Nonhistone human chromatin protein PC4 is critical for genomic integrity and negatively regulates autophagy

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Multifunctional human transcriptional positive co-activator 4 (PC4) is a bona fide nonhistone component of the chromatin and plays a pivotal role in the process of chromatin compaction and functional genome organization. Knockdown of PC4 expression causes a drastic decompaction which leads to open conformation of the chromatin, and thereby altered nuclear architecture, defects in chromosome segregation and changed epigenetic landscape. Interestingly, these defects do not induce cellular death but result in enhanced cellular proliferation, possibly through enhanced autophagic activity. Moreover, PC4 depletion confers significant resistance to gamma irradiation. Exposure to gamma irradiation further induced autophagy in these cells. Inhibition of autophagy by small molecule inhibitors as well as by silencing of a critical autophagy gene drastically reduces the ability of PC4 knockdown cells to survive. On the contrary, complementation with wild-type PC4 could reverse this phenomenon, confirming the process of autophagy as the key mechanism for radiation resistance in the absence of PC4. These data connect the unexplored role of chromatin architecture in regulating autophagy during stress conditions such as radiation.

Introduction

The nonhistone chromatin associated proteins modulate the dynamicity of the genome organization and thereby the gene expression by directly interacting with the different components of the chromatin. However, their role as responders of stress and maintenance of cellular homeostasis remains to be understood. We have shown that multifunctional human transcriptional co activator, positive co-activator 4 (PC4) is a bona fide nonhistone component of the chromatin [1] and is essential for life. However, brain-specific knock-out of PC4 leads to reduced neurogenesis with defects in memory extinction [2].

Abbreviations
3-MA, 3 methyl adenine; AMPKα1, 5’AMP-activated protein kinase catalytic subunit alpha-1; AMPKα2, 5’AMP-activated protein kinase catalytic subunit alpha-2; Baf, bafilomycin A1; BCL2, apoptosis regulator Bcl-2; BECN1, Beclin1; DRAM1, DNA damage regulated autophagy modulator 1; GABARAPL1, gamma-aminobutyric acid receptor-associated protein-like 1; GABARAPL2, gamma-aminobutyric acid receptor-associated protein-like 2; MAP1LC3B/LC3, microtubule-associated protein 1B light chain 3B; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase; RT-PCR, real time PCR; shRNA, short hairpin RNA; ULK1, Unc-51 Like autophagy activating kinase 1; ULK2, Unc-51 Like autophagy activating kinase 2.
The human genome is efficiently safeguarded against several environmental stresses and other DNA damage inducing agents by the coordinated functions of chromatin-associated proteins (acting as sensors), altered histone modifications and thereby a changed transcriptional output [3]. Macro-autophagy (herein autophagy), a well conserved cellular self-eating process, is a lysosome-dependent degradative pathway known to maintain the cellular homeostasis by getting rid of unwanted organelles and toxic proteins. Autophagy has a prosurvival role as it responds to stress such as starvation and genomic insult due to radiation. Several studies have highlighted the cytoprotective role of autophagy upon radiation stress in tumor cells [4]. A study in glioma stem cells upon exposure to ionizing radiation led to induction of autophagy which helped in maintaining cellular viability. Treatment with small molecule inhibitors like Bafilomycin A1 (Baf) and upon silencing of autophagy-related genes like ATG5 and Beclin1 (BECN1), these cells became sensitized to gamma irradiation which led to a significant decrease in the cell number [5].

The mechanism of autophagy induction under different stress stimuli is yet to be elucidated. Aside from the transcription factors, there have been also evidences of epigenetic modifications and thereby the chromatin state playing a critical role in altering the expression of key regulators of autophagy. Although autophagy is majorly a cytosolic event which requires an arsenal of cytosolic core and effector proteins for its functioning, there is a growing interest to know the role of the nucleus in modulating this phenomenon [6,7]. A network of diverse epigenetic modifiers like miRNAs [8] and histone modifications along with transcription factors relay a highly intricate cellular signalling which influences or regulates autophagy-mediated cell death or survival. Although the role of transcription factors, HATs and HDACs were known for a long time to be regulators of autophagy, their functions were only limited to the cytoplasmic interactions with autophagy-related proteins. Recent studies throw light on their nuclear impact which regulates autophagy at the transcriptional level [6]. Stress-responsive transcription factors like p53, STAT3 are known to regulate autophagy at transcriptional level [9,10]. Recent advances in the field also show the important role of other general transcription factors like pTEFB, FOXO in regulating several of the autophagy-related genes [11,12]. However, the cumulative effects of these transcription factors along with the alterations in the chromatin remain to be elucidated in the context of autophagy. Chromatin modulators like acetyltransferase, KAT5/TIP60 can be activated by glycogen synthase kinase-3, which in turn directly acetylates Unc-51 Like autophagy activating kinase 1 (ULK1) and induces autophagy [13]. Also the master lysine acetyltransferase p300 is known to negatively regulate autophagy, by its direct interaction with Atg7 in the cytoplasm [14]. Knocking down of EP300 activates autophagy, whereas overexpression of EP300 inhibits starvation-induced autophagy. Other than the epigenetic enzymes certain chromatin associated proteins like HMGA, and PARP has been shown to affect the autophagy process [15]. There has been evidence that mammalian cells can degrade nuclear components by the process of autophagy to maintain the nuclear function and integrity [16]. Autophagy is known to play a cytoprotective role under conditions of DNA damage, by regulating the dNTP pool levels [17], which are essential for DNA replication and repair. Thus, autophagy induction and initiation by various stressful environmental conditions is quite unique and is intricately regulated by the chromatin state, nuclear, cellular composition, and architecture along with various modifications which the chromatin harbors. However, how these multiple factors act together in a concert and thereby balance the fate of death and survival of the cells in the context of autophagy remains unknown.

In this study, we generated a stable PC4 knockdown cell line to explore the significance of PC4 in the physiological context. The PC4-depleted cells harbor an open or decompacted chromatin, and a significantly altered epigenetic landscape with severe morphological as well as segregation defects. In spite of acquiring such cellular defects, the PC4-deficient cells not only proliferated faster but also became more resistant to gamma irradiation-induced cytotoxicity. Significantly, the PC4 knockdown cells show greater induction of autophagy which further gets enhanced upon exposure to gamma irradiation. The gene network underlying the induction of this process of autophagy in the absence of PC4 has also been investigated and it is shown to be mitigated through the global alteration of the epigenetic landscape. Our results highlight the critical role of autophagy in survival and radiation resistance of these PC4-depleted cells.

Results

Knockdown of PC4 leads to altered nuclear and chromosomal morphology and results in cell segregation defects

Positive co-activator 4, was found to be a bonafide component of chromatin and it induced chromatin
compaction both in vitro and in vivo [2]. To explore the significance of PC4 in the physiological context, we generated a stable PC4 knockdown in HEK293 cell line (sh-PC4) using a lentivirus-mediated short hairpin RNA (shRNA) delivery system. Different shRNAs (marked as #) targeting different regions of PC4 gene were used to check the level of silencing of PC4 both at transcript and protein levels (Fig. 1A,B). The shRNA #5 was used to make a stable PC4 knockdown cell line as it showed maximum downregulation both at transcript and protein levels (Fig. 1A,B,C). We observed that in the absence of PC4, (in the shRNA-mediated knockdown cells) the individual metaphase chromosomes could not be spread unlike the chromosomes from the control cells. The typical metaphase chromosome shape was found to be altered in these cells (Fig. 2A). The shape and size of the nuclei was found to be highly variable and significantly deformed in the PC4 knockdown cells. Significantly the nuclear lamina also was invaginated when PC4 was absent in the nuclei, indicating the role of PC4 in the maintenance of the global nuclear architecture (Fig. 2B). The sh-PC4 cells also showed formation of anaphase bridges when binucleate analysis was performed upon nuclear staining with DAPI (Fig. 2C,D). The control cells, however, showed no such defects in the form of nuclear shape and size, and anaphase bridge formation also could not be detected. To visualize the segregation defects in greater detail at the chromosome level, telomere PNA-FISH (Peptide nucleic acid fluorescence in situ hybridization) was performed. In agreement with the anaphase bridge analysis, we observed binucleates with equal number of PNA foci as green signal in the control cells whereas in sh-PC4 cells, unequal number of PNA foci was predominant in the binucleates (Fig. 2E). Quantitation of the PNA signal showed that the unequal distribution of the foci in PC4 knockdown cells is statistically significant as compared to the near complete equal distribution in control cells (Fig. 2F). This unequal distribution of chromosomes in the newly divided daughter nuclei could be attributed to segregation defects. To visualize these structural anomalies in the PC4-depleted cells in a greater detail, both HEK293 and sh-PC4 HEK293 cells were subjected to transmission electron microscopy. The images showed abnormal nuclei with altered shape and accumulation of large number of double membraned vesicles around the nucleus of PC4 knockdown cells (Fig. 2G) which were completely absent in the control cells. Collectively, these results indicate that depletion of PC4 alters the nuclear as well as chromosomal morphology which in turn might also alter other cellular processes, thereby exhibiting an unexpected stressful cellular environment.

**PC4 knockdown perturbs the epigenetic status**

The ordered chromatin structure plays a vital role in maintaining the cellular homeostasis and is critical for efficient functioning of a cell. Considering the role of PC4 as a chromatin organizer, the compaction state of the chromatin upon knockdown of PC4 was analyzed

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**Fig. 1.** Generation and validation of PC4 knockdown stable cell lines. (A) Expression of PC4 protein analyzed by western blotting using whole cell extracts prepared from indicated cell lines with anti PC4 antibody (Top panel) GAPDH antibodies were used for loading control across the samples (Bottom panel). Each number represents a different clone of shRNA. (B) Transcript analysis for PC4 expression by RT-PCR using different shRNAs against PC4 gene. (C) Indirect immunofluorescence staining in indicated cell lines using anti PC4 antibodies (red).Nuclei were stained with DAPI (blue). [Colour figure can be viewed at wileyonlinelibrary.com]
by digesting the nuclei of the knockdown cells with micrococcal nuclease in a time-dependent manner. Compared to the control cells, the knockdown cells exhibit a decompacted chromatin as is evident by the presence of mono-nucleosomes in the digested pattern of MNase-treated nuclei (Fig. 3A). When nuclei from both the cells were digested with equal units of MNase for similar time points as of 10 and 15 min, preponderance of mono-di-nucleosomes could be observed in the sh-PC4 cells as compared to the control cells (Fig. 3A). It was also intriguing to observe that at 0 min time point of MNAse digestion (where the

Fig. 2. Knockdown of PC4 leads to altered nuclear and chromosomal morphology and defects in segregation. (A) Metaphase chromosome spreads reveal abnormal chromosomal structures in PC4 knockdown (sh-PC4) cells as compared to control cells. Scale bar 1 μm. (B) Changes in nuclear shape and size with PC4 knockdown (sh-PC4) as compared to control cells, with top bottom panels depicting changes to nuclear lamina (green) and DNA (blue) as indicated by arrows. Scale bar 2 μm. (C) Analysis of nuclear division showing irregular segregation and formation of Anaphase Bridges in the sh-PC4 cells compared to control cells. Scale bar 1 μm. (D) Quantitative representation of genome instability in terms of number of anaphase bridges formed per 100 nuclear divisions. Data are presented as means ± SEM **P < 0.001, ***P < 0.0001. (E) Telomere PNA-FISH analysis showing irregular segregation in sh-PC4 cells in terms of differing number of PNA foci in daughter nuclei as compared to control. Green fluorescence represents Telomere-PNA probe. The horizontal dotted line denotes axis of segregation while the vertical line denotes axis of cell division. Scale bar 1 μm. (F) Quantitative representation of the PNA hybridization in daughter nuclei in terms of the mean fluorescence intensity in a.m.u. As shown, there is differing signal intensity in the daughter nuclei in sh-PC4 as compared to control cells. Data are presented as means ± SEM (n = 3). **P < 0.001, ***P < 0.0001. (G) Representative image of electron micrographs from control versus sh-PC4 cells. sh-PC4 cells harbour nucleus of irregular shape and shows presence of vesicles near the nucleus. Scale bar 1 μm. [Colour figure can be viewed at wileyonlinelibrary.com]
reaction was immediately stopped after addition of the enzyme), chromatin from sh-PC4 cells showed presence of mono-nucleosomes whereas the chromatin from the control cells showed no digestion pattern, as evident by the smear signifying higher molecular weight bands (Fig. 3A). This signifies the integral role of PC4 in maintaining the compacted chromatin state in cells. Subsequently, owing to the opening of the chromatin in the PC4 knockdown cells, we investigated the alteration in the epigenetic landscape especially in the context of histone modifications. Most of the transcriptional activation associated histone modification marks analyzed, namely histone acetylation marks like H3K9Ac, H3K27Ac, H3K18Ac, and H4K8Ac were found to be enhanced in the sh-PC4 cells as compared to the control cells (Fig 3B). Interestingly, we also find upregulation of H3R17me2 mark which is in lieu with a previous report, of CARM1 regulating autophagy [11]. Taken together, these data establish that stable knockdown of PC4 not only alter the nuclear architecture but also the global chromatin organization toward a more open, and possibly transcriptionally amenable genome. In order to analyze the precise sites of differential accessible chromatin loci in PC4 knockdown cells an assay for transposase-accessible chromatin using sequencing (ATAC-seq) analysis was carried out using 50 000 intact cells as input (both control and PC4 knockdown). Upon closely analysing the sequencing results both from control and sh-PC4 cells, 1091 differential peaks were obtained in the knockdown cells. This corresponded to the specific open chromatin regions generated due to the depletion of PC4 in the cells. Mapping the differential ATAC-seq peaks across the whole genome showed highest occurrence in Chromosome 1 (Fig. 4A), also it was noted that no significant peaks were found in the Y chromosome. Significant proportion of the differential peaks obtained were found to be present on the promoter, intergenic regions (Fig. 4B), and CpG islands (Fig4C). This signifies that the novel chromatin accessibility sites upon PC4 knockdown mapped to functional regions of the genome which possibly affected expression of various number genes nearby to the peaks. In depth analysis of the molecular functions of the closely associated genes and extensive pathway analysis, revealed interesting conduits of gene expression relevant to cellular physiology. Several genes belonging to vesicular transport, transcriptional regulation function as well as antioxidant activity was found to be closely associated with the open chromatin regions in the PC4 knockdown cells (Fig. 4E). It was also intriguing to note that certain essential cellular pathways for example p38 MAPK pathway, EGFR pathway were found to be closely allied with the ATAC-seq peaks in the PC4 knockdown cells. (Fig. 4D).

**Knockdown of PC4 exhibits higher cell migration and enhanced survival upon exposure to gamma irradiation**

We found that PC4 knockdown cells were able to divide and grow well in multiple passages; infact knockdown cells appeared to grow faster than control cells. To understand the rate of proliferation upon PC4 knockdown, we performed wound healing assays by creating equal sized scratch by a sterile tip. Healing

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Fig. 3. Absence of PC4 causes global chromatin decondensation, and consequent changes in epigenetic state. (A) Micrococcal nuclease digestions were carried out at four different time points (0, 5, 10 and 15 min) with the nuclei isolated from control cells (lanes 1–4) and from sh-PC4 cells (lanes 5–8). (B) Comparison of histone modification marks between control and sh-PC4 cells. Core histones extracted from control cells and knockdown cells. Transcription activation associated histone modifications were examined by immunoblotting using highly specific antibodies against specific histone modifications as indicated.
Fig. 4. ATAC-seq analysis to analyze the differential opening of the chromatin in the absence of PC4: Differential peaks were analyzed with respect to the control cells. (A) Percentage of differential peaks obtained upon knockdown of PC4 as mapped to the different chromosomes of the human genome (hg19). (B) Annotation of differential ATAC-seq peaks in sh-PC4 cells across the different functional regions of the genome. (C) Fine Annotation of differential ATAC-seq peaks in sh-PC4 cells across the different functional regions of the genome. (D) Nearest genes obtained from mapping of the differential ATAC-seq peaks were clustered and analyzed for pathway analysis using online PANTHER ontology tools (http://www.pantherdb.org/). (E) Nearest genes obtained from mapping of the differential ATAC-seq peaks were clustered and analyzed for molecular functions using online PANTHER ontology tools (http://www.pantherdb.org/). [Colour figure can be viewed at wileyonlinelibrary.com]
was much faster in case of sh-PC4 compared to control cells (Fig. 5A). The migratory ability of cells was also compared upon passing high current through the cells. After 12 h, impedance of sh-PC4 cells was still higher than the control cells indicating PC4 knockdown cells have higher migration ability (Fig. 5B). The faster healing of wound could also be attributed to higher proliferation rate of sh-PC4 apart from its greater cell migration ability than control cells. The sh-PC4 cells indeed showed higher proliferation rate as is evident by the clonogenic assay (Fig. 5C). On seeding equal number of cells for both sh-PC4 and control cells, PC4-depleted cells not only showed greater number of colonies after 10 days of growth as compared to the control cells but the colonies also appeared to be larger in size and shape, morphologically. However, increased proliferation despite the several defects in sh-PC4 cell nuclei was puzzling. To get an insight, we further exposed these cells to a genotoxic stress like gamma irradiation and then compared the proliferation rates as well as cell death in control and PC4 knockdown cells. Remarkably, greater survival was observed in case of PC4 knockdown cells upon increased gamma radiation as compared to control cells as evidenced by appearance of significantly greater number of live colonies after 10 days of exposure to gamma irradiation (Fig. 5D,E). We also investigated the state of apoptosis in the sh-PC4 cells upon exposure to gamma irradiation by flow cytometry analysis using Annexin V/Cy3.18 dye. Using Annexin V staining coupled to flow cytometry, we observed that sh-PC4 cells apoptosed significantly lesser than the control cells upon exposure to gamma irradiation even after 6 h of exposure (Fig. 5F). The control cells showed a significant increase in the percentage of apoptotic cells 6 h after exposure to gamma irradiation (4.76–11.43%) while the percentage of apoptotic cells in sh-PC4 cell population showed no significant alteration in their population even after exposure to gamma irradiation (Fig. 5G). Immunoblotting analysis for apoptosis markers like cleaved PARP and caspase 3 revealed the presence of them in control cells post gamma irradiation but not in the sh-PC4 cells (Fig. 5H lane 3 vs 4). These results collectively signify that PC4 depletion confers radiation resistance to the cells.

**PC4 knockdown cells exhibit enhanced autophagy levels**

In order to elucidate the molecular mechanism of the unique ability of sh-PC4 cells to survive better, we hypothesized that a compensatory cellular pathway such as autophagy might be operating rendering the property of enhanced proliferation and resistance to gamma irradiation to these cells. Autophagy, a well conserved, cellular degradative process is known to act as a potent survival mechanism for cells especially under stress or toxic conditions [18]. We thus looked into the autophagy levels in sh-PC4 cells, by monitoring the levels of microtubule-associated protein light chain 3B (LC3) protein (an autophagy marker). The western blotting analysis of cell lysates from PC4 knockdown and control cells show that in sh-PC4 cells the levels of LC3II (lipidated form of LC3) was significantly enhanced as compared to the control cells (Fig. 6A). Furthermore, when cells were grown in a nutrient deficient media for 2 h to induce autophagy, sh-PC4 cells showed further increase in the levels of LC3II (Fig. 6A). The increase in the LC3II level can also be attributed to the accumulation of autophagy vacuoles due to a defective autophagy pathway; to investigate this possibility Baf inhibition assay was carried out. Baf Baf disrupts the autophagic flux by

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**Fig. 5.** Absence of PC4 renders radioresistance upon exposure to gamma irradiation. (A) Microscopic images of wound healing assay performed toward comparison of proliferation rate between control and sh-PC4 cells. Equal wound created in both cell lines (0 h) was monitored at regular time intervals till complete healing was achieved in either of the cells (12 h). (B) The representation of the normalized impedance for the control versus sh-PC4 cell population healing the wound for the monitored period using real time cell analyzer. Data are presented as means ± SD (n = 3). **P < 0.001, ***P < 0.0001. (C) Representative images of the crystal violet stained colonies of control and sh-PC4 cells shows greater proliferation rate in PC4 knockdown cells. (D) Representative images of the crystal violet stained colonies of control and sh-PC4 cells sustained after exposure to increasing doses of the gamma radiation. (E) Graph plot depicting surviving fraction in percentage. Data are presented as means ± SEM (n = 3). **P < 0.001, ***P < 0.0001. (F) Apoptosis analysis was performed in control vs sh-PC4 cells. Cells were irradiated at 2 Gy and after 6 h of exposure; cells were analyzed by FACS after staining the cells with Annexin V. Annexin-Cy3.18 (AnnCy3) binds only to apoptotic cells which can be detected as red fluorescence which has been represented as blue in the upper quadrant (Fig. 3F). The live cells were pseudocolored as red. The upper right and left quadrant represents apoptotic cells. Since sh-PC4 cells harbor a GFP expressing shRNA plasmid, the upper left quadrant in sh5 panel represents apoptotic GFP positive cells. (G) Total percentage of apoptotic cells were counted from the FACS analysis and represented in the bar graph. Data are presented as means ± SEM (n = 3). **P < 0.001, ***P < 0.0001. (H) Immunoblotting of apoptosis-related proteins like Caspase 3 and PARP to measure the induction of apoptosis in both control and sh-PC4 cells post 6 h of gamma irradiation (2 Gy). * and ** in the PARP panel represents total PARP and cleaved PARP levels respectively. [Colour figure can be viewed at wileyonlinelibrary.com]
inhibiting the lysosomal proton pump V-ATPase, resulting in a defect in autophagosome-lysosome fusion [19]. Upon treatment with Baf inhibitor in increasing concentrations of 50 nM and 100 nM there was a significant increase in the LC3II levels in the sh-PC4 cells over and above the induced LC3II levels.
(Fig. 6B). These data argue for the fact that the induced LC3II level observed in the sh-PC4 cells is indeed due to enhanced autophagy, which is abrogated in the presence of an inhibitor, Baf. Rescuing PC4 expression in the knockdown cells (Fig. 6C upper panel) resulted in significant downregulation of LC3 at protein level (Fig. 6C lower panel). Degradation of SQSTM1/p62, another well-known autophagy marker, was also analyzed in the sh-PC4 cells both in rich or 6 h starved conditions with or without 100 nm Baf inhibition (Fig. 6D). p62 was detected at significantly low levels in sh-PC4 cells upon starvation induced autophagy. However, upon treatment with Baf we do find an increase in its expression, indicating that this higher autophagic flux in the PC4 knockdown cells could be significant (Fig. 6D). To detect the cellular signatures of autophagy in the PC4 knockdown cells, both the control and PC4 knockdown cells were subjected to electron microscopy. In agreement with the biochemical data, the cellular visualization showed accumulation of large number of double membranated vesicles in the sh-PC4 cells which are characteristics of autophagy vesicles (Fig. 6E, left panel). Visualization of such vesicles was not observed in the control cells even after imaging several fields of electron micrographs (Fig. 6E, right panel). Presence of autophagy like double membranated vesicles even in the absence of autophagy inducers in the sh-PC4 cells further validate the fact that basal autophagy level is elevated in the absence of PC4. This induction of autophagy could be due to the alteration of cellular homeostasis including greater DNA damage condition. In agreement to this, we indeed find accumulation of DNA damage as evidenced by the enhanced formation of γH2A.X and 53BP1 foci in PC4 knockdown cells (Fig. 7).

Induced autophagy in PC4-depleted cells harbors gamma radiation resistance to the PC4 knockdown cells

Since sh-PC4 cells show enhanced autophagy without any exogenous stimuli of starvation, stress or any chemical autophagy enhancer, we wanted to investigate the functional role of autophagy in conferring radiation resistance to PC4 knockdown cells. For this purpose, both the control and sh-PC4 cells were exposed to increasing doses of gamma irradiation. Western blot analysis of cell lysates from sh-PC4 cells and control cells after 24 h of gamma irradiation showed enhanced LC3II levels in sh-PC4 cells as compared to the control cells suggesting greater induction of autophagy in the PC4-depleted cells as compared to control cells (Fig. 8A). This reduced expression of PC4 might further lead to induction of autophagy even in the control cells. To fortify the protective role of autophagy in sh-PC4 cells, we pretreated the cells with two known small molecule autophagy inhibitors, 3-methyladenine (3-MA) and Baf. 3-MA is known to inhibit autophagy by blocking autophagosome formation by inhibiting the type III Phosphatidylinositol 3-kinases (PI-3K), making it an early inhibitor of the autophagic pathway [19]. Pre-treatment with both 3-MA and Baf alone led to significant decrease in the number of live sh-PC4 cells as compared to the control cell line (Fig. 8C,E). Administration of autophagy

**Fig. 6.** Absence of PC4 induces autophagy. (A) Induction of autophagy in sh-PC4 cells upon starvation: Control and sh-PC4 cells were starved for 2 h in EBBS media. Cell lysates from both control and sh-PC4 cells in rich and starvation media were analyzed for LC3 I and LC3 II levels. Tubulin used as the loading control. Representative graph showing the change in LC3 II level upon PC4 knockdown both in rich and starvation media is shown. Data are presented as means ± SEM (n = 2). *P < 0.01, **P < 0.001, ***P < 0.0001. (B) Effect of Baf (50 nm, 100 nm) on control and sh-PC4 cells. Accumulation of LC3 II levels upon Baf treatment for 2 h signify an inhibition in the process of autophagy. Corresponding, representative graph shows the change in LC3 II level upon Baf inhibition both in control as well as sh-PC4 cells. Data are presented as means ± SEM (n = 2). *P < 0.01, **P < 0.001, ***P < 0.0001. (C) Flag PC4 was transiently expressed to rescue the expression of PC4 in PC4 knockdown cells and the expression was checked after 48 h post transfection by immunoblotting with anti-Flag antibody (Upper panel), (Lower panel) LC3II levels were checked in PC4 Knockdown cells lane 1 and PC4KD cells transfected with Flag PC4 (rescue; lane 2). (D) Absence of PC4 induces higher autophagy flux in the cells as evidenced by p62 levels. Cells were starved for 6 h in EBBS media in the presence or absence of Baf (100nm) as indicated. Higher degradation of p62 in sh-PC4 cells indicates greater influx of autophagy which is inhibited upon treatment with Baf. [*] represents nonspecific bands. Corresponding bar graph to show p62 levels both in control as well as sh-PC4 cells in different conditions. Data are presented as means ± SEM (n = 2). *P < 0.01, **P < 0.001, ***P < 0.0001 (E) Electron micrographs of sh-PC4 cells (right panel) show huge accumulation of double membranated vesicles as indicated by yellow arrows which are characteristics of autophagy vacuoles. No such vacuoles were observed in control cells (left panel). Scale bar as denoted in figure. [Colour figure can be viewed at wileyonlinelibrary.com]
inhibitors thus led to a significant decrease in the viability of sh-PC4 cells which were exposed to gamma irradiation, signifying the critical role of autophagy in conferring radiation resistance to the PC4 knockdown cells (Fig. 8D,F). To further validate that autophagy is critical for enhanced survivability of sh-PC4 cells, we
resorted to genetic inhibition of autophagy pathway by administering cells with specific shRNA against ULK1 gene (Fig. 8G). ULK1 is known to be act at the initiation of the autophagosome formation; its inhibition has been shown to shut down autophagy in several cell lines. Interestingly, we find that shRNA-mediated knockdown of ULK1 drastically reduced the proliferation rate of the sh-PC4 cells in comparison to the control cells. (Fig. 8H,I). Collectively these data indicate that enhanced autophagy is one of the key mechanisms of better survivability and enhanced proliferation of PC4-depleted cells.

PC4 knockdown induces transcription of autophagy-related genes in an epigenetic manner

Absence of PC4 creates an open chromatin architecture in the nucleus with a transcriptionally favourable epigenetic state of the genome. To investigate whether depletion of PC4 has a direct transcriptional output of autophagy-related genes we followed the possible gene expression signature in PC4 knockdown cells both before and after exposure to gamma irradiation. Figure 6A summarizes the result obtained for gene

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**Fig. 8.** Exposure to gamma irradiation in the absence of PC4 induces autophagy further: (A) Induction of autophagy in sh-PC4 cells upon gamma irradiation: Control and PC4 knockdown sh-PC4 cells were irradiated with different doses of gamma rays as indicated. Cell lysates from both cell lines were harvested after 24 h post irradiation and western blot analysis were carried out with specific antibodies. LC3 antibody was used to analyze the process of autophagy while tubulin was used as loading control. Lower panel shows graph representative of the change in LC3 II level upon gamma irradiation both in control and sh-PC4 cells is shown. Data are presented as means ± SEM (n = 2). *P < 0.01, **P < 0.001, ***P < 0.0001. (B) Expression of PC4 upon gamma irradiation. Control cells were irradiated with increasing dose of gamma irradiation. Cell lysates were harvested after 24 h post irradiation and western blot analysis were carried out with specific antibodies to evaluate the expression of PC4 against a tubulin control. Lower panel shows graph representative of the change in PC4 level upon gamma irradiation. Data are presented as means ± SEM (n = 2). *P < 0.01, **P < 0.001, ***P < 0.0001. (C) Autophagy inhibition sensitizes PC4-depleted cells to gamma irradiation: Control and sh-PC4 cells were treated with small molecule inhibitors of autophagy 10 mM 3-MA (C) and 100 nM Baf (E) 2 h prior to exposure to 2Gy of gamma irradiation. Cells were grown in the presence of the respective inhibitor for 24 h post irradiation. Colonies were grown for 10 days. (C) and (E) Representative images of the crystal violet stained colonies of control and sh-PC4 cells sustained after treatment with 3MA and Baf and exposure to gamma irradiation. (D, F) Bar Graph depicting surviving fraction in percentage of the control and sh-PC4 cells after autophagy inhibition. Data are presented as means ± SEM (n = 2). *P < 0.1, **P < 0.01, ***P < 0.001, ****P < 0.0001. (G) Western blotting to confirm ULK1 knockdown 48 h post transfection with specific shRNA against ULK1 in increasing concentrations. (H) Control and sh-PC4 cells were transfected with 2 µg of shRNA for 48 h, and then plated for colony formation assay. Representative images of the crystal violet stained colonies of control and sh-PC4 cells sustained after transfection of ULK1shRNA. (I) Bar Graph depicting surviving fraction in percentage of the control and sh-PC4 cells after autophagy inhibition. Data are presented as means ± SEM (n = 2). *P < 0.01, **P < 0.001, ***P < 0.0001. [Colour figure can be viewed at wileyonlinelibrary.com]
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Chromatin organization critical for autophagy

A. Gamma irradiation (Gy) effect on LC3I and LC3II expression.

B. PC4 expression upon gamma irradiation.

C. Effect of 3MA and 2Gy on cell morphology.

D. Surviving fraction of cells treated with 3MA and 2Gy.

E. Effect of Baf and 2Gy on cell morphology.

F. Surviving fraction of cells treated with Baf and 2Gy.

G. ULK1 shRNA effect on cell morphology.

H. Scr and ULK1shRNA effect on cell morphology.

I. Surviving fraction of cells treated with Scr and ULK1shRNA.
expression of each autophagy-related genes as obtained from real time PCR (RT-PCR) analysis. The RT-PCR analysis reveals significant upregulation of most autophagy genes like 5'AMP-activated protein kinase catalytic subunits alpha-1 and 2 (AMPKα1, AMPKα2), ULK1 and ULK2 and microtubule-associated protein 1B (MAP1LC3B) in sh-PC4 cells as compared to the control cells (Fig. 9A, compare first group to second group of each color-coded gene) which gets further upregulated upon exposure to gamma irradiation in sh-PC4 cells (Fig. 9A). It is also to be noted that in accordance with the western data in Fig. 8A, the control cells showed induction of autophagy upon gamma irradiation also at the transcript level. Interestingly, these irradiated control cells also demonstrated dose dependent downregulation of PC4 expression suggesting the negative regulation of autophagy by PC4. This further verifies the fact that the induction of autophagy is a prime factor that might be responsible for mediating the cell survivability even in the case of control cells as the gene expression analysis was carried out from the live population of control cells.

In order to establish the direct role of PC4 in regulating ATG gene expression at the transcript levels, we investigated the binding of PC4 at the autophagy gene promoters. The ChiP qPCR data (Fig. 9B) confirms significant occupancy of Flag tagged-PC4 at autophagy gene promoters specifically that of AMPK, ULK1, and BECN1 but not at HNF4α promoter. This observation reinforces the fact that PC4 is a direct regulator of ATG-related genes at the basal level.

Since absence of PC4 enhanced the histone acetylation related to transcriptional activation, we also investigated the level of histone H3K9 acetylation (H3K9Ac) on the promoter sites of different upregulated genes by chIP assay using highly specific antibodies for H3K9Ac. We investigated the occupancy of the active histone H3K9Ac marks at the promoters of five autophagy-related genes which were known to be essential and critical in the initiation of the autophagic pathway in the cells. It was found that the occupancy of the H3K9Ac mark at the promoters of the autophagy genes in sh-PC4 cells is enhanced as compared to the control cells (Fig. 9C). In the promoters of all the five autophagy genes that we tested, the H3K9Ac occupancy was enhanced in the sh-PC4 cells as compared to the control cells. However, the occupancy of the active H3K9Ac mark do not alter at the promoters of genes like GAPDH and HNF4α (genes nonrelated to autophagy pathway) signifying that the occupancy of the specific active mark is somewhat specific in coherence with the transcriptional output result summarized before. These results are in agreement with the fact that H3K9Ac mark is highly upregulated in absence of PC4 in the cells which gets possibly enriched at specific promoter sites in the genome leading to an altered cellular physiology. Taking cue from our ATAC-seq study we correlated the gene signature of autophagy-related genes to the differential peaks obtained from sh-PC4 cells. It was found that 12 genes directly overlapped between the two gene sets (Fig. 9D). This directly corroborates with our hypothesis that the open chromatin state due to depletion of PC4 might be directly correlated to the aberrant gene expression observed in the sh-PC4 cells and thereby also the phenomenon of enhanced autophagy. Further to reinforce the phenomenon of epigenetic regulation of autophagy-related gene expression, the ATAC-seq peaks directly overlapped with the histone acetylation active mark (H3K4me3) chIP peak at their promoters (Fig. 9D lower panel). To understand the global

Fig. 9. PC4 regulates autophagy-related genes at the transcriptional level by binding directly to its promoter: (A) Alteration of autophagy-related genes as determined by qPCR in ctrl versus sh-PC4 cells before and after gamma irradiation. Different color represents different autophagy genes; expression of each gene is denoted by four groups, first represents expression in control cells, second fold change in gene expression in sh-PC4 cells, third represents the fold change in gene expression after 6 h of gamma irradiation (2Gy) in control cells and fourth represents the same treatment but in sh-PC4 cells. Data are presented as means ± SEM (n = 2). Actin is used as an internal control. *P < 0.1. (B) Occupancy of PC4 at the promoters of the selected autophagy genes were evaluated in HEK293 cells. HEK293 cells were transfected with a pCDH vector control represented as Control and pCDH Flag-PC4 construct represented as F-PC4. Flag Chip qPCR was carried out and the results are represented as Fold change Over IgG. Data are presented as means ± SEM (n = 2). *P < 0.01, **P < 0.001, ***P < 0.0001. (C) Enrichment of H3K9Ac at the promoters of selected autophagy-related genes were assayed in both control and sh-PC4 cells. The ChiP qPCR results are represented as the percent input; second bar for each individual color-coded gene represents enrichment in sh-PC4 cells while the first bar is representative of H3K9Ac occupancy in the control cells. Data are presented as means ± SEM (n = 2). **P < 0.001, ***P < 0.0001. (D) Correlation analysis of genes mapped with the differential ATAC-seq peaks and the set of human autophagy-related genes obtained from the Human autophagy database HADb (http://autophagy.lu/). Genes marked in red signifies genes which either belong to the core autophagy machinery or as master transcriptional regulators. Lower panel: Sorted aligned files for autophagy-related genes and the histone activation mark (H3K4me3) from ENCODE were visualized in IGV. (E) Altered gene network upon PC4 knockdown shows alteration in autophagy-related genes and thereby the associated gene network. Downregulated gene is color coded as green while genes showing upregulation is denoted as red. [Colour figure can be viewed at wileyonlinelibrary.com]
Chromatin organization critical for autophagy
change in the genome architecture and thereby the transcriptional alteration in PC4-depleted conditions, we resorted to in silico analysis of pathways regulated by the different altered genes (protocol described in the materials and methods). This in silico network analysis of pathways interconnected several genes possibly regulated by PC4, and thereby providing an insight to the cellular pathways which might get altered in PC4-depleted cells. The analysis provides a broader overview of the altered physiological state of the PC4-depleted cells which might be due to the concerted effect of the alteration of the several pathways as denoted on the line interconnecting the nodes. Most of the genes to be altered belonged to the autophagy regulatory pathway referred in red circular nodes (Fig. 9E). It was also interesting to observe that perturbation of PC4 levels not only led to altered expression of autophagy genes, but also affected other essential pathways for cell migration, proliferation and homeostasis.

Discussion

Human transcriptional coactivator, PC4 has been previously reported to be an integral component of the chromatin. Depletion of PC4 at cellular level alters global organization of the nucleus as well as leads to an open chromatin state establishing it as one of the most important chromatin architectural protein. Here, we establish a novel role of the chromatin associated protein PC4 in cell segregation, chromosomal morphology, and maintenance of the epigenetic state of the cell. PC4 knockdown cells show irregular nuclear structure as is evidenced by the nuclear staining indicating the possible role of PC4 in maintenance of proper nuclear shape and function. It will be thus interesting to delve into the mechanistic detail of PC4’s direct role in interacting with nuclear lamins and establishing it as an important nuclear architectural protein. PC4-depleted cells also show abnormal cellular segregation, enhanced hyper acetylation of histones and distorted shaped chromosomes. Interestingly, despite harboring severe cellular defects, PC4-depleted cells shown higher proliferation rate than normal cells, and were also resistant to genotoxic stress like gamma irradiation, quite similar to the phenotype of an oncogenic transformed cells. To delineate the molecular mechanism of such a phenotype, we found that PC4 is a novel regulator of the well conserved cellular process of self-eating, called autophagy. Autophagy levels were found to be highly elevated in absence of PC4. Electron micrographs of PC4 knockdown cells showed presence of various double membraned autophagy like vacuoles. It was also interesting that PC4-depleted cells showed appearance of multivesicular bodies (large number of vacuoles engulfed in one vesicle). Appearance of multivesicular bodies has been reported in a particular type of autophagy process [20]. The appearance of these multivesicular bodies and other double membraned vesicles especially near the nucleus in PC4-depleted cells thus might be possibly an indication of a cellular cue which might be operating via the altered nuclear architecture to a cytosolic process like autophagy. The enhanced autophagy levels in the PC4 knockdown cells play a critical role in its survivability possibly by maintaining cellular homeostasis in an otherwise physiological chaotic state [4,21].

When the autophagy levels were depleted by administration of small molecule inhibitors or by knockdown of an essential autophagy gene ULK1, it highly compromised the survival rate of PC4 knockdown cells and upon gamma irradiation. This establishes the significant role of autophagy in attributing the property of enhanced growth rate to PC4 knockdown cells. PC4 knockdown significantly upregulated several autophagy-related genes, like the AMPK and ULK genes which were previously reported to be prime regulators of the autophagy pathway. AMP-activated protein kinase (AMPK) is a well conserved energy sensing serine threonine kinase which is activated upon energy depleted or upon cellular stress conditions. This kinase further activates a cascade of proteins including the Unc-51-like kinase 1 and 2 (ULK1 and ULK2) which is an essential component in the formation and maturation of autophagosomes [22]. We also find upregulation of MAP1LC3B gene in the absence of PC4 which is involved in the final step of autophagosomes maturation and thereby degradation. This signifies that PC4 knockdown in cells not only leads to just an enhanced initiation of the self-eating process, but the degradative pathway is complete and functional up to its final step. When we complemented PC4 in the knockdown background, we found significant down-regulation of LC3 at protein levels (Fig. 6C) signifying it’s direct role in regulation of autophagy. Furthermore, PC4 was found to be enriched at the promoters of autophagy-related genes at basal condition, thus reinforcing the fact that PC4 is involved in the direct negative regulation of autophagy (Fig. 9B). The altered transcriptome in the PC4-depleted cells is possibly due to the differential opening of the chromatin state (ATAC-seq of sh-PC4) and reformed epigenetic landscape which is now permissive to enhanced transcription. The role of histone modifications in autophagy has not been deciphered in great details;
Fig. 10. Model depicting the role of PC4 in autophagy regulation: (A) Control cells with basal autophagy undergo apoptosis upon gamma irradiation. (B) Enhanced autophagy in PC4 knockdown cells renders gamma irradiation resistance to these cells. (C) Genetic and chemical inhibition of autophagy makes the PC4-depleted cells susceptible to gamma irradiation mediated cell death. The figures have used illustration templates from the website somersault1824 (http://www.somersault1824.com) available under a Creative Commons Attribution-Noncommercial-Share Alike License (CC BY-NC-SA 4.0). [Colour figure can be viewed at wileyonlinelibrary.com]
however, recent advances in the field, signify the role of the HDAC inhibitors like butyrate and suberoylanilide hydroxamic acid which enhanced autophagic cell death in various human cancer cell lines. Thus, there is increasing interest to look into histone hyper-acetylation state and induction of autophagy [23]. Here in this report we try to directly co relate the histone acetylation level to autophagy induction in absence of PC4. PC4 depletion resulted in increased levels of histone modification marks related to transcriptional activation, like H3K9Ac, H3K27Ac, H3K18Ac, and H3R17me2. We here show that PC4 knockdown enhanced the enrichment of the activation histone mark H3K9Ac mark at the promoters of AMPK and ULK1 and ULK2 genes, which possibly leads to an enhanced autophagy process. Thus, PC4 knockdown epigenetically upregulates the autophagy process by directly enhancing the autophagy-related genes at the transcript level through an open chromatin structure (Fig. 10). However, altered nuclear architecture and morphology may also contribute to the process of enhanced autophagy. This study provides one of the first direct evidences of interlink among chromatin, nuclear architecture, chromatin state, and the process of autophagy.

Materials and methods

Reagents and media

Dulbecco’s Modified Eagle’s Medium (DMEM; with 4500 mg L−1 glucose, l-glutamine, and 25 mm HEPES; Sigma D1152, St. Louis, MO, USA), sodium bicarbonate (Sigma S5761), Penicillin-Streptomycin-Amphotericin B solution (Himedia, A002A, Mumbai, India), FBS heat inactivated (Life Technologies 04-121-1A, Thermofisher Scientific, Carlsbad, CA, USA), 3-Methyl adenine (Sigma 59281), and puromycin for three passages to establish the cell line.

Cell culture and treatment

Embryonic kidney cell line HEK293, HEK293T, and HEK293 sh-PC4 were grown in the DMEM supplemented with sodium bicarbonate, 100 U·mL−1 Penicillin 0.1 mg·mL−1 Streptomyacin, 0.25 µg·mL−1 Amphotericin B and 10% heat inactivated FBS at 37 °C in 80% humidifier air and 5% CO2. For the starvations experiments, cells were allowed to grow up to 70% confluence and then were shifted to a low nutrient media of Earle’s Balanced Salt solution for 2/6 h. For the small molecule inhibition assays the cells were grown to 70% confluence and then treated with different concentrations of 3-Methyl Adenine and Baf for 2/6 h.

PC4 knockdown stable cell line

This was generated using 10 µg pGPZ lentiviral shRNAs targeting PC4 (Open Biosystems, Dharmacon, Lafayette, CO, USA) and helper plasmids (5 µg psPAX2, 1.5 µg pRSV-Rev, 3.5 µg pCMV-VSV-G). Ten microgram of sh-plasmid was mixed with helper plasmids (5 µg psPAX2, 1.5 µg pRSV-Rev, 3.5 µg pCMV-VSV-G) and were co transfected into HEK293T cells using calcium phosphate method. Forty-eight hours post transfection media containing assembled virus was collected and its titre was estimated. Desired cell line (here HEK293) was infected with 106 IU·mL−1 virus. Infected cells were subjected to selection pressure 72 h post transfection. Cells were grown in the presence of 3 µg·mL−1 puromycin for three passages to establish the cell line.

Antibodies

anti-PC4 (in-house-generated polyclonal antibody), anti-histone H3 (in-house-generated polyclonal antibody), anti-H3K9ac (Abcam, ab16635, Cambridge, UK), anti-H2AK5ac (Cell Signalling, 2576S, Danvers, MA, USA), H4K16ac (Abcam, ab109463), anti-Tubulin (Calbiochem, Merck DM1A+, Burlington, MA, USA) and anti- GAPDH (in-house-generated polyclonal antibody), anti-FLAG (Sigma, F1804, Lot no. SLBK1346V), anti-53BP1 (Santa Cruz SC-22760, Lot no. K0315, Dallas, TX, USA), Goat Anti-Rabbit IgG H&L (HRP; Abcam, Cat no. ab97051, Lot no. GR288027-9), Goat Anti-Mouse IgG H&L (HRP; Abcam, Cat no. ab97023, Lot no. GR298142-12), F(ab’)2-Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Life Technologies, Cat no. A11070, Lot no. 1618692), Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 (Life Technologies, Cat no. A21052, Lot no. 1712097).

Gamma irradiation experiment

HEK293 and HEK293 sh-PC4 cells were seeded onto a 35 mm dish and grown till 80% confluency. The dishes were exposed to different doses of radiation by setting the time in the irradiator (Blood Irradiator-2000, BIR2000, BRTT, Navi Mumbai, India), JNCASR, which has the rate of irradiation as 5.463 Grey units (Gy) per minute as follows: 1 Gy-14 s, 2 Gy-29 s, 3 Gy-43 s. After exposure to radiation, fresh media was given to the cells and incubated for 24 h at 37 °C in a 5% CO2 supply and an 80% relative humidity of a CO2 incubator. After 24 h of irradiation the cells were harvested, and lysates were prepared for western blotting.
Clonogenic assay

Clonogenic assay was performed using 200 cells (single cell population) seeded in 100 mm tissue culture dishes 24 h prior to gamma irradiation. Cells were subjected to different doses of irradiation namely 0, 1, 2, 3 Gy. Media was replaced with fresh complete media soon after the irradiation. Plate was kept in 37 °C incubator with 5% CO2 for 10 days to monitor colony formation. Colonies were stained with Crystal violet solution, counted in both the cases and surviving fraction was calculated as (No. of colonies at a particular dose/No. of colonies at 0Gy)*100. For Clonogenic assay with autophagy inhibitors, cells were seeded onto a 35 mm dish and grown till 70% confluency, cells were then pre-treated with autophagy inhibitors for 2 h, prior to exposure to 2 Gy gamma irradiation. After exposure to radiation, fresh media was given to the cells and incubated for 24 h at 37 °C in a 5% CO2 supply and an 80% relative humidity of a CO2 incubator. Cells were pre-treated with autophagy inhibitors were also supplemented with fresh media containing the desired concentration of inhibitors after exposure to gamma irradiation. After 24 h of irradiation, cells were trypsinized and then seeded for colony formation. For better quantitation of the effect of autophagy inhibitors on PC4 knockdown cells, control cells/plates which had lesser variation in the number with sh-PC4 cells in the control condition (without inhibitor/radiation) were considered.

shRNA mediated knockdown of ULK1

A specific shRNA against human ULK1 gene (Addgene #27633, Watertown, MA, USA; shRNA sequence: ACATCGAGAACGTCACCAAGT) was transfected in the control cells as well as sh-PC4 cells. After 48 h of transfection, the cells were trypsinized and seeded for colony formation assay as described earlier. Lysates collected from the shRNA transfected cell lines were subjected to immunoblotting after 48 h of transfection against a specific antibody for ULK1.

Real time-PCR

RNA was extracted from ~ 2 × 10⁴ cells. 0.5 μg of RNA was reverse transcribed using MMLV reverse transcriptase (Sigma, M1302). Resulting cDNAs were quantified by RT-PCR using KAPA SYBR FAST Universal qPCR master mix (KAPA Biosystems, KK4601, Wilmington, MA, USA) on the Stepone Plus RT-PCR platform (Life Technologies). Amplification with specific gene primers was performed. The housekeeping gene, β-actin was used for normalization. The primers used are given in Table S1. Relative expression was normalized to the endogenous control Actin using the 2⁻ΔΔCt method. Experiments were carried out in technical triplicates, and biological duplicates. Primers used are listed in the table below.

| Gene     | Primer sequence 5’-3’          |
|----------|--------------------------------|
| ULK1 Fwd | GTCACACGCCACATAACAGA          |
| ULK1 Rev | CCACTAAAGTGGATGGAAGAAG       |
| ULK2 Fwd | CCTCCCGAACGTCAGTGTAG          |
| ULK2 Rev | TCTCATGTTGGTCTCTGTAG          |
| GABARAPL1 Fwd | AGGACAGACTTGTGAGTTG          |
| GABARAPL2 Rev | CCTCGGACACCTATGAAAC          |
| GABARAPL2 Fwd | GTCAAGGGTGGTGGCATATT         |
| AMPKalpha1 Fwd | GTCAAGGGCAGCCAAATGGA      |
| AMPKalpha1 Rev | GTACAGGGCAGGCCAGAATT        |
| AMPKalpha2 Fwd | CAGGCGCTAAAGTGCCAGTAA      |
| AMPKalpha2 Rev | AAAAGCTGTCGGAGGTGCTGA       |
| DRAM1 Fwd | GTCAGCCGGCCTTATTACT         |
| DRAM1 Rev | ACTCTTCTGAGGTGGTTGTTTC      |
| BECN1 Fwd | CCCGTTGAAATGGAATAGGATTA     |
| BECN1 Rev | CCGTAAAGGGAAACATGGGATAC     |
| PTEN Fwd | TGATACAAAGGCGAATGCTAA       |
| PTEN Rev | AGCATGGAGGCGAATGATTTAG       |
| BAX Fwd | GGAGGAGTCGCAATGTTCCG        |
| BAX Rev | GGTTGTGCACCCCTTTTCTAC       |
| BCL2 Fwd | AGATGGAGCAGTAATGGTAGTACTG    |
| BCL2 Rev | TCTGTGCTAGCTGATGTATG       |

Immunofluorescence

Assay was performed in desired cell lines and probing was carried out using different antibodies as indicated. DNA was stained with 0.1 μg/mL Hoechst 33258 (Sigma).

Apoptosis analysis and cell sorting

Annexin V-Cy3 Apoptosis Detection Kit (Sigma APOAC; utilizes the sharp and bright Cy3 red fluorescence dye conjugated to Annexin V. Annexin V-Cy3 binding can be detected by flow cytometry (Ex = 488 or 543 nm; Em = 570 nm) using the phycoerythrin emission signal detector (usually FL2). Detection of apoptosis was carried out as suggested by manufacturer's kit protocol. Briefly, the cells were washed twice with Annexin Binding buffer IV (100 mM HEPES/NaOH, pH 7.5, containing 1.4 mM NaCl and 25 mM CaCl2). Cells were dissolved in the buffer such that it is 10⁶ cells·mL⁻¹. One hundred microliter of cell suspension was taken and to this 1.5 μL of Dye was added. Thereafter, the suspension was incubated for 15 min in dark and then added to 400 μL of Annexin Buffer. This was used for analysis. For sorting 2 million cells were taken (100 μL suspensions containing 10⁶ cells stained with 1.5 μL of Dye). The volume of the suspension was made up to 2ml with the Annexin Buffer. Cells which stained Annexin V positive were excluded and only the live population was collected in sterile 1X PBS buffer post sorting.
**Impedance measurement for proliferation/wound healing.**

The impedance across 8W10E array plates seeded with 10 000 cells per well was monitored in realtime using the ECIS-Z0 (Applied BioPhysics, Troy, NY, USA) under multi-frequency mode. After saturation in the proliferation curve analyses were carried out using ECIS-SOFTWARE-v1.2.8.PC and Excel 2007 (Microsoft, Redmond, WA, USA) for the impedance values of grouped wells and plotted the average of triplicates for each population. At the end of saturation point wounds of equal size were created (at 5000 μA; 60 000 Hz; 20 s) and monitored the proliferation status over the next 12 h. The normalized impedance was analyzed as described previously.

**Scratch assay**

This was performed using 80% confluent cells which were scratched with a sterile pipette tip in four separate places and image was captured after indicated time points.

**Cell lystate preparation**

Cell pellets washed in 1x PBS from the desired cell line after the mentioned treatment were lysed in 1x SDS Buffer [50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol]. Cell lysates were then heated at 90°C for 10 min and then subjected to immunoblotting.

**chIP**

chIP was performed using HEK293 cells and HEK293 sh-PC4 cells, using the protocol described [24]. The primers for the ChIP data were designed in accordance to the ENCODE data against the region in the promoters for each gene which showed a peak for H3K9Ac. The primers used for chIP qPCR are as follows: ULK1-Fw: 5’- GATTC CCAACCCGGATCAT-3’ and ULK1-Rev: 5’- CGTGC TCTGGAAGCCAAAC-3’. ULK2-Fw: 5’- GCAAGATT TCAAGACACCTA-3’ and ULK2-Rev: 5’- GGTACTCT CGTACAGCAAGAG-3’. AMPKα1-Fw: 5’- CTCGGA CGAACATGGCTTTA-3’ and AMPKα1-Rev: 5’- CTGTA GAGGCGCTGTGATTAC-3’. AMPKα2-Fw: 5’- CGCT GCACCGGTGGTAG-3’ and AMPKα2-Rev: 5’- CACG TAGTGCTCCATCTTC-3’. DNA damage regulated autophagy modulator 1 (DRAM1)-Fw: 5’- AGCGGTGGT GTAAAGTGAT-3’ and DRAM1-Rev: 5’- GGTC AGCTCCAGAGAATT-3’. HNF4a-Fw: 5’- CCTGC TGCTGCTGATAATG-3’ and HNF4a-Rev: 5’- CAGC TAGTGCTCCATCTTC-3’.

For PC4 chIP analysis, HEK293 cells stably expressing a Flag-tagged PC4 or only pCDH vector were taken. The stable cells were generated as mentioned in Dhansekaran, et.al [25]. Flag chIP was carried out using a highly specific Flag antibody, followed by qPCR for the promoters of ATG genes using primers as mentioned above. The primer for BECN1 promoter was designed from the region as mapped from ATAC-seq of sh-PC4 cells. BECN1-Fw: 5’-GACTCCCTTCAAAGGTCCTAA-3’ BECN1-Rev: 5’-GAAGACAGACGGATGTTC-3’.

**Analysis of segregation defects**

Wild-type and PC4 knockdown HEK293 were grown to 60% confluence and treated with 26 μg·mL⁻¹ Cytochalasin B for 3 h in 37 °C incubator with 5% CO₂. Cells were harvested by pipetting, washed once with 1x PBS and pellet was fixed in 1 mL of Carnoy’s Fixative (methanol : glacial acetic acid 3 : 1). Fixed cells can be kept at 4 °C for storage. Fixed cells were gently agitated and slides were prepared by the slide drop technique using Pasteur pipette. Slides were air dried, stained with 1 μM of DAPI (4’,6-diamidino-2-phenylindole) and sealed with a coverslip. Imaging was carried out using Olympus (Shinjuku, Japan) BX-61 Fluorescence Microscope. Segregation defect was analyzed in terms of the number of anaphase bridges formed per 100 binucleates counted for both wild-type and PC4 knockdown HEK293 and a graph was plotted using GRAPHPAD PRISM™.

**FISH analysis of segregation defects**

Wild-type and PC4 knockdown HEK293 were grown to 60% confluence and both were treated with 26 μg·mL⁻¹ Cytochalasin B and 2.6% Colcemid for 3 h in 37 °C incubator with 5% CO₂. Cells treated with Colcemid were harvested for chromosome analysis and treated with 1 mL of 0.54% Potassium Chloride for 20 min at 37 °C water bath. Pellet was then fixed in 1 mL of Carnoy’s Fixative (methanol : glacial acetic acid 3 : 1) and stored at 4 °C. Cytochalasin B-treated cells for binucleate analysis were harvested, washed, fixed in 1 mL of (methanol : glacial acetic acid 3 : 1) and stored at 4 °C. Slides for chromosomes and binucleates were prepared by the slide drop technique, air dried and aged in a dust free environment overnight. Fluorescence in-situ hybridization of telomere PNA probe was performed with chromosomes and binucleates as per the protocol [26]. Signal obtained was observed under Olympus BX 61 fluorescent microscope and quantified using IMAGEJ software [27]. A graph was plotted in GRAPHPAD PRISM™ for the binucleate FISH in terms of the signal positivity in the daughter nuclei.

**Assay for transposase-accessible chromatin using sequencing**

Library for ATAC-seq was prepared according to a published protocol (Buenrostro et al., 2013) [28] using 50 000 intact cells as input. Cells were washed and lysed to generate a crude nuclei preparation. Tn5 transposase tagmentation simultaneously fragments the genome and tags the resulting DNA with...
Illumina sequencing adapters. DNA fragments were PCR amplified and subsequently purified using Qiagen MinElute PCR Purification Kit (Qiagen, Hilgen, Germany). Final library quality and quantity were analyzed and measured by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and Life Technologies Qubit 3.0 Fluorometer (Life Technologies), respectively. The final libraries were sequenced using 150 bp paired-end reads on Illumina HiSeq 4000 (Illumina Inc., San Diego, CA, USA). The reads were first mapped to the reference genome set (hg19) using BOWTIE version 1.2.0. [29] and the unique mapped reads were used for peak calling by MACS2 [30]. All discordant and unpaired reads were removed during alignment and then sorted and indexed. Homer annotatePeaks.pl was used for annotation of peaks. Common genes between the ATAC-Seq analysis and Human autophagy database were identified using custom R, (R - programming language), Auckland, New Zealand Script. PANTHER was used for the Gene Ontology analysis. Correlation with the already available ENCODE chIP-Seq data was done by aligning sorted files of sh-PC4 ATAC-seq peaks and the histone activation mark (H3K4me3) in HEK293 cells from ENCODE were visualized in integrative genome browser (IGV).

**Gene network analysis**

For generating the core network representative of autophagy, significantly enriched biological categories/gene ontology/pathways harbouring differentially expressed genes upon treatment as compared to the control cells were subjected to network identification using BRIDGEN ISLAND Software (Bionivid Technology Pvt. Ltd., Bangalore, India), resulting in identification of key nodes and edges. Output of Bridge Island Software was used as input to CYTOSCAPE V 2.8, Seattle, WA, USA. Force directed spring embedded layout under yFiles algorithm was used to visualize the network that encompasses biological categories, differentially expressed genes that were significantly enriched. All the genes in the network were color coded based on their fold change upon PC4 knockdown, compared to control cells.

**Statistical analysis**

All values are expressed as the mean ± SEM. Graphs were plotted in GraphPad PrismTM. For the statistical analysis, results were analyzed using unpaired t test or one-way or two-way ANOVA and differences were considered significant if P < 0.01/defined otherwise. All experiments were done in triplicates with a biological replicate represented as n.

**Electron microscopy**

HEK293 and sh-PC4 cells were fixed in suspension with 4% glutaraldehyde in 0.1 m cacodylate buffer (pH 7.3) after harvesting, overnight at 4 °C. Cells were dehydrated with a graded series of ethanol, and embedded in epoxy resin. Then the areas containing cells were cut into ultrathin sections, stained and observed on transmission electron microscope Tecnai G2 F-30, a 300 Kv TEM/STEM equipped with a schottky field emission source and a point - point resolution of 2.2.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

SS performed most of the experiments, designed a few, analyzed the data, and prepared the manuscript. SK identified the radiation resistance property of PC4 knockdown cells. PM performed western blotting experiments. NR performed the nuclear lamin immunofluorescence studies. SP, AS, and BB performed and analyzed the chromosome segregation studies. UB analyzed the ATAC-seq data. RM designed and analyzed experiments related to autophagy and contributed in the manuscript preparation and defending. TKK conceived the project, designed the experiments, analyzed and interpreted the data, and contributed in the writing of the manuscript.

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