SUMO E3 ligase CBX4 regulates hTERT-mediated transcription of CDH1 and promotes breast cancer cell migration and invasion

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Running Title: Regulation of hTERT-mediated gene transcription by CBX4
Abstract

hTERT, the catalytic component of human telomerase enzyme, is regulated by post-translational modifications, like phosphorylation and ubiquitination by multiple proteins which remarkably affects the overall activity of the enzyme. Here we report that hTERT gets SUMOylated by SUMO1 and polycomb protein CBX4 acts as the SUMO E3 ligase of hTERT. hTERT SUMOylation positively regulates its telomerase activity which can be inhibited by SENP3-mediated deSUMOylation. Interestingly, we have established a new role of hTERT SUMOylation in repression of E-cadherin gene expression and consequent triggering on the epithelial-mesenchymal-transition (EMT) program in breast cancer cells. We also observed that catalytically active CBX4, leads to retention of hTERT/ZEB1 complex onto E-cadherin promoter leading to its repression through hTERT-SUMOylation. Further through wound healing and invasion assays in breast cancer cells, we showed the tumour promoting ability of hTERT was significantly compromised upon overexpression of SUMO-defective mutant of hTERT. Thus our findings establish a new post-translational modification of hTERT which on one hand is involved in telomerase activity maintenance and on the other hand plays a crucial role in regulation of gene expression thereby promoting migration and invasion of breast cancer cells.

Key words: EMT, Polycomb, SUMOylation, Telomerase, E-cadherin, Mutation
**Abbreviations:**

| Abbreviation | Description |
|--------------|-------------|
| ALT          | Alternative lengthening of telomeres |
| A.U.         | Arbitrary units |
| BMI1         | B-cell specific Moloney murine leukemia virus integration site 1 |
| CBX4         | Chromobox 4/ Chromobox like protein 4 |
| CDH1         | Cadherin 1 |
| CHAPS        | 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| E-cadherin   | Epithelial cadherin |
| EMT          | Epithelial-mesenchymal transition |
| EZH2         | Enhancer of zeste homolog 2 |
| HEPES        | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hTERT        | Human telomerase reverse transcriptase |
| N-cadherin   | Neural cadherin |
| NEM          | N-ethylmaleimide |
| NP40         | Nonidet P-40 |
| Pc2          | Polycomb 2 homolog |
| SDS          | Sodium dodecyl sulfate |
| SUMO         | Small Ubiquitin-like modifier |
| SENPs        | Sentrin-specific proteases |
| TRAP         | Telomeric repeat amplification protocol |
| ZEB1         | Zinc Finger E-box Binding Homeobox 1 |
Introduction

Telomeres are the repetitive, non-coding hexameric DNA sequences that protect linear chromosomal end from degradation and inappropriate processing as damaged DNA. Despite this protective role, telomere shortening occurs in the normal somatic cells due to intrinsic limitations of the replication machinery, and this shortening causes cellular senescence. However, cancer cells are able to avoid this shortening and thereby senescence by activating the ribonucleoprotein enzyme telomerase, which can extend telomeres. The human telomerase enzyme contains three essential components necessary for its function [1]: telomerase reverse transcriptase (hTERT), the catalytic subunit which is an RNA dependent DNA polymerase; the telomerase RNA component (hTERC), which acts as a template for the enzyme telomerase to synthesize the telomeric DNA; and the protein dyskerin which plays a crucial role in maintaining the stability of hTERC. Several epigenetic modifications in cancer cells have been reported to be critically important for preserving telomeric integrity. For example, methylation status of the sub-telomeric DNA repeats is reported to be inversely correlated with telomere length and telomere recombination in various human cancers [2], and histone methylation on the promoter of hTERT was also found to control the catalytic activity of human telomerase [3]. Additionally, several studies have highlighted the importance of post-translational modifications (PTMs) of telomeric proteins in maintaining the telomere length and telomeric integrity. SUMOylation is one such PTM which has been reported to be important in telomere maintenance. Studies in different yeast mutants revealed that SUMOylation of different telomere associated proteins like Yku70/80, Sir4, Sir2 and Cdc13 limits the accessibility of telomeres to telomerase and SUMO-defective mutants effectively show abnormally lengthened telomeres [4,5]. In humans, crosstalk between SUMOylation and ubiquitination is needed for the stability of an important member of shelterin complex, TRF2 [6]. Another important protein of the human telomerase enzyme complex, dyskerin also gets SUMOylated and disruption of dyskerin SUMOylation leads to impaired telomerase activity and eventually causes a fatal premature ageing syndrome, dyskeratosis congenital (DKC) [7]. Furthermore, the role of SUMOylation has also been well studied for maintaining telomere length in the cells which follow the Alternative lengthening of telomeres (ALT) pathway for telomere elongation. For the formation of ALT-associated PML bodies
(APB) and the localization of telomeres into it, the SUMOylation of TRF1 and TRF2 by SUMO E3 ligase MMS2 found to be an indispensable requirement [8].

Polycomb group (PcG) complex is a well known transcriptional repressor complex that regulates the expression of the target genes either by direct repression of the transcription machinery or by regulating the higher-order chromatin structures. Interestingly, earlier studies have indicated an association of polycomb group of proteins with telomere maintenance. In *Drosophila*, PcG proteins were found to bind with telomeric associated sequences (TASs) taking significant part in telomeric position effect (TPE) [9]. BMI1, an important polycomb group member and well-studied oncogene, was found to induce telomerase enzyme and helped in immortalization of the human mammary epithelial cells [10]. Correlation between BMI1 and telomerase activity was also observed in case of human ovarian cancer [11] and as well as in hTERT induced epithelial to mesenchymal transition (EMT) of oral epithelial cells [12]. Our protein of interest, CBX4 (or Pc2) is also an integral component of canonical polycomb repressive complex 1 (cPRC1). It was discovered as a SUMO E3 ligase which enhances the SUMOylation status of the transcriptional co-repressor CtBP in both *in vitro* and *ex vivo* context by recruiting the substrate protein and the E2, Ubc9 within the PcG body [13]. Later-studies demonstrated that CBX4 can SUMOylate several other proteins like BMI1 itself, HNRNPK, HIPK2 etc. and recruit them at the DNA damage site by initiating p53 mediated DNA damage response (DDR) pathway [14–16]. Being a polycomb group member, the role of CBX4 has been well documented in transcriptional repression. CBX4-mediated SUMOylation of ‘de novo’ methyltransferase Dnmt3a and zinc finger protein CTCF contributes significantly to their repressive activity [17,18].

CBX4 mostly acts as a tumor promoting gene in hepatocellular carcinoma and osteosarcoma by modulating several biological pathways through its SUMO E3 ligase activity [17–19]. But evidences suggested that it might also exert non-canonical function as a tumor-supressor in colorectal carcinoma independent of its SUMO E3 ligase, chromodomain and polycomb complex activity [22]. Recently the role of this protein has also been shown in breast cancer through activation of Notch 1 signaling pathway mediated by miR-137 [20]. Although, the individual roles of CBX4 and telomerase have been well-studied in the context of cancer progression, to our knowledge, there has been no report of a functional association between the above two proteins. The current study investigates the role of SUMO E3 ligase CBX4 in the regulation of telomerase activity and for the first time we show that hTERT gets SUMOylated by
SUMO1 and CBX4 acts as the SUMO E3 ligase of hTERT. Fascinatingly, we found that CBX4 mediated SUMOylation of hTERT does not only alter the telomerase activity but also plays an important role in hTERT/ZEB1 mediated transcriptional regulation of E-cadherin and in turn epithelial-to-mesenchymal (EMT) progression. Further in depth analysis has revealed the epigenetic significance and relevance of this regulation in the context of breast cancer.

Materials and Methods

Cloning and expression of constructs of CBX4

We got FLAG-tagged constructs of CBX4 (FLAG-CBX4/FLAG-CBX4 ΔSIM1/ΔSIM2/ΔSIM1/2) as kind gifts from Prof. David Wotton. FLAG-SENP1, FLAG-SENP2, RGS-SENP3, 3xHA-hTERT were procured from non-profit plasmid repository ‘addgene’ (Cambridge, MA, USA). SENP3 was cloned into pCMV-FLAG backbone by using specific primers. Site directed mutagenesis was done on HA-hTERT template (K710R) with the help of Dpn1 (NEB, Ipswich, MA, USA) restriction enzyme as described elsewhere [24] by using specific primers. In case of each construct, after transformation plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) as per manufacturer’s protocol and were resolved in 1% agarose gel to check the integrity of the DNA.

Cell culture and treatments

HeLa and MCF-7 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). MDA-MB-231 and MDA-MB-468 cells were maintained in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All cell lines were supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and penicillin/streptomycin (10uL/mL of medium, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% (v/v) CO₂.

Over expression and siRNA transfection

HeLa cells were transiently transfected with FLAG-CBX4 (for TRAP assay, coimmunofluorescence and Co-IP experiments), FLAG-CBX4 ΔSIM1/ΔSIM2/ΔSIM1/2 (for TRAP assay and Co-IP experiments), FLAG-SENP1/SENP2/SENP3 (for TRAP assay and Co-IP experiments), WT/HA-K710R hTERT (for Co-IP). MCF7, MDA-MB-468 and MDA-MB-231...
cells were transiently transfected with FLAG-CBX4 and FLAG-CBX4 ΔSIM1/2 (for ChIP, qRT PCR, wound healing scratch assay and transwell invasion assay respectively). All the transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) by following manufacturer’s protocol.

HeLa cells were treated with CBX4 siRNA (Genex India Bioscience Pvt. Ltd., Chennai, Tamil Nadu, India) or negative control siRNA (Thermo Fisher Scientific, Waltham, MA, USA) using INTERFERin transfection reagent (Polyplus-transfection SA, Illkirch-Graffenstaden, France) according to the manufacturer’s protocol and incubated for 24 hours prior doing experiments.

Co-immunoprecipitation (Co-IP) and Western blot analysis

Co-immunoprecipitation assay, was performed as described elsewhere [25]. In brief, 20 mM of freshly prepared N-ethylmaleimide (NEM) was added to the lysis buffer prior to cell lysis to prevent the degradation of SUMOylation by isopeptidases. After that cell lysis buffer (50mM HEPES (pH7.5), 150mM NaCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.5% Sodium deoxycholate, 5% Glycerol, 1mM DTT and complete protease inhibitor cocktail) was added to the cells and incubated on ice for 1hr followed by centrifugation at 13000 rpm for 10mins at 4°C. After pre-clearing the lysates with normal sheep serum, immunoprecipitation was done with the respective antibodies, followed by washes with the same buffer and analysis by western blotting. The antibodies used in Co-IP and Western blot analysis are listed in Table 1.

Coimmunofluorescence and confocal microscopy

Coimmunofluorescence staining was performed following standard protocol [26]. In brief, after fixing the cells with 4% Paraformaldehyde, permeabilization was done with 1% Triton X-100 followed by 3% BSA blocking. Cells were then incubated with with anti-FLAG (Sigma-Aldrich, St. Louis, Missouri, USA) and anti-hTERT (Santacruz Biotechnology, Dallas, Texas, USA) antibodies for 1 hour. The cells were subsequently washed thrice with PBST. Anti-mouse Alexa fluor 488 and anti-rabbit Alexa fluor 594-conjugated secondary antibodies were then added to the cells and incubated for 1hr at room temperature. The coverslips were again washed with PBST and mounted with DAPI-containing Prolong Gold antifade mountant (Thermo Fisher Scientific, Waltham, MA, USA) before imaging. Confocal imaging was done with Andor
Spinning Disk Ti-E confocal scanning microscope with A1RMP scanner head (Nikon, Minato, Tokyo, Japan) using 100x objective.

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed as described elsewhere [27]. Briefly, the cells were crosslinked with 1% formaldehyde and then 0.125M Glycine was added to stop the reaction. Cells were lysed in Cell lysis buffer [5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP40 (with fresh PI)] and the segregated nuclei were re-suspended in nuclei lysis buffer [50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS (with fresh PI)]. After ultrasound shearing the lysate was taken and pre-clearing was done with Normal sheep serum. Pre-blocked DYNA beads were put for binding to pull chromatin complex with specific antibodies listed in Table 1. Subsequently, RIPA buffer, high-salt buffer, LiCl buffer and TE buffer were added consecutively for washing the immunoprecipitated beads. After that, RNase A and Proteinase K treatment were given to the same. The beads were then de-crosslinked at 65°C overnight. Next, following Phenol-chloroform extraction the DNA was precipitated using ethanol. The DNA pellet was solubilized in water and subjected to qPCR analysis using gene specific primers mentioned in Table 2.

**RNA Extraction & Quantitative real time PCR (qRT-PCR)**

Total cellular RNA was extracted using TRIZOL reagent (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer’s instructions and RNA quantity, quality was determined using Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Complementary DNA was synthesized from RNA using Revertaid Fast strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s protocol followed by qRT-PCR using ABI-SYBR GREEN mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and the primers enlisted in Table 2. ABI 7500 Fast Real-time PCR machine (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was used for qRT-PCR.

**Telomerase activity assay**

Telomerase activity in cell extracts was measured by performing TRAP (Telomeric repeat amplification protocol) assay with TRAPeze RT Telomerase Detection Kit (S7710) (MilliporeSigma, Burlington, MA, USA) as mentioned elsewhere [28]. Briefly, cell lysates were
prepared by CHAPS lysis buffer followed by setting up the assay with TRAPEze RT Reaction mix, PCR grade water, Taq DNA polymerase (GoTaq Flexi DNA polymerase, Promega Corporation, Madison, Wisconsin, USA) and cell lysate. Additionally, a dilution series was prepared with TSR8 control template in CHAPS lysis buffer to serve as standard curve. Samples were then subjected to PCR amplification cycle using an ABI 7500 Fast Real-time PCR machine. Data points were fitted to a linear regression plot and from that arbitrary telomerase units were calculated relative to the TSR8 amplification.

Wound healing assay
Wound healing assay was performed as described elsewhere [29]. Briefly, MDA-MB-231 cells were seeded in a 6-well dish and at around 80% confluence, cells were transfected with indicated plasmids. After 24 hours, cell surface was scratched with a 200 μl sterile tip and this time point was mentioned as 0hr. Images were captured with Nikon T1 E100 microscope (Nikon, Minato, Tokyo, Japan) at 0hr, 24hrs and 48 hrs (post-scratch) to determine the wound healing by the cells reflecting their migratory ability. The images were further analyzed using Image J software (NIH, Bethesda, Maryland, USA) to quantify the percentage of wound recovery by the cells. Statistical analysis were done based on three replicate experiments.

Transwell invasion assay
Transwell chambers (Corning Inc., Corning, New York, USA) coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used to perform invasion assay following the protocol as delineated elsewhere [29]. In brief, MDA-MB-231 cells were transfected with indicated plasmids and kept for 24 hours prior seeding for invasion assay. After 24 hours, 0.5X 10⁶ transfected cells were trypsinized and seeded in the upper well of the transwell chamber containing serum free medium and allowed to invade towards the serum enriched bottom chamber for 20 hours. After incubation, invaded cells were stained with 0.5% crystal violet in ethanol, imaged with Nikon T1 E100 microscope (Nikon, Minato, Tokyo, Japan), counted with Image J software (NIH, Bethesda, Maryland, USA) to quantify the percentage of invaded cells. Three independent experiments were performed in each case to determine statistical significance.
SUMOylation-site prediction:

To identify the probable SUMOylation site of human telomerase reverse transcriptase (hTERT), the amino acid sequence was retrieved from UniProt (UniProt id: O14746) and the occurrence of SUMO-interaction motif (SIM) was determined by using two individual SUMOylation site prediction tools with high threshold values: (a) JASSA-V4 (Joined Advanced Sumoylation Site and Sim Analyser) [30] and (b) GPS-SUMO-V2 (Prediction of SUMOylation sites and SUMO-binding motifs) [31].

Statistics:

Statistical significance was determined performing Student’s two-tailed unpaired t-test as mentioned in the figure legends. GraphPad Prism 6.02 (GraphPad software Inc., La Jolla, CA) was used for the analysis. At least three (n=3) individual experiments were done in each case and data were represented as mean ± SD. p-value < 0.05 were considered to be statistically significant.

Results

CBX4 interacts with hTERT, catalytic component of human telomerase enzyme

CBX4, a well-studied member of polycomb repressive complex 1 (PRC1), plays significant role in DNA damage response and transcriptional regulation through its SUMO E3 ligase activity [14–16,32]. Similar to other PcG proteins like BMI1, CBX7 and EZH2 [33], CBX4 shows tumor-promoting effect in several types of cancer [20,21]. We also observed higher expression of CBX4 at mRNA levels in cervical cancer cells (HeLa), Luminal B type breast cancer cells (MCF-7) and basal type breast cancer cells (MDA-MB-468) as compared to non-tumorigenic breast epithelial cells (MCF 10A) (Supplemental figure S1A). Previously it was shown that oncogenic function of BMI1 intricately depends on the activation of telomerase enzyme activity [10]. The activation of BMI1 was also found to be essential in case of hTERT-induced epithelial to mesenchymal transition (EMT) of oral epithelial cells [12]. Interestingly, CBX4 has been identified as the SUMO E3 ligase of BMI1 and helps to recruit the protein at the DNA damage site [14]. Recently, the role of CBX4 was also observed in the proliferation and metastasis of lung cancer cells through BMI1 regulation [35]. Together these observations led us to examine the role of CBX4 in telomerase activity regulation. As endogenous telomerase activity is higher even in the non-dividing sub-population of HeLa cells [35] and it showed
considerable CBX4 expression (Supplemental figure S1A), we selected this cell line for our further biochemical studies. To measure the relative telomerase activity, telomeric repeat amplification protocol (TRAP) was performed either by over expressing or knocking down CBX4 in HeLa cells. Significantly high telomerase activity was found upon CBX4 over expression and the knock-down showed reverse effect (Figure 1A and 1B). The activity of telomerase enzyme greatly depends on the function of the catalytic component, hTERT which is able to add new hexameric telomere sequences at the end of the chromosome by using hTERC as template. So we subsequently performed co-immunoprecipitation assay to verify whether there is any physical association between CBX4 and hTERT. Indeed we found hTERT interacts robustly with CBX4 (Figure 1C). Reciprocal co-immunoprecipitation also showed similar association (Supplemental figure S1B). However, we observed additional higher molecular weight bands which were indicative of posttranslational modification of CBX4 and hTERT respectively. Further we performed co-immunofluorescence experiment after transiently transfecting FLAG-CBX4 in HeLa cells where CBX4 appeared as nuclear foci, a typical characteristic feature of PcG bodies [13] (Figure 1D). Interestingly, hTERT followed the same distribution pattern and showed prominent co-localization with FLAG-CBX4 as the calculated Pearson’s coefficient was greater than 0.5 (Figure 1D and Supplemental figure S1C, panel I and II). These results clearly indicate towards the involvement of CBX4 in regulating telomerase activity through interaction with catalytic component of human telomerase enzyme, hTERT.

**hTERT gets SUMOylated by SUMO 1**

The maintenance of telomeric integrity is tightly regulated by multiple factors, including several epigenetic modifications. Along with methylation of sub-telomeric DNA, SUMOylation has also been observed to regulate telomeric length and telomeric integrity at different molecular levels. Studies in different yeast mutants revealed that accessibility of telomeres to telomerase enzyme also depends on the SUMOylation status of the cells [4]. In addition to that, previous report on phosphorylation and ubiquitination of hTERT itself also showed remarkable effect on the overall activity of telomerase enzyme [36,37]. Given our finding that hTERT interacted with the well-studied SUMO E3 ligase CBX4 (Figure 1C), we sought to determine whether hTERT itself is SUMOylated. Indeed through co-immunoprecipitation and reciprocal co-immunoprecipitation, significant interaction was observed between hTERT and SUMO1 (Figure 2A and Supplemental
To further study the robustness of this interaction, prospective target lysine was identified at 710 position of conserved central catalytic RT domain of hTERT [38] within a consensus SUMO motif (Figure 2B) and the chance of SUMOylation was predicted with the help of JASSA-V4 and GPS-SUMO-V2 online tools. Both the tools indicated Lysine 710 as a potential SUMOylation site with high confidence. Subsequently we generated the SUMO-defective mutant K710R by replacing lysine with arginine and transfected HeLa cells with HA-tagged wild type and mutant constructs. Co-immunoprecipitation was then performed by anti-HA antibody and interestingly, we observed a considerable reduction in the association of hTERT with SUMO1 (presence of lesser number of higher molecular weight bands) as a result of this mutation confirming the involvement of this particular lysine residue (Figure 2C) in this process.

**CBX4 acts as SUMO E3 ligase for hTERT**

CBX4 functions as SUMO E3 ligase and SUMOylates different proteins involved in diversified pathways. The interaction of hTERT with CBX4 and subsequent interaction of the former protein with SUMO1 prompted us to speculate that CBX4 can act as SUMO E3 ligase for hTERT. Previous report has shown that CBX4 contains two non-covalent SUMO-interaction motifs, SIM1 and SIM2 which are critical for its activity as SUMO E3 ligase and ablation of these two motifs (ΔSIM1/2) greatly affects the enzymatic activity of CBX4 [39]. Therefore to investigate the catalytic role of CBX4 in the SUMOylation of hTERT, we transiently transfected wild type and different catalytic mutants of CBX4 (FLAG-CBX4 ΔSIM1, ΔSIM2 and ΔSIM1/2) in HeLa cells and checked the interaction between hTERT and SUMO1. Although no considerable reduction in the interaction of hTERT with SUMO1 was observed upon over expression of SIM1 and SIM2 deleted constructs (FLAG-CBX4 ΔSIM1 & ΔSIM2), prominent reduction in SUMOylation was found in the cells overexpressing the double mutant (FLAG-CBX4 ΔSIM1/2) with compared to wild type CBX4 (Figure 2D, panel I). Next we attempted to observe the effect of catalytic mutants of CBX4 on telomerase activity. TRAP assay was performed to measure the activity after transiently transfecting the mutants into HeLa cells. Unlike wild type CBX4, no significant change was found in relative telomerase activity upon over expression of catalytic mutants of CBX4 and all of them showed a basal level of telomerase.
activity as of vector control cells (Figure 2D, panel II). HeLa whole cell lysates (WCL) and TRAP lysates were further subjected to immunoblotting to check the expression of FLAG tagged proteins (Supplemental Figure S2A and S2C). So, together these results certainly validate the function of CBX4 as a SUMO E3 ligase for hTERT and also indicate that the catalytic activity of CBX4 is essential for the regulation of telomerase activity.

Regulation of SUMO deconjugation from its substrate has an immense importance in SUMO system and it ensures plasticity of protein interaction network [40]. Six SUMO specific proteases or Sentrin-specific protease (SENPs) are reported so far having role in various disease development [41]. After we found hTERT gets SUMOylated by SUMO E3 ligase CBX4 (Figure 2D), we wanted to check the effect of most well studied deSUMOylases, SENP1, SENP2 and SENP3 on hTERT SUMOylation. So, HeLa cells were transiently transfected with FLAG-SENP1, SENP2 and SENP3 and interaction was studied between hTERT and SUMO1. However, substantial reduction in the interaction of hTERT with SUMO1 was observed after over expression of all three SUMO-specific proteases, but the most significant effect was found with SENP3 as compared to SENP1 and SENP2 (Figure 2E, panel I). Further TRAP assay was performed after over expressing the SENPs in the cells to check the effect of deSUMOylases on telomerase activity. Interestingly, major reduction in telomerase activity was found upon FLAG-SENP3 over expression but SENP1 and SENP2 showed no considerable effect (Figure 2E, panel II). Protein expression of FLAG-SENP1, SENP2 and SENP3 was confirmed from HeLa whole cell lysates and TRAP-lysates by immunoblotting (Supplemental Figure S2B and S2D). These results indicate that SENP3 acts as the effective deSUMOylase for hTERT and also helps to maintain the activity of the telomerase enzyme.

**CBX4 regulates the recruitment of hTERT/ZEB1 complex as a SUMO E3 ligase and represses E-cadherin in breast cancer**

Studies in past few years have shown that hTERT has several other functions apart from lengthening of telomeres which are necessary for transformation of normal cells to cancer cells [42]. In case of colorectal cancer, hTERT was found to regulate the expression of E-cadherin, forming complex with an important nuclear transcription factor ZEB1 and showed positive effect in epithelial-to-mesenchymal transition (EMT) [43]. We sought to investigate the role of CBX4
as a SUMO E3 ligase in this transcriptional regulation. However, E-cadherin expression is known to be silenced by DNA methylation in various cervical cancer cell lines including HeLa [44,45]. Contrarily, the Luminal B type breast cancer cells MCF-7 shows considerable E-cadherin expression [46] and has been used for our further in-depth analysis. MCF-7 cells were transiently transfected with FLAG-CBX4 or FLAG-CBX4 ΔSIM1/2 to observe the CDH1 (E-cadherin) gene expression followed by protein expression. While wild type CBX4 over-expression showed significant reduction in E-cadherin expression, comparatively compromised effect was seen in case of catalytic mutant of the same (Figure 3A, panel I and panel II). A rescue experiment was then performed by overexpressing FLAG-CBX4 or FLAG-CBX4 ΔSIM1/2 after knocking down hTERT in the background. Although de-repression of CDH1 gene was observed after knocking down hTERT (Figure 3B), wild type and mutant CBX4 failed to show any significant effect on CDH1 expression in hTERT-depleted condition (Figure 3B). This result indicates that hTERT is the main molecular player involved in this mechanism. To further elucidate the mechanism of this regulation, occupancy of hTERT was then examined on to the E-box region of CDH1 (E-cadherin) gene promoter. Interestingly, significant reduction in the enrichment of hTERT was observed on that site through chromatin Immunoprecipitation (ChIP) assay upon FLAG-CBX4 ΔSIM1/2 over-expression (Figure 3C) as compared to FLAG-CBX4. Since E-box region of CDH1 has a canonical ZEB1 binding site, we tested for its recruitment at that region and observed differential regulation in the occupancy of the protein upon FLAG-CBX4 and FLAG-CBX4 ΔSIM1/2 over-expression (Supplemental figure S3A). This clearly indicates that the catalytically active wild type CBX4 facilitates the recruitment of hTERT/ZEB1 complex onto CDH1 gene promoter through its SUMO E3 ligase activity. Further, the recruitment of SUMOylated hTERT was also checked on the above promoters through sequential ChIP and indeed considerable decrease in the enrichment of the complex was observed after FLAG-CBX4 ΔSIM1/2 over-expression (Figure 3D). These result reconfirmed that the SUMOylation of hTERT is instrumental for the transcriptional repression of CDH1 gene. Previous reports have already shown the positive effect of hTERT in EMT [47]. We also observed notable reduction in the CDH1 expression upon HA-hTERT over-expression (Figure 3E). However, the effect was significantly compromised with SUMO-defective mutant of hTERT (HA-hTERT K710R). To further ascertain the importance of hTERT SUMOylation in the repression of CDH1, we over-expressed CBX4 along with wild type hTERT and increasing
concentrations of SUMO-defective mutant of hTERT (HA-hTERT K710R) in MCF7 cells (Figure 3F). It is evident from the result that CBX4 showed significant repression of CDH1 when co-expressed with wild type hTERT but with hTERT K710R mutant, gradual compromised effect was observed with increasing DNA concentration (1μg, 2 μg, 3 μg) (Figure 3F). This result suggests that CBX4 effectively inhibits CDH1 gene expression via hTERT SUMOylation.

It was hypothesized that besides acting as SUMO E3 ligase CBX4 also undergoes SUMOylation via an auto-regulatory mechanism [48]. As a member of well-known gene repressive complex PRC1, CBX4 was found to bind the H3K27Me3 mark in a SUMOylation-dependent manner [49]. We observed compromised recruitment of CBX4 itself onto CDH1 promoter upon catalytic mutant over-expression (Supplemental figure S3B) which prompted us to check the enrichment of H3K27Me3 at that site. Interestingly H3K27Me3 and the specific methyl transferase EZH2 showed similar pattern like CBX4 (Supplemental figure S3C and S3D). These observations support the notion that CBX4 acts as a SUMO E3 ligase for itself and auto-SUMOylation helps in the recruitment of CBX4 to chromatin and gene repression function.

**hTERT enhances cell migration rate and invasiveness in breast cancer cells in a SUMOylation-dependent manner**

Epithelial-to-mesenchymal transition (EMT) plays a critical role in the metastatic progression of different types of cancer [50]. It is a highly regulated program by several developmental transcription factors and signaling pathways which eventually lead to the repression of epithelial genes along with the activation of mesenchymal genes. Interestingly, we observed a differential expression of mesenchymal genes CDH2 and VIM and EMT-related transcription factors ZEB1 and SNAI1 upon overexpression of FLAG-CBX4 and FLAG-CBX4 ΔSIM1/2 in both the luminal-B (MCF-7) (Supplemental Figure S4) and basal (MDA-MB-468) type breast cancer cells (Supplemental Figure S5). After confirming the effect of SUMO E3 ligase activity of CBX4 in EMT initiation, we wanted to examine the function of the protein in the regulation of migration and invasion in triple negative and highly invasive breast cancer cells, MDA-MB-231. To check the effect of SUMO E3 ligase activity of CBX4 on cell migration ability, monolayer wound healing assay was performed after over-expressing FLAG-CBX4 and FLAG-CBX4 ΔSIM1/2 in MDA-MB-231 cells. While over expression of wild type CBX4 showed considerably faster wound healing compared to vector transfected cells, there was no such effect.
with FLAG-CBX4 ΔSIM1/2 (Figure 4A, panel I, II,III and IV). Further through transwell invasion assay we found over-expression of wild type CBX4 resulted in significant increase in the invasiveness of the cells but the overexpression of catalytic mutant had similar effect as vector control cells (Figure 4B, panel I, II and III and IV). Next we performed wound healing and transwell invasion assay to investigate the direct effect of hTERT-SUMOylation on migratory property and invasiveness of the breast cancer cells. While over-expression of HA-hTERT showed faster rate of wound closure (Figure 5A, panel I, II and IV) and increased invasiveness (Figure 5B, panel I, II and IV) of cancer cells, surprisingly HA-hTERT K710R transfected cells behaved quite similarly as empty vector transfected cells (Figure 5A and 5B, panel I, III and IV). All together these data confirmed the role of SUMOylation in hTERT-mediated EMT through cellular migration and invasion and clearly identified CBX4 as the SUMO E3 ligase of hTERT.

Discussion

CBX4, an integral component of canonical polycomb repressive complex 1 (cPRC1) is a well-studied SUMO E3 ligase which is able to SUMOylate several proteins of different biological pathways and thus plays a major role in DNA damage response and cancer. Although tumour-promoting function of CBX4 has been quite well investigated in different cancers like hepatocellular carcinoma, osteosarcoma, breast cancer [19,21,23], report suggests that it also suppresses metastasis by interacting with HDAC3 in colorectal carcinoma [22]. Interestingly, by interacting with HDAC1, CBX4 exert oncogenic function by suppressing KLF6 in clear cell renal carcinoma [51]. Therefore, it is quite evident that CBX4 has an ability to interact with different members of various multiprotein complexes which in turn differentially regulates its oncogenic activity. Previously, the effect of Bmi1, another important oncogenic member of PRC1 complex, was observed in the induction of telomerase enzyme activity and immortalization of the human mammary epithelial cells by regulating hTERT promoter [10]. As CBX4 acts as a SUMOE3 ligase for Bmi1, these observations led us to investigate the role of CBX4 itself in the maintenance of telomerase activity and its association to telomerase complex. Interestingly, through our study we have established a novel interaction between hTERT and SUMO E3 ligase CBX4 which ultimately results in altered telomerase activity (Figure 1A and B). Previously, the interaction of hTERT and its subsequent effect has been studied with several
E3 ubiquitin ligases like MKRN1, CHIP and Plk1[37,52,53]. As ubiquitination and SUMOylation often work in a coordinated manner we got interested to see whether hTERT undergoes SUMOylation. Indeed we found hTERT gets SUMOylated by SUMO1 (Figure 2A). It is quite well known that for SUMOylation, the target lysine needs to be present within a consensus SUMOylation motif [54]. Through bioinformatics analysis we identified that specific lysine residue at 710 position of RT domain of hTERT and confirmed its involvement by point-mutational approach (Figure 2B and 2C). Overexpression of K710R mutant of hTERT showed reduced hTERT-SUMO1 association as several high-molecular weight (HMW) bands corresponding to SUMOylated hTERT was diminished (Figure 2C). But, a few HMW bands remained unaltered even in the presence of hTERT K710R mutant, indicating that other potential SUMOylation sites could be involved in this process. Previously mutations in the hTERT promoter have been extensively studied in various cancers [55]. Interestingly, some natural mutations (G682D, P721R, T726M, and K902N) are also found at the RT domain of hTERT in the patients with hematologic disorders and their functional characterization was done [56]. But although two other point mutations (E705Q and V711A) are reported in the TCGA database by the vicinity of our newly identified residue, no detailed analysis has been performed [57, 58].

Along with covalent attachment of SUMO to the target lysine residue, the presence of SUMO-interaction motif (SIM) is also necessary for non-covalent SUMO interaction [39,59]. Astonishingly, SIM can be found in different components of SUMOylation machinery ranging from E1 activating enzyme, members of SUMO E3 ligase family to even in the SUMO-substrates [60]. CBX4 contains two SIMs which are involved in its SUMO E3 ligase activity as well as mediating non-covalent SUMO-interaction with other proteins [39]. Recently, it has been shown these two SIMs are also responsible for its auto-SUMOylation property [48]. In this current study, we used different SIM deletion mutants of CBX4 to check the effect on hTERT and SUMO1 interaction. Over-expression of SIM1 and SIM2 double mutant of CBX4 (FLAG-CBX4 ΔSIM1/2) showed significant reduction in the hTERT SUMOylation and failed to affect the telomerase activity which confirmed the role of CBX4 as the SUMO E3 ligase of hTERT (Figure 2D, panel I and panel II). De-SUMOylation plays a major role to maintain the SUMO pool inside the cells [40]. SUMO or Sentrin specific proteases (SENPs) are unique group of isopeptidase enzymes that help not only in the maturation process of SUMO by C-term proteolytic cleavage but also in the deconjugation of SUMO from the substrate protein [41]. So,
additionally we checked the effect of SENP1, 2 and 3 on hTERT SUMOylation. Considerable reduction in the interaction of SUMO1 with hTERT and consecutive decrease in the telomerase activity upon different SENP over-expression indicated that the conjugation of SUMO1 with hTERT is instrumental for proper functioning of the enzyme (Figure 2E, panel I and II). Nevertheless, reduction in the level of HMW bands was more prominent with over-expression of FLAG-CBX4 ΔSIM1/2 (Figure 2D, panel I). This observation further strengthens the requirement of SIMs in CBX4 for the SUMOylation process of hTERT.

hTERT, being the catalytic subunit of human telomerase enzyme, prevents shortening of telomere primarily caused due to ‘end-replication problem’ and catalyzes the addition of telomeric DNA repeats (TTAGGG) at the chromosomal end by using hTERC as a template. However, recent evidences have suggested the importance of several non-canonical functions of hTERT apart from telomere lengthening, which eventually regulate different hallmarks of cancer[61]. Previous report has shown that hTERT promotes epithelial-to-mesenchymal transition (EMT) in gastric cancer [46]. Recently the role of hTERT has also been studied in colorectal cancer through ZEB1 mediated E-cadherin repression and EMT progression [43]. ZEB1 mediated EMT was reported in breast cancer also, where multiple factors are involved [62]. Through our study we found that wild type CBX4 over-expression resulted in hTERT/ZEB1 mediated repression of E-cadherin (Figure 3A, panel I and II) and the induction of EMT in breast cancer cells which is evident from the altered expression of EMT markers and EMT-associated transcription factors (Supplemental Figure S4 and S5). But the catalytic mutant of CBX4 failed to show any significant effect (Figure 3 and Supplemental Figure S4 and S5). Interestingly, when the same experiment was repeated after depleting hTERT in the system, de-repression of CDH1 was found and wild type as well as catalytic mutant of CBX4 failed to show any significant alteration in the CDH1 expression (3B). Further the direct effect of hTERT SUMOylation in this process has also been checked by using increasing concentrations of SUMO-defective mutant of hTERT (HA-hTERT K710R) in the presence of over-expressed CBX4 (3E and F). But our results always indicate that CBX4 is only able to repress CDH1 through hTERT SUMOylation and hTERT acts as the predominant factor. Compromised recruitment of SUMOylated hTERT at the CDH1 (E-cadherin) gene promoter upon overexpressing FLAG-CBX4 ΔSIM1/2 further supported the role of the protein as a SUMO E3 ligase of hTERT in this process (Figure 3D). These findings indicate that SUMOylation through
CBX4 augments the hTERT/ZEB1 mediated repression of CDH1 and consequent EMT progression.

Besides acting as SUMO E3 ligase for variety of proteins, CBX4 also contains a consensus sumoylation motif VKPE ranging from amino acid residue 491 to 494 [48] and itself gets SUMOylated. Further studies have shown that though the SUMO E3 ligase for CBX4 is not identified till date, over-expression of catalytically defective mutant of CBX4 showed significant reduction in the SUMOylation status of the protein itself indicating the presence of an auto-regulatory mechanism [48]. It was also reported that CBX4 binds to H3K27Me3 in a SUMOylation mediated manner as a part of repressive PRC1 complex and helps in the repression of several developmental genes [49]. We observed significant reduction in the occupancy of CBX4 itself and H3K27Me3 at CDH1 gene promoter upon over expression of FLAG-CBX4 ΔSIM1/2 (Supplemental figure S3B and S3C). Furthermore, substantial decrease in the enrichment of H3K27Me3-specific histone methyltransferase EZH2 was also observed at CDH1 gene promoter upon FLAG-CBX4 ΔSIM1/2 over-expression (Supplemental figure S3D) with compared to FLAG-CBX4 which ascertained that the auto-regulation of SUMOylation by CBX4 itself helps in the chromatin recruitment of the protein as a part of repressive chromatin complex.

Induced migratory property and invasiveness of cancer cells were often found as a result of EMT in several types of cancer [50]. We found migration and invasiveness of breast cancer cells majorly depends on SUMOylation of hTERT by SUMO E3 ligase CBX4 (Figure 5A and B, panel I, II, III and IV). Fascinatingly, both the catalytic mutant of CBX4 and the SUMO-defective mutant of hTERT showed similar compromised effect on migration and invasiveness of breast cancer cells which further re-confirms CBX4 as a SUMO E3 ligase of hTERT (Figure 4A, 4B, 5A and 5B, panel I, II, III and IV) and strengthens the fact that requirement of hTERT-SUMOylation is essential for EMT progression.

In conclusion, through our present study we have found SUMOylation as a novel posttranslational modification of hTERT and identified polycomb group protein CBX4 as the SUMO E3 ligase for hTERT in this process. Further in depth analysis has revealed the biological significance of hTERT and SUMO1 association in the process of hTERT/ZEB1 mediated EMT in breast cancer cells. Altogether our findings suggest a unique cross talk between repressive
polycomb complex and the enzyme telomerase which is essential for the proper regulation of the CDH1 gene and associated EMT during breast cancer progression.

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Author Contributions
C. Das, S. Sengupta (Bandyopadhyay) and S. Sanyal conceived of, designed and analysed the study followed by data interpretation. S. Sanyal, P. Mondal and S. Sen performed the experiments. C. Das and S. Sanyal wrote the manuscript. All the authors have read, discussed and approved the final manuscript.

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Figure Legends:

Figure 1: SUMO E3 ligase CBX4 interacts with hTERT, the catalytic component of human telomerase enzyme. (A) Telomeric Repeat Amplification Protocol (TRAP) was performed by transiently transfecting HeLa cells with FLAG-CBX4 (panel I) and (B) knocking down CBX4 by specific siRNA (panel I). FLAG and CBX4 expressions were checked by immunoblotting (A panel II & III and B, panel II). Empty FLAG vector was used as vector control and scrambled siRNA was used as control siRNA in this experiment. At least 3 separate experiments were performed. Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010). (C) Endogenous CBX4 was co-immunoprecipitated with hTERT from HeLa cells and subsequently immunoblotted with anti-CBX4 and anti-hTERT antibodies. IgG was used as negative control. (D) HeLa cells were transiently transfected with FLAG-CBX4 and co-immunostained with anti-FLAG and anti-hTERT antibodies. Calculated Pearson’s coefficients was >0.5.

Figure 2: hTERT gets SUMOylated by SUMO1: (A) Endogenous hTERT was subjected to co-immunoprecipitation from HeLa cells and subsequently immunoblotted with anti-hTERT, anti-SUMO1 antibodies. IgG was served as negative control. (B) Amino acid sequence of hTERT depicting the consensus SUMOylation motif in blue and the target lysine residue is marked in red. (C) HeLa cells were transfected with 3xHA tagged hTERT wild type and SUMO defective mutant HA-hTERT K710R and co-immunoprecipitation was done with anti-HA tag antibody followed by immunoblotting with anti-HA and anti-SUMO1 antibodies. (D) HeLa cells were transiently transfected with empty FLAG vector (as vector control), FLAG-CBX4 and FLAG-CBX4 ΔSIM1/ΔSIM2/ΔSIM1/2 respectively and whole cell lysates were subjected to immunoprecipitation with endogenous anti-hTERT antibody and subsequently immunoblotted with anti-hTERT and anti-SUMO1 antibodies (Panel I). Telomeric Repeat Amplification Protocol (TRAP) was performed to measure the relative telomerase activity after transiently transfecting empty FLAG (as vector control), wild type CBX4 and different catalytic mutants of CBX4 into HeLa cells ) (Panel II). Experiments were repeated thrice. Error bars show standard deviation. Unpaired student’s t-test was used to determine p value (*p<0.05, **p<0.010). (E) HeLa cells were transiently transfected with empty FLAG vector (as vector control), FLAG-SENP1/SENP2/SENP3 respectively and whole cell lysates were subjected to
immunoprecipitation with endogenous anti-hTERT antibody and subsequently immunoblotted with anti-hTERT and anti-SUMO1 antibodies (Panel I). Telomeric Repeat Amplification Protocol (TRAP) was performed to measure the relative telomerase activity after transiently transfecting empty FLAG (as vector control) and different SENPs into HeLa cells (Panel II). At least 3 separate experiments were performed. Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010).

**Figure 3: CBX4 represses CDH1 expression by SUMOylating hTERT in breast cancer.**

(A) Alteration in *CDH1* (E-cadherin) gene expression (panel I) and protein expression (panel II) was checked after transiently transfecting Empty FLAG Vector, FLAG-CBX4 and FLAG-CBX4 ΔSIM1/2 in MCF-7 cells. In case of gene expression, relative abundance of mRNA was plotted after normalizing to 18s rRNA. At least 3 separate experiments were performed. Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010). In case of protein expression β-tubulin was used as loading control (B) Alteration in *CDH1* (E-cadherin) gene expression was checked after knocking down hTERT and then transiently transfecting Empty FLAG Vector, FLAG-CBX4 and FLAG-CBX4 ΔSIM1/2 in MCF-7 cells. Only Empty vector (without knocking down hTERT) transfected cells were used as a vector control. Relative abundance of mRNA was plotted after normalizing to 18s rRNA. At least 3 separate experiments were performed. Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010). (C) ChIP assay was performed in MCF-7 cells with α-hTERT antibody to show the enrichment onto *CDH1* gene promoter. (D) ChIP-re-chIP assay was done in MCF-7 cells with α-hTERT antibody followed by pulling the complex with α-SUMO1 to show the recruitment at specific region of *CDH1* gene promoter. Two separate regions indicated within the E-box of the promoter were selected for primers. Quantitative PCR was done and relative fold was plotted normalizing to IgG. At least 3 separate experiments were performed. Error bars show standard deviation. Unpaired student’s t-test was used to determine p value (*p<0.05, **p<0.010). (E) Alteration in *CDH1* (E-cadherin) gene expression was checked after transiently transfecting empty HA vector, HA-hTERT and HA-hTERT K710R. Relative abundance of mRNA was plotted after normalizing to 18s rRNA. At least 3 separate experiments were performed. Error bars show standard deviation. Unpaired student’s t-test was used to determine p value (*p<0.05, **p<0.010). (F) Alteration in *CDH1* (E-
cadherin) gene expression was observed after transiently transfecting FLAG-CBX4 in combination with HA-hTERT and/or increasing concentrations (1μg, 2μg and 3μg) of HA-hTERT K710R. Empty vector treated cells were used as control. Relative abundance of mRNA was plotted after normalizing to 18srRNA. At least 3 separate experiments were performed. Error bars show standard deviation. Unpaired student’s t-test was used to determine p value (*p<0.05, **p<0.010).

Figure 4: CBX4 regulates cell migration rate and invasiveness of breast cancer cells though SUMO E3 ligase activity. (A) Monolayer wound healing assay was performed after transiently transfecting MDA-MB-231 cells with empty vector (panel I), FLAG-CBX4 (panel II) and FLAG-CBX4 ΔSIM1/2 (panel III). Scratching a wound after 24hrs of transfection (t = 0h), cells were allowed to grow for another 48 hours and images were taken after each 24 hrs (t = 24hrs and t = 48 hrs). Wound healing rate was quantified in term of percentage recovery of wound and graphically represented with statistical significance (panel IV). At least 3 separate experiments were performed. Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010). (B) Transwell invasion assay was performed after transiently transfecting empty vector (panel I), FLAG-CBX4 (panel II) and FLAG-CBX4 ΔSIM1/2 (panel III) in MDA-MB-231 cells. Cells at three independent fields for each well were counted and graphically represented (panel IV). Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010).

Figure 5: Cell migration rate and invasiveness of breast cancer cells depends on SUMOylation status of hTERT. (A) Monolayer wound healing assay was performed after transiently transfecting MDA-MB-231 cells with empty HA vector (panel I), HA-hTERT (panel II) and HA-hTERT K710R (panel III). Scratching a wound after 24hrs of transfection (t = 0h), cells were allowed to grow for another 48 hours and images were taken after each 24 hrs (t = 24hrs and t = 48 hrs). Wound healing rate was quantified in term of percentage recovery of wound and graphically represented with statistical significance (panel IV). At least 3 separate experiments were performed. Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010). (B) Transwell invasion assay was performed after transiently transfecting empty vector (HA) (panel I), HA-hTERT (panel II) and HA-hTERT
K710R (panel III) in MDA-MB-231 cells. Cells at three independent fields for each well were counted and graphically represented (panel IV). Error bars show standard deviation. Un-paired student’s t-test was used to determine p value (*p<0.05, **p<0.010).
Figure 2

A.

| Vector control | FLAG CBX4 | FLAG CBX4 ΔSIM1 | FLAG CBX4 ΔSIM2 | FLAG CBX4 ΔSIM1/2 |
|----------------|-----------|-----------------|-----------------|-------------------|
| IgG           | IgG       | IgG             | IgG             | IgG               |
| IP-hTERT      | IP        | IP              | IP              | IP                |

D.

| kDa | Vector control | FLAG CBX4 | FLAG CBX4 ΔSIM1 | FLAG CBX4 ΔSIM2 | FLAG CBX4 ΔSIM1/2 |
|-----|----------------|-----------|-----------------|-----------------|-------------------|
|     | IgG            | IgG       | IgG             | IgG             | IgG               |
| 150-|                |           |                 |                 |                   |
| 300-|                |           |                 |                 |                   |

B. hTERT 701 DPPPELYFKVDVTGAYDTI 720

C. HA- hTERT HA- hTERT K710R

E.

| Vector Control | FLAG SENP1 | FLAG SENP2 | FLAG SENP3 |
|----------------|------------|------------|------------|
| IgG            | IgG        | IgG        | IgG        |
| IP-hTERT       | IP         | IP         | IP         |

II) TRAP ASSAY

Relative telomerase activity DNA copy number (A.U. x 10^11)

** NS
Figure 3

A. qRT PCR: CDH1

B. qRT PCR: CDH1

C. ChIP: hTERT

D. ChIP-re-ChIP: hTERT-SUMO1

E. qRT PCR: CDH1

F. qRT PCR: CDH1
**Figure 4**

**A.**

- 0 hr
- 24 hrs
- 48 hrs

**I.** Vector control

**II.** FLAG-CBX4

**III.** FLAG-CBX4 ΔSIM1/2

**B.**

- Vector control
- FLAG-CBX4
- FLAG-CBX4 ΔSIM1/2

**IV.**

- % Recovery
  - 0 h
  - 48 h

**IV.**

- % of Invaded cells
  - 0 h
  - 48 h

*Note: The images and graphs show the effects of different treatments over time. The bars indicate the percentage of recovery or invaded cells with statistical significance indicated by asterisks.*
**Figure 5**

**A.**

0 hr  
24 hrs  
48 hrs  

I) Vector control (HA)  
II) HA-hTERT  
III) HA-hTERT K710R  

**B.**

I) Vector control (HA)  
II) HA-hTERT  
III) HA-hTERT K710R  

**IV.**

|          | 24h | 48h |
|----------|-----|-----|
| Vector control (HA) | ![Graph](#) | ![Graph](#) |
| HA-hTERT | ![Graph](#) | ![Graph](#) |
| HA-hTERT K710R | ![Graph](#) | ![Graph](#) |

- *p < 0.05  
- **p < 0.01
Table 1. The list of antibodies used in Western blotting, Co-IP and ChIP experiments.

| Antibody            | Company            | Catalogue No. |
|---------------------|--------------------|---------------|
| Anti-CBX4           | Abcam              | ab4189        |
| Anti-β tubulin      | Abcam              | ab6046        |
| Anti-hTERT          | Abcam              | ab 32020      |
| Anti-H3             | Abcam              | ab18521       |
| Anti-γH2AX          | Abcam              | ab11174       |
| Anti-E-cadherin     | Cell Signaling Technology | 24E10       |
| Anti-FLAG Monoclonal| Sigma-Aldrich      | F1804         |
| Anti-SUMO1          | Sigma-Aldrich      | S8070         |
| Anti-FLAG HRP       | Sigma-Aldrich      | A8592         |
| Anti-HA tag         | Abcam              | ab9110        |
| Anti-ZEB1           | Abcam              | ab 124512     |
| Anti-H3K27Me3       | Abcam              | ab192985      |
| Anti-EZH2           | Abcam              | ab186006      |
Table 2. The list of primers used in qRT PCR and ChIP experiments.

| Gene Name | Sequence |
|-----------|----------|
| **CBX4**  | Fw 5’-GGGCAGAGTGAGGTATCTGG-3’  
|           | Rv 5’-GGTCAGGACATTGGAACGAC-3’ |
| **CDH1**  | Fw 5’-GTCACTGACACCAACGATAATCCT-3’  
|           | Rv 5’-TTTCAGTGTGGTGATTACGACGT-3’ |
| **CDH2**  | Fw 5’-CCATCAAGCCTGTGGGAATC-3’  
|           | Rv 5’-GCAGATCGGACCGGATACTG-3’ |
| **VIM**   | Fw 5’-ACACCCTGCAATCTTTCAGACA-3’  
|           | Rv 5’-GATTCCACTTGGCGTCAAGGT-3’ |
| **SNAI1** | Fw 5’-TCGGAAGCCTAACTACAGCGA-3’  
|           | Rv 5’-AGATGAGCATTGGCAGCGAG-3’ |
| **ZEB1**  | Fw 5’-CCTCACCACATAGCTGGCAG-3’  
|           | Rv 5’-TTCTTTGCCCTTTGGAACATT-3’ |
| **18srRNA** | Fw 5’-GCTTAATTTGACTCAACACGGA-3’  
|           | Rv 5’-AGCTATCAATCTGGTCAATCTTGC-3’ |
| **CDH1** (80-55)  | Fw 5’-CTGTGGCAGCGAGCTGAAC-3’  
| (ChIP Primer) | Rv 5’-GGAGAGTCACCGCAGCCTTGA-3’ |
| **CDH1** (29-24)  | Fw 5’-AACCCAGTGGAATCAGAACC-3’  
| (ChIP Primer) | Rv 5’-CAGATACGCTCCGGCCAC-3’ |