NIH Public Access
Author Manuscript
Oncogene. Author manuscript; available in PMC 2009 June 8.

Published in final edited form as:
Oncogene. 2007 March 15; 26(12): 1714–1722. doi:10.1038/sj.onc.1209988.

PHC3, a component of the hPRC-H complex, associates with E2F6 during G_0 and is lost in osteosarcoma tumors

Amit M. Deshpande[1,*], **Jennifer D. Akunowicz**[1], **Xavier T. Reveles**[2], **Bina B. Patel**[1], **Elizabeth A. Saria**[1], **Richard G. Gorlick**[3], **Susan L. Naylor**[2], **Robin J. Leach**[2], and **Marc F. Hansen**[1]

1*Center for Molecular Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030*

2*Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas 78229*

3*Department of Pediatrics, The Children's Hospital at Montefiore, Bronx, New York 10467*

Abstract

Polyhomeotic-like 3 (PHC3) is a ubiquitously expressed member of the polycomb gene family and part of the human polycomb complex hPRC-H. We found that in normal cells PHC3 associated with both hPRC-H complex components and with the transcription factor E2F6. In differentiating and confluent cells, PHC3 and E2F6 showed nuclear co-localization in a punctate pattern that resembled the binding of polycomb bodies to heterochromatin. This punctate pattern was not seen in proliferating cells suggesting that PHC3 may be part of a E2F6-polycomb complex that has been shown to occupy and silence target promoters in G_0. Previous loss of heterozygosity (LoH) analyses had shown that the region containing *PHC3* underwent frequent LoH in primary human osteosarcoma tumors. When we examined normal bone and human osteosarcoma tumors we found loss of PHC3 expression in 36 of 56 osteosarcoma tumors. Sequence analysis revealed that *PHC3* was mutated in 9 of 15 primary osteosarcoma tumors. These findings suggest that loss of *PHC3* may favor tumorigenesis by potentially disrupting the ability of cells to remain in G_0.

Keywords

tumor suppressor gene; osteosarcoma; polycomb gene; polyhomeotic-like; cell cycle regulation

Introduction

The Polycomb group genes (PcG) encode a diverse set of regulatory proteins that are involved in maintenance but not initiation of expression patterns that control development (Lund & van Lohuizen, 2004; Ringrose & Paro, 2004). First identified in Drosophila (Lewis, 1978), they have been found throughout higher eukaryotes. PcG complexes are responsible for long-term silencing of genes by altering chromatin structure through deacetylation of histone tails and by inhibiting ATP-dependent chromatin remodeling (Shao et al., 1999; van der Vlag & Otte, 1999).

Studies have demonstrated that PcG proteins divide into two separate complexes (Ali & Bender, 2004; Cao & Zhang, 2004). The first complex which includes homologues of the Drosophila *esc* and *E(z)* genes, has histone deacetylase activity (Sewalt et al., 1998; van

Correspondence Address: Center for Molecular Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, Phone: (860) 679-2261, Fax: (860) 679-7639, Email: E-mail: mhansen@nso2.uchc.edu.
*Present address: Molecular Oncology Research Institute, Tufts-New England Medical Center, Boston, MA 02111

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Lohuizen et al., 1998). The second PcG complex (dPRC-1 in Drosophila, mPRC1 in mouse and hPRC-H in humans), contains homologues of the Drosophila *Pc*, *Psc*, *Ph*, *RING* and *Scm* genes (Alkema et al., 1997; Franke et al., 1992; Satijn et al., 1997; Shao et al., 1999) and can mediate silencing of target genes by interfering with SWI/Snf chromatin remodeling machinery, blocking transcriptional initiation and the recruitment of additional silencing activities (Dellino et al., 2004; Francis et al., 2001; King et al., 2002; Lavigne et al., 2004; Levine et al., 2002; Shao et al., 1999).

In humans, there are three orthologs of the Drosophila *Ph* gene: *PHC1*, *PHC2* and *PHC3* (Gunster et al., 1997; Levine et al., 2002; Tonkin et al., 2002). Database screening has shown that all three are evolutionarily conserved in vertebrates (Isono et al., 2005). Each of the PHC genes encodes a (Cys)4-type Zinc coordination domain (Deatrick et al., 1991), and a sterile alpha motif (SAM) domain (Ponting, 1995) at the C-terminus. The SAM domain is common to several PcG genes and has been shown to be involved in protein-protein interactions (Isono et al., 2005; Kim et al., 2002).

We set out to characterize the function of PHC3. As expected, we found that PHC3 interacted with members of the hPRC-H complex. We also found that PHC3 interacted with E2F6, possibly as part of a complex that occupies E2F- and Myc-responsive genes in G_0 (Ogawa et al., 2002). *PHC3* maps to a region of human chromosome 3q26 that has been found to undergo tumor-specific LoH in human osteosarcomas (Kruzelock et al., 1997). Because of its potential role in regulating gene expression during G_0 and its localization within a region of frequent loss, we hypothesized that PHC3 might be a tumor suppressor gene. We found PHC3 expression was present in normal bone but absent in a majority of primary osteosarcoma tumors. When we examined DNA from primary osteosarcomas, we discovered somatic intragenic inactivating mutations. These findings were consistent with the role of *PHC3* as a tumor suppressor gene in human osteosarcoma.

Results

PHC3 is part of the hPRC-H PcG complex but also binds E2F6

Co-immunoprecipitation analyses confirmed that Phc3 co-immunoprecipitated with proteins YY1 and M33 as previously reported (Levine et al., 2002) (Figure 1). We found that Phc3 also co-immunoprecipitated with E2F6 and Bmi1. As a negative control, Phc3 did not co-immunoprecipitate with CtBP or RB1 *(data not shown)* that have been shown to be part of a different PcG complex (Dahiya et al., 2001) or with the unrelated protein Sox9. The data confirmed that PHC3 was part of the hPRC-H complex, which has previously been shown to contain PHC3, YY1 and M33 (Levine et al., 2002) and demonstrated that PHC3 may be part of an E2F6-containing PcG complex (Ogawa et al., 2002; Trimarchi et al., 2001).

Subcellular localization of PHC3 is dependent on cell cycle stage

PcG proteins form distinct nuclear foci called polycomb bodies that associate with heterochromatin (Saurin et al., 1998). PHC1 and PHC2 are both found in these polycomb bodies (Gunster et al., 1997). Analysis of PHC3 expression in NHOst cells showed that PHC3 was diffusely localized in the nuclei of proliferating cells. Following induction of differentiation, a punctate pattern of nuclear distribution for PHC3 was observed as soon as Day 2 following induction (Figure 2). This punctate pattern continued through differentiation.

A similar pattern of punctate localization was observed in confluent cells. In nonconfluent cells, PHC3 was diffusely nuclear. As cells became confluent, PHC3 showed an increasingly punctate sublocalization (Figure 3). The punctate pattern of localization of PHC3 was similar to that seen for heterochromatin-associating polycomb bodies.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

PHC3 and E2F6 colocalize in confluent and differentiating cells

To test whether PHC3 was part of an E2F6-containing complex, we examined the co-localization of PHC3 and E2F6. hMSC cultures were analyzed for subcellular localization of PHC3 and E2F6 at 50% confluency and at >80% confluency. In cells that were 50% confluent, both PHC3 and E2F6 were expressed diffusely in the nucleus. In contrast, when the cells were >80% confluent, PHC3 and E2F6 were co-localized in the nucleus in the punctate pattern (Figure 4). Similar patterns of co-localization were seen when the cells were induced to differentiate along the osteogenic lineage *(data not shown)*. This punctate pattern of co-localization was similar to that seen for PHC3 alone in differentiating and confluent cells (Figures 2 and 3). This strongly suggested that PHC3 and E2F6 were part of a G_0-specific DNA-binding complex (Ogawa et al., 2002).

Absence of expression of *PHC3* in primary osteosarcoma tumors

If PHC3 was part of an E2F6 G_0-regulating complex, then loss of PHC3 function from this complex might affect cells entering or remaining in G_0, favoring tumorigenesis. Significantly, *PHC3* maps to a region of human chromosome 3q26.2 that was shown to undergo frequent loss of heterozygosity in osteosarcomas (Kruzelock et al., 1997). Since LoH is frequently associated with homozygous inactivating mutations that lead to loss of expression of tumor suppressor genes (Haber & Harlow, 1997), we tested for PHC3 and E2F6 expression in human osteosarcoma tumor tissue arrays. 56 informative cases of osteosarcoma were examined. As a positive control, we examined co-expression in a dpc12.5 embryonic mouse. Our analysis showed that Phc3 was expressed in the cells of the embryonic mouse skeleton and PHC3 expression was absent in 36 of 56 (64%) osteosarcoma tumors. E2F6 was present in all of the tumors and the developing mouse skeleton. Examples of the analysis are shown in Figure 5.

Somatic intragenic mutations disrupt PHC3 in human primary osteosarcoma tumors

As PHC3 expression was absent in a majority of osteosarcoma tumors, we sequenced matched sets of normal and tumor DNAs from 15 patients with osteosarcoma to see if mutations could be found in the tumors. In one tumor sample the PCR amplification for exon 2 revealed two bands (Figure 6A). We sequenced the PCR fragments from this tumor. The larger fragment proved to be due to a tandem duplication that resulted in a 196bp insertion into the coding region of exon 2 (Figure 6B). The insertion was not present in the matched normal DNA sample from the same patient suggesting that the mutation arose somatically in the tumor. It is not clear whether the mutation was actually heterozygous in the tumor or a result of contamination of the tumor sample with normal cells during DNA extraction.

Sequence analysis of the remaining 14 samples revealed that seven tumors had homozygous 1–4bp insertions/deletions that caused frameshift mutations (Figure 6C). The most proximal mutation was a 1bp deletion in codon 2 that would eliminate the entire protein. The most distal mutation was a 4bp deletion at codon 799 that would eliminate the SAM domain of the protein. None of the mutations were found in the matched normal DNA suggesting that all had arisen somatically in the tumors.

One tumor had a C to T substitution that led to a TAG stop codon at codon 419 (Figure 6C). This mutation appeared to be heterozygous in the tumor. The mutation was not found in the DNA from the matched normal sample suggesting that the mutation arose somatically in the tumor. As in the first tumor, it is not clear whether the wildtype allele was actually present in the tumor or whether the presence of the wildtype allele was due to contaminating normal cells.

A map of the mutations in relation to the conserved domains of the protein is shown in Figure 6D. The mutations showed no evidence for hotspots suggesting that the mutations likely inactivated the gene product rather than activated a novel function. This is consistent with the

NIH-PA Author Manuscript

concept that loss of function is the critical event for tumor suppressor genes in tumorigenesis (Haber & Harlow, 1997).

Discussion

In mice, *Phc1* has a critical role in mediating PcG-dependent bridging of distant chromatin templates (Lavigne et al., 2004). Co-immunoprecipitation of PHC1 and PHC2 proteins from HeLa and U2OS cell extracts suggests that the polymerization of SAM domains of the PHC proteins may be involved in mediating PcG-dependent silencing through higher-order chromatin structures (Gunster et al., 1997; Levine et al., 2002).

During the initiation of silencing by the PcG complexes, specific subunits of the PRC1 complex must interact with chromatin to recruit the free nucleosome arrays. Proteins interactions have been shown to be mediated through the SAM domain (Ph) and the RING domain (RING and Bmi1) which may also play a critical role in recruitment (Lavigne et al., 2004; Levine et al., 2002). Studies have shown the PRC1 complex does not significantly alter nucleosomal position or access of nucleases to the nucleosomal arrays (Dellino et al., 2004; Lavigne et al., 2004). This suggests the PRC1 complex can inhibit chromatin remodeling by blocking interaction with SWI/Snf without making the template inaccessible. This may be important, as it would permit cells to reactivate silenced genes if needed.

The PHC orthologues are not completely interchangeable, as PHC1 has a more restricted tissue distribution than either PHC2 or PHC3 (Isono et al., 2005; Tonkin et al., 2002). Moreover, mutations in *PHC1* or *PHC2* are not equivalent as the double mutant *Phc1/Phc2* has a synergistic effect (Isono et al., 2005; Takihara et al., 1997). Thus PHC3 either cannot substitute for the other PHC proteins or mutant forms of PHC1/PHC2 may affect the oligomerization of PRC1 complexes containing PHC3 or PHC3 may not be present in sufficient quantities to allow oligomerization of PRC1 complexes on DNA (Isono et al., 2005).

PHC3 is part of an hPRC-H complex that associates with E2F6

We found that in addition to members of the hPRC-H complex, PHC3 co-immunoprecipitated with E2F6. This suggested that the PHC3 might be part of an E2F6-containing complex that contributes to long-term silencing of genes in quiescent cells (Ogawa et al., 2002). The idea that PHC3 may play a role in regulation of transcription silencing during G_0 was supported by localization analysis. In proliferating cells PHC3 had a diffuse nuclear localization. During differentiation PHC3 shifted to a punctate nuclear localization. Similar results occurred when the cells were allowed to become confluent. These data were consistent with localization of PHC3 to the polycomb bodies during G_0.

Punctate co-localization of PHC3 and E2F6 in confluent and/or differentiating cells supported the model of a PHC3-containing hPRC-H complex binding DNA during G_0; our data suggested by extension that this complex was also the E2F6-containing complex. The role of PHC3 in the complex may be to regulate binding to the DNA or compaction of the nucleosome arrays. As with other PHC proteins, it is possible that the SAM or (Cys)4-type Zinc coordination domains may play an important functional role in PHC3. Loss of PHC3 function could lead to inability to maintain long-term G_0 silencing mediated by the complex and allow cells to continue proliferating.

PHC3 in osteosarcoma tumorigenesis

Thus, several lines of data suggest that loss of *PHC3* function would favor tumorigenesis. First, other PcG genes have been implicated in tumorigenesis (Gil et al., 2005; Leung et al., 2004; Nowak et al., 2006; Pasini et al., 2004; Raaphorst, 2005). PcG complexes have been shown to

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

regulate control of cellular proliferation and loss of this control would favor tumorigenesis (Bracken et al., 2006; Dahiya et al., 2001; Pasini et al., 2004).

Second, the discovery that the E2F6 complex present in HeLa cells cannot occupy target genes (Ogawa et al., 2002) suggests that the E2F6-containing complex is incomplete in some form. PHC3 was missing from the HeLa cell E2F6-containing complex suggesting that in its absence the complex cannot bind DNA or regulate transcription.

The discovery that *PHC3* lies within a region of human chromosome 3q26.2 shown to undergo LoH at high frequency in osteosarcomas (Kruzelock et al., 1997) suggests *PHC3* as a possible tumor suppressor. Expression analysis showed PHC3 was present in cultured human mesenchymal stem cells, osteoblasts and embryonic mouse skeleton but absent in a significant fraction of osteosarcoma tumors.

Finally, tumor suppressor genes characteristically have homozygous intragenic tumor-specific inactivating mutations that result in an absence of functional protein (Haber & Harlow, 1997). When we examined DNA from matched tumor and normal tissues, we found somatic mutations in 9 of the osteosarcoma tumors. These frameshift and nonsense mutations showed no evidence of clustering suggesting that the mutations led to loss of function rather than alteration in function.

When taken together, these data are consistent with *PHC3* as a tumor suppressor gene. In conjunction with the loss of checkpoints controlled by the *RB1* and *p53* pathways in osteosarcoma (Wang, 2005), the end result would be loss of the ability of the cell to exit cell cycle or remain in quiescence, thus strongly promoting tumorigenesis.

Materials and Methods

Patient Materials and cell lines

Human osteoblasts (NHOst) and mesenchymal stem cells (hMSC) (Cambrex Corp., East Rutherford, NJ) were grown according to the manufacturer's recommendations.

All patient tumor and normal DNA samples used in this study were collected with Institutional Review Board-approved Informed Consent as part of Phase III trials conducted by the Childrens Oncology Group (Arcadia, CA). All samples were completely anonymous with no patient identifiers. DNA was isolated from tumors and blood and/or adjacent normal bone using the Wizard Genomic DNA purification kit (Promega Corp.; Madison, WI). For immunofluorescent analysis, commercial tumor microarrays (HistoArrays, Imgenex Corp., San Diego, CA) were used.

PHC3 Antibody

A peptide sequence (ERELRDVRIRKMPENSD) found in both human and mouse PHC3, but not PHC1 or PHC2, was conjugated to KLH (Research Genetics/Invitrogen, Carlsbad, CA) and used to generate mouse monoclonal antibodies (Hybridoma Center for Agriculture and Biological Sciences (HYCABS), Oklahoma State University (http://www.ento.okstate.edu/hycabs/)). The specificity of the anti-PHC3 antibody was confirmed by western analysis. The antibody detected a single protein band of approximately 110–115kd, which is the same size as previously described (Levine et al., 2002) (data not shown).

NIH-PA Author Manuscript

Co-Immunoprecipitation

Proteins were extracted from snap-frozen mouse femurs and immunoprecipitation performed as previously described (Harlow & Lane, 1988). Western detection was done using the appropriate second antibody as previously described (Harlow & Lane, 1988). The antibodies used were Bmi1 (H95), CtBP (H440), E2F6 (E20), RB1 (IF8), YY1 (H10) and Sox9 (H90) (Santa Cruz Biotechnology, Inc.).

Immunofluorescence and co-localization analysis

For confluency analysis, cells were plated on chamber slides at 50% confluency. The cells were fed with growth media (Cambrex Corp.; East Rutherford, NJ) following the manufacturer's protocol. The cells became >80% confluent within 48 hours. Confluent cultures continued to be fed with appropriate growth media for 7 days. For analysis of differentiating cells, cells were grown on chamber slides and induced to differentiate by feeding with osteogenic differentiation media (Cambrex Corp.; East Rutherford, NJ) following the manufacturer's protocol.

Immunofluorescence analysis was done as previously described (Harlow & Lane, 1988) using our mouse monoclonal antibody to PHC3. Goat polyclonal antibody to E2F6 (E-20, Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) was used for co-localization assays. All immunofluorescently labeled samples were viewed on a BX-60 microscope (Olympus America, Inc.; Melville, NY) and images captured with a digital cooled-CCD camera (Hamamatsu Corp.; Bridgewater, NJ) and OpenLab image analysis software (Improvision, Inc.; Lexington, MA). Co-localization was determined using the OpenLab Co-localization software module. All photographs were taken at 200X magnification, unless otherwise noted.

To show terminal differentiation of the osteoblast lineage, cells were stained with Alizarin Red (Sigma-Aldrich; St. Louis, MO) as recommended by the manufacturer.

Tumor microarray analysis

Analysis of tumor arrays was performed as previously described (Harlow & Lane, 1988) except that de-paraffinized array slides were first treated with 4N HCl for 10 minutes prior to incubation with mouse monoclonal antibody to PHC3 and goat antibody to E2F6 followed by incubation with Alexafluor-labeled secondary antibodies (Anti-mouse IgG and Anti-goat IgG, Invitrogen/Molecular Probes, Inc.; Eugene, OR) for detection. 5μm sections of paraffin-embedded 12.5dpc mouse embryo sections were also used as positive controls for PHC3 and E2F6 expression.

Samples were viewed and images captured as described above. Samples with less than 50 tumor cells in the microscope field were excluded from the analyses. Fluorescent signal intensities for both PHC3 and E2F6 were compared to positive (mouse embryo) control using OpenLab software. Loss of expression of PHC3 had occurred if the fluorescent intensity of PHC3 in the tumor sample was ≤ 15% of the fluorescent intensity of PHC3 in the positive control and if the fluorescent intensity of E2F6 in the tumor sample was ≥ 85% of the fluorescent intensity of E2F6 in the positive control.

Sequence analysis

Primers for each exon were designed from flanking introns of the *PHC3* gene (Supplemental Table 1). Each exon was amplified by PCR and the product gel-purified using Sephadex-G50 columns. DNA sequencing was performed by the University of Connecticut Health Center sequencing core. Comparative analysis of the sequences was performed using CodonCode Aligner, (Codon Code Corp., Dedham, MA).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Drs. A. Lichtler, Y.-F. Huang, D. Pathmanathan, M. Sciaudone and A. Arnold for their helpful discussions and P. Vachon, A. Marren and V. DuPaul-McGloin for their administrative support. We would also like to thank the Childrens Oncology Group for their assistance. This work was supported by grants from the National Institutes of Health (CA074802 and AR049940), the Charlotte Geyer Foundation and the Murray-Heilig Foundation.

Literature Cited

Ali JY, Bender W. Cross-regulation among the polycomb group genes in Drosophila melanogaster. Mol Cell Biol 2004;24:7737–47. [PubMed: 15314179]

Alkema MJ, Bronk M, Verhoeven E, Otte A, van 't Veer LJ, Berns A, van Lohuizen M. Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian polycomb complex. Genes Dev 1997;11:226–40. [PubMed: 9009205]

Bracken AP, Dietrich N, Pasini D, Hansen KH, Helin K. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev 2006;20:1123–36. [PubMed: 16618801]

Cao R, Zhang Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 2004;14:155–64. [PubMed: 15196462]

Dahiya A, Wong S, Gonzalo S, Gavin M, Dean DC. Linking the Rb and polycomb pathways. Mol Cell 2001;8:557–69. [PubMed: 11583618]

Deatrick J, Daly M, Randsholt NB, Brock HW. The complex genetic locus polyhomeotic in Drosophila melanogaster potentially encodes two homologous zinc-finger proteins. Gene 1991;105:185–95. [PubMed: 1937015]

Dellino GI, Schwartz YB, Farkas G, McCabe D, Elgin SC, Pirrotta V. Polycomb silencing blocks transcription initiation. Mol Cell 2004;13:887–93. [PubMed: 15053881]

Francis NJ, Saurin AJ, Shao Z, Kingston RE. Reconstitution of a functional core polycomb repressive complex. Mol Cell 2001;8:545–56. [PubMed: 11583617]

Franke A, DeCamillis M, Zink D, Cheng N, Brock HW, Paro R. Polycomb and polyhomeotic are constituents of a multimeric protein complex in chromatin of Drosophila melanogaster. EMBO J 1992;11:2941–50. [PubMed: 1353445]

Gil J, Bernard D, Peters G. Role of polycomb group proteins in stem cell self-renewal and cancer. DNA Cell Biol 2005;24:117–25. [PubMed: 15699631]

Gunster MJ, Satijn DP, Hamer KM, den Blaauwen JL, de Bruijn D, Alkema MJ, van Lohuizen M, van Driel R, Otte AP. Identification and characterization of interactions between the vertebrate polycomb-group protein BMI1 and human homologs of polyhomeotic. Mol Cell Biol 1997;17:2326–35. [PubMed: 9121482]

Haber D, Harlow E. Tumour-suppressor genes: evolving definitions in the genomic age. Nat Genet 1997;16:320–2. [PubMed: 9241260]

Harlow, E.; Lane, D. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1988.

Isono K, Fujimura Y, Shinga J, Yamaki M, J OW, Takihara Y, Murahashi Y, Takada Y, Mizutani-Koseki Y, Koseki H. Mammalian polyhomeotic homologues Phc2 and Phc1 act in synergy to mediate polycomb repression of Hox genes. Mol Cell Biol 2005;25:6694–706. [PubMed: 16024804]

Kim CA, Gingery M, Pilpa RM, Bowie JU. The SAM domain of polyhomeotic forms a helical polymer. Nat Struct Biol 2002;9:453–7. [PubMed: 11992127]

King IF, Francis NJ, Kingston RE. Native and recombinant polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro. Mol Cell Biol 2002;22:7919–28. [PubMed: 12391159]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Kruzelock RP, Murphy EC, Strong LC, Naylor SL, Hansen MF. Localization of a novel tumor suppressor locus on human chromosome 3q important in osteosarcoma tumorigenesis. Cancer Res 1997;57:106–9. [PubMed: 8988049]

Lavigne M, Francis NJ, King IF, Kingston RE. Propagation of silencing; recruitment and repression of naive chromatin in trans by polycomb repressed chromatin. Mol Cell 2004;13:415–25. [PubMed: 14967148]

Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P, Van Lohuizen M, Marino S. Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. Nature 2004;428:337–41. [PubMed: 15029199]

Levine SS, Weiss A, Erdjument-Bromage H, Shao Z, Tempst P, Kingston RE. The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. Mol Cell Biol 2002;22:6070–8. [PubMed: 12167701]

Lewis EB. A gene complex controlling segmentation in Drosophila. Nature 1978;276:565–70. [PubMed: 103000]

Lund AH, van Lohuizen M. Polycomb complexes and silencing mechanisms. Curr Opin Cell Biol 2004;16:239–46. [PubMed: 15145347]

Nowak K, Kerl K, Fehr D, Kramps C, Gessner C, Killmer K, Samans B, Berwanger B, Christiansen H, Lutz W. BMI1 is a target gene of E2F-1 and is strongly expressed in primary neuroblastomas. Nucleic Acids Res 2006;34:1745–54. [PubMed: 16582100]

Ogawa H, Ishiguro K, Gaubatz S, Livingston DM, Nakatani Y. A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. Science 2002;296:1132–6. [PubMed: 12004135]

Pasini D, Bracken AP, Helin K. Polycomb group proteins in cell cycle progression and cancer. Cell Cycle 2004;3:396–400. [PubMed: 14752272]

Ponting CP. SAM: a novel motif in yeast sterile and Drosophila polyhomeotic proteins. Protein Sci 1995;4:1928–30. [PubMed: 8528090]

Raaphorst FM. Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumors. Hum Mol Genet 2005;14(Suppl 1):R93–R100. [PubMed: 15809278]

Ringrose L, Paro R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu Rev Genet 2004;38:413–43. [PubMed: 15568982]

Satijn DP, Gunster MJ, van der Vlag J, Hamer KM, Schul W, Alkema MJ, Saurin AJ, Freemont PS, van Driel R, Otte AP. RING1 is associated with the polycomb group protein complex and acts as a transcriptional repressor. Mol Cell Biol 1997;17:4105–13. [PubMed: 9199346]

Saurin AJ, Shiels C, Williamson J, Satijn DP, Otte AP, Sheer D, Freemont PS. The human polycomb group complex associates with pericentromeric heterochromatin to form a novel nuclear domain. J Cell Biol 1998;142:887–98. [PubMed: 9722603]

Sewalt RG, van der Vlag J, Gunster MJ, Hamer KM, den Blaauwen JL, Satijn DP, Hendrix T, van Driel R, Otte AP. Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. Mol Cell Biol 1998;18:3586–95. [PubMed: 9584199]

Shao Z, Raible F, Mollaaghababa R, Guyon JR, Wu CT, Bender W, Kingston RE. Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell 1999;98:37–46. [PubMed: 10412979]

Takihara Y, Tomotsune D, Shirai M, Katoh-Fukui Y, Nishii K, Motaleb MA, Nomura M, Tsuchiya R, Fujita Y, Shibata Y, Higashinakagawa T, Shimada K. Targeted disruption of the mouse homologue of the Drosophila polyhomeotic gene leads to altered anteroposterior patterning and neural crest defects. Development 1997;124:3673–82. [PubMed: 9367423]

Tonkin E, Hagan DM, Li W, Strachan T. Identification and characterisation of novel mammalian homologues of Drosophila polyhomeoticpermits new insights into relationships between members of the polyhomeotic family. Hum Genet 2002;111:435–42. [PubMed: 12384788]

Trimarchi JM, Fairchild B, Wen J, Lees JA. The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. Proc Natl Acad Sci (USA) 2001;98:1519–24. [PubMed: 11171983]

van der Vlag J, Otte AP. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet 1999;23:474–8. [PubMed: 10581039]

van Lohuizen M, Tijms M, Voncken JW, Schumacher A, Magnuson T, Wientjens E. Interaction of mouse polycomb-group (Pc-G) proteins Enx1 and Enx2 with Eed: indication for separate Pc-G complexes. Mol Cell Biol 1998;18:3572–9. [PubMed: 9584197]

Wang LL. Biology of osteogenic sarcoma. Cancer J 2005;11:294–305. [PubMed: 16197719]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

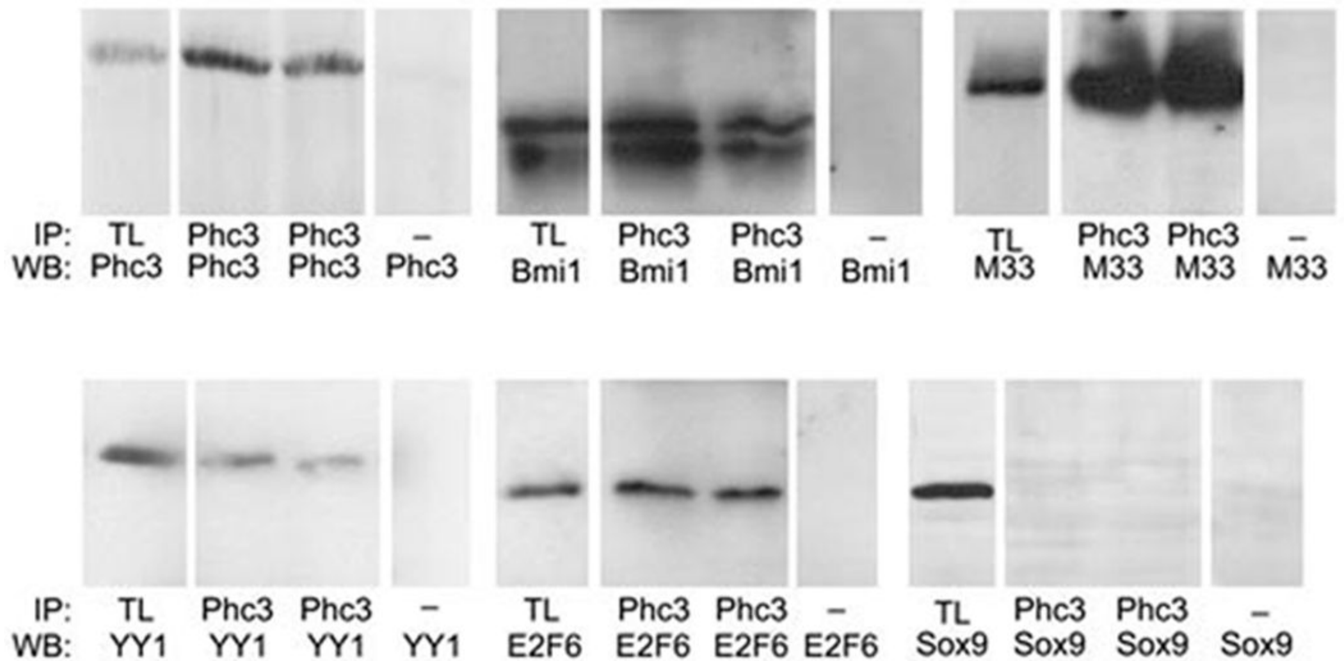

Figure 1.
Co-immunoprecipitation analysis. Total protein lysates from adult mouse bone were immunoprecipitated with antibody against Phc3, followed by western blot with a second antibody. Western blotting with antibodies against Bmi1, M33, YY1 and E2F6, detected the presence of these proteins complexed with Phc3. No association was detected using an antibody against Sox9. IP – antibody used for immunoprecipitation, WB – antibody used for Western blot detection, TL – total protein lysate, — – negative control.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

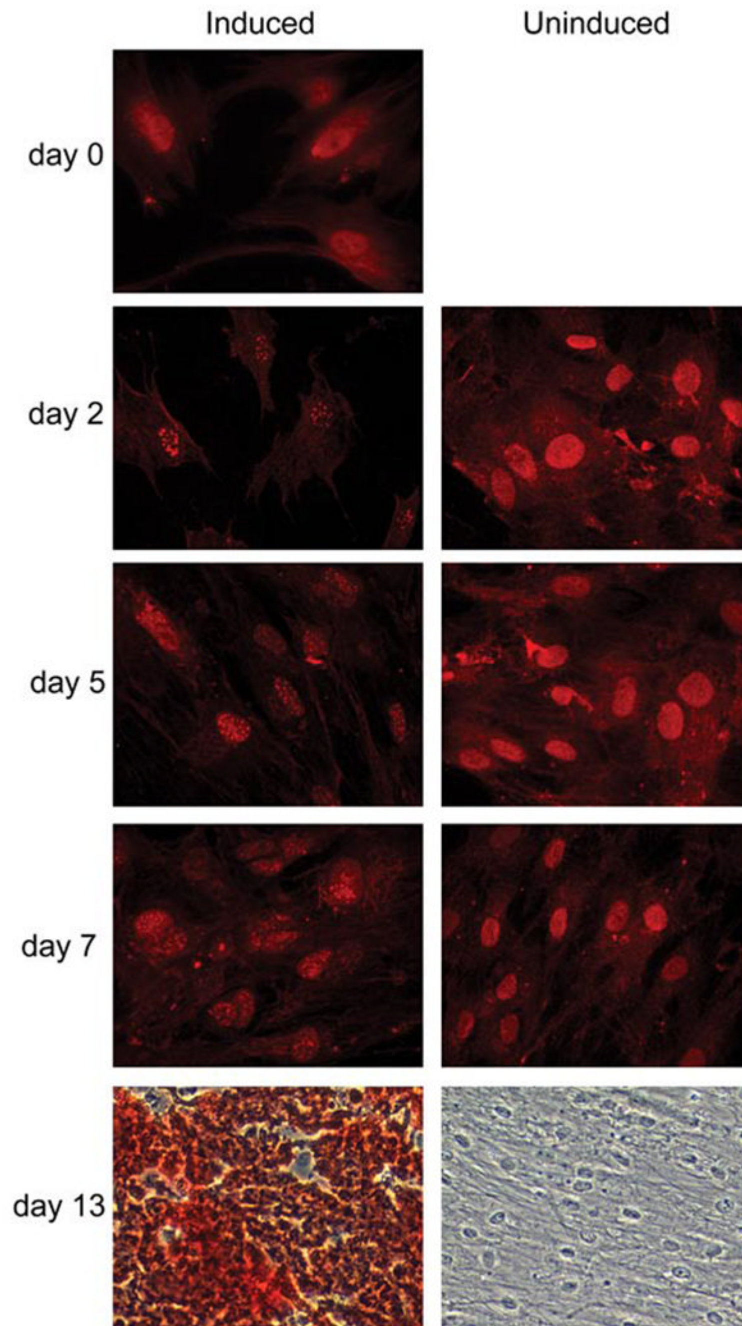

Figure 2.

Subcellular localization of PHC3 in differentiating NHOst cells. Cells seeded at low density were induced to terminally differentiate and PHC3 expression was analyzed at days 0, 2, 5 and 7 days post-induction. Analyses of uninduced cells were performed at the same times. Induction of differentiation resulted in a shift to a punctate pattern of PHC3 expression with punctate staining becoming more pronounced through day 7 (left panels). Punctate PHC3 expression was not seen in uninduced cells until confluency was achieved at approximately day 7 (right panels). At day 13 post-induction, induced and uninduced NHOst cells were stained using Alizarin Red to demonstrate mineralization, a function of terminal osteoblast differentiation (100X magnification).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

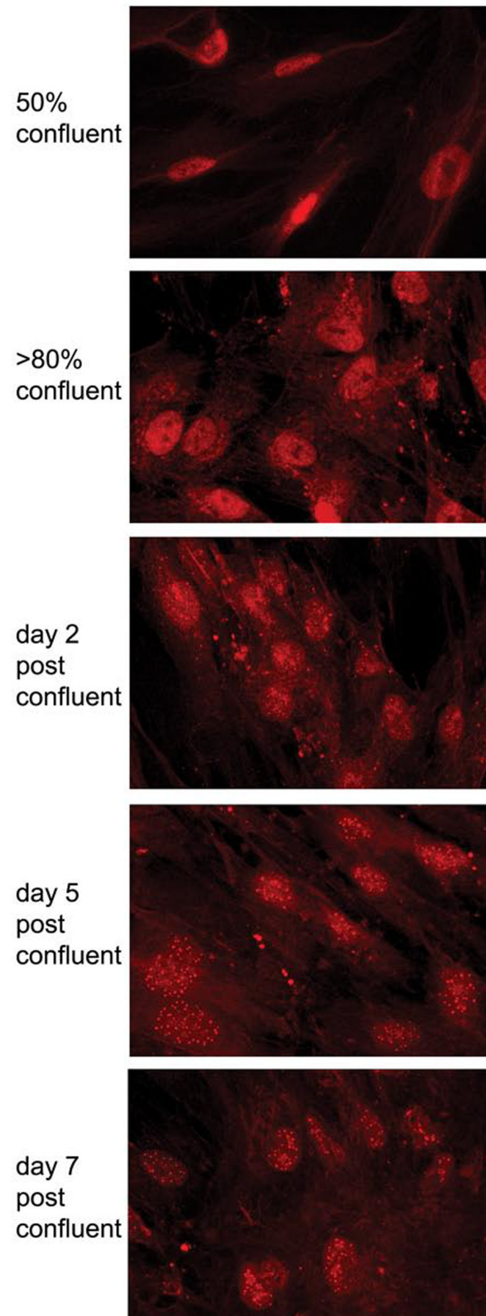

Figure 3.

Immunofluorescence analysis of confluent NHOst cells. Cells were analyzed for PHC3 localization at 50% confluency and at >80% confluency as well as days 2 and 5 following confluency. PHC3 was localized in the nucleus in a punctate expression pattern in the confluent cells.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

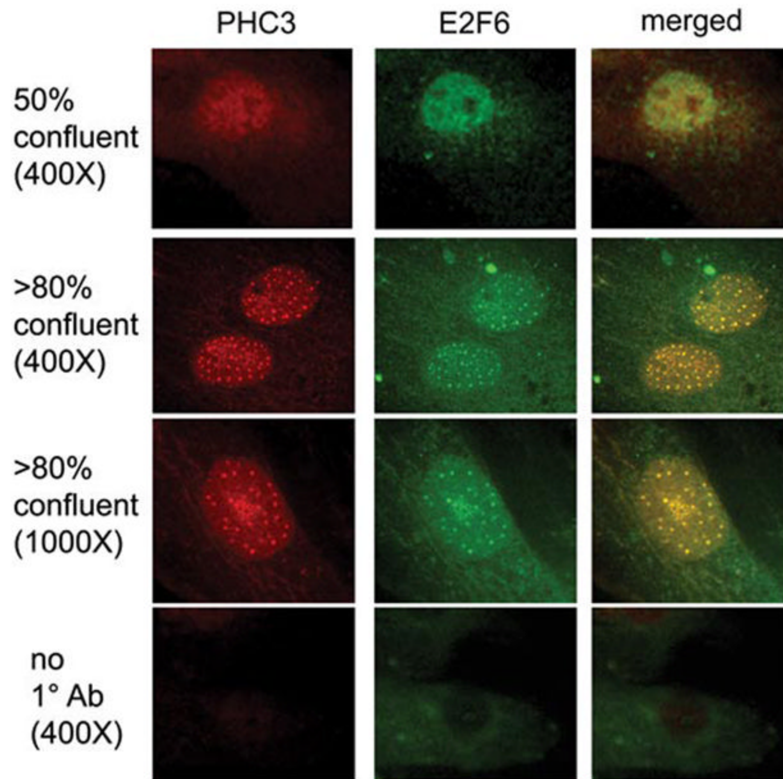

Figure 4.

Co-immunofluorescence analysis of PHC3 and E2F6 in proliferating and confluent cells. hMSC cells were analyzed for PHC3 and E2F6 localization by immunofluorescence at 50% confluency and >80% confluency. At 50% confluency (proliferating cells), both PHC3 and E2F6 are diffusely nuclear. In the >80% confluent cells, both PHC3 (red) and E2F6 (green) co-localize (yellow) in the nucleus in a punctate pattern.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

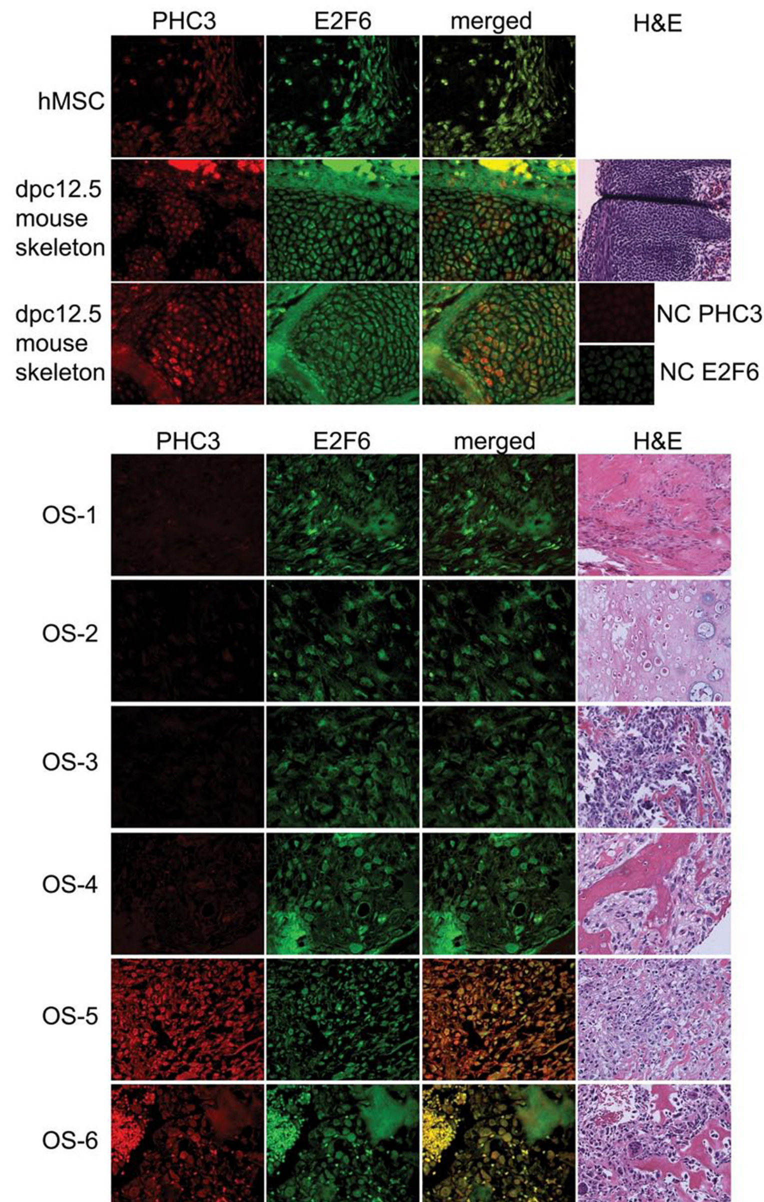

Figure 5.

Immunofluorescent analysis of PHC3 expression. Representative examples of normal (hMSC and dpc12.5 mouse skeleton) and primary osteosarcoma tumors (OS-1 through OS-6) were tested for PHC3 (red) and E2F6 (green) expression by immunofluorescence imaging. H&E stains of serial sections from the same mouse skeleton and tumors are also shown. Positive PHC3 staining can be seen in the hMSC cell line and the developing mouse skeleton vertebrae. Osteosarcoma tumors OS-1 through OS-4 are examples of $PHC3^{-}$ tumors while OS-5 and OS-6 are examples of $PHC3^{+}$ tumors. All H&E stained photos were taken at 100X. NC – negative controls where no primary antibody was used for staining mouse skeleton.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

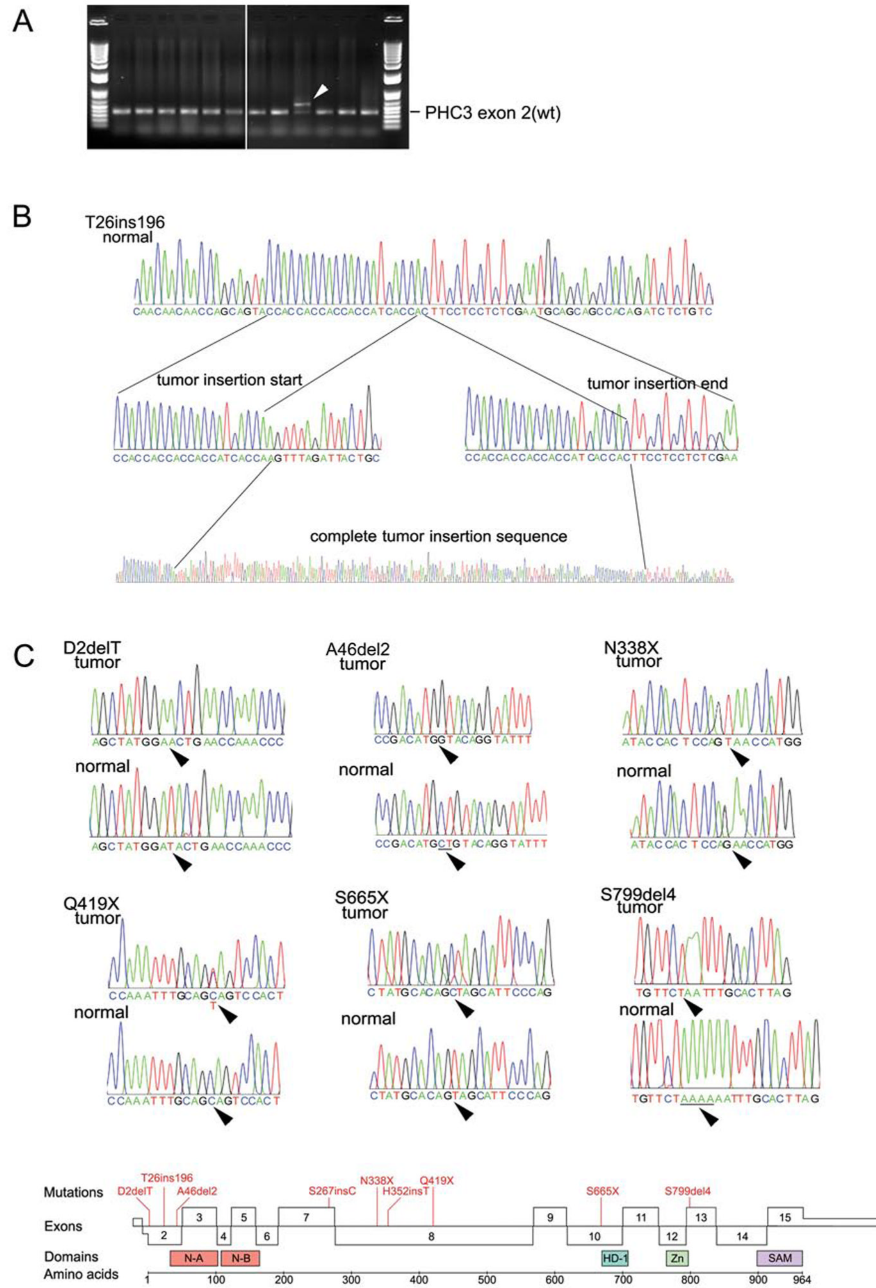

Figure 6.

Mutational analysis of *PHC3.* **A)** Agarose gel of exon 2 PCR-amplified tumor DNAs showing two amplification products in one tumor sample (arrow). **B)** Sequence analysis of the larger fragment from exon 2 of the tumor shown in (A) revealed a 196bp tandem repeat at the beginning of exon 2 in the tumor DNA which was absent in DNA from the matched normal tissue. **C)** Representative examples of sequence analysis from osteosarcoma and matching normal DNA samples. The majority of alterations were 1–4bp insertions/deletions that resulted in frameshift and nonsense mutations. Arrows indicate the alterations. One tumor had a C/T transition in codon 419 that resulted in a heterozygous nonsense mutation. **D)** Map of the mutations found in osteosarcoma tumors. The exon structure of *PHC3* is shown as alternating boxes. Half height boxes show noncoding portions of the mRNA. N-A and N-B – N-terminal

homology domains shared by human and mouse PHC3, HD-1 – homology domain shared amongst PHC proteins, Zn – putative Zn coordination domain, SAM – Sterile Alpha Motif protein interaction domain.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript