Nucleophosmin interacts with FOXM1 and modulates the level and localization of FOXM1 in human cancer cells

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Running title: Interaction between FOXM1 and NPM in cancer cells

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Keywords: NPM, FOXM1, co-localization, interaction, cancer cells, tumorigenesis
Background: FOXM1 and NPM are over-expressed in human cancers.

Results: Knockdown of NPM leads to the suppression of FOXM1 expression in cancer cells.

Conclusion: NPM interacts with FOXM1, and their interaction is required for sustaining the level and localization of FOXM1.

Significance: Targeting the interaction between FOXM1 and NPM by peptides or small molecules may represent a novel therapeutic strategy against cancer.

Summary
Using mass spectrometric analysis we found that oncogenic transcription factor FOXM1 that is over-expressed in a majority of human cancers interacts with multifunctional protein NPM, which is also over-expressed in a variety of human tumors. Coimmunoprecipitation and glutathione S-transferase pull-down experiments demonstrated that NPM forms a complex with FOXM1 and also identified the regions responsible for their interaction. Immunofluorescence microscopy confirmed the interaction between FOXM1 and NPM in cancer and immortal cells. Furthermore, knockdown of NPM in immortal and cancer cells led to significant down-regulation of FOXM1 similar to its levels in normal cells, suggesting that NPM might modulate FOXM1 level. In addition, in OCI/AML3 leukemia cells where mutant NPM is localized in the cytoplasm we found that typically nuclear FOXM1 was predominantly co-localized with NPM in the cytoplasm, while NPM knockdown led to the disappearance of FOXM1 from the cytoplasm, suggesting that NPM may also determine intracellular localization of FOXM1. Knockdown of FOXM1 or NPM in MIA PaCa-2 pancreatic cancer cells inhibited anchorage-dependent and independent growth in cell culture, and tumor growth in nude mice. In addition, over-expression of FOXM1 reversed the effect of NPM knockdown in vitro. Our data suggest that in cancer cells NPM interacts with FOXM1 and their interaction is required for sustaining the level and localization of FOXM1. Targeting the interaction between FOXM1 and NPM by peptides or small molecules may represent a novel therapeutic strategy against cancer.

Introduction
Forkhead box (Fox) M1, FOXM1, is a transcription factor of the Forkhead family that induces the expression of genes involved in the execution of the mitotic program (1,2). FOXM1 is expressed in dividing mammalian cells and in transformed/tumor cells, but it is not expressed in terminally differentiated or quiescent cells (3-6). As a potential oncogene FOXM1 is activated by oncogenic Ras-MAPK (7), Sonic Hedgehog (8), NF-kB (9) and EGFR (10) pathways, and is negatively regulated by tumor suppressor p53 (11). Furthermore, FOXM1 is overexpressed in anaplastic astrocytomas and glioblastomas (12,13), breast cancer (10,14), basal (8) and squamous (15) cell carcinomas, colorectal cancer (16), gastric cancer (17), hepatocellular carcinomas (18), malignant mesothelioma (19), pancreatic carcinomas (20) and in many other human cancers.

Downregulation of FOXM1 in pancreatic (21) and breast (22) cancer cells by RNA interference led to the inhibition of proliferation, migration and invasion of cancer cells. In addition, FOXM1 is overexpressed in metastatic prostate cancer cells (23) and FOXM1 knockdown in prostate cancer cell lines led to a significant reduction in cell proliferation and anchorage-independent cell growth on soft agar (24,25). Furthermore it has been shown that FOXM1 is a potent activator of tumor metastasis (26). All these data suggest that FOXM1 may be required for human tumor growth and metastasis, implicating a role for FOXM1 as the “Achilles’ heel” of cancer (27).

Nucleophosmin (NPM) belongs to the nucleophosmin/nucleoplasmin family of chaperones, which are ubiquitously
expressed in mammalian cells (28,29). NPM is a phosphoprotein that shuttles between the nucleus and the cytoplasm. Multiple cellular proteins with different functions directly interact with NPM, suggesting that NPM may be involved in various cellular processes (30-33). For example, it has been shown that NPM directly interacts with c-Myc and acts as a key cofactor for the transforming activity of c-Myc (30). Moreover, similarly to FOXM1 the expression of NPM is associated with cell proliferation, showing increased NPM levels in transformed/tumor cells compared to normal cells (34). In addition, NPM is overexpressed in gastric (35), colon (36), breast (37) and prostate (38) carcinomas, as well as in thyroid tumors (39).

In this study, we show that FOXM1 interacts with NPM in human cancer cells. We also found that knockdown of NPM leads to the down-regulation of FOXM1 protein level. And we also demonstrate that FOXM1 or NPM knockdown impair the ability of MIA PaCa-2 pancreatic cancer cells to form colonies in cell culture and to form tumors in vivo. Taken together, our data suggest that in cancer cells the interaction between FOXM1 and NPM is necessary for sustaining the level and localization of FOXM1 and it may be required for cancer progression.

Materials and Methods

Cell lines and chemical compounds. U2OS-C3, a clone of U2OS human osteosarcoma cells with doxycycline inducible FOXM1-GFP fusion protein (40); U2OS-FOXM1, subline of U2OS constitutively expressing T7-FoxM1(41); MIA PaCa-2 human pancreatic (ATCC) and HEK-293 human embryonic kidney (ATCC) cells were grown in DMEM medium (Invitrogen). MCF-7 human breast cancer cells (ATCC) were grown in RPMI 1640 medium (Invitrogen). The media were supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Invitrogen). Stable cell lines using the ATCC obtained parental cells were generated by transduction of control and FOXM1 (Sigma) or NPM (Thermo Scientific) shRNA lentiviral particles followed by selection with puromycin (Sigma). OCI/AML3 leukemia cells (42) were grown in Alpha MEM (Invitrogen) supplemented with 20% fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Invitrogen). BJ wild type and immortal human foreskin fibroblast cells (43) were grown in Knockout DMEM (Invitrogen) supplemented with Medium 199 (Invitrogen), 15% fetal bovine serum (Atlanta Biologicals), 1% glucose (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). The cell lines were incubated at 37º C in 5% CO₂. Thioestrepton (Sigma) was dissolved in DMSO and Doxycycline (LKT Laboratories) in PBS.

Plasmids, shRNAs and siRNAs. GST-NPM full length (1-295) and truncated plasmids (1-117, 1-186, 1-259, 187-295, 187-259 and 260-295) were a kind gift from Dr. Dawn E. Quelle (University of Iowa) (31) and Dr. Stephen R. Hann (Vanderbilt University Medical Center) (30). GFP-FOXM1 full length, and T7-FOXM1 full length and truncated plasmids were a kind gift of Dr. Pradip Raychaudhuri (University of Illinois at Chicago). Five TRC human lentiviral shRNA clones targeting NPM and the control shRNA pLKO.1 were obtained from The Open Biosystems Expression Arrest™TRC Library (Thermo Scientific). Control and FOXM1 shRNA lentiviral particles, and also control small interfering RNA (siRNA) and siRNA specific to FOXM1 were purchased from Sigma.

Immunoblot analysis. Cells were harvested and lysed in IP buffer (20 mM HEPES, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM NaF, 10 mM Na₂P₂O₇, 1 mM sodium orthovanadate, 0.2 mM PMSF supplemented with protease inhibitors and protein concentration was determined by the Bio-Rad Protein Assay reagent (BIO-RAD). Fifty µg of the cell lysates were electrophoresed on 8% SDS-polyacrylamide mini gels and transferred to polyvinylidine difluoride (PVDF) membrane. Immunoblotting was performed with antibodies specific for FOXM1 (sc-500
and sc-502, Santa Cruz, and the rabbit polyclonal antibody against FOXM1 was described previously (7), NPM (sc-47725, Santa Cruz), T7 (69522, Novagen) and β-actin (AS441, Sigma).

**Immunoprecipitation-immunoblotting.** Mid-log cells were harvested, lysed in IP buffer supplemented with protease inhibitors and protein concentration was determined by the Bio-Rad Protein Assay reagent (BIO-RAD). Five hundred μg of the lysates in 500 μl were pre-cleared by incubating on a rotary mixer with 1 μg of mouse or rabbit IgG and 30 μl of protein-A-agarose (sc-2001, Santa Cruz) for 30 minutes at 4°C. The tubes were centrifuged for 1 min at 0.8 g and the clarified lysates were transferred to fresh microcentrifuge tubes. One-two μg of NPM or FOXM1 antibodies and 30 μl of protein A-agarose were added and the tubes were incubated on a rotary mixer overnight at 4°C. The agarose beads were washed 4 times with IP buffer containing protease inhibitors, each time centrifuging for 1 min at 0.8 g at 4°C. The beads were then boiled with 100 μl 2X Laemmli sample buffer for 3 min, centrifuged and the supernatant was separated on an 8% SDS-polyacrylamide gel and immunoblotted with NPM or FOXM1 specific antibodies.

**Preparation of Glutathione-S-transferase (GST)-NPM Fusion Proteins.** Plasmids containing full length and truncated NPM cDNAs fused to pGEX-2T (encodes GST, from Pharmacia) were transformed in competent E. coli DH5α (New England Biolabs) and overnight cultures were prepared from single colonies in LB media (containing 100 μg/ml ampicillin). The overnight cultures were diluted 1:10 with LB media containing ampicillin and grown for 2 hrs at 37°C, then 0.2 mM Isopropyl-β-D-thio-galactopyranoside (IPTG) was added and the growth was continued with shaking at 30°C for 4 hrs. The bacteria were lysed by sonication at 4°C in phosphate buffered saline containing 1 mM phenylmethyl sulfonyl fluoride (PMSF) and protease inhibitors. The fusion proteins were purified by binding to glutathione-sepharose beads (GE-Healthcare) as described previously (44). The purity and relative amounts of the GST fusion proteins were checked by 8-10% SDS/PAGE followed by silver staining (Thermo-Scientific).

**GST-pull down assay.** Full length and truncated T7-tagged FOXM1 expression plasmids were transfected into MIA PaCa-2 or HEK-293 cells and grown for 48 hrs. Clear lysates of the cells were prepared in IP buffer containing protease inhibitors. Five-hundred μg of lysates were mixed with glutathione-sepharose beads containing the respective GST-NPM protein in microfuge tubes and rotated overnight at 4°C. The beads were collected by centrifugation, washed 4 times with IP buffer supplemented with protease inhibitors. The beads were suspended in 100 μl of Laemmli sample buffer, boiled for 3 minutes, separated by 8-10% SDS-PAGE, transferred to PVDF membrane and blotted with T7 specific antibody.

**Transient transfection.** The control shRNA pLKO.1 and five TRC human lentiviral shRNA clones targeting NPM gene or the control small interfering RNA (siRNA) and siRNA specific to FOXM1 were transfected into U2OS-C3 or MIA PaCa-2 cancer cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s recommendations.

**Immunofluorescence and confocal microscopy.** Cells grown on cover slips were fixed and permeabilized with methanol and acetone (1:1) at -20°C for 10 min. Fixed cells were incubated in blocking buffer (1% bovine serum albumin in PBS) for 30 min, then with primary antibodies in blocking buffer for 1 hr at room temperature. Next, cells were washed 3 times in PBS and incubated with Alexa Fluor fluorochrome conjugated secondary antibodies (Invitrogen) for 1 hr at room temperature. Then, cells were washed 3 times in PBS and cover slips were mounted using ProLong® Gold antifade mounting medium with DAPI (Invitrogen). Fluorescence was detected using confocal laser scanning microscope (Zeiss, Inc.) with a magnification of 63X. The following antibodies were used: FOXM1 (Santa Cruz), NPM (Santa Cruz),...
T7 (Novagen), Alexa Fluor® F(ab′)2 fragment of goat anti-mouse IgG(H + L) 633 and goat anti-rabbit IgG(H + L) 488 (Invitrogen), both at 1:1000 dilution in blocking medium.

**Anchorage-dependent growth assay (colony-forming assay).** 2 x 10³ cells were plated on 100 mm dishes in duplicates. After 10 days cells were stained with crystal violet. Quantification of colonies was done by using ImageJ.

**Anchorage-independent growth assay (soft agar assay).** 1.5 x 10⁴ cells per well were plated in triplicates in six-well plates in 0.35% agarose on a 0.7% agarose bed. After 15 days colonies were counted.

**Xenograft animal experiment.** Four weeks old male athymic nude mice were purchased from Taconic. Bilaterally, 1x10⁶ MIA PaCa-2 vector control and FOXM1 (shFOXM1) or NPM (shNPM) stable knockdown cells per site in 100 µl matrigel (BD Biosciences) were injected subcutaneously in the flank region. After tumors became palpable tumor size was measured once a week using a Vernier caliper and tumor volume was calculated with the formula: (length x width x height)/2. At the end of the study, mice were sacrificed by CO₂ inhalation followed by cervical dislocation, and tumors were excised. All animal experiments were in accordance with and approved by the Animal Care and Use Committee of the University of Illinois at Chicago.

**Statistical analysis.** Statistical analysis was performed with Microsoft Excel using the Student t test (two-tailed). P values of <0.05 were considered to be statistically significant.

**Results and Discussion**

**FOXM1 and NPM interact in human cancer cell lines.**  
To identify proteins that may interact with FOXM1 we utilized human osteosarcoma U2OS-C3 cell line with doxycycline-inducible FOXM1 (40). Cells were treated with thiazole antibiotic/proteasome inhibitor thioestrepton (45) to increase exogenous FOXM1 level. Following treatment, cells were immunoprecipitated with FOXM1 specific antibody and the immunoprecipitates were purified by TCA-acetone and analyzed by mass spectrometry. The results of this experiment suggested that FOXM1 interacts with NPM. In order to confirm the interaction between FOXM1 and NPM, DMSO (control) and thioestrepton treated U2OS-FOXM1 cells with constitutive expression of FOXM1 protein were immunoprecipitated with NPM specific antibody and the SDS-PAGE separated immunoprecipitates were immunoblotted for FOXM1 and NPM. Thiazole antibiotic/proteasome inhibitor thioestrepton stabilizes exogenous, but suppresses endogenous FOXM1 (45,46). As shown in Fig. 1A NPM is immunoprecipitated in both control and drug-treated cells. In thioestrepton treated cells, stabilized exogenous FOXM1 co-immunoprecipitates with NPM confirming their interaction.

To determine whether FOXM1 and NPM may interact physiologically in human cancer cells, we performed reciprocal co-immunoprecipitations with FOXM1 and NPM antibodies under stringent conditions followed by immunoblotting for NPM and FOXM1. In MIA PaCa-2 human pancreatic cells, immunoprecipitation with NPM specific antibody pulled-down FOXM1 along with NPM, while FOXM1 specific antibody pulled down NPM along with FOXM1 (Fig. 1B). These data suggest that NPM and FOXM1 interact in human cancer cells. To identify the regions of FOXM1 that are responsible for interactions with NPM, we performed pull-down experiments. T7-tagged full length (1-748) and truncated (1-688 and 1-195) FOXM1 plasmids were transiently transfected into MIA PaCa-2 cells. Cell lysates were used in pull-down assays with GST-NPM (FL) bound to glutathione-sepharose beads. The pulled-down product was detected by immunoblotting with T7 specific antibody. As shown in Fig. 1C, GST-NPM binds to both full length (1-748) and C-terminal deleted (1-688) FOXM1, but not to the N-terminal 1-195 part of FOXM1. These data
suggest that the region of FOXM1 located between amino acids 195 and 688 is responsible for binding to NPM (Fig. 1C, F).

To identify the regions of NPM that are responsible for interacting with FOXM1, we performed additional pull-down assays using full length and truncated mutants of NPM protein fused to GST. T7-tagged full length FOXM1 plasmid was transiently transfected into MIA PaCa-2 cells. Cell lysates were used in pull-down assays with full length and truncated GST-NPM bound to glutathione-sepharose beads. Immunoblotting with T7 specific antibody was used to detect the pulled-down product. While the full length (1-295) and truncated (1-259, 187-295, 187-259) GST-fused NPM proteins pull down T7-FOXM1, GST-NPM 1-117, 1-186 and 260-295 or GST alone do not. This clearly shows that the heterodimerization domain of NPM (residues 187-259) (Fig. 1D, E) is essential for interaction with FOXM1. This region of NPM is also crucial for binding to several other proteins such as c-Myc (30), p21 (33), p53 (32), and ARF (31). Taken together, these data suggest that NPM via its heterodimerization domain binds to sequences located in the transactivation domain (TAD) of FOXM1 (Fig. 1E, F).

**NPM modulates FOXM1 level in human cancer cell lines.**

Since we found that NPM and FOXM1 interact in different cell types, we explored whether modulating NPM protein levels may affect FOXM1. Five different NPM shRNA plasmid constructs (#68-72), as well as a control vector (pLKO.1) were transiently transfected in U2OS-C3 cells and cell extracts were analyzed for NPM and FOXM1 protein levels by immunoblotting. We found that the NPM shRNAs downregulate NPM protein expression to different levels with shRNA#70 being the most potent (Fig. 2A). In addition, we found that knockdown of NPM by the different shRNAs leads to the downregulation of FOXM1 protein level (Fig 2A).

Using lentiviral transduction of shRNA#70, NPM was also knocked down transiently in both breast MCF-7 and pancreatic MIA PaCa-2 human cancer cell lines. We found that transient NPM knockdown results in marked downregulation of FOXM1 protein levels in both cell lines (Fig. 2B - E). Similarly, stable NPM knockdown in these cell lines leads to the suppression of FOXM1 protein expression (Suppl. Fig. 1). These data suggest that binding to NPM may be required for sustaining FOXM1 protein levels in human cancer cells.

**FOXM1 and NPM co-localize in human cancer cell lines, but not in normal cells.**

To examine the interaction between FOXM1 and NPM in normal cells we employed BJ foreskin normal human fibroblasts. In this cell line we observed very weak expression of both FOXM1 and NPM (Fig. 3A) by immunofluorescent staining. Immortalization of the cell lines leads to increasing expression of FOXM1 and NPM, and to their co-localization (Fig. 3A). Interestingly, transient NPM knockdown in immortal BJ cells (BJ-IM-shPM) leads to significant down-regulation of FOXM1 expression, which is comparable to the levels observed in wild type BJ cells (Fig. 3A). These results suggest that NPM may be required for FOXM1 expression in immortal (and cancer) cells (Fig. 3A).

Next, we examined the localization of FOXM1 and NPM in human cancer cell lines. We found that FOXM1 is predominantly localized in the nucleus in MIA PaCa-2 pancreatic and MCF-7 breast human cancer cell lines (Fig. 3B). NPM is mainly distributed in nucleolar and nucleoplasmic regions, which agreed with previous reports (34) (Fig. 3A-B). Co-localization of FOXM1 and NPM is mostly found in the nuclear regions (yellow color) of MIA PaCa-2 and MCF-7 cancer cell lines (Fig. 3B). To further confirm that NPM knockdown leads to downregulation of FOXM1 we utilized MCF-7 breast cancer cell line with stable NPM knockdown (Suppl. Fig. 1) and performed immunofluorescent staining. We found that knockdown of NPM leads to downregulation of FOXM1 (Fig. 3B). These results support the notion that binding...
to NPM may be required for FOXM1 protein expression in cancer cells. To validate co-localization of FOXM1 and NPM we used two T7-tagged FOXM1 constructs, T7-FL-FOXM1 and T7-688-FOXM1 (Fig. 1) that interacted with NPM and one construct T7-195-FOXM1 (Fig. 1) that did not. Using T7 and NPM antibodies we found that only the constructs (T7-FL-FOXM1 and T7-688-FOXM1) that interacted with NPM shown by previous experiments (Fig. 1) co-localize strongly with NPM (yellow color; Fig. 4). In contrast, the construct T7-195-FOXM1 that was not able to interact with NPM does not co-localize with NPM shown by confocal microscopy (Fig. 4). Taken together, these data suggest that FOXM1 and NPM indeed co-localize in the nucleus of cancer cells.

To further examine if NPM regulates cellular localization of FOXM1 we utilized OCI/AML3, a leukemia cell line where NPM is mutant and it is localized in the cytoplasm (42). In OCI/AML3 cells, FOXM1 has unusual cytoplasmic localization and it mainly co-localizes with mutant NPM in the cytoplasm (Fig. 5A). However, in the NPM-knockdown OCI/AML3 cells (Fig. 5A, B) it appears to be a change in the distribution as well as amount of FOXM1 (Fig. 5A, B), suggesting that NPM modulates FOXM1 localization and expression in human cancer cells.

**FOXM1 or NPM knockdown inhibits anchorage-dependent and independent growth in cell culture, and tumor growth in vivo**

The potential role of FOXM1 in tumorigenesis has been extensively documented in the literature. We examined how FOXM1 or NPM knockdown may affect the tumorigenicity of MIA PaCa-2 highly aggressive human pancreatic cancer cell line. First, we tested the colony forming ability of the pancreatic cells following transient depletion of FOXM1 or NPM by RNA interference in anchorage-dependent and independent conditions. Knockdown of FOXM1 or NPM inhibit colony formation in comparison with control (Fig. 6A). To assess how knockdown of FOXM1 or NPM may affect the anchorage-independent growth of pancreatic cells we performed soft agar assay. FOXM1 and NPM knockdown cells form less colonies compared to control cells. Furthermore, overexpression of FOXM1 in NPM knockdown cells reverses the decreased colony forming ability of MIA PaCa-2 pancreatic cancer cells (Fig 6A, B). These data suggest that depletion of FOXM1 or NPM has similar effects on the anchorage-dependent or -independent growth of human pancreatic cancer cells (Fig. 6A, B) and consequences of NPM depletion may be partially explained by its effect on FOXM1 stability. To evaluate the effect of FOXM1 or NPM knockdown on tumor growth in vivo, male nude mice were injected with 1x10^6 MIA PaCa-2 FOXM1 (shFOXM1) or NPM (shNPM) knockdown and with the corresponding control vector cells subcutaneously in the flank region on both sides. Tumor size was measured once a week after tumors became palpable. We found that FOXM1 or NPM knockdown strongly impairs the ability of pancreatic cancer cells to induce xenograft tumors in nude mice (Fig. 6C, D). These data suggest that FOXM1 expression is required for tumorigenicity of MIA PaCa-2 cells in vivo. It is possible that FOXM1 suppression as a result of NPM knockdown is partially responsible for the inhibition of xenograft tumor growth, suggesting that FOXM1 or FOXM1/NPM interaction may be considered as promising targets for anticancer therapy.

It has been shown that FOXM1 (2) and NPM (34) are overexpressed in a broad range of human malignancies. Our data suggest that the interaction between FOXM1 and NPM in human cancer cells may be necessary for FOXM1 expression. Since FOXM1 is considered as an oncogene that can induce tumorigenesis (13,47) the interaction between FOXM1 and NPM could be required for cancer progression. Therefore, targeting this interaction may represent a novel strategy for cancer treatment. Additional experiments are needed to validate this approach and to find
out whether it is suitable for patient treatment.

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Footnotes
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2. Abbreviations: FOXM1: Forkhead Box M1; NPM: Nucleophosmin; Thio: Thiostrepton; BJ-IM: BJ immortal; GST: Glutathione-S-transferase; GFP: Green fluorescence protein; ATCC: American Type Culture Collection; FL: Full length

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Figure legends
Figure 1. FOXM1 interacts with NPM in different human cancer cell lines.
A. U2OS-FOXM1 cells were treated with 10 µM of thiostrepton, lysed, immunoprecipitated with NPM antibody or control mouse IgG and immunoblotted for FOXM1 and NPM. B. Mid-log MIA PaCa-2 cells were lysed, immunoprecipitated with NPM or FOXM1 specific antibodies or mouse/rabbit IgG (as controls) and immunoblotted for NPM and FOXM1. C. T7-tagged full length (1-748) and truncated (1-688 and 1-195) FOXM1 constructs were transiently transfected into MIA PaCa-2 cells for 48 hrs. Cell lysates were pulled down by GST-NPM (FL)-sepharose beads, separated by SDS-PAGE and blotted with T7 specific antibody. D. Full length T7-FOXM1 plasmid was transiently transfected into MIA PaCa-2 cells for 48 hrs. Cell lysates were pulled down with full length and truncated GST-NPM-sepharose beads, separated by SDS-PAGE and immunoblotted with T7-specific antibody. E. Schematic of the domain structure of full length and various deletion mutant GST-NPM constructs. HoD: homodimerization domain; NLS: nuclear localization signal; HeD: heterodimerization domain; NBD: nucleic acid binding domain. F. Schematic of the domain structure of full length and different truncated deletion mutant T7-tagged FOXM1 constructs. DBD: DNA binding domain; TAD: transactivation domain.

Figure 2. NPM knockdown leads to downregulation of FOXM1.
A. U2OS-C3 cells were transfected with either pLKO1 (control) or NPM shRNA constructs #68-72. Forty-eight hrs after transfection cells were lysed and immunoblotted for FOXM1 and NPM. β-actin was used as the loading control. B. Protein levels of FOXM1 and NPM following transient NPM knockdown by lentiviral transduction in MCF7 human breast cancer cell line were determined by immunoblotting. β-actin was used as the loading control. C. The graphs show mean values ± SEM of three independent experiments. D. Protein levels of FOXM1 and NPM following transient NPM knockdown by lentiviral transduction in MIA PaCa-2 human pancreatic cancer cell line was determined by immunoblotting. β-actin was used as the loading control. E. The graphs show mean values ± SEM of three independent experiments.

Figure 3. FOXM1 and NPM show co-localization in the nucleus in immortal and cancer, but not in normal cells. A. BJ wild type, immortal and transient NPM knockdown human foreskin fibroblast cells were seeded on cover slips in 6-well plates and immunostained for FOXM1 and NPM. The confocal images were subjected to deconvolution and then merged. FOXM1 (green) and NPM (red) co-localized (yellow) in the nuclei. Nuclei were counterstained with DAPI (blue). B. MIA PaCa-2, MCF7 and MCF7 NPM knockdown cells were seeded on cover slips in 6-well plates and immunostained for FOXM1 and NPM. The confocal images were
subjected to deconvolution and then merged. FOXM1 (green) and NPM (red) co-localized (yellow) in the nuclei. Nuclei were counterstained with DAPI (blue).

**Figure 4. Co-localization of FOXM1 full length and truncated mutant constructs with NPM.** MCF7 breast cancer cells were transiently transfected with T7-tagged full length and truncated (1-1688 and 1-195) FOXM1 constructs. Forty-eight hrs following transfection immunostaining was performed for T7 and NPM. The confocal images were subjected to deconvolution and then merged. T7 (green) and NPM (red) co-localized (yellow) in the nuclei. Nuclei were counterstained with DAPI (blue).

**Figure 5. FOXM1 co-localizes with mutant NPM in the cytoplasm of human cancer cells.** A. Following transient NPM knockdown by lentiviral transduction in NPM mutant OCI/AML3 human leukemia cells immunostaining was carried out for FOXM1 and NPM. The confocal images were subjected to deconvolution and then merged. FOXM1 (green) and NPM (red) co-localized (yellow) in the cytoplasm. Nuclei were counterstained with DAPI (blue). B. Protein levels of FOXM1 and NPM following transient NPM knockdown by lentiviral transduction in OCI/AML3 NPM mutant human leukemia cell line were determined by immunoblotting. β-actin was used as the loading control.

**Figure 6. FOXM1 or NPM knockdown inhibits tumorigenesis in vitro and in vivo.** A. For the anchorage-dependent growth assay, FOXM1 and NPM were transiently knocked down, and also FOXM1 was transiently overexpressed in MIA PaCa-2 pancreatic cancer cells and 48 hrs after transfection 2x10^3 cells were plated in duplicates. Cells were stained with crystal violet after 10 days. Graph shows quantification of a representative experiment done in duplicates and representative plates are shown. B. For the anchorage-independent growth assay, FOXM1 and NPM were transiently knocked down, and also FOXM1 was transiently overexpressed in MIA PaCa-2 pancreatic cancer cells and 48 hrs after transfection 1.5x10^6 cells per well were plated on soft-agar plates in triplicates. Colonies were counted after 15 days. Graph shows the quantification of a representative experiment done in triplicates. C. MIA PaCa-2 pancreatic cells with stable FOXM1 knockdown and the corresponding vector control cells (1x10^6) were injected subcutaneously into the flank region on both sides of 4 week old male nude mice. Tumor size was recorded weekly by caliper measurement. Graph demonstrates differences in rates of tumor growth over the study period. Representative picture of the excised tumors is shown. D. MIA PaCa-2 pancreatic cells with stable NPM knockdown and the corresponding vector control cells (1x10^6) were injected subcutaneously into the flank region on both sides of 4 week old male nude mice. Tumor size was recorded weekly by caliper measurement. Graph demonstrates differences in rates of tumor growth over the study period. Representative picture of the excised tumors is shown.
Fig. 5

A

|        | Vector | shNPM |
|--------|--------|-------|
| FOXM1  |        |       |
| NPM    |        |       |
| Merge  |        |       |
| DAPI   |        |       |

B

| Oci/AML3 |        |       |
|----------|--------|-------|
| Vector   |        |       |
| shNPM    |        |       |
| FOXM1    |        |       |
| NPM      |        |       |
| β-actin  |        |       |
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