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HN1 expression contributes to mitotic fidelity through Aurora A-PLK1-Eg5 axis

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Abstract

Hematological and neurological expressed 1 (HN1) is homolog of Jupiter protein from *Drosophila melanogaster* where it functions as a microtubule-associated protein. However, in mammalian cells, HN1 is associated partially with y-tubulin in centrosomes, Stathmin for stabilizing microtubules, and Cdh1 for regulating Cyclin B1 for cell cycle regulation. Moreover, HN1 overexpression leads to early mitotic exit as well. Other molecular functions and interactions of HN1 are not clear yet. Here, based on our previous analysis where HN1 was shown to cluster supernumerary centrosomes and maintain mitotic spindle assembly, we further investigated the role of HN1 in centrosome maintenance and mitotic fidelity in PC-3 prostate and MDA-MB231 mammary cancer cell lines. The maturation-associated roles of HN1 during cell division by examining the AuroraA-PLK1 axis involving a plus end kinesin, Eg5 as well as pericentriolar matrix protein (PCM1) as components of centrosomes were established. We found that HN1 co-localized to centrioles with Eg5 and Aurora A to suppress aberrant spindle formation to ensure the fidelity of centriole/centrosome duplication when overexpressed. Consistently, depleting the HN1 expression using siRNA or shRNA resulted in an increased number of dysregulated mitotic spindle structures, where Aurora A as well as PLK1 co-localizations with Eg5 and PCM1 were disrupted. Further, the PLK1 and Aurora A kinase's phosphorylations also decreased, confirming the hypothesis that the cells struggle in mitotic progression, display nuclear and cytokinetic abnormalities with supernumerary but immature mononucleated centrosomes. In summary, we described the role of HN1 in centrosome nucleation/maturation in PLK1-Eg5 axis and concomitant mitotic spindle formation in human cells.

KEYWORDS
Aurora a, cell cycle, Eg5 kinesin, HN1, PLK1

Abbreviations: APC/C, anaphase promoting complex/cyclosome; ATCC, American Type Culture Collection; BSA, bovine serum albumin; Cdk1, cyclin-dependent kinase 1; DMEM, Dulbecco's modified Eagle medium; Eg5/Kif11, kinesin 11; FBS, fetal bovine serum; HN1 ORF, HN1 open reading frame; HN1, hematological neurological expressed 1; IF, immunofluorescence; KD, knockdown; MAP, microtubule-associated protein; MTOC, Microtubule Organizing Center; OE, overexpression; PBS, phosphate buffered saline; PCM1, pericentriolar matrix protein 1; PI, propidium iodide; PLK1, polo-like kinase.

1 | INTRODUCTION

Initially identified in mouse embryonic tissue by Tang et al. (1997), Hematological and Neurological Expressed 1 (HN1) was recognized for its crucial function in mouse brain neurodevelopment. Subsequent studies have confirmed high levels of HN1 expression in multiple human tissues, with particularly prominent expression in the human brain. Also referred to as Jupiter microtubule-associated homolog 1 (JPT1), is a highly conserved protein with ubiquitous expression across multiple tissues (Karpova et al., 2006; Zhou et al., 2004). Notably, HN1 has been found to be upregulated in various cancers, producing growing interest in its role in tumorigenesis and progression (Li et al., 2024). It exhibits a high degree of conservation across vertebrates (Varisli et al., 2011) plays a pivotal role in regulating signaling pathways, that is essential for cell growth and development through its control of cell cycle and apoptosis (Li et al., 2024). Significantly, HN1 has been linked to the tumor growth and progression implying its contribution to tumorigenesis with its overexpression (OE) commonly observed in various cancers (Pan et al., 2021). The oncogenic effects of HN1 are mediated through multiple mechanisms including signaling pathway activation, gene expression regulation, cytoskeletal dynamics modulation, and RNA processing regulation (Casey et al., 2018; Goto et al., 2006, 2012).

The HN1 gene expresses a ubiquitously encoded small protein (154-amino acid [aa]) in vertebrates (Varisli et al., 2012; Zhou et al., 2004). No previously defined functional domains or motifs have been clarified in the primary structure of the HN1. The protein is also notable for the absence of an HN1 homolog other than a protein called HN1L (with a 30% homology). Previous studies have reported that the knockdown (KD) of HN1 using siRNA-mediated silencing increases melanin secretion in melanoma cells (Laughlin et al., 2009) and delays the G1/S transition in both melanoma and prostate cancer cells (Laughlin et al., 2009; Varisli et al., 2011). In addition, these studies have shown that p21, cyclin D1, and cyclin B1 levels are increased upon partial HN1 depletion (Laughlin et al., 2009; Varisli et al., 2011). HN1 has previously been reported as a partially centrosome-localized protein, interacting with γ-tubulin for maintaining the integrity of microtubule organizing centers (MTOCs) in Prostate cancer cells (Varisli et al., 2021).

The correlation of HN1 expression being linked to tumorigenesis and its involvement in biological processes such as cycle regulation, microtubule dynamics, and de-differentiation pathways, prompted us to investigate HN1 further with cellular models of aggressive cancer such as PC-3 for Prostate cancer and MDA-MB231 for Breast cancer. These models lacking p53, are prone to centrosome aberrations and are considered good candidates to study centrosome-related cell survival mechanisms (Conduit et al., 2015). Centrosomal proteins are screened to exhibit disordered regions and coiled coil regions with relatively higher number of exons (Dos Santos et al., 2013; Kuhn et al., 2014). HN1 does exhibit disordered structure with secondary coiled coil domains (Varisli et al., 2021); however, it is a relatively smaller protein as compared to generic centrosome-related proteins. With disordered proteins, it is well known that their interactions are dynamic and not rigid (Csizmok et al., 2016), therefore, even though HN1 was physically found to be associated with centrosome-components throughout cell cycle, not all HN1 is localized on centrosome solely (Varisli et al., 2021). However, functional evidence does indicate that HN1 is involved in centrosome maintenance, but the mechanism is unclear yet.

HN1 interacts with Stathmin 1 for destabilizing microtubules in aggressive thyroid cancer cells (Pan et al., 2021). HN1 protein levels are also regulated via microtubule dynamics as shown previously in Neuroblastoma cells (Özar et al., 2024). Even though HN1 homolog in *Drosophila melanogaster* is a protein named Jupiter, which is a microtubule-associated protein (MAP), HN1 has not been shown to interact with microtubules in biochemical assays. HN1 co-expression network has microtubule motor proteins including kinesins which are plus end-directed microtubule motor proteins (Varisli et al., 2021). Moreover, HN1 protein is phosphorylated in mitosis and its temporally induced OE leads to early mitotic exit as well (Javed et al., 2023). Most recently, a genetic screen identified HN1 as a player in regulating mitotic tubulin levels. The same study demonstrated that HN1 together with its ortholog HN1L are required for microtubule destabilization as their depletion increases mitotic spindle tubulin mass (Su et al., 2024). Therefore, apart from centrosome maintenance, HN1 has a role in spindle or microtubule dynamics as well.

HN1 interacts with Cdh1 for Cyclin B1 degradation and itself is a potential substrate for Cdk1 (Javed et al., 2023). Cdk1 together with PLK1 kinase acts for centrosome maturation to ensure mitotic fidelity (X. Zhang et al., 2009). As mentioned previously, we identified that HN1 co-expresses with mitotic motor proteins, therefore from our preliminary analysis, we chose Eg5 as it is required for centrosome maintenance (She et al., 2022), to clarify the mechanistic axis of HN1 involvement in mitotic regulation. Moreover, Eg5 regulation of centrosome separation and maturation is controlled via activities of PLK1 and Cdk1 (Smith et al., 2011). However, the mechanism for centrosome maturation is still required to be understood further with possibly unexplored molecular players missing from this pathway. Hence, HN1 is an interesting protein with possibly multiple functions where centrosome maintenance role of HN1 could be due to microtubule stabilization and centrosome maturation. Therefore, in this study, we used the PC-3 and MDA-MB231 cell lines, to demonstrate the role of HN1 on centrosome maturation and polarization of the microtubules in mitotic division.

2 | MATERIALS AND METHODS

2.1 | Cell culture propagation and synchronization

PC-3 and MDA-MB231 (both p53-negative) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were propagated as recommended in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2. Cells

were checked routinely for mycoplasma contamination. Cells were treated with cell cycle inhibitors to induce arrest at either S or G2/M using 20 μM Thymidine (double block as appropriate) and/or 165 nM nocodazole (at 16–24 h), respectively. Although it is known that most cells arrest at the G2/M transition after nocodazole treatment (due to microtubule depolarization), some cells remain able to progress beyond the checkpoint. To separate the cells at G2 and prometaphase, a mitotic shake-off was performed after the nocodazole treatment, when appropriate. Round cells that had detached from the surface were collected, examined under a light microscope, and used for further studies.

2.2 | Establishment of stable HN1 KD and HN1 OE cell lines

HN1 ORF was inserted into the pcDNA4-HisMax backbone as described previously (Javed et al., 2023; Varisli et al., 2021). SmaI and EcoRV (New England Biolabs (NEB), UK) enzymes were utilized to digest out the HN1 ORF from pcDNA4-HisMax-HN1 and inserted into pCW57.1 (a gift from Dr. Şerif Şentürk at IBG, Izmir, Turkey) which was prepared by deleting the CcdB fragment by using SalI (NEB) and NheI (NEB) enzymes. Blunt end ligation was performed using T4 DNA ligase (NEB), followed by the transformation into chemically competent DH5α cells. The pCW57-HM-HN1 plasmid was obtained after isolation using the Midi-prep kit (Qiagen, Germany). pCW57-HM-HN1 as a transfer vector along with the viral packaging plasmids obtained from Addgene such as pMDLg/pRRE, pRSV-Rev, and pMD2G were transfected into HEK293T cells grown in DMEM/F12 media (supplemented with 5% FBS) and normal cell culture conditions using PEI (1:3 DNA to reagent ratio optimized) as a transfection reagent (Transporter 5, Polysciences, Germany). Chloroquine (Sigma Aldrich, Europe) at the final concentration of 25 μM was added to the culture media to block endosome formation and increase the transfection efficiency. The viral supernatant was harvested at 72 h of posttransfection, pelleted by centrifugation at 16,000*g* overnight, and re-suspended in 100 μL phosphate buffered saline (PBS) solution. Polybrene (Sigma Aldrich, Europe) at a final concentration of 10 μg/mL was used to transduce target cells with pCW57-HM-HN1 lentiviral particles. Twenty-four to 48 h after transduction, the cells were allowed to grow in normal conditions for 24 h before puromycin (Sigma Aldrich, Europe) selection (2 μg/mL) for 6 days (determined after performing a kill-curve with titrating Puromycin concentrations from 1 to 10 μg/mL).

Optimized 1 μg/mL Doxycycline (Sigma Aldrich, Europe) was used for inducing HN1 expression in HN1-inducible stable cells.

For cell lines stably depleted for HN1, shHN1 in lentiviral backbone plasmid bought from Santacruz Biotechnology, was packaged in lentiviruses using the method explained above. The cells were transduced with shHN1 particles also as described for HN1 stably expressing cells. Following the selection, both cell lines were tested for OE as well as KD of HN1 expression through western blot analysis (Figure S1).

Additionally, HN1 ORF was fused with a Venus fluorescence expressing tag sequence to determine the HN1 localization within Doxycycline inducible cells. Briefly, HN1 was amplified using a forward primer with an EcoR1 site and reverse primer (without Stop codon) with a Not1 site and inserted into a pcDNA4-TO-Venus-Puro plasmid obtained from Addgene (44118). Moreover, the HN1-Venus fragment was then digested and inserted into the pCW57 backbone to generate a Tetracycline inducible HN1-Venus construct, from which stabilized PC3 cells were generated after lentiviral transduction and antibiotic selection as described above (Javed et al., 2023; Varisli et al., 2021).

2.3 | Antibodies

Antibodies against HN1 were purchased from Invitrogen (Carlsbad, USA). Antibodies against Cdk1, p-Cdk1$^{(T14Y15)}$, Cyclin B1, pH 3$^{(S10)}$, Cdc25C, p-PLK1, p-PLK1$^{(T210)}$, Aurora A, p-Aurora A$^{(T288)}$, Cdc20, Cdh1, Centrin2, Eg5, PCM1, Pericentrin and γ-tubulin, as well as non-specific IgG antibodies were purchased from Santa Cruz Biotechnology Inc. (Germany). The β-tubulin antibody was purchased from BioVision (Mountain View, CA, USA), and the β-actin antibody was from Sigma (UK, Europe). Anti-mouse and anti-rabbit Alexa405, 488, 532, and 594 conjugated antibodies were purchased from Invitrogen (Carlsbad, USA). The antibodies were used at concentrations of 0.2 to 1 μg/mL, where appropriate.

2.4 | Immunoblotting

Cells were lysed with ice-cold RIPA buffer (1% Nonidet P-40, 50 mM Tris–HCl [pH 7.4], 0.25% Na-deoxycholate and 150 mM NaCl) containing 1 mM NaF, 1 mM EDTA, 1 mM $Na3VO_4$ and complete protease and phosphatase inhibitors and cocktails (Roche, Germany), unless otherwise indicated. Proteins were separated on 10–15% SDS-polyacrylamide gels and were transferred to PVDF membranes (Amersham, UK) using a wet transfer blotter. Briefly, the membranes were blocked using TBS-T (Tris-buffered saline containing 0.1% Tween-20) containing 5% skim milk (w/v). The antibody incubations were performed using TBS-T containing 0.5% dry milk at RT for 1 h or at 4°C overnight. The membranes were developed using the ECL Plus reagent (Amersham, UK) for 5 min and were photographed using Kodak x-ray film in a dark room.

2.5 | Immunoprecipitations

Protein lysates were obtained for immunoprecipitation as mentioned for immunoblotting. The amount of protein lysate used for each immunoprecipitation reaction was 0.5 mg with one-hour preclearance performed in the IP-matrix (40 μL) obtained from Santa Cruz Biotechnology Inc. or Dynabeads protein G (10003D, Thermo Fischer Scientific, USA) and used according to the manufacturer's

recommendations. Briefly, lysates divided into two were used for the reaction with either a specific antibody against a protein of interest or a nonspecific immunoglobulin G for 4 h at 4°C. After the reaction, the IP matrix (40 μL) or magnetic beads (20 μL) were used to form complexes with the antibody in the reaction of the antibody and the lysate overnight at 4°C before washing with a RIPA-modified buffer. The complexes were washed thoroughly in a RIPA-modified buffer four times before denaturation of the samples in a Laemmli buffer (25 μL) for 5 min at 95°C. The solution was then run on SDS gel for protein separation and immunoblotted for target antibodies as specified previously (Javed et al., 2023).

2.6 | Flow cytometry

To determine the cell cycle distribution upon treatment with various inhibitors and transfections, propidium iodide (PI) staining was performed on the cells harvested by trypsinization and fixed in 70% ethanol in PBS and stored at −20°C for at least 24 h. Briefly, fixed cells were washed with PBS and incubated with 0.2% Triton X-100 in PBS for 5 min at room temperature on a shaker. The cells were pelleted by resuspending in 20 μg/mL RNAse A solution in PBS and incubated at 37°C for 30 min. Cells were pelleted again and resuspended in 1 μg/mL PI in PBS. Afterward, the cells were analyzed for DNA content on the C6 BD Accuri flow cytometer (Becton Dickinson, USA) and analyzed for cell cycle distribution on ModFit LTTM or Flowjo v10 software.

2.7 | Immunofluorescence labeling and microscopy

For immunofluorescence (IF) labeling and subsequent microscopy, after concluding the specific treatments, cells grown on coverslips were fixed in either cold methanol (100%) at −20°C for 30 min or paraformaldehyde (4% in PBS) at room temperature for 30 min, were washed with PBS, and were permeabilized in 0.2% Triton X-100 in PBS for 5 min at the shaker, before another washing. The 1% bovine serum albumin (BSA) prepared in PBS was used for blocking the coverslip for 5 min of incubation on a shaker at room temperature. Primary antibody dilutions were prepared in 1% BSA in PBS and added to the cells followed by incubation for 1 h in a humidified chamber at 37°C. Cells were washed with PBS four times before secondary antibody incubation for 20 min in the same conditions as primary antibody incubations. The secondary antibodies were either Alexafluor 594 (anti-mouse)- or Alexafluor 488 (anti-rabbit)-conjugated antibodies (Invitrogen, Carlsbad, USA). After incubation, cells were washed with PBS four times and treated with 70% ethanol for 1 min and 100% ethanol for 1 min. The cover glasses were then air-dried and mounted on glass slides with 0.5 to 1 μg/mL DAPI in 30% glycerol in PBS and analyzed immediately under a DM4000 LED B fluorescence microscope (Leica, Germany) as described previously (Javed et al., 2023). Leica imaging software was used with a 5.5 Mpix digital camera for capturing images.

2.8 | Statistical analysis

The data values are presented as the means ± standard error of the mean. The statistical analysis was conducted on either Microsoft Excel or GraphPad Prism 5 software packages. Differences in mean values between groups were analyzed using a two-tailed Student's *t*-test or ANOVA, where $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | HN1 is required for centrosome integrity

In our previous studies, we reported that the microtubule nucleation was influenced by HN1 expression (Varisli et al., 2021), whereas another study showed that HN1 regulates tubulin acetylation through STMN1 and induces migration in cancer cells (Pan et al., 2021). HN1 was first reported in *Drosophila melanogaster* as a microtubule-associating molecule, named JPT1. Since the kinesins are co-expressed with HN1/JPT1 as classified in in silico studies, and the mitotic polar MAPs required for sister chromatid separation (Varisli et al., 2021), we first studied the Eg5 (Kif11) as a putative HN1/JPT1 regulated kinesin required for centrosome stability. Eg5 localization and Aurora A (T288) phosphorylation at centrosomes were examined in HN1-OE (Figure 1a) and HN1-KD (Figure 1b) MDA-MB231 cells. In both conditions, Eg5 and Aurora A expressions were found to be decreased while the spindle poles clearly scattered in HN1 KD cells in comparison to controls. Both the vector control and shControl cells exhibiting bipolar nucleation sites were clearly identifiable with Eg5 expression at centrosome structures, with co-localizing of Aurora A (T288) phosphorylation in OE (HM-HN1) cells with higher HN1 expression. Concomitantly, the cells with KD-depleted HN1 expression showed disrupted smaller centrosome structures with Eg5 and p-Aurora $A^{(T288)}$ co-localizations. The results suggested that the HN1 expression abundance contributes to bipolar spindle formation for appropriate cell division. Furthermore, Eg5 immunoprecipitation with γ-tubulin was tested when HN1 was depleted and found that the HN1 KD resulted in disrupted Eg5 interaction with γ-tubulin in PC3 cells (Figure 2a). If this occurrence is related to decreased expression of Eg5, Cdk1 biochemical interactions with PLK1 and p-$PLK1^{(T210)}$ (Figure 2b), as well as Aurora A and Eg5 with PLK1 immunoprecipitations were also examined (Figure 2c). To be certain, Aurora A with PLK1 interaction was also re-examined in KD cells in comparison to HN1 OE PC3 cells (Figure 2d). Accordingly, we found that the HN1 depletion severely reduced the expressions as well as the interactions of the mitotic regulators. Since HN1 is a component of centrosomes (Varisli et al., 2021) and we consistently observed that the HN1-depleted cells have disrupted centrosomes, we investigated the subcellular localizations of Eg5, PLK1 as well as Aurora A by using IF staining in MDA-MB231 and PC3 cells as well. When shHN1 and shControl stabilized MDA-MB231 cells were used as genetic background, we observed that the Aurora A and Eg5 co-localizations to centrosomes are severely affected in HN1 depleted cells in

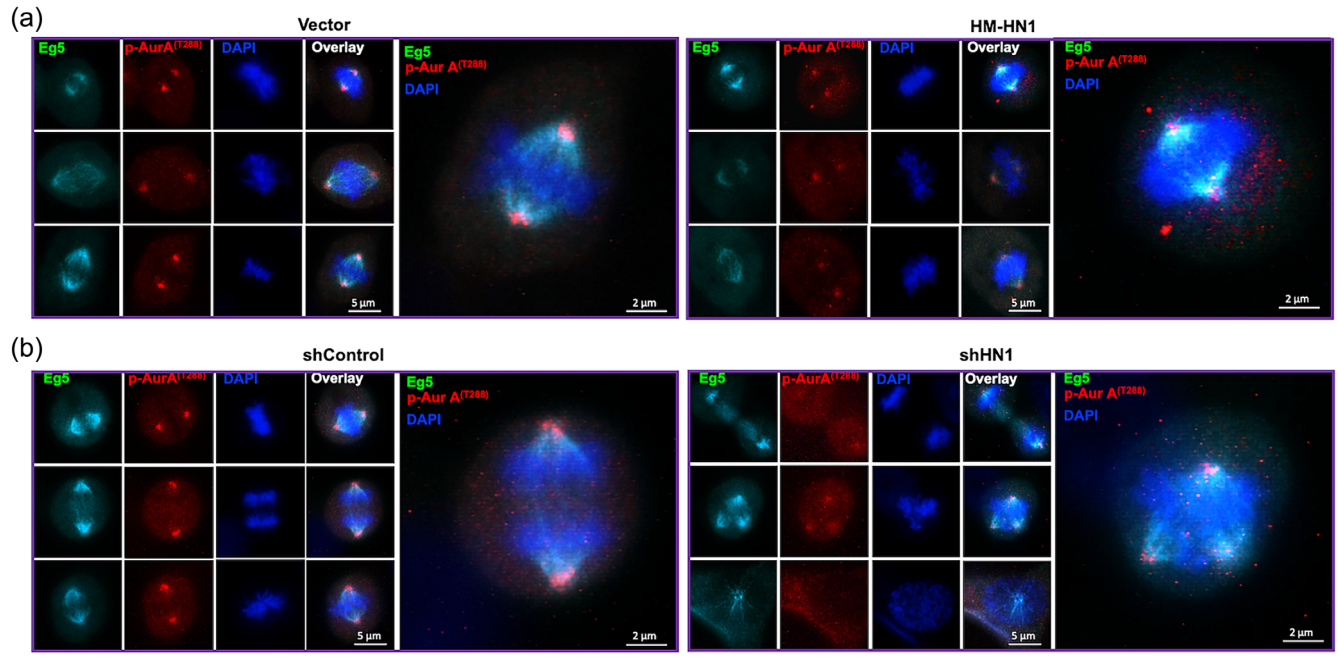

FIGURE 1 HN1 expression is critical in association of centrosome components for bipolar nucleation. (a) HN1 stabilizes p-Aurora A(T288) co-localization with Eg5 for centrosome maintenance and its overexpression does not change the polarity. (b) However, when HN1 is depleted using shRNA, centrosome number varies (usually more than 2) and these cells having centrosomes exhibit scattered Aurora A and Eg5 localizations to centrosomes. Also, mono- and multinucleated microtubule structured cells having chromatin alterations increase in MDA-MB231 cells.

comparison to controls (Figure 2e). Further, a centrosome stability component, PCM1 co-localization with Aurora A was studied, and found that the PCM1 localizations were also disrupted. The data suggesting that the centrosome stability and structural associations change in shHN1 cells in comparison to controls (Figure 2f) and corresponding enlarged images. Moreover, γ-tubulin and PCM1 co-localizations were examined in PC3 cells, the HN1 depletion negatively influenced the PCM1 localization to centrosomes (Figure 2g). To be certain that the localization of the components to centrosome is altered due to depletion of HN1 expression, PCM1 localization was restored using wtHN1 OE (Figure 2h) in prostate cancer cell line PC3. Overall, we suggest that HN1 is a centrosome stability factor that is required for reverting the phenotype if the cells suffer from centrosome anomalies at mitotic progression.

3.2 | HN1 contributes to bipolar spindle formation via Eg5 localizations

To investigate the link between perturbed HN1 expression and the formation of multiple centrosome cores with disrupted spindles, PC-3 cells were depleted for HN1 expression and blocked with nocodazole for 16 h. The cells were fixed and labeled with HN1 and Eg5-specific antibodies 1 h after being released from the nocodazole block. Interestingly, polar spindles marked with Eg5 were observed more frequently disrupted in the HN1-depleted cells (HN1 KD cells) than the controls (Figure 3a,b). Because mono- or multipolar spindle formations are subsequent to abnormal series of events that usually occur in the absence of centrosome duplication (Faragher & Fry, 2003; Haren et al., 2006) the data suggested that the HN1 depletion leads to abnormal spindles by abrogating the appropriate centrosome duplication or maturation. Remarkably, despite the presence of bipolar Eg5 localizations, most of the HN1-depleted dividing cells displayed multi Eg5 nucleation sites with spindles originating denser from one of the cores (Figure 3b). Presumably, this might be due to the disruption of the association between centrosome components where the Eg5 has an important role that was restored in wtHN1 expression in HN1-depleted cells (Figure 3c). These findings were also consistently observed in Venus-HN1 restorations when compared with control Venus' expression (Figure 3d), in which Aurora A localizations were also restored by Venus-HN1 (Figure 3e). Consistent with our previous observations, the depletion of HN1 produced scattered nucleation sites in mitotic poles, where microtubules nucleated heavily from one of these MTOCs (Varisli et al., 2021), that were partially but significantly restored by wtHN1 ($p < 0.05$; Figure 3f), or HN1-Venus expressions ($p < 0.01$; Figure 3g), which were carried out using lentiviral transductions.

Whether the excess centrosome centers are associated with impaired cellular and nuclear structures, we also studied the centrosome components, Centrin 2 as well as C-Nap1 localizations in PC-3 and MDA-MB-231 cells. In the depletion (KD) and presence (OE) of HN1 expression, the control cells showed well-organized Centrin 2 as well as C-Nap1 localizations at centrosome cores, while HN1-OE cells exhibited scattered and distorted formations indicating errors in centrosome and/or centriole structure (Figure 4a,b). In addition to corresponding distortions in terms of localizations, the HN1-OE cells

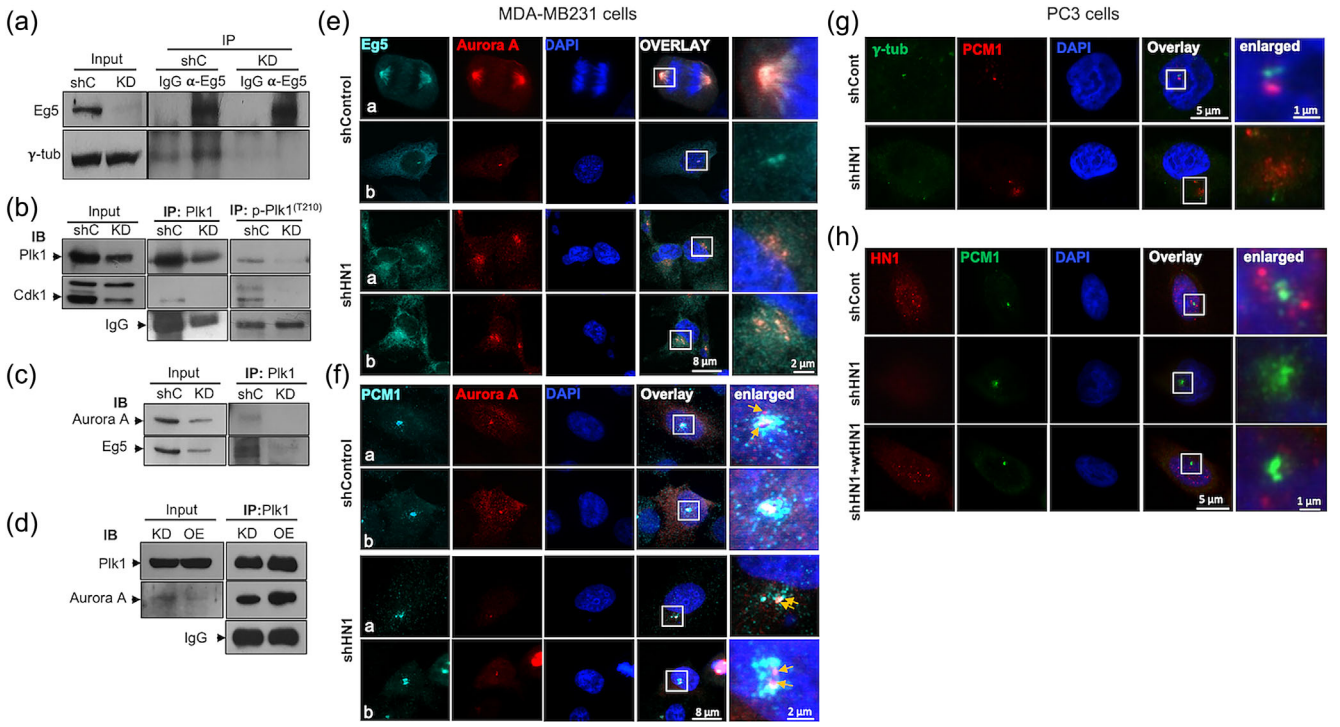

FIGURE 2 HN1 is required for centrosome maintenance. (a) Eg5 immunoprecipitation with y-tubulin was studied when HN1 was depleted. (b) Whether the HN1 depletion influences the putative Cdk1 biochemical interactions with p-PLK1(T210) and PLK1, (c) as well as Aurora A and Eg5 with PLK1 immunoprecipitations were performed at shControl, shHN1(knockdown [KD]) in PC3 cells. (d) Aurora A interaction was also re-tested in KD lysates in comparison to HN1 overexpression (OE) in PC3 cells. (e) shHN1 and shControl stabilized MDA-MB231 cells were used as genetic background. When HN1 was depleted in these cells, Aurora A and Eg5 co-localizations were disrupted. (f) The HN1 depletion led to diffuse PCM1 localization though the Aurora A co-localizes at centrosome. (g) y-tubulin and PCM1 co-localizations were also dramatically altered by HN1 depletion. (h) Venus-HN1 and HM-HN1 were used for transient restorations where the PCM1 disrupted localization in shHN1 cells were partially restored in both ectopic expressions of HN1. DAPI was used for DNA staining. The a-b are images of different cells from same experiment. Also, the images from white squares were cropped and enlarged. Scale bars are given.

showed a remarkable enlargement and accumulation at the proximal localizations, where the HN1-KD cells showed scattered and distorted C-Nap1 expressions far from each other suggesting that the centrosome components not only get disrupted structurally but also altered in the molecular interactions as well as the expression abundance (Figure 4c). This might lead to the loss of spindle cell morphology together with bipolarity, which was examined in PLK1 association with HN1 in nocodazole synchronized cells (Figure 4d). The y-tubulin association clearly decreased in C-Nap1 in KD cells in comparison to controls (Figure 4e). Overall, the data suggest that HN1 depletion results in supernumerary centrosomes in either nocodazole synchronized or asynchronized breast and prostate cancer cell lines, that can be restored by its OE.

3.3 | HN1 contributes to centrosome maturation

As the eukaryotic centrosomes are attached to perinuclear membrane and their maturation requires Separase at G2 phase of the cell cycle as well as Nek2 expression, we compared the expression patterns and found that the Separase was decreased in asynchronized cells, whereas increased in later hours of mitosis when HN1 was overexpressed. This suggested that the Separase expression might be lower at some cell cycle phases rather than the mitosis, though it is a critical event for the mitotic progression. Consistently, Nek2 expression slightly increased at 0 hours of Nocodazole release whereas the Cyclin B was found lower in comparison to control and continued through mitosis in HN1-OE cells in comparison to controls (Figure 5a). Furthermore, we examined the Separase, Eg5, Aurora A and PLK1 expressions as well as Aurora A and PLK1 phosphorylations in the mitotic and interphase cell lysates from KD and OE cells versus their controls (Figure 5b). As the competent centrosome requires temporal regulation of functional components throughout the cell cycle, HN1 regulates the subcellular localizations as well as the abundancy of the factors that are playing roles in centrosome maturation as well as their separation. HN1 OE stabilized NEK2 expression in both interphase and mitotic cells (Figure 5a), whereas HN1 depletion resulted in dramatic mis-localization of NEK2 from speckled shape to diffused pattern (Figure 5c) implying that the cells with higher or lower expression of HN1 are prone to carry centrosome defects into next cell cycle phases. It is perhaps accomplished by the mitotic division but with errors, which is observed in Nocodazole, and Thymidine synchronized cell with deregulated HN1 expression. The pronounced phenotypic effects such as the ratio of the

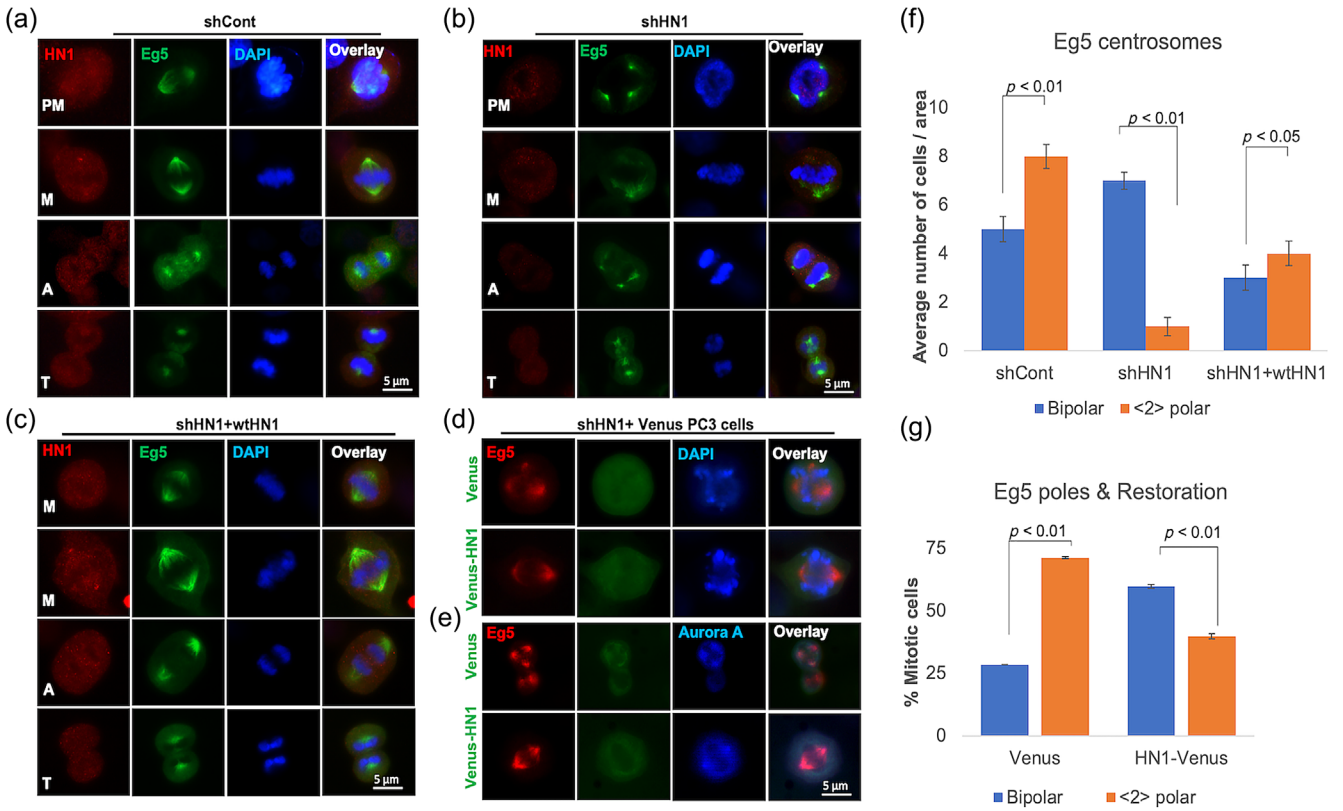

FIGURE 3 Eg5 plays an important role in microtubule polarity, which requires wtHN1 expression. (a) shControl stabilized PC3 cells having bipolar nucleated microtubules are shown as control in comparison to knockdown (KD) and restorations. (b) When HN1 is depleted (KD) in PC3 cells, Eg5 expressions and localizations demonstrate that the polarity is affected, which is examined at different phases of cell cycle. (c) When HN1 depletion is restored by wtHN1 ectopic expression, Eg5 localizations and polarized microtubule structures are restored. (d) Also, Venus and Venus-HN1 were also used for transient restorations. DAPI was used for DNA staining. (e) Moreover, Aurora A co-localized with restored Eg5 when Venus-HN1 were transiently restored. Aurora A is stained with Alexa405 and visualized at DAPI channel. (f) Bipolar versus mono- and multipolar cells were counted and analyzed statistically in shControl ($n = 114$), shHN1 ($n = 125$) cells and found statistically different ($p < 0.01$). When the HN1 restoration was performed, the number of cells with bipolar microtubules increased significantly ($p < 0.05$). (g) Additional restoration was carried out using either Venus-HN1 (71/554 mitosis/cell count) or Venus-HN1 (153/988 mitosis/cell count) in PC3 cells and found that the significant number of bipolar centrosomes were restored ($p < 0.01$). Different images are from different phases of mitosis. Scale bars were given.

ploidy were profoundly increased in shHN1 population in comparison to controls (Figure 6).

4 | DISCUSSION

HN1 since its discovery has been studied in the context of neural development or nerve regeneration (Goto et al., 2012) and tumorigenesis (Laughlin et al., 2009). It promotes dedifferentiation of retinal pigment epithelial cells (Goto et al., 2006) and is implicated in carcinogenesis (Zhang et al., 2014) and migration of cancer cells (Varisli et al., 2015). However, the precise mechanisms through which it exerts its activity remain elusive. The *D. melanogaster* homolog of HN1 called Jupiter, is considered a MAP and a microtubule marker. But HN1 has not been reported to be associated directly with microtubules. Recent reports suggested that HN1 proteins levels are dependent on microtubule stability (Özar et al., 2024) and its depletion results in stabilized microtubules (Su et al., 2024). Moreover, HN1 rescue after its depletion results in centrosome clustering in advanced Prostate cancer cells (Varisli et al., 2021), and its OE is linked with early mitotic exit (Javed et al., 2023). Here, we investigated HN1 in the context of centrosome stability and its maturation pathway where other mitotic players play important role in cell cycle progression and mitotic fidelity.

Centrosome maturation is an intricate process involving the timely localizations of important kinases and microtubule-associated motor proteins at MTOCs for subsequent formation of mitotic spindle assembly (Blanco-Ameijeiras et al., 2022; van Ree et al., 2016). One of such factors is Eg5 microtubule motor protein functioning as a kinesin motor for positioning of centrosomes and resultant polarity (Yu et al., 2022). HN1 previously was shown to co-express with kinesins (Varisli et al., 2021). Eg5 functions in centrosome maturation together with PLK1 and CDK1 (Smith et al., 2011), which were influenced significantly when HN1 expression is altered. Therefore, we performed functional experiments with changing HN1 levels to investigate Eg5 localizations with a mature centrosome component p-Aurora $A^{(T288)}$.

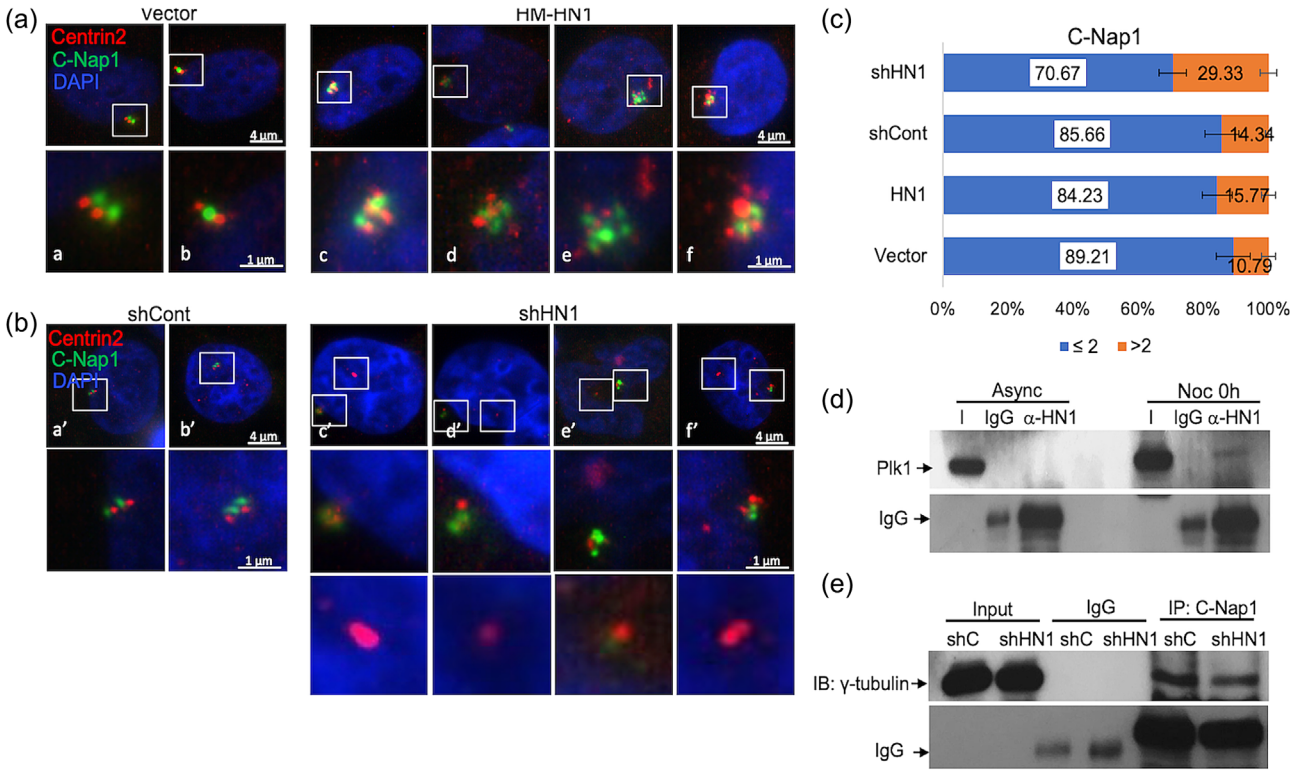

FIGURE 4 HN1 is required for centriole separation timely. (a) Vector and HM-HN1 cells exhibit that they have more Centrin 2 and C-Nap1 in larger centrosomes. Upper panels show the cells from different examples. Lower panels show the enlarged images of the white brackets more focused into centrosome structures. (b) Although shControl cells have less Centrin2 as well as C-Nap1 expressions, these cells represent bipolarity having exact numbers of Centrin2 and C-Nap1 labeled centrosomes. (c) Average number of cells having significantly less C-Nap1 localized centrioles in both shControl ($n = 114$) and shHN1 ($n = 125$) cells. HN1 depletion resulted significant number of disrupted centrioles. The mother centriole is stained with Centrin2 (Alexa594). C-Nap1 is stained with Alexa488 and DAPI was used for DNA staining. (a–f) and (a′–f′) are different images from different cells. The images from white squares were cropped and enlarged at bottom images. Scale bars were given. (d) Whether the HN1 depletion influences the putative PLK1 biochemical interaction as well as, (e) C-Nap1 interaction with y-tubulin, immunoprecipitations were performed in shControl, shHN1(knockdown [KD]) PC3 cells.

Both HN1 OE and depletion resulted in mis-localization of p-Aurora A$^{(T288)}$ and Eg5 with defective mitotic spindle prominent in HN1 depleted mitotic cells (Figure 1). Eg5 recruitment to centrosomes is required for centrosome maturation via Aurora A phosphorylation (Mierke, 2020). Moreover, Eg5 interaction with centrosome core protein y-tubulin is reduced in HN1-depleted cells, further implying that Eg5 is not recruited completely at centrosomes in HN1-depleted cells (Figure 2).

The major cell cycle and centrosome maturation player PLK1 is involved in regulating these processes via direct activity on Aurora A (Kalous & Aleshkina, 2023). Here, we showed reduced Aurora A interaction with PLK1 in HN1-depleted cells as compared to HN1 overexpressed cells, further demonstrating that HN1-depleted cells show a certain phenotype with centrosome pathway defects. These observations are consistent with previous findings where temporally induced expression of HN1 at G2, led to early mitotic exit, and consistently HN1 depletion may delay mitotic progression (Varisli et al., 2021) due to effects observed here. To properly coordinate the mitotic progression, mitotic activators, and their substrates with centrosomal and spindle processes must be appropriately forwarded to their localizations (Hoffmann, 2021). HN1 depletion results in multiple centrosomal defects, including collapsed spindles and multiple poles, disrupted chromatin structure, micronucleus formations including numerical defects (Javed et al., 2023; Su et al., 2024; Varisli et al., 2021).

We observed defective mitotic spindles when we studied HN1-depleted cells, and this phenotype could only be partially restored in HN1 rescue experiments (Figure 3). We examined structural components of centrosomes and found that PCM1 localization to y-tubulin is disturbed when HN1 is depleted via shHN1 (Figure 2), which we also observed in our previous studies (Varisli et al., 2021). Moreover, C-Nap1 and Centrin 2 were also investigated for their localizations, and it was observed that HN1-depleted cells exhibited distorted mother centrioles (Figure 4).

We further examined different mitotic factors and their temporally changing levels with HN1 OE and found that Nek2, as a centrosome player was also altered when HN1 was overexpressed. Nek2 is a crucial mitotic kinase that targets centrosomes and kinetochores, and regulates centrosome separation, formation of bipolar spindles, kinetochore-spindle attachments, and spindle assembly checkpoint

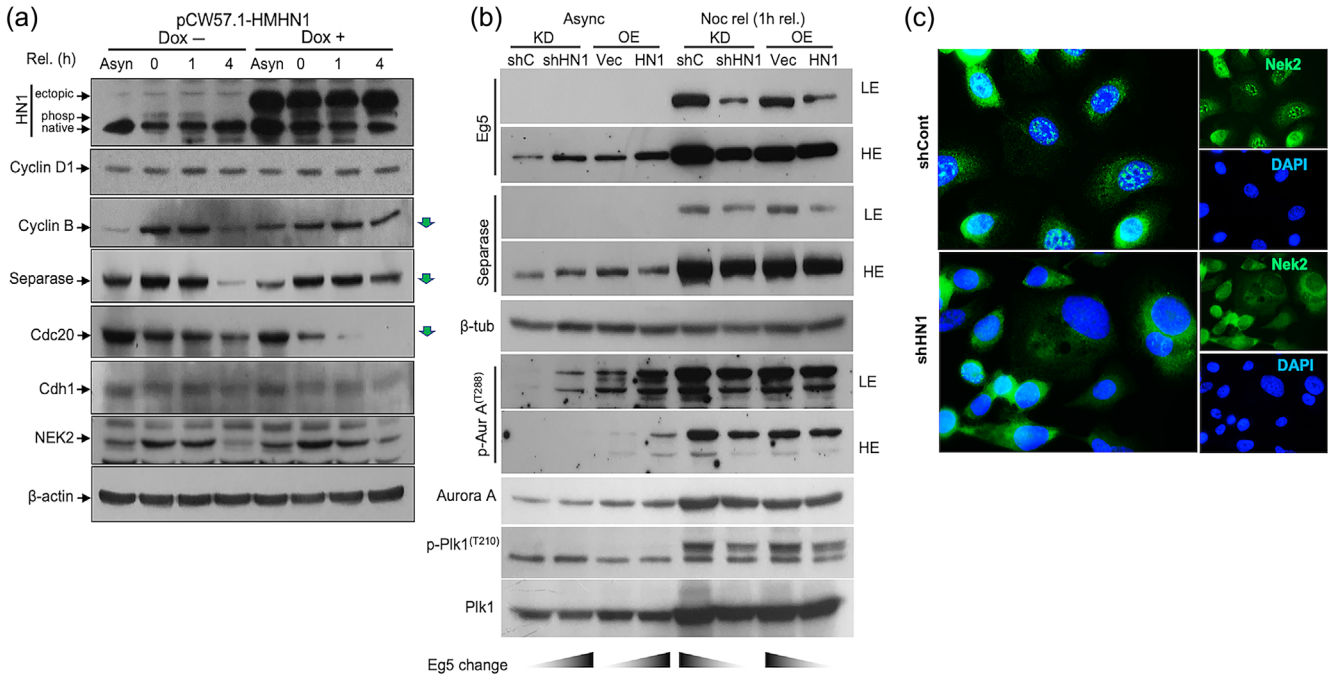

FIGURE 5 HN1 changes expression levels of Mitosis Promoting Complex (MPF) components from degradation and influence cell cycle as well as centrosome duplication in prostate cancer cell line PC3. (a) Cyclin D1, Cyclin B1, Separase, Cdc20 Cdh1 and Nek2 expressions were studied in doxocycline inducible HN1 expressing PC3 cells w and w/o doxocycline. When HN1 is overexpressed and released from G2/M transition, Cyclin D1 does not change while Cyclin B1 and Separase expressions as well as native HN1 expression stabilize through mitotic transition at late hours (4 h of nocodazole release). Also, Nek2 expression slightly increases in Nocodazole 0 time point in overexpression (OE) cells. (b) PC3 cells were either untreated (asynchronized cells) or treated with Nocodazole (16 h), washed and released 1 h before harvest. The cells were lysed and Eg5, Separase, B-tubulin, Aurora A, PLK1 expressions as well as p-Aurora A (T288) and p-PLK1(T210) phosphorylations were examined in western blots from shcontrol, shHN1 (knockdown [KD]), Vector and HM-HN1 (OE) cell lysates. (c) When HN1 is knocked down using shHN1, Nek2 nuclear localization substantially changed in comparison to shControl cells, where nuclei were stained with DAPI.

(Cappello et al., 2014). HN1 depletion leads to dramatically shifted phenotype for Nek2 localization and cellular diffusion (Figure 5). Phenotypes for spindle errors, mitotic irregularities, and centrosome maturation defects associated with HN1 depletion in mammalian cells, could be due to Nek2 activity as previously, therefore we suggested that HN1 has a putative substrate sequence for Nek2 kinase (Varisli et al., 2021). This can be an interesting hypothesis to study the molecular axis of HN1 activity comprehensively in future in the context of centrosome pathway.

The cell cycle dynamics were explored in HN1-depleted cells, and we confirmed that HN1-depleted cells exhibit delayed mitotic progressions in cells released from thymidine block (Figure 6). The data are consistent with our previous reports demonstrating that HN1 induction after G2 leads to early mitotic exit. Moreover, the nocodazole treated HN1-depleted cells showed the lethal accumulation of polyploid cells, which complies to chromosome and centrosome errors shown in previous studies as well (Javed et al., 2023; Varisli et al., 2021). Previously, we showed that HN1 as an interactor of Cdh1, one of the cofactors of APC/C, E3 ubiquitin ligase complex functioning as a major mitotic regulator. We explored Cyclin B1 degradation because of HN1 activity where HN1 binding to Cdh1 enhanced Cyclin B1 degradation (Javed et al., 2023). Now, a similar mechanism could be present for Eg5 degradation because Cdh1 is known to bind with multiple interacting partners for assisting in their degradation. Moreover, Eg5 is known to be degraded via Parkin E3 ligase which also function partly via Cdh1. These data lead to interesting hypothesis which can be explored in future.

In summary, this study showed that HN1 reduces interactions between Eg5 and Aurora A, where HN1 depletion results in mitotic spindle errors arising from mis-localization of Eg5 onto centrosome. HN1 depletion phenotypes further included dysregulated PLK1-Aurora A interaction and instability of PCM1-γ-tubulin binding. Moreover, HN1 was found in interaction with PLK1 during mitosis as well, where Nek2 speckles were lost when HN1 was depleted. Therefore, we conclude that HN1 is a centrosome component in mammalian tumor cell lines, which is required for the formation of bipolar microtubule spindles from functional centrosomes. When it is depleted in cell lines, localizations of centrosome components such as Eg5, Aurora A and Nek2 change, whereas Centrin 2, C-Nap1 and PCM1 dissociate from each other as well. The observations of these phenotypes are novel for HN1 depletion and therefore imply that HN1 plays important roles in mitotic spindle architecture and the control of centrosome function by contributing to Aurora A-PLK1-Eg5 localizations at the centrosomes.

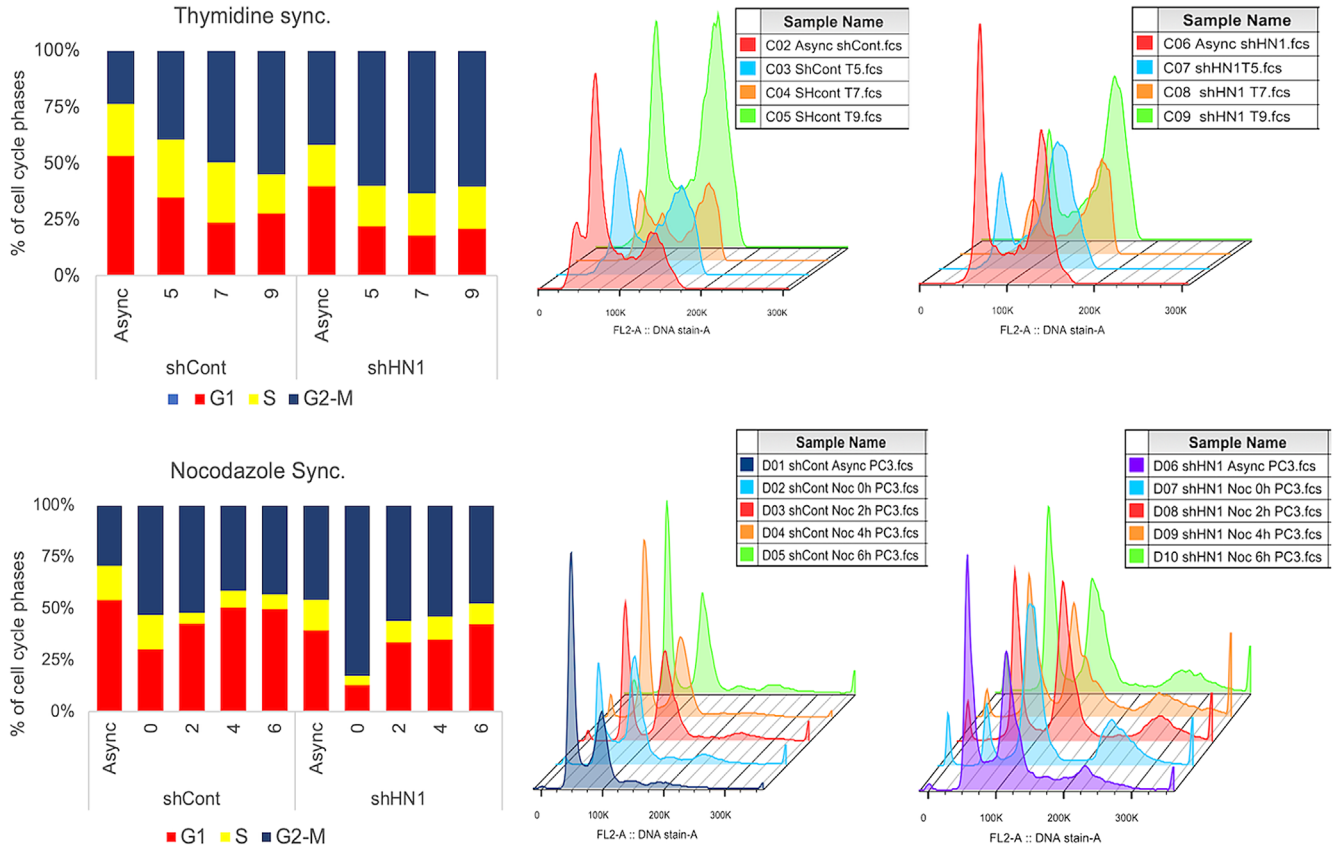

FIGURE 6 Flow cytometry data from (a) Thymidine and (b) Nocodazole synchronized cells after release represents significant percentage of cells are exhibiting ploidy when HN1 is depleted.

AUTHOR CONTRIBUTIONS

Conceptualization: K.S.K. *Methodology*: G.Ö. and F.S. *Software analysis*: G.Ö. and F.S. *Validation*: G.Ö. *Formal analysis*: F.S., G.Ö., and K.S.K. *Writing—original draft preparation—review and editing*: A.J. and K.S.K. *Visualization supervision*: K.S.K. *Project administration*: K.S.K. *Funding acquisition*: K.S.K. All authors have read and agreed to the published version of the article.

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The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The article does not report any data generation or analysis from previous published data.

CONSENT FOR PUBLICATION STATEMENT

Corresponding author is sole correspondent on behalf of coauthors.

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