C truncations and hTERT. hTERT was found in the anti-myc immunoprecipitate but not in anti-myc negative control, this again confirmed the association of these two proteins inside the cells (Figure 4.3A). Besides, hTERT can be co-immunoprecipitated with myc- tagged a.a. 95-293, a.a. 128-293, a.a.1-130 and a.a. 1-174, which have shown inhibitory effects on telomerase activity (Figure 4.4). This indicated that there was association between telomerase inhibition and interaction. However, myc-tagged C a.a.95-174 failed to express. This domain was then cloned into pcDNA3.1-mycHisA and transfected into HEK293 with improved expression level. It was found that hTERT can be co-immunoprecipitated with this mycHis-tagged a.a.95-174 (Figure 4.3B). The interaction was much weaker when compared with the wild type, this may be due to the improper folding of the short fragment or the lower level of myc His-tagged C a.a.95-174 being immunoprecipitated. This weaker interaction may explain the decrease in telomerase inhibition of C a.a.95-174 observed in the TRAP assay. In a short summary, the above interactions confirmed the association between hTERT with C a.a.95-174 and further supported the importance of this domain in the telomerase inhibition and interaction. However, as the myc-tagged hnRNP C truncations were precipitated by a different degree, it was difficult to compare the strength of their interaction with hTERT. Myc- tagged a.a 260-293 failed to express in any conditions probably due to its small size.



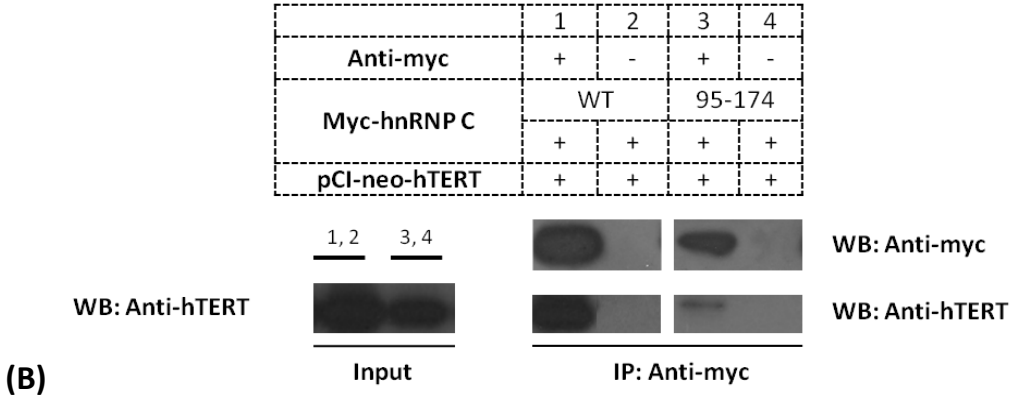


Figure 4.3 hTERT associates with the a.a.95-174 of hnRNP C. (A) Immunoprecipitation was carried out by transfecting different myc-hnRNP C truncations and hTERT in to HEK293T. The presence of hTERT in the myc-precipitates was analyzed by western blot. Negative control was performed by using the cell lysate expressing full length hTERT without adding anti-myc antibody. **(B)** Co-immunoprecipitation of myc-hnRNP C and mycHis-hnRNPC a.a95-174 with hTERT in HEK293.

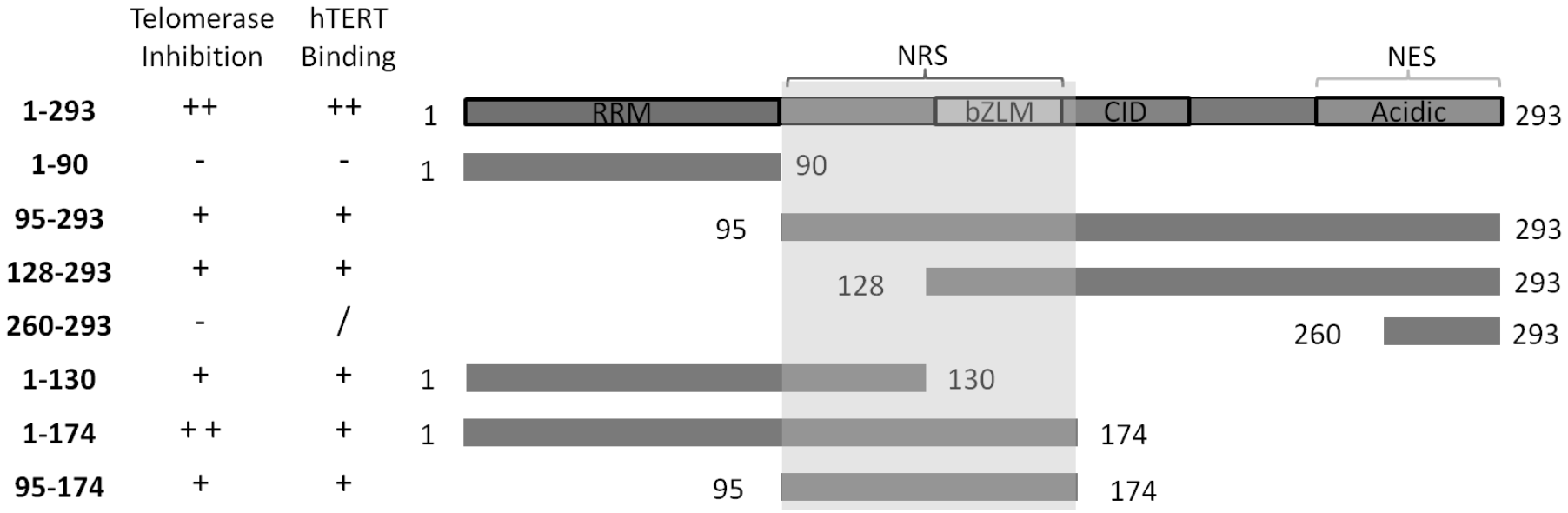


Figure 4.4 hnRNP C inhibits and interacts with the telomerase with the same domain. Map summarizing the inhibitory effect of hnRNP C on telomerase activity and the interaction between hnRNP C and hTERT. '-' is representing the lack of inhibitory effect or hTERT binding, the number of '+' indicates the strength of inhibitory effect and the hTERT binding. The shaded region indicated the region responsible for both the interaction and inhibition.

4.2.4 Role of RNA binding activity on telomerase interaction

From the co-immunoprecipitation assay, it was shown that RRM (a.a.1-90) did not interact with hTERT. However, as suggested in the TRAP, the RRM was required for the inhibition of telomerase activity and may be related to the RNA binding activity assay. To confirm the role of RNA binding activity in hnRNP C/hTERT interaction, RNA binding activity was removed by point mutation on the RRM (Wan et al, 2001). One of the mutants was made by changing phenylalanine to serine at a.a.19 (F19S). In the other mutant, guanine was mutated to a thymine at amino acid 56 (Q56H).

In the co-immunoprecipitation between myc-tagged hTERT and FLAG-tagged hnRNP C wild type and mutants, both the wild type and mutated-hnRNP C can be co-immunoprecipitated with hTERT. However, interaction between hTERT and mutated-hnRNP C was less than that with the wild type. It revealed that RNA binding activity of hnRNP C to some extend is required for the interaction between hnRNP C and telomerase (Figure 4.5).

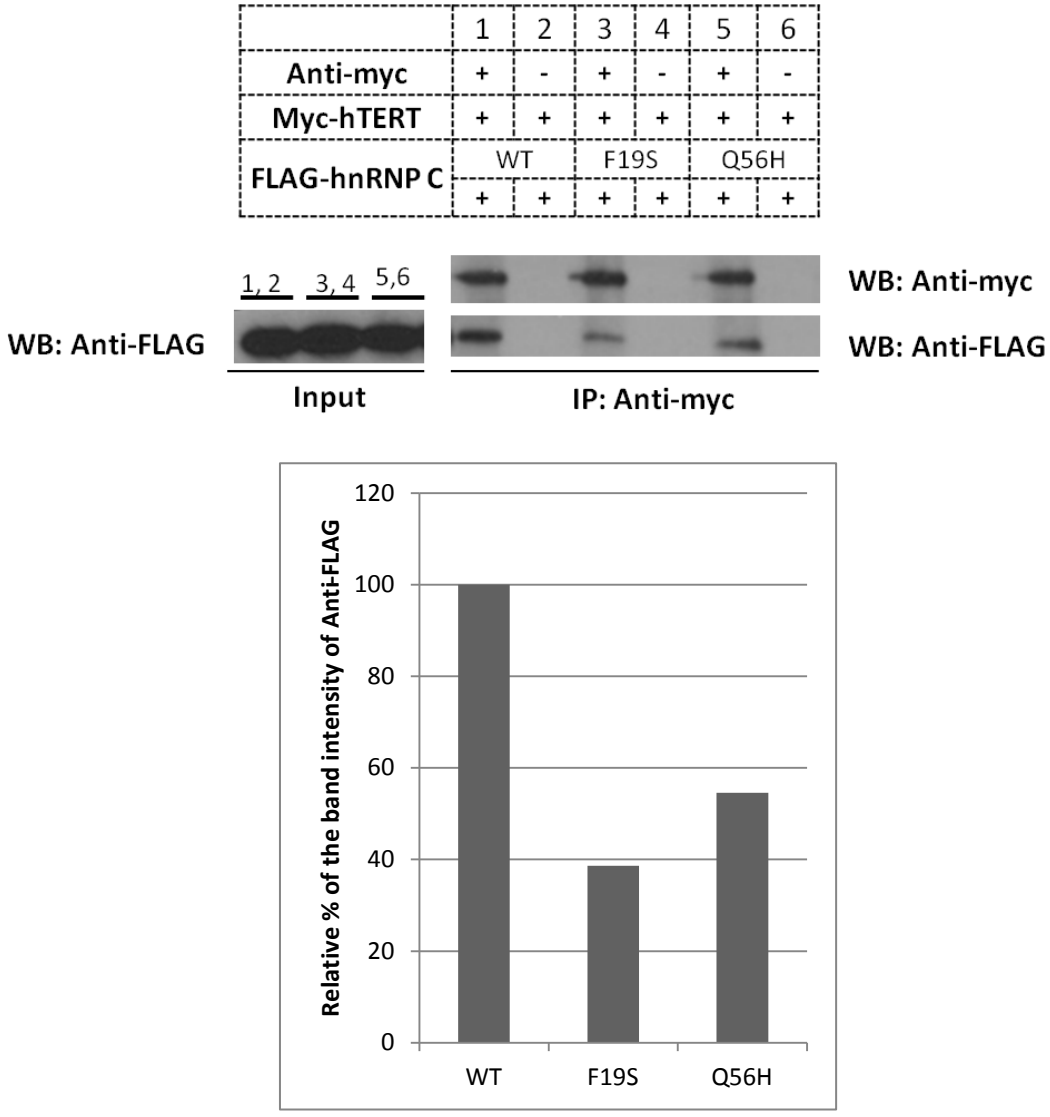


Figure 4.5 RRM mutated hnRNP C can associate with hTERT. Co-immunoprecipitation was performed by co-transfecting myc-tagged hTERT with wild type or RRM-mutated FLAG-hnRNP C into HEK293T. Myc-tagged hTERT was used as bait. Presence of hnRNP in the immuneprecipitate was detected by western blot.

4.3 Discussion

hnRNP C has been found in association with the endogenous human telomerase complexes and overexpression of hnRNP C results in telomere shortening (Fu and Collins 2007). Our data showed that in hnRNP C silenced cells, telomerase activity could be increased, which further support the role hnRNP C as a negative telomere and telomerase regulator. As a RNA binding protein involved in mRNA translation, it is possible that hnRNP C can bring some downstream effects which cause the above observations. However, as it was demonstrated in the TRAP assay, hnRNP C could directly inhibit the telomerase activity *in vitro*; it suggested a direct regulation on telomerase activity. This inhibitory effect on telomerase activity gives a possible reason for telomere shortening in hnRNP C-overexpressed cell line.

By subjecting the hnRNP C truncations into the TRAP assay and co-immunoprecipitation assay, domain that is responsible for the telomerase inhibition and interaction was identified. It was shown a.a 95-174 was an important domain for the inhibition and interaction. Truncations containing a.a 95-174 were able to associate with hTERT and also have the ability to inhibit the telomerase activity. This suggested that hnRNP C may inhibit the telomerase activity through its association with the hTERT.

However, out of those truncations, only a.a.1-174 can give an inhibitory effect on telomerase activity that was similar to the wild type. It may due to the requirement of the canonical RRM and bZLM to form a single RNA binding domain (McAfee, Shahied-Milam et al. 1996). Moreover, hnRNP C was found to associate directly with the integral RNA component of telomerase by binding to a 6-base uridylate tract of the hTR. Mutation of this tract on hTR resulted in the inability for hnRNP C to precipitate the telomerase activity (Ford, Suh et al. 2000). All these suggested that association of hTERT and hnRNP C is dependent on the binding of hnRNP C to hTR. However, our

study found that mutation on the hnRNP C RRM to remove the RNA binding activity did not totally disrupt the association between hnRNP C and hTERT, suggesting other possible linkage between hnRNP C and hTERT.

From the data in chapter 3, hnRNP C can associate with hTERT at a.a.170-325, which is a linker region of hTERT that joins the extreme N-terminal domain (RID1) of hTERT to the rest of the protein containing the RID2-RT-C-terminal domain (Figure 3A). This linker region is poorly conserved upon the six major regions in the N termini of 10 TERT family members and the function of it is not well characterized (Moriarty et al., 2002; Autexier and Lue, 2006). In addition, it was believed that hTERT can form dimeric or multimeric complexes and the flexibility of the linker allow the multimerization of hTERT. It was suggested that the N terminus containing the RID1 of one hTERT molecule can complement another truncated hTERT containing the RID2-RT-CTE region, to restore the activity of telomerase (Beattis et al., 2001; Autexier and Lue, 2006). However, more investigations have to be done to find out the role and significance of the linker on telomerase conformation and multimerization. Based on the above information, it suggested a possibility that hnRNP C interferes the telomerase activity by disrupting the conformation of the hTERT and also the multimerization of hTERT through the binding to the linker region. Besides, as the linker region is found between the two RIDs of hTERT, it is possible that hnRNP C associates with hTR and binds to hTERT at this region.

Chapter 5

Conclusion

5.3 Concluding Remarks

hnRNP C was confirmed as a novel interacting partner of PinX1. The interaction between PinX1 and hnRNP C and their effect on telomerase activity were characterized. It was found that PinX1 and hnRNP C can inhibit the telomerase activity independently. The role of hnRNP C/PinX1 interaction on telomerase regulation remains to be investigated.

The role of hnRNP C as a telomerase inhibitor was confirmed. It was found that hnRNP C can inhibit the telomerase activity, and telomerase activity can be increased in hnRNP C-silenced cells. hnRNP C a.a. 95-174 was shown to be an important domain for both the telomerase inhibition and interaction. hnRNP C was suggested to inhibit the telomerase activity through its association with hTERT. Given that only hnRNP C a.a. 1-174, which consists of the two important domains for the RNA binding, can give an inhibitory effect similar to that of the wild type hnRNP C, the binding of hnRNP C on the hTR is believed to be involved in the association between hnRNP C and hTERT.

As mentioned, hnRNP C has the ability to regulate cancer progression through its regulation on mRNA translation. Increase in hnRNP C mRNA expression was observed in various lung cancer cells, indicating an alteration in this mRNA processing protein (Pino et al., 2003). It seems that although hnRNP C has the ability to inhibit the telomerase activity through direct interaction with it, no significant suppression of

hnRNP C expression was observed in cancer cells. Therefore, in the future, it will be important to find out under what circumstance hnRNP C will inhibit the telomerase activity and the mechanism for the recruitment of hnRNP C on hTERT.

5.4 Future Prospects

5.4.1 Cell-cycle dependent interaction between hnRNP C and hTERT

Telomere synthesis occurs in S-phase and it involves the cell cycle dependent localization of hTERT and hTR. hnRNP C has the ability to inhibit the telomerase activity, however the time and location that this inhibition occurs are not yet determined. Inhibition of telomerase activity by hnRNP C involves the association of hnRNP C with hTERT and also hTR, cell cycle dependent localization of these proteins may take part in this association process. To gain insight on that, the interaction between hnRNP C, hTERT and hTR at different stages of cell cycle can be investigated. Cells will be synchronized and collected at different stages of cell cycle. Immunofluorescence on these proteins will be done to find out their subcellular localization. This helps to investigate the cell-cycle dependency of the interaction between hnRNP C, hTERT and hTR.

5.4.2 Importance of PinX1/hnRNP C interaction

It is found that PinX1 and hnRNP C can interact with each other. However, there is no significant importance for this interaction on telomerase activity inhibition. Efforts have to be put to find out the reasons for hnRNP C-PinX1 interaction. PinX1 has multiple functions in telomerase regulation; for example, mediating hTERT and TRF1 localization and the recruitment of telomere and telomerase. Involvement of hnRNP C in these regulations is a direction for future investigation. It can be achieved by silencing or overexpressing hnRNP C to see if there are changes on the above PinX1 functions. Besides, as hnRNP C has the ability to co-localize with TRF1, the interaction between hnRNP C, PinX1, TRF1 can also be studied.

5.4.3 Effect of hnRNP C and hnRNPs on telomerase activity

Several hnRNPs family members were found to associate with telomere and telomerase and have implication in telomere regulation. For example, hnRNP A1 can enhance the telomerase activity through recruitment and unfolding of the quadruplex of telomeric DNA. It was believed that hnRNPs has a positive role in telomere regulation and has the ability to bridge the association of telomerase to the telomere. (Ford et al., 2002). However, in this study, hnRNP C was found to have a negative role in telomerase regulation. How these proteins interact and make a balance on telomere and telomerase regulation may be an important direction for future study.

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