Investigation of Nek2 Kinase Targets with Focus on Centrosome Clustering

by

Batuhan Mert Kalkan

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Koç University

Graduate School of Health Sciences

This is to certify that I have examined this copy of a doctoral dissertation by

Batuhan Mert Kalkan

and have found that it is complete and satisfactory in all respects,

and that any and all revisions required by the final

examining committee has been made.

Committee Members:

Prof. Ceyda Açılan Ayhan (Advisor)

Assist. Prof. Gözde Korkmaz

Assoc. Prof. Elif Nur Fırat Karalar

Assoc. Prof. Umut Şahin

Assoc. Prof. Hilal Yazıcı Malkoçoğlu

22-11-2023

I dedicate my thesis

To Mauro Emanuel Icardi Rivero

ABSTRACT

Investigation of Nek2A Kinase Targets with Focus on Centrosome Clustering

Batuhan Mert Kalkan

Doctor of Philosophy in Cellular and Molecular Medicine

Cancer cells, unlike normal cells, usually have extra centrosomes, which form multipolar spindles (MPS) and cause cell death. Nonetheless, they divide successfully and avoid the lethal implications of uneven genetic material segregation by clustering their extra centrosomes into two poles. Nek2A is a mitotic kinase that regulates a variety of mitotic events. In this study, we demonstrate that while reduction of Nek2A activity via knock-out, silencing or using specific inhibitors favours centrosome clustering, its overexpression unclusters extra centrosomes making cancer cells vulnerable to cell death. Interestingly, none of the centrosomal targets of Nek2 (C-Nap1, Rootletin or Gas2L1) or its targets that can induce genomic instability (TRF1 or HEC1) appeared to responsible for its action on clustering, suggesting that other targets may be involved in this process.

To investigate whether the effect of Nek2A on MPS is a novel pathway or acted in concert with other known unclustering factors, we tested HSET and NuMA. Our findings revealed that HSET's unclustering activity was complementary to NEK2A, implying an independent process, whereas the suppression of NuMA could reverse this effect. We also performed TurboID proximity labelling analysis, uncovering several potential Nek2A targets that were either situated at the centrosome or along microtubules, including NuMA. Although NuMA was in proximity, it did not co-IP with Nek2A. Intriguingly, we identified KIF2C as a new interaction partner of Nek2A and our subsequent analysis indicated that silencing it attenuated Nek2A activity on centrosome clustering.

In conclusion, Nek2A's role in centrosome clustering and the identification of new interaction partners like KIF2C provides a deeper comprehension of cancer cell biology and may offer innovative avenues for targeted cancer therapy.

ÖZET

Sentrozom Kümesi Üzerine Odaklı Nek2A Kinaz Hedeflerinin İncelenmesi

Batuhan Mert Kalkan

Hücresel ve Moleküler Tıp, Doktora

Normal hücrelerin aksine, kanser hücreleri genellikle ekstra sentrozomlara sahiptirler ve bu ekstra sentrozomlar çok kutuplu iğlerin (MPS) oluşmasına yol açar, bu da hücre ölümünü tetikler. Bununla birlikte, bu hücreler başarılı bir şekilde bölünmeyi başarır ve genomik materyalin eşitsiz ayrılmasının ölümcül sonuçlarından kaçınırlar, çünkü ekstra sentrozomlarını iki kutba birleştirirler. Nek2A, çeşitli mitotik süreçleri düzenleyen bir mitotik kinazdır. Bu çalışmada, Nek2A aktivitesinin azaltılmasının (knock-out, susturma veya özel inhibitörler kullanılarak) sentrozomların kümelendirilmesini teşvik ettiğini, aşırı ifadesinin ise ekstra sentrozomları kümesizleştirdiğini ve kanser hücrelerini hücre ölümüne karşı savunmasız hale getirdiğini gösterdik. İlginç bir şekilde, Nek2'nin sentrozomal hedefleri (CNAP1, Rootletin veya Centlein) veya genomik istikrarsızlığa neden olabilen hedefleri (TRF1 veya HEC1) kümelendirme üzerindeki etkisinden sorumlu gibi görünmedi, bu da diğer hedeflerin bu süreçte rol oynayabileceğini göstermektedir.

Nek2A'nın MPS üzerindeki etkisinin yeni bir yol olup olmadığını veya bilinen diğer kümesizleştirme faktörleri ile birlikte çalışıp çalışmadığını incelemek için HSET ve NuMA'yı test ettik. Bulgularımız, HSET'in kümesizleştirme aktivitesinin NEK2A'ya ek olarak olduğunu, bağımsız bir süreci ima ettiğini gösterdi, oysa NuMA'nın bastırılması bu etkiyi tersine çevirebilirdi. Ayrıca TurboID yakınlık işaretleme analizi yaptık ve sentrozom veya mikrotübüller boyunca bulunan çeşitli potansiyel Nek2A hedeflerini ortaya çıkardık, bunlar arasında NuMA da vardı. NuMA, yakınlıkta olsa da Nek2A ile birlikte çekim yapmamıştır. İlginç bir şekilde, KIF2C'yi Nek2A'nın yeni bir etkileşim ortağı olarak tanımladık ve ardından yaptığımız analiz, onun susturulmasının Nek2A'nın sentrozom kümesi üzerindeki etkisini azalttığını gösterdi.

Sonuç olarak, Nek2A'nın sentrozom kümesi üzerindeki rolü ve KIF2C gibi yeni etkileşim ortaklarının tanımlanması, kanser hücre biyolojisinin daha derinlemesine anlaşılmasının yolunu açabilir ve hedefe yönelik kanser tedavisi için yenilikçi yöntemler sunabilir.

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ABBREVIATIONS

CA	Centrosome Amplification
CDK	Cyclin Dependent Kinase
CE	Centriole Elongation
CIN	Chromosomal Instability
Co-IP	Co-Immunoprecipitation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DOX	Doxycycline
GFP	Green Fluorescent Protein
gRNA	Guide RNA
IF	Immunofluorescence
KD	Knock-down
KIFC1	Kinesin Family Member C1
KO	Knock-out
MIN	Microsatellite Instability
MPS	Multipolar Spindle
NEK2	NIMA related Kinase 2
OX	Overexpression
PCM	Pericentriolar Material
PCR	Polymerase Chain Reaction

PFA	Paraformaldehyde
PLK4	Polo-like Kinase 4
RNAi	RNA Interference
RT-qPCR	Real-time Quantitative PCR
SAC	Spindle Assembly Checkpoint
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
WT	Wild Type

1 INTRODUCTION

1.1 Centrosome

Centrosomes are the principal microtubule-organizing centers of animal cells, and they are well known for their function in microtubule nucleation regulation (Bornens, 2012). The centrosome is a tiny non-membranous organelle made up of two barrel-shaped structures known as centrioles. Each centriole is made up of a unique arrangement of nine sets of microtubule triplets (Fu et al., 2015). The intercentriolar linkage connects the elder centriole, known as the mother, and the younger centriole, known as the daughter, at their proximal ends (Kenney et al., 1997). The connection between mother and daughter centrioles has been demonstrated to be flexible and dynamic (Piel et al., 2000). Procentrioles arise from the side wall at the proximal ends of centrioles during centriole duplication. The mother centriole differs from the daughter centriole in that it has distal and subdistal appendages (Marshall, 2001).

Depending on their cell cycle phase, animal cells typically have one or two centrosomes. Mitosis produces two daughter cells, each of which inherits one centrosome from the mother cell. A mature and functional centrosome is made up of two centrioles, one dubbed the mother and the other the daughter, which are surrounded by a network of fibers and different regulatory proteins that make up the PCM (Doxsey, 2001). During the S phase of the cell cycle, centriole duplication occurs on the wall of the mother centriole, resulting in the formation of two centrosomes, each with a pair of centrioles. Phosphorylation of linker proteins between mother centrioles separates them at the conclusion of the G2 phase, and centrosomes function as the cell's mitotic spindle poles, facilitating bipolar and symmetrical division to ensure equitable distribution of genetic material (Conduit et al., 2015).

Centrosomes also influence other cellular processes in interphase cells, such as morphology and polarity (Godinho et al., 2009). In quiescent cells, centrosomes also serve as a foundation for the primary cilium (Sánchez & Dynlacht, 2016).

1.2 Centrosome Cycle and Its Regulation

Centrosome duplication happens once every cell cycle and is controlled by a variety of processes. Centrioles are replicated during S phase by the formation of a pro-centrioles. Centrosome duplication, like DNA replication, is semi-conservative. The templated model of pro-centriole creation, which proposes that the new centriole is created from an existing structure on the maternal centriole (Kochanski & Borisy, 1990).

The activity of the cyclins and the cyclin-dependent kinases (CDK) is involved with centrosome cycle regulation (Lacey et al., 1999). Cyclin expression levels change throughout the cell cycle; for example, CyclinE expression peaks at the G1-S, whereas CyclinA and CyclinB expression peaks during the G2-M phases (Siu et al., 2012; Yam et al., 2002). The CyclinE/CDK2 complex is one of the key regulators of centrosome duplication, which occurs in late G1-S (Lacey et al., 1999). CDK2 regulates both centrosome duplication and DNA replication and is activated by hyper-phosphorylation of Retinoblastoma protein (pRB) (Matsumoto et al., 1999). The CyclinE/CDK2 complex phosphorylates and dissociates nucleophosmin, delaying centriole splitting until late G1 (Okuda et al., 2000). CyclinA/CDK2 activity is also required for centrosome duplication (Meraldi et al., 1999). CyclinB/CDK1-mediated phosphorylation of Eg5 and Nek2-mediated phosphorylation of centriole linker proteins govern centriole linkage dissociation at the G2/M transition (Blangy et al., 1995; Fry, Meraldi, et al., 1998; Mardin et al., 2011; Mardin & Schiebel, 2012). Phosphorylation of pRB is also required for centrosome duplication in animal cells. Overexpression of E2F, a downstream effector of pRB, was found to be sufficient to overcome the effect of expression of a non-phosphorylatable mutant of pRB, demonstrating that E2F is the primary effector of the pRB pathway on centrosome duplication (Meraldi et al., 1999). Apart from being a fundamental regulator of the cell cycle, centrosomal proteins also control centrosome duplication. PLK4 is a centrosome duplication master regulator, and its activity is essential for the formation of a procentriole (Habedanck et al., 2005; J. Kleylein-Sohn et al., 2007). Many distinct centrosomal proteins have been discovered as being involved in pro-centriole formation at various stages, including HsSAS-6, CPAP, CEP135, CP110, and γ-tubulin at various phases of procentriole formation (J. Kleylein-Sohn et al., 2007).

STIL is another essential protein that contributes to centriole biogenesis, and PLK4, STIL, or HsSAS-6 depletion has been demonstrated to impede centrosome duplication (Arquint & Nigg, 2016; Arquint et al., 2012; Vulprecht et al., 2012). Independent research has shown that the interaction between PLK4 and STIL is essential for appropriate centriole biogenesis (Kratz et al., 2015; Moyer et al., 2015; Ohta et al., 2014). Furthermore, CEP63 has been found to be crucial in early centriole biogenesis, followed by HsSAS-6 recruitment (Brown et al., 2013).

Centriole elongation (CE), which occurs after procentriole development, is another critical phase in the centrosome cycle. CE is influenced by elements such as CPAP, CP110, and CEP120, as well as their actions and interactions. CPAP (centrosomal protein 4.1-associated protein) is essential for procentriole microtubule incorporation, while CP110, a distal end-capping protein, operates in CE (Pelletier et al., 2006; Schmidt et al., 2009; Yadav et al., 2016). CEP120 also interacts with CPAP and positively controls CE (Lin et al., 2013). FOP, CAP350, HsSAS-6, CEP170, ninein, C-Nap1, and CEP97 were discovered as CE regulators using a siRNA screen for centrosomal proteins (Korzeniewski et al., 2010).

Because centrosome biology is influenced by numerous variables, a disruption in any of them could result in abnormal centrosome numbers or centrosomal structural abnormalities, resulting in aneuploidy and chromosomal instability. Because centrosome amplification (CA) is a prevalent characteristic of tumors, it is critical to understand the underlying mechanisms that cause CA.

1.3 Centrosome Amplification (CA) in Cancer

Boveri (Boveri, 2008) discovered centrosome amplification in human cancers more than a century ago, but it wasn't until more than eighty years later that the connection between p53 loss and centrosome amplification was discovered (Fukasawa et al., 1996;

Tarapore & Fukasawa, 2002). Subsequent research built on this revelation, resulting in a more complete understanding of the variables that contribute to centrosome amplification in cancer. Supernumerary centrosomes have been found in clinical tumor samples (Chan, 2011), precancerous lesions (Pihan et al., 2003), and cancer cell lines (Marteil et al., 2018). Furthermore, centrosome amplification is associated with a poor prognosis in patients (Chan, 2011). The identification of specific mechanisms responsible for centrosome amplification in malignancies holds hope for the development of inhibitors targeting these CA mechanisms, which might be used to suppress cancer growth.

The long-standing debate has centered on whether centrosome amplification (CA) is a cause or a result of cancer. Recent study has thrown light on this subject, indicating that centrosome amplification can indeed promote carcinogenesis and tumor invasion under certain conditions (Sabat-Pospiech et al., 2019). One study, for example, found that CA can cause cancer in flies (Basto et al., 2008). Similarly, a recent study found that an increase in centrosome numbers increases tumorigenesis in a mouse model (Levine et al., 2017). Surprisingly, the presence of supernumerary centrosomes alone is sufficient to cause aneuploidy and the spontaneous formation of malignancies across multiple tissues (Levine et al., 2017). CA is also connected with increased tumour invasiveness. A fascinating study found that cells with additional centrosomes can drive the invasion of surrounding cells in breast cancer via paracrine signalling, with released IL-8 being identified as a key component for generating invasion via HER2 activation (Arnandis et al., 2018). This work demonstrates that supernumerary centrosomes can have a disproportionately large influence on tumour progression even in a heterogeneous tumour mass where only a small number of cells have them. Given that CA is increasingly connected to numerous aspects of tumour biology, understanding the processes that cause CA in tumours is critical.

The mechanisms underlying centrosome amplification in cancer can be divided into two categories: the overduplication of centrosomes caused by a loss of strict control over the centrosome biogenesis process due to genetic mutations and/or the overexpression of centrosome duplication factors (direct centrosome amplification); and other cellular events that lead to indirect centrosome amplification, such as cell fusions, PCM fragmentation and cytokinesis failure (**Figure 1.1**)

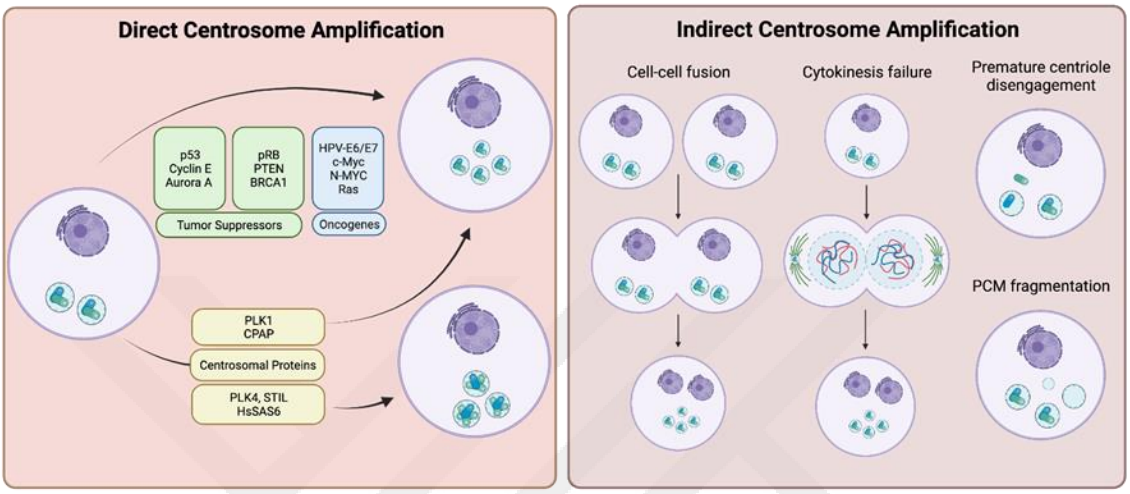

Figure 1.1: The modes of centrosome amplification in cancer cells. Image created with BioRender. Taken from (Kalkan et al., 2022)

The key mechanism leading to the presence of supernumerary centrosomes in cancer revolves around centrosome duplication cycle dysregulation (Kalkan et al., 2022). This cycle is extensively regulated by a slew of positive and negative regulators, including tumor suppressors such as p53, pRB, PTEN, and BRCA1. These genes' loss-of-function mutations have been associated to centrosome amplification (CA) in a variety of tumor types. Furthermore, CA is linked to the misexpression of proto-oncogenes such as E6, E7, Myc, and Ras. Furthermore, changes in the genetic and epigenetic landscape within malignancies can result in the overexpression of centrosome duplication factors such as PLK4, STIL, and HsSAS-6, directly leading to CA by the overproduction of additional centrioles.

Polo-like kinases (PLK1–5) are a family of five Ser/Thr kinases that play important roles in cell cycle progression, the centrosome cycle, and mitosis. Surprisingly, all PLK isoforms have been found to be centrosome-localized in humans (Zitouni et al., 2014). PLK4 is a pivotal centrosomal duplication protein that has been found to be overexpressed in cancers of various tissues and organs, including the colon, stomach, breast, prostate, and brain (Chan, 2011; Korzeniewski et al., 2012; Li et al., 2016; Liao et al., 2019; Shinmura et al., 2014; Tian et al., 2018). PLK4 expression has been found to be decreased in hepatocellular carcinoma (Pellegrino et al., 2010) and hematological malignancies (Ward et al., 2015). PLK4 mRNA expression is regulated by p53, which involves the recruitment of HDACs to the PLK4 promoter area (Li et al., 2005). The loss of p53 in malignancies is thought to contribute to enhanced PLK4 expression (Godinho & Pellman, 2014). Tumours that develop as a result of PLK4-induced centrosome amplification (CA) have lower p53 expression (Levine et al., 2017). Furthermore, the expression of the HPV-16 E7 oncoprotein has been shown to increase PLK4 levels (Korzeniewski et al., 2011). Several studies in p53-deficient mice have shown that overexpression of PLK4 can accelerate tumours growth in the epidermis by causing CA, tissue hyperplasia, and the loss of primary cilia (Sercin et al., 2016).

PLK4 activity is crucial in centriole biogenesis, with an increase in PLK4 levels resulting in the formation of additional centrioles surrounding a mother centriole, forming a distinct structure known as a rosette (or flower) centrosome due to its unique configuration (Cosenza et al., 2017; J. Kleylein-Sohn et al., 2007). Habedanck et al. followed up by reporting the production of "flower-like structures" in cells overexpressing PLK4 (Habedanck et al., 2005). Multiple centrioles within rosettes emerge during the S phase and remain throughout the S and G2 phases, demonstrating that rosette centrosomes act as cohesive structures that cycle in the same way as conventional centrosomes (J. Kleylein-Sohn et al., 2007). It is also reported that overexpressing PLK4, HsSAS6, and SAS4 in CHO cells at the same time resulted in the creation of rosette centrosomes (Kuriyama, 2009). Furthermore, researchers reported rosette centrosomes in primary tumour samples from multiple myeloma, glioblastoma, and colon cancer, demonstrating that the formation of these structures is observable in naturally occurring tumours rather than being a result of genetic or pharmacological cell manipulations (Cosenza et al., 2017). Their research also shown that overexpression of STIL can result in the formation of rosette centrosomes (Cosenza et al., 2017).

The aforementioned gene expression changes are direct pathways that lead to centrosome amplification (CA), however supernumerary centrosomes can also develop indirectly (Kalkan et al., 2022). Cell-cell fusion, cytokinesis failure, premature centriole disengagement, and PCM fragmentation are among the mechanisms involved. These methods induce CA by mechanisms other than enhanced centriole biogenesis or duplication. Cell-cell fusion occurs when two cells with their normal centrosome complement fuse, resulting in a single cell with double the quantity of DNA and twice the number of centrosomes. Cytokinesis failure occurs when a cell that has completed both DNA replication and centrosome duplication fails to complete cytokinesis near the end of mitosis, resulting in a single cell with twice the number of DNA and centrosomes. Premature centriole disengagement causes the daughter centriole to separate from the mother centriole too soon, resulting in a cell with normal DNA content but more than two centrosomes, at least two of which contain just a single centriole. PCM fragmentation occurs when large numbers of PCM components collect at random in the cytoplasm.

Fluorescent imaging has been used by researchers to determine the presence, prevalence, and impact of certain CA types. Antibodies specific to centriole proteins and centrosome components in fixed cells, as well as fluorescently-tagged proteins in living cells, have shown to be invaluable tools for distinguishing between CA types (Maiato & Logarinho, 2014). This dual method, which employs both live and fixed cells, is vital; for example, distinguishing between cell-cell fusion and cytokinesis failures involves live cell imaging and is thus critical to acquiring full insights.

Depletion of spindle-kinetochore proteins such as SKA3, CENP-E, or Cdc20 revealed an alternate mechanism for premature centriole disengagement. This depletion disrupted coordinated sister chromatid separation; a condition known as cohesion fatigue. Premature centriole disengagement and the development of multipolar spindles resulted from cohesion fatigue (Daum et al., 2011; Maiato & Logarinho, 2014). Furthermore, recovery from microtubule pharmacological inhibition by drugs such as colcemid or nocodazole resulted in the development of multipolar spindles with atypical centrioles at spindle poles. These poles might be either mono-centriolar or acentriolar. Furthermore, therapies including nitrous oxide exposure (Brinkley & Rao, 1973) and heat shock (Vidair et al., 1993) produced comparable results.

1.4 Consequences of Centrosome Amplification

Despite the negative impact that supernumerary centrosomes have on cell viability, the persistence of this phenotype in cancer cells prompts an investigation into the underlying causes and suggests that centrosome amplification (CA) may confer advantages on these cells or potentially turn a negative situation into a favourable one. If cancer cells survive, they gain benefits such as chromosomal instability, changes in cell polarity, increased motility, and intracellular signaling. All of these components can be helpful to cancer cells, enhancing their survival and proliferation potential. For example, CA-induced genomic instability might increase tumor heterogeneity, perhaps increasing compatibility with the microenvironment (Thompson & Compton, 2011). As a result, keeping extra centrosomes could be viewed as a survival and fitness mechanism for cancer cells, where the benefits of CA-induced changes balance the downsides of having extra centrosomes.

Extra centrosomes have been discovered in several cancer cells, both in situ and in culture, with assertions that they contribute to both genetic instability and tumor growth. Genomic instability, a hallmark of cancer progression, is mostly caused by chromosomal instability (CIN), while microsatellite instability (MIN) has been proposed as a possible contributor to CIN. CIN is a term that refers to the rate of change in chromosome number or structure. It is closely related to aneuploidy, which is defined as an aberrant deviation in chromosome count from the cell's normal ploidy level. Although numerous causal factors have been identified, the mechanisms underlying CIN and its function in aneuploidy remain unknown. CA has emerged as a significant player in CIN, alongside disruptions in mitotic checkpoints, incomplete chromosome condensation, and defective microtubule-kinetochore attachments. Whether CA directly initiates tumorigenesis or emerges as a result of other cell cycle aberrations remains unknown. Numerous studies have found CA in a variety of tumor types, including breast, prostate, ovarian, head and neck, lung, and bone, and have connected it to higher rates of CIN and aneuploidy. This shows that CA may be a significant contribution to carcinogenesis (Chan, 2011). Furthermore, the centrosome cycle involves a large variety of oncoproteins and tumour suppressors, raising the likelihood that cancer may cause changes in centrosome quantity and function, promoting CA and tumour growth (Fukasawa, 2007).

Although aneuploidy and centrosome amplification (CA) regularly co-occur in cancer cells (Zyss & Gergely, 2009), the existence of supernumerary centrosomes may appear to be a disadvantage to the cell at first. Extra centrosomes can cause multipolar divisions, in which a diploid cell is likely to lose genes critical to its survival. However, because cancer cells are frequently tetraploid, multipolar division is more likely to produce not just viable offspring but also progeny with beneficial chromosomal rearrangements (Nigg, 2002). CA-induced multipolarity would normally result in non-viability in diploid cells, however the clustering of these extra centrosomes into a bipolar spindle allows for continuing proliferation. These clustered spindles not only continue the proliferative cycle for an extended period of time, but they also act as the principal mechanism for inducing chromosomal instability (CIN) and aneuploidy (Ganem et al., 2009).

Centrosome defects are common in most aggressive cancers, where both CIN and cell cycle abnormalities caused by extra centrosomes can contribute to tumour formation. CA can increase CIN in the early stages of cancer, resulting to the accumulation of oncogenic mutations and the loss of tumour suppressors. It has been reported that centrosome anomalies may cause the progression of pre-invasive and early-stage lesions into aggressive and high-grade malignancies in cervical, breast, and prostate tissues (Pihan et al., 2003). Similarly, D'Assoro et al. claimed that CA can be used to predict the prognosis of breast cancers with genetic instability, indicating their aggressiveness (D'Assoro et al., 2002). Similar results were seen in bladder cancer and chronic myeloid leukaemia, demonstrating that CA-driven genomic instability enhances tumour growth, raises the clinical stage of the tumour, and predisposes it to recurrence (Giehl et al., 2005; Yamamoto et al., 2004).

CA's role in carcinogenesis has been demonstrated in mouse models (Sercin et al., 2016). PLK4 overexpression caused CA during epidermal development in both mice, resulting in p53 stabilization, apoptosis, and skin abnormalities. Mice became more susceptible to skin cancer when PLK4 overexpression was paired with loss of p53. Another study using transgenic mice that overexpressed PLK4, producing CA, found that carcinogenesis was accelerated in the absence of p53: cells in pancreatic and skin tissues demonstrated rapid proliferation, which was correlated with an increase in centrosome numbers (Coelho et al., 2015). CA was found to be a significant contributor to tumour growth in the absence of p53 function, and its impact was magnified significantly.

While the centrosome clustering is beneficial to cell viability, it is accompanied with aberrant chromosomal and microtubule attachments. Cancer cells containing additional centrosomes can produce transitory multipolar spindle assemblies that then shift to a pseudo-bipolar state via centrosome clustering (Nigg, 2002). This clustering mechanism culminates in the production of merotelic kinetochore attachments, which avoid detection by the spindle assembly checkpoint (SAC) and cause segregation problems (Silkworth et al., 2009). Merotelic attachments that escape the SAC most usually result in lagging chromosomes (Cimini, 2008), which are known to be a significant source of aneuploidy and CIN (Janssen et al., 2011). Furthermore, lagging chromosomes have been shown to give rise to micronuclei, which contribute to both DNA replication mistakes and DNA damage in daughter cells (Crasta et al., 2012; Hatch et al., 2013; Janssen et al., 2011). As previously stated, greater DNA damage is expected to enhance mutagenesis, which is a driving force in cancer. As a result, centrosome clustering following CA promotes tumor cells toward aneuploidy. As a result, CIN is a two-edged sword for cancer cells: it can either boost proliferation or restrict growth. The CIN-dependent increase in tumorigenesis rate is most likely due to an increased frequency of mutations in developing cancer cell subpopulations (Schvartzman et al., 2010). This condition of genetic heterogeneity caused by CIN allows cancer cells to acquire emergent characteristics that improve their chances of survival (Thompson & Compton, 2011). As a result, the degree of CIN becomes the key predictor of cancer cell survival. Lower degrees of genomic instability are tolerated and even beneficial to tumor growth, whereas larger levels of CIN are deleterious and potentially deadly to cancer cells (Weaver et al., 2007). To summarize, CIN may act as a strong selection pressure on cancer cells, causing them to retain their extra centrosomes.

1.5 Centrosome Clustering and Its Targeting

Centrosome clustering is commonly regarded as the key mechanism by which cells resist multipolar divisions, whether in normal or malignant tissue. As a result, understanding the complex mechanism of centrosome clustering has become a focus of intensive research. The phenomena of centrosome clustering was first identified in N1E-115 mouse neuroblastoma cells during both interphase (Brinkley et al., 1981) and mitosis (D. Ring et al., 1982), which have significantly elevated amounts of supernumerary centrosomes. Numerous review articles have discussed the potential therapeutic implications of centrosome clustering, with suggestions that inhibiting clustering with small molecules could induce multipolar divisions in cells with extra centrosomes, increasing the likelihood of cell death and decreasing tumorigenesis (**Figure 1.2**). To fulfil this therapeutic promise, a better knowledge of the precise mechanisms driving centrosome clustering is required.

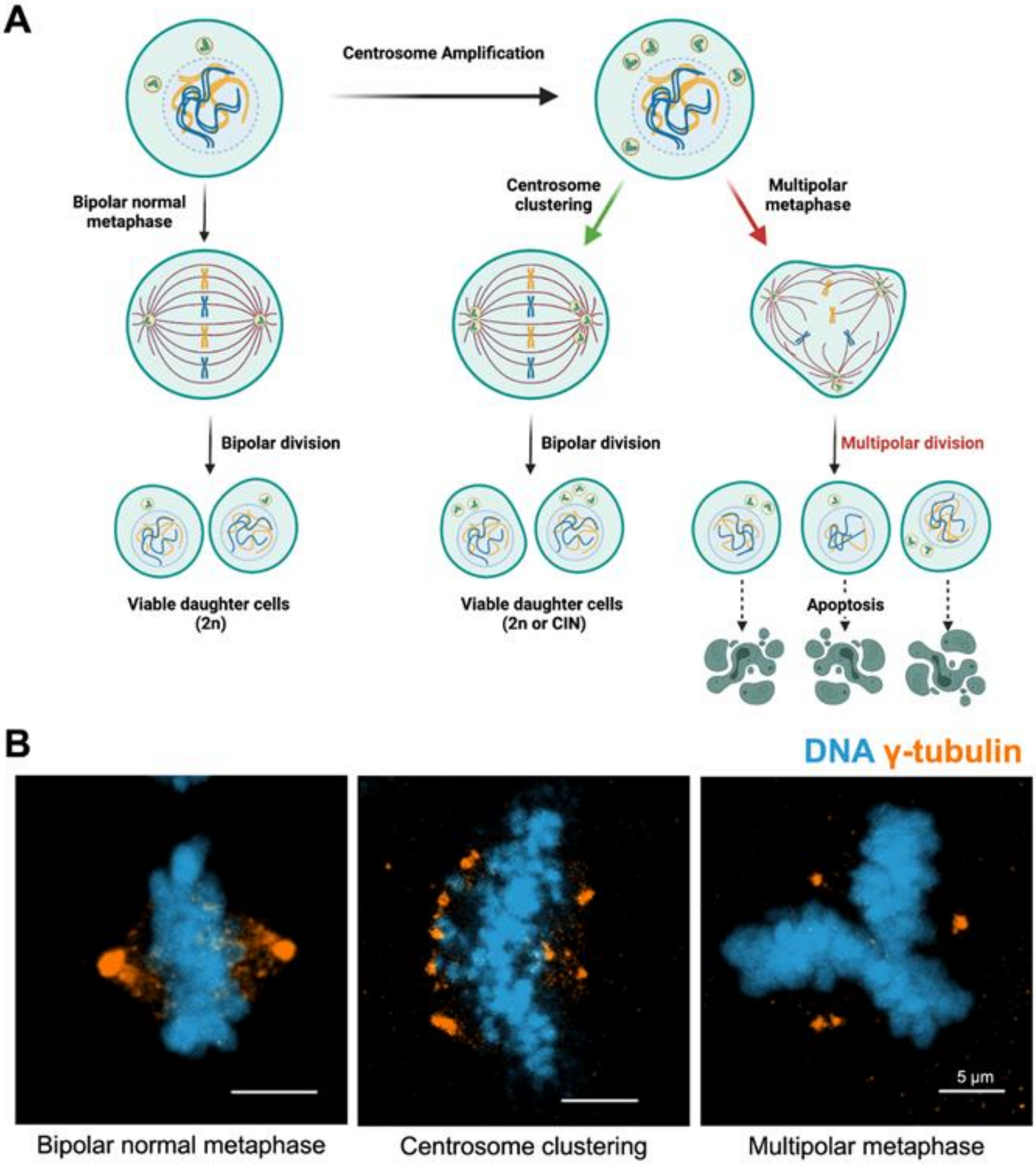

Figure 1.2: The fate of cancer cells with supernumerary centrosomes. (**A**) Extra centrosomes have the ability to create extra poles during cell division, causing multipolar divisions, potentially resulting in the loss of vital genetic material and eventually activating pathways that lead to cell death. Conversely, cancer cells can avoid this outcome by grouping their excess centrosomes and successfully undergoing bipolar division**.** Image created with BioRender. (**B**) Representative images normal bipolar, bipolar clustered and multipolar metaphases. Cyan: DNA (DAPI), Orange: centrosomes (γ-Tubulin).Taken from (Kalkan et al., 2022).

One of the early studies into the mechanisms of centrosome coalescence revealed the critical role of the microtubule motor cytoplasmic dynein; overexpression of spindle proteins such as NuMA resulted in dynein mislocalization, resulting in the formation of multipolar spindles (Quintyne et al., 2005). Two genome-wide screens were performed to uncover additional target proteins responsible for centrosome clustering, one in Drosophila (Kwon et al., 2008) and one in human tumour tissues (B. Leber et al., 2010). The latter study, an RNAi screen, evaluated the frequencies of multipolar spindle formation in cancer cells to normal fibroblasts and prioritized cancer-specific targets. These studies have sparked efforts to identify specific factors involved in clustering, which can be divided into three categories proteins involved in cell division control and spindle tension, proteins associated with spindle pole structure and centrosomes, and proteins involved in actin organization and cell adhesion. **Figure 1.3** depicts an overview of various regulators of centrosome clustering.

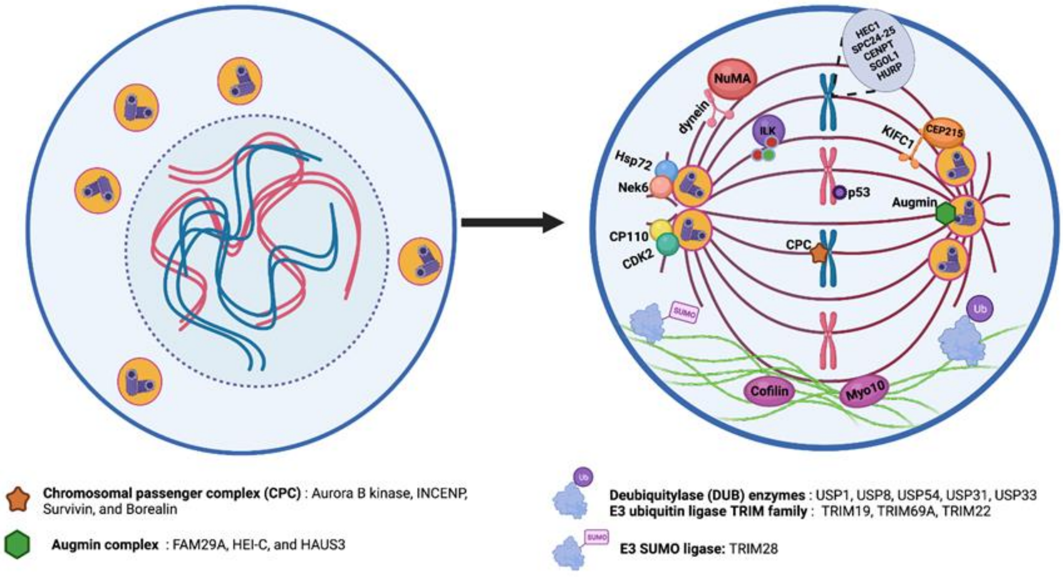

Figure 1.3: A summary of mechanisms leading to centrosomal clustering. Image was created with BioRender. and originally taken from (Kalkan et al., 2022).

The first group of factors identified in these screens is called cell division proteins, and it includes SAC components like the chromosomal passenger complex, chromatid cohesion factors, kinetochore components, and spindle tension proteins like the augmin complex (B. Leber et al., 2010). The Aurora B kinase, INCENP, Survivin, and Borealin chromosomal passenger complex is known as the master regulator of cell division (Carmena et al., 2012). The augmin complex, which consists of eight subunits, interacts with the -tubulin ring complex (γ-TuRC) to generate spindle microtubules (R. Uehara et al., 2009). Depletion of any member of the chromosomal passenger complex increases the rate of multipolar metaphases, according to screen results. Depletions of augmin complex proteins such as FAM29A, HEIC, and HAUS3 have also been linked to decreased clustering capacity (B. Leber et al., 2010). Disrupting spindle tension via mechanisms such as diminished chromatid cohesion or improper kinetochore attachment has been shown to be sufficient to obstruct centrosome clustering. Various proteins, including HEC1, SPC24, SPC25, CENPT, Sororin, and Shugoshin (SGOL1), have been shown to have this impact (Kwon et al., 2008; B. Leber et al., 2010).

Furthermore, HURP, a tension-sensitive kinetochore stabilizing factor (Koffa et al., 2006), has recently been discovered as another important element essential for centrosome clustering (Breuer et al., 2010).

The structural spindle pole and centrosome proteins are the second group of proteins required for centrosomal clustering. The C-terminal kinesin motor KIFC1, discovered in the Drosophila screen, was the first hit in this category (Kwon et al., 2008). Despite the fact that KIFC1 was not found in the human cancer screen, many subsequent investigations have shown its significance in centrosome clustering in human tumour cells. KIFC1, a kinesin motor, works by attaching to microtubule minus ends and crosslinking neighboring microtubules near the spindle pole (She & Yang, 2017). Importantly, KIFC1 has been discovered as a direct CEP215 binding partner, highlighting its critical involvement in microtubule attachment to centrosomes (Chavali et al., 2016). The CEP215-KIFC1 complex also promotes centrosome clustering (Chavali et al., 2016; Sabat-Pospiech et al., 2019). Furthermore, KIFC1 has been discovered as a dependence protein in breast cancer malignant cells (Patel et al., 2018). Intriguingly, KIFC1 has been demonstrated to be phosphorylated by both ATM and ATR in response to DNA damage, inducing centrosome clustering and eventually tumour recurrence (G. Fan et al., 2021). Inhibiting this phosphorylation mechanism restored both phenotypes, indicating strong support for KIFC1 inhibition as a possible therapy option in malignancies with enlarged centrosomes. AZ82 (Wu et al., 2013), CW069 (C. A. Watts et al., 2013), and SR31527 (W. Zhang et al., 2016) are three selective KIFC1 inhibitors that have recently been identified. In BT-549 breast cancer cells with increased centrosomes, AZ82 was discovered to bind selectively to KIFC1, resulting in centrosome declustering (Wu et al., 2013). CW069 only enhanced multipolarity in cells with extra centrosomes (C. A. Watts et al., 2013), and SR31527 inhibited centrosome clustering in triple-negative breast cancer cells while decreasing colony formation and cell viability (W. Zhang et al., 2016). All three inhibitors showed a strong selectivity for cells with additional centrosomes vs cells with a normal centrosome complement, indicating that KIFC1 is a particular target for centrosome declustering. KIFC1 has been demonstrated to induce tumour growth through centrosome-independent activities in addition to its role in centrosome clustering (Pannu et al., 2015).

Other centrosome and spindle pole structural proteins, in addition to KIFC1, have been identified as critical components of centrosome clustering. Integrin-linked kinase (ILK), which plays a function in actin and mitotic microtubule structure and has been identified as a clustering factor in multiple cell lines generated from breast and prostate tissues with additional centrosomes (Fielding et al., 2011), is one of these. ILK participates in centrosome clustering by modulating the microtubule-associated proteins TACC3 and chTOG (Fielding, Dobreva, McDonald, et al., 2008). In cancer cells, disrupting ILK function resulted in multipolar anaphases, mitotic arrest, and cell death. The study also discovered that ILK modulates Aurora A-dependent phosphorylation of TACC3, which is required for centrosome clustering (Fielding et al., 2011).

CP110, a centrosome duplication regulatory factor, has also been found to play a function in centrosome clustering. CDK2 phosphorylation of CP110 has been shown to be required for the development of multipolar spindles (S. Hu et al., 2015). Furthermore, CEP164 depletion causes spindle pole disintegration and the development of acentrosomal spindle poles (B. Leber et al., 2010). Another work (Sampson et al., 2017) demonstrated the role of Nek6-dependent phosphorylation of Hsp72 in centrosome clustering. Previous work from this group demonstrated that Nek6 was required to direct Hsp72 to the centrosome, where it participated in kinetochore fiber formation by recruiting TACC3 and chTOG (Fielding, Dobreva, McDonald, et al., 2008; O'Regan et al., 2015). Hsp72 depletion increased multipolarity but did not result in the development of acentrosomal poles. As a result, multipolar spindles were detected in cancer cells with extra centrosomes, while in normal cells, loss of either Hsp72 or Nek6 activity had no effect on spindle formation (Sampson et al., 2017).

The final group of proteins found in large-scale centrosome clustering screens are those that link actin and microtubules. Myo10 (also known as Myosin X) and Cofilin are two major proteins found in this group (Kwon et al., 2008). Myo10, an N-terminal myosin, modulates spindle pole orientation via interacting with microtubules [233]. Myo10 also helps to link microtubules to centrosomes, which aids in centrosome orientation towards retraction fibers (Kwon et al., 2015). Myo10 is also involved in the binding of microtubules to centrosomes, allowing centrosomes to be properly oriented towards retraction fibers (Kwon et al., 2015). Myo10 depletion enhanced the incidence of multipolarity in cells containing extra centrosomes, notably in S2 and N1E-115 cells, suggesting its role in centrosome clustering (Kwon et al., 2008). Three tiny proteins in the actin-depolymerizing factor (ADF)/Cofilin family regulate actin dynamics by promoting filament severing (Kanellos & Frame, 2016). A pharmacological screen for centrosome clustering inhibitors yielded two medicines, CP-673451 and crenolanib, which encouraged multipolar spindle formation by disrupting the actin network via Cofilin activation. This not only found two potential tumor-targeting drugs, but also demonstrated the importance of cortical actin instability as a method to block centrosome clustering (Konotop et al., 2016). Cortical actin, particularly cortical contractility, is also important in epithelial cells expressing large levels of E-cadherin. These cells have limited cortical contractility, which is linked to their inability to cluster amplified centrosomes. E-cadherin expression levels fall during the epithelial-to-mesenchymal transition, which is accompanied by an increase in cortical contractility, allowing for KIFC1-dependent centrosome clustering (Rhys et al., 2018).

Several more proteins, in addition to the three previously described categories, have been discovered as critical elements for centrosomal clustering. The Deubiquitylase (DUB) enzymes, which are responsible for eliminating ubiquitin from proteins, are one such protein family. The siRNA screen in Drosophila identified two DUBs, USP8 and USP31, as essential for centrosome clustering (Kwon et al., 2008). A previous siRNA screen on human cancer cells identified USP54 as another protein needed for additional centrosome clustering (Quesada et al., 2004). Furthermore, different DUBs have been shown to be required for different stages of the centrosome cycle: USP1 and USP33 are required for centrosome replication, whereas USP44 is essential for centrosome separation (Darling et al., 2017). This shows that the DUB family is involved in a complex regulatory network of centrosome duplication, control, and clustering mechanisms, laying the groundwork for future research to obtain a better understanding of these processes.

Members of the TRIM family of E3 ubiquitin ligases, in addition to deubiquitylating enzymes (DUBs), play a role in centrosome amplification and clustering (Venuto & Merla, 2019). TRIM28, an ARF-binding protein and an E3 SUMO ligase, for example, alters NPM1 via SUMOylation, increasing its localisation to centrosomes and preventing centrosome amplification (Neo et al., 2015). TRIM19 inhibits Aurora A kinase activity, which in turn inhibits centrosome amplification (Xu et al., 2005). Mitotic abnormalities occur when TRIM69A, an important component linked with spindle poles and critical for mitotic spindle formation, is reduced. Interestingly, increasing TRIM69A levels can counteract HAUS1-induced spindle multipolarity, underlining its role as an effective clustering facilitator (Sinnott et al., 2014). Furthermore, using RNA interference (RNAi) to decrease TRIM69A has been demonstrated to inhibit tumour growth in vivo (Sinnott et al., 2014). Furthermore, TRIM22, an interferon-inducible gene, localizes to centrosomes and promotes centrosome clustering irrespective of the cell cycle (Petersson et al., 2010; Venuto & Merla, 2019).

Furthermore, two drug-based screenings were carried out in order to discover possible targets for reducing centrosome clustering. The first screening (Rebacz et al., 2007) found griseofulvin as a possible candidate using fungal extract libraries. Griseofulvin was discovered to stabilize microtubules at low concentrations, and studies demonstrated that its binding location on microtubules coincides with that of paclitaxel, implying a common mechanism for producing multipolarity (Godinho & Pellman, 2014; Rathinasamy et al., 2010). GF-15, a synthetic derivative of griseofulvin, was found to be a powerful inhibitor of centrosome clustering in cancer cells and to have promising anticancer effects both in vitro and in vivo (Raab et al., 2012). In the second pharmacological screening, fourteen compounds were found to prevent centrosome clustering in BT-549 cells, a breast cancer cell line containing additional centrosomes. Furthermore, these chemicals caused mitotic arrest. Among the chemicals found, CCCI-01 showed great potential due to its significant differential impact on cancer cells and noncancerous mammary epithelial cells. CCCI-01 caused a significant rate of multipolarity and cell death in BT-549 cells, but not in normal cells, indicating CCCI-01 a possible anti-cancer therapeutic candidate targeting the centrosomal clustering mechanism (Kawamura et al., 2013).

Finally, a p53 mutation hampered the clustering of extra centrosomes in tetraploid cells through affecting the RhoA/ROCK signalling pathway. This finding implies that functional p53 is required for centrosomal clustering, which is required for producing viable progeny from cells with additional centrosomes (Yi et al., 2011). Based on research with non-malignant cell lines, the findings suggest that the processes underlying centrosome clustering in p53 WT cells may differ dramatically from those in cancer cells without p53 activity. This understanding is especially important as we continue to identify potential centrosome clustering inhibitors with the goal of developing drugs that selectively target tumors while sparing normal tissues, especially given the importance of centrosome clustering in tissues that require tetraploid cell generation, such as hepatocytes (Gentric et al., 2012; Wang et al., 2017).

1.6 NIMA-related Kinase 2 (Nek2)

Nek2A is one of the most widely studied members of the mammalian Nek family. Nek2A is a serine/threonine kinase with a catalytic domain at the N-terminus and a regulatory domain at the C-terminus (Fry, 2002). The C-terminal domain, in particular, has a coiled coil motif that is responsible for centrosomal localisation (Fry et al., 1999). In terms of cell cycle regulation, Nek2A has low levels of activity throughout G1 and mitosis but peaks during the S/G2 phase (Fry, 2002). Nek2A is essential for centrosome separation during mitotic start by phosphorylating two centrosomal coiled coil proteins, C-Nap1 and Rootletin, at certain serine and threonine residues (Helps et al., 2000; Yang et al., 2002). Interestingly, investigations have shown that overexpression of Nek2A or silencing of C-Nap1 causes centrosomes to separate prematurely (Fry et al., 2000). Furthermore, Nek2A plays an important role in centrosome maintenance and assembly, with overexpression resulting in additional centrosomes, centrosome dispersion, and morphological defects (Gräf, 2002). Emerging data suggests that Nek2A has a role in the SAC and chromosome segregation. Phosphorylation events involving Hec1, as well as regulatory interactions with Mad1 and Mad2, highlight the significance of Nek2A activity in these processes, while it may not be completely necessary for normal chromosomal segregation (Chen et al., 2002a; Lou et al., 2004). Because it targets the kinetochore protein Sgo1, Nek2A has an impact on kinetochore and microtubule connections during mitosis (Fu et al., 2007).

Nek2A expression along with its kinase activity are closely controlled via transcriptional and post-translational processes. Nek2A mRNA levels stay low during the M and G1 phases of the cell cycle but peak during the S and G2 phases (Twomey et al., 2004). Notably, transcription factors such as E2F4 and FoxM1 regulate its expression (Ren et al., 2002; Wonsey & Follettie, 2005). Nek2A is degraded at the protein level, which is aided by a particular destruction motif situated near its C-terminal domain. This degradation mechanism is driven by polyubiquitylation by APC/C (Hayes et al., 2006).

C-Nap1, a centrosome-localized linker protein, was one of the first Nek2A targets identified (Fry, Mayor, et al., 1998). Nek2A and C-Nap1 colocalize at the proximal ends of centrioles, where Nek2A phosphorylates both the N-terminus and the C-terminus of C-Nap1, eventually dissociating C-Nap1 from the centrosome (Mayor et al., 2002). Rootletin, another intercentriolar linker protein that is required for centrosome cohesion, interacts with Nek2A and C-Nap1 (S. Bahe et al., 2005). Rootletin, like C-Nap1, is found at the proximal ends of centrioles and acts as a physical linker. Centlein, another centriolar linker protein, was discovered to be a Nek2A target later on (Fang et al., 2014). Rootletin, like C-Nap1, is found at the proximal ends of centrioles and acts as a physical connection. Centlein, another proteinaceous linker between parental centrioles, forms a complex with C-Nap1 and Cep68 at the proximal ends of the centrioles. During interphase, centlein helps to molecular interactions between C-Nap1 and Cep68. Nek2A also interacts directly with telomeric repeat binding factor 1 (TRF1), a recognized DNA-binding protein implicated in telomere maintenance (Lee & Gollahon, 2013). TRF1 has also been linked to mitotic progression and cell cycle regulation (Nakamura et al., 2001; Shen et al., 1997).

The importance of Nek2A extends to cancer progression and prognosis. Numerous studies have found Nek2A overexpression in malignancies ranging from breast to lung to pancreas to prostate (T. Kokuryo et al., 2019). High Nek2A expression has been linked to increased cell proliferation (Cappello et al., 2014; Wang et al., 2012; Zeng et al., 2015) and invasion in cancer cells (Kokuryo et al., 2016; Tsunoda et al., 2009). Nek2A promotes metastasis by regulating β-catenin expression and location. Surprisingly, Nek2A overexpression contributes to treatment resistance in cancer. Notably, Nek2A overexpression has been linked to drug resistance in cancer cells, possibly via genes such as aldehyde dehydrogenase 1 family member A1 (ALDH1A1) and ATP binding cassette subfamily G (ABCG) (Lin et al., 2016; Yang et al., 2014). As a result of its varied participation in the cell cycle and potential as a broad-spectrum target for intervention, Nek2A appears as a prospective candidate for cancer therapy.

1.7 Aim of the Study

During the process of cell division known as mitosis, strict control mechanisms ensure that each daughter cell receives the correct amount of genetic material. This is achieved through precise attachments of chromosomes to the well-organized spindle fibers, resulting in a symmetrical separation in a bipolar manner. However, there are instances of cell divisions that involve more than two poles, leading to abnormal chromosome numbers. This condition, known as multipolar spindle (MPS) formation, is a common defect observed in numerous cancer cell lines, often associated with an increase in the number of centrosomes. In normal cells, the centrosome count typically does not exceed two, but cancer cells frequently possess supernumerary centrosomes (more than two), potentially giving rise to MPS.

Apart from the increased risk of tumorigenesis associated with chromosomal gain, MPS can also have detrimental consequences by resulting in the loss of crucial genetic material. To ensure the survival of the offspring, cancer cells have adapted to cope with the presence of extra centrosomes by forming pseudo-bipolar spindle structures, a process referred to as "centrosomal clustering." Recently, there has been significant research interest in preventing centrosomal coalescence, and novel drug candidates have been explored as potential therapies for cancer, particularly targeting mitotic cells displaying supernumerary centrosomes. Therefore, the primary objectives of this study are to identify the specific molecules involved in centrosomal clustering and to unravel the underlying mechanism.

Many of the chemotherapeutic agents currently in use suffer from the drawback of causing harmful side effects on healthy tissues due to their inability to selectively target tumor cells. In contrast to normal cells, tumor cells frequently possess extra centrosomes, which tend to give rise to multipolar spindles during cell division. This, in turn, can lead to the death of some cells due to an insufficient amount of genetic material, triggering cell death pathways. Nevertheless, cancer cells have developed a mechanism for successful division by bringing together their extra centrosomes into two functional poles.

Nek2 kinase is a pivotal molecule that regulates various aspects of mitosis, including the centrosome cycle, kinetochore attachment, microtubule organization, and the spindle assembly checkpoint. Previous studies from our research group have demonstrated that disrupting Nek2 activity results in centrosomal clustering and bipolar divisions, while overexpression of Nek2 can disperse centrosomes, leading to MPS. In this research proposal, our primary objective is to identify novel Nek2 targets and ascertain which of these targets play a role in centrosome clustering. This approach may open up possibilities for selectively eliminating cancer cells displaying supernumerary centrosomes by targeting these specific proteins.

2 MATERIALS AND METHODS

2.1 Cell Culture

Mouse neuroblast cell line N1E115 (ATCC, CRL-2263), human breast cancer cell line MDA-MB-231 (ATCC, HTB-26), human osteosarcoma cell line U2OS (ATCC, CRL3455), human pancreatic carcinoma cell line SU86.86 (ATCC, CRL1837) and human embryonic kidney cell line 293T (ATCC, CRL-3216) were maintained in DMEM, which was supplemented with 10% FBS and 1% Pen/Strep at 37°C and 5% CO_2 incubator.

2.2 Chemicals and Reagents

Nocodazole (CAS 31430-18-9) was purchased from Sigma-Aldrich and cells were treated with 100 ng/ml final concentration for 16 hours. Doxycycline hyclate (CAS 24390-14-5) was purchased from Sigma-Aldrich and cells were treated with 1 µg/ml final concentration for 24-72 hours to induce Nek2A and PLK4 overexpressions. INH154 was purchased from MedChemExpress (Cat. No.: HY-117154) and dissolved in DMSO. Cells were treated with 2.5 and 5 µM final concentrations. JH295 was purchased from Merck (480017) and dissolved in DMSO. Cells were treated with 1 µM final concentration.

2.3 Cell Viability Assay

WST-1 assay was used for cell viability. Briefly, cells were seeded as 3 x 10^3 cell/well in 96 well plates and allowed to adhere for 16 hours. Afterwards, cells were incubated 30 minutes with WST-1 reagent (Roche). The absorbance was measured at 440 nm using microplate reader (Synergy H1 Reader, Biotek, USA)., viability was calculated relative to the control group.

2.4 Virus Packaging and Transduction

Virus packaging was used for LentiCRISPR v2, Nek2A and PLK4 overexpressions and fluorescent tagging of the cells for competition assays. Briefly,

2×10^6 293T cells were seeded per 10cm petri dish. 2500 ng transfer vector, 2250 ng packaging vector (psPAX2 for lentivirus, pUMVC for retrovirus) and 250 ng envelope vector (pCMV-VSV-G) is mixed with 20 µL Fugene 6 (Roche, USA) diluted in OptiMEM. Cell were transfected with the mixture prepared. Medium containing virus was collected at 48- and 72-hours post-transfection and 100X concentrated by 50% (w/v) PEG 8000 (P2139, Sigma).

1×10^5 cells were seeded in 6 well plates. Infections were performed using 10 µL virus and 8 µg/ml protamine sulphate in 2 ml culture medium. Cells transduced with LentiCRISPR v2 and Dox-inducible Nek2 overexpression vector were selected with 2 µg/ml puromycin and cells transduced by retroviral Nek2 expression vector were selected with 20 µg/ml blasticidin.

2.5 siRNA Transfection

siRNA transfection for silencing Nek2 and its targets were performed as the following. 2x105 cells were seeded in 6 well plates and 100 pmol siRNA, purchased from SIGMA, and 7,5 µl of Lipofectamine 3000 (Thermo) were introduced to culture media according to manufacturer’s protocol. Knock down efficiencies were analysed by western blot or RT-qPCR. The list of siRNAs used in this study is given below.

Table 1: List of siRNAs used in this study.

Gene	Brand	Product No
Nek2	MERCK	EHU109951
Gas2L1	MERCK	EHU078871
TRF1	MERCK	EHU114821

NuMA	MERCK	EHU059141
KIFC1	MERCK	EHU148011
KIF2C	MERCK	EHU046211

2.6 Cloning

Three guide RNA sequences targeting early exons of Nek2, TRF1, C-Nap1, Rootletin and Centlein were cloned into LentiCRISPR v2 (Addgene, 52961) as previously described (Sanjana et al., 2014). Briefly, ssDNA oligos for each guide RNA were annealed and 5' end phosphorylation was done using T4 Polynucleotide Kinase (NEB). LentiCRISPR v2 vector was digested with BsmBI (NEB), dephosphorylated by Antarctic Phosphatase (NEB) and NucleoSpin Gel & PCR Clean-up kit (Macherey Nagel) was used for gel purification. Linearized backbone and the inserts were ligated using T4 DNA Ligase (NEB).

Nek2 in pJP1563 (retroviral expression vector) was purchased from DNASU and Nek2 cDNA sequence was subcloned into pCW57-RFP-P2A-MCS (Addgene, 78933) lentiviral doxycycline inducible vector. Briefly, Nek2 coding sequence was amplified by PCR using primers which PstI and BamHI restriction sites inserted. PCR product and cloning plasmid were both digested with PstI and BamHI, followed by gel purification and ligation steps.

2.7 Site-Directed Mutagenesis

Kinase-dead version (K37R) of Nek2 was derived from WT Nek2 expressing retroviral vector using Q5 Site-Directed Mutagenesis Kit (NEB). In order to design mutagenic primers and determine Q5-optimized annealing temperatures, NEBaseChanger™, the online NEB primer design software, was used. PCR amplification was performed according to kit manual. Next, PCR products were treated with Kinase, Ligase & DpnI (KLD) reaction mixture to phosphorylate and ligate while eliminating the template DNA. Transformation was performed using NEB 5-alpha Competent E. coli included by the kit. Plasmids were purified by MiniPrep (Macherey Nagel) and sent to sequencing to confirm desired mutation. Oligo sequences used for site-directed mutagenesis are provided below.

Table 2: List of oligos used for site-directed mutagenesis.

Name	**Sequence**
NEK2_SDM_K37R-F	TGA TGG CAA GAT ATT AGT TTG GAG AGA ACT TGA CTA TGG CTC
NEK2_SDM_K37R-R	GAG CCA TAG TCA AGT TCT CTC CAA ACT AAT ATC TTG CCA TCA

2.8 Centrosome Amplification by Nocodazole Treatment

$2x10^5$ cells were seeded on 15x15 mm coverslips. Nocodazole (SIGMA M1404) (100 ng/ml) treatment was done for 24 hours to achieve metaphase arrest and mitotic skip, resulting in duplicated centrosomes. Culture media was replenished without Nocodazole, and cells were incubated 24 hours to allow cells to recover and re-enter cell cycle with amplified centrosomes.

2.9 Scoring for Centrosome Amplification, Centrosome Clustering and Multipolarity

Cells were stained for γ-Tubulin in order to determine the presence of centrosomes at the poles. Multipolarity is scored in cells at metaphase, and the decision will be made based on DNA shape. Centrosomal unclustering is scored when at least three poles are positive for centrosomal markers. Minimum of 300 metaphases is scored per experiment and each experiment is repeated at least twice. Centrosome amplification scoring is done based on the centrosome number per cell at interphase. Cells bearing more than 2 centrosomes are marked as centrosome amplification positive.

2.10 Competition Assays

The long-term of given conditions on cell survival and proliferation were assessed by performing dual-color competition assays *in vitro* and *in vivo.* Cells were tagged either with PGK-H2BmCherry (Addgene #21217) or PGK-H2BeGFP (Addgene #21210) and mixed 1:1 ratio. For the *in vitro* competition assays, 5 x 10^4 total cells were seeded to 6-well plates. Cells were passaged 1:5 when the confluency was reached. Plates were imaged using Agilent BioTek Cytation 5 imaging platform at day 0, day 5, day 10 and day 15. mCherry-positive cells were quantified for each well using the Cytation 5 software. Alternatively, cells were harvested at given days above and mCherry-positive cells were quantified using flow cytometry.

Competition assay *in vivo* was conducted as the following. Cells were mixed 1:1 in dPBS to final concentration of 2 x 10^7 cells / ml. For each injection, 50 µl of cell suspension was mixed with 50 µl Matrigel (Corning). 100 µl mixture of cells and Matrigel were injected to 8-10 weeks old male SCID mice subcutaneously. After 8 weeks of post-injection, mice were sacrificed, and tumors were collected. Collected tumors were fixed with a 10% formalin for 2 days. After this period, the tissues, which were rinsed in distilled water, were processed for further procedures. For dehydration, the tissues were passed through 50%, 70%, and 90% ethanol series for 2 hours each, and then they were left overnight in absolute ethanol. Subsequently, the tissues were subjected to a 3-hour clearing process with xylene, followed by a 4-hour incubation in paraffin at 55°C in an oven. Once the follow-up process was completed, the tissues were turned into paraffin blocks using a casting device (Diapath). Sections of 2µm thickness were obtained from the resulting paraffin blocks using a microtome (Leica RM2245).

2.11 Immunofluorescence Staining

Cells grown on coverslips were fixed with ice-cold methanol for 15 minutes, washed 3 times with DPBS-T and blocking was done with 5% (w/v) BSA for 30 minutes. γ-Tubulin staining was performed using SIGMA mouse monoclonal antibody (1:1000 dilution in 1% BSA in PBS) incubated 4ºC overnight followed by 1:1000 diluted secondary antibody (Alexa flour 488, Thermo) incubation at RT for 2 hours. Cell nuclei was labelled by DAPI containing mounting medium (Vectashield). Imaging was performed using Zeiss Axio Imager M1.

2.12 RT-qPCR

Total RNA is isolated using NucleoSpin RNAII kit (Macherey-Nagel) following manufacturer’s instructions. 1000 ng total RNA is reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen) cDNA synthesis kit following the manufacturer's protocol. RT-qPCR is performed using The LightCycler 480 SYBR Green I (Roche) and the reaction is run at LightCycler 480 Instrument II (Roche). Samples are normalized to beta-actin expression. To confirm amplification specificity, the PCR products are subjected to a melting curve analysis and subsequent agarose gel electrophoresis. The list of qPCR primers used throughout this study is provided below.

Table 3: List of primers used for RT-qPCR analysis.

NAME	**SEQUENCE**
NEK2_qPCR-F	TTG GAG CAG AAA GAA CAG GAG C
NEK2_qPCR-R	TCC CCA CTG AAA TGA ACT TTC TTC
ACTB-F	TCA CCA TGG ATG ATG ATA TCG C

ACTB-R	ATA GGA ATC CTT CTG ACC CAT GC
GAPDH-F	CTG ACT TCA ACA GCG ACA CC
GAPDH-R	GTT GTC ATA CCA GGA AAT GAG C
PLK4-QPCR-F	GGC CAA GGA CCT TAT TCA CCA
PLK4-QPCR-R	TGT GGC ATG CCC ACT ATC AA
TERF1-F	CAG CGC AGA GGC TAT TAT TCA TGG
TERF1-R	AGG GCT GAT TCC AAG GGT GT
GAS2L-1-F	GAC ACG CTG GAG CAT TAC CT
GAS2L-1-R	TGG AGA AAA GGT GCA GAC CC
HSET-F	TTG GTA CTG CTC AGG CCA AC
HSET-R	GAT AGC CCT GGG ACA TGG TG
NuMa-F	CAG GTG GAA ACT AAT TCT AAG CCA G
NuMa-R	GTC ACT CCA ATG CGC CTC CT
KIF2C-qPCR-F	CGC GTT TCT CTT CCT TGC TG
KIF2C-qPCR-R	CCT TTG TGG CAC CTC CTT CT

2.13 Western Blot

Cells were cultured in 10 cm plates as 1 x 10^6 cells per plate. Upon reaching 80% confluence, the cells were harvested through trypsinization and subsequently centrifuged at 1500 rpm for 5 minutes. The resulting cell pellets were washed with PBS, subjected to another round of centrifugation, and then stored at -80 ˚C.

For the isolation of whole-cell proteins, the cells were lysed using a buffer containing NP-40 (1% NP-40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 7.8, 1 mM NaF, 1 mM PMSF, and 1X protease inhibitor cocktail from Complete Protease Inhibitor Cocktail Tablets by Roche). This lysate was incubated on ice for 30 minutes, followed by a centrifugation step at 14,000 rpm for 10 minutes at 4 ˚C. The supernatant, containing the proteins, was collected for subsequent experiments. The concentration of proteins was determined using the BCA Protein Assay Kit from Thermo Scientific, USA, following the manufacturer's instructions.

For further analysis, 50 µg of protein from each sample was mixed with 4X Laemmli sample buffer (Biorad, 1610747, USA), supplemented with a 1:10 ratio of β-mercaptoethanol, and incubated at 95 ˚C for 10 minutes to denature the proteins. Subsequently, the samples were placed on ice and loaded onto a 4-12% Mini Protean TGX Precast Gel (Biorad, 456-1044, USA). The proteins were then transferred to a PVDF membrane using the Biorad Trans-Blot Turbo Transfer System (Biorad, USA).

The membranes were stained with Ponceau-S (PS05, EcoTech, Turkey) after transfer, rinsed with TBS-T, and blocked by incubating with 5% non-fat dry milk in TBS-T. After blocking, the membranes were probed with specific antibodies, as detailed in Table 2.9, and left to incubate overnight on a shaker at 4 ˚C. Following the 16-hour incubation, the membranes were washed three times with TBS-T and then incubated with the appropriate secondary antibodies for one hour. Subsequently, the membranes were washed thrice with TBS-T. SuperSignal West Femto Maximum Sensitivity Substrate

(34095, Thermo Scientific, USA) or Pierce ECL (32209, Thermo Scientific, USA) was used for detection.

2.14 Annexin V Apoptosis Assay

The Annexin V staining procedure was conducted using the Muse® Annexin V & Dead Cell Kit from Luminex (MCH100105), following the provided manufacturer's guidelines.

The cells were subjected to centrifugation at 1200 rpm for 5 minutes, after which the supernatant was removed, and the cell pellet was resuspended in 500 µl of cold PBS containing 1% FBS. Following this, the cell suspension underwent another round of centrifugation, and the pellet was resuspended in 75 µl of cold PBS with 1% FBS. Subsequently, 75 µl of Annexin V & Dead Cell Reagent was added to the cell suspension. The samples were left to incubate at room temperature for 20 minutes and were later analyzed using the Muse Cell Analyzer (Merck, Darmstadt, Germany) with a total of 5000 events recorded per sample. The gating strategy was determined in accordance with control groups.

2.15 Cell Cycle Assay

Cell cycle analysis was performed using the Muse Cell Cycle Assay Kit (MilliporeSigma). Cells were seeded in 6 well plates. After treatment, cells were harvested, washed with PBS, and fixed in 70% ethanol at -20 ºC. Fixed cells were then stained with the Muse Cell Cycle Reagent, following the manufacturer's instructions. Flow cytometry analysis was conducted using the Muse Cell Analyzer, and the data were processed using Muse analysis software to determine cell cycle distribution.

2.16 Caspase 3/7 Assay

Caspase 3/7 activity was assessed using the Muse Caspase 3/7 Assay Kit (MilliporeSigma). Cells were seeded in 6 well plates. After treatment, cells were harvested and resuspended in the Muse Caspase 3/7 Reagent. The cells were then incubated at 37 ºC for 30 minutes. Flow cytometry analysis was conducted using the Muse Cell Analyzer, and the data were processed using Muse analysis software to determine caspase 3/7 activity.

2.17 Co-immunoprecipitation (Co-IP)

Cells were harvested via trypsinization (0.05% trypsin). Pelleted cells were fixed with 1% paraformaldehyde (PFA) for 7 minutes, followed by centrifugation to remove excess PFA, and quenched with ice-cold 1.25mM glycine. After quenching, glycine was removed by centrifugation. For non-PFA immunoprecipitation, cells were lysed directly using ice-cold immunoprecipitation (IP) buffer (Thermo) supplemented with protease inhibitor, phosphatase inhibitor, and PMSF, and maintained on ice for 45 minutes. Protein concentration was determined using a BCA assay. Protein G magnetic beads were washed thrice with PBS and incubated with cell lysates for pre-clearance for 30 minutes. Magnetic beads were separated, and protein samples were subsequently incubated with either FLAG antibody or IgG control for 2 hours at 4°C. Following this incubation, the antibody-protein mixture was further incubated with protein G magnetic beads overnight. After overnight incubation, beads underwent four washes, and proteins were eluted by denaturation with 4x Laemmli buffer containing 5% DTT at 95°C for 15 minutes. Western blotting was employed for subsequent protein analysis.

2.18 Turbo-ID Proximity Labeling

TurboID expression-induced U2OS-TurboID-Nek2A-WT and Nek2A-KD cells, as well as the control group cells, were treated with 500 µM biotin (Life Technologies, Cat. No. B1595) 30 minutes before the end of the synchronization period. The cells were rinsed five times with ice-cold PBS solution to stop biotinylation, and they were collected by pipetting with PBS solution. Cell pellets were obtained by centrifugation. Protein lysates were prepared by adding protease inhibitor to the cell pellets in lysis buffer (10mM TrisCl pH7.6, 0.5% SDS, 2% NP40, 150 mM NaCl, 1 mM EDTA, 10 mM Iodoacetamide). Protein concentrations were measured using the BCA method, and the obtained proteins were incubated with Streptavidin beads (Pierce; Cat No. 53117) overnight at +4°C on a tube rotator. After incubation, the tubes were centrifuged at 1000 rpm for 5 minutes, and the supernatant was stored as the 'unbound fraction' (for checking complete binding). Beads and the proteins bound to them were washed twice for 10 minutes each with the following washing solutions:

Wash solution 1: 2% SDS

Wash solution 2: 50 mM Hepes pH 7.5, 2% deoxycholate, 1% Triton-X, 50 mM NaCl, 1 mM EDTA

Wash solution 3: 10 mM Tris pH8.1, 1 mM EDTA, 500 mM NaCl, 1% Triton-X, 0.5% deoxycholate, 0.5% NP-40

Wash solution 4: 50 mM Tris pH7.4, 50 mM Tris pH 7.4

Samples for Mass Spectrometry analysis were kept in wash solution 4, and the protocol for direct protein cleavage was followed. When Western blotting was applied for the confirmation of Mass Spectrometry experiments, after the final wash, 200 µl of Laemmli buffer containing 1M DTT was added to bead-protein complexes and heated at 98°C for 10 minutes.

Prior to protein cleavage, 55 mM iodoacetamide prepared in 100 mM ammonium bicarbonate (100 µl) was added to the mass spectrometry samples, and they were incubated in the dark for 1 hour. Subsequently, a trypsin solution suitable for mass spectrometry (Pierce; Cat. No.: 90058) was applied to the samples, and they were incubated overnight at 37°C. Then, formic acid was added to the samples to achieve a final concentration of 5%, and they were centrifuged at 14,000 × g for 30 minutes. The supernatant was collected for analysis in the mass spectrometer. Thermo Scientific Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer was used for the analysis, and peptides were analyzed using Thermo Fisher Scientific Proteome Discoverer and MaxQuant software. Known contaminant proteins (e.g., keratin) were excluded from the analysis, and proteins identified with at least two different specific peptides were considered significant in terms of quantity.

2.19 Microscopy

Leica DMI8 SP8 microscope was used for confocal imaging. LASX software was utilized for image processing. Experiments involving in metaphase scoring was performed with Carl Zeiss Axio Imager M1. Live-cell imaging-based competition assays were performed by using BioTek Cytation 5 Cell Imaging Multimode Reader.

2.20 Statistical Analysis

All experiments were conducted as biological repeats, and statistical analysis was performed using GraphPad Prism version 9.0. The student's t-test was employed to compare two groups, while the two-way ANOVA was used to compare more than two groups for parametric variables. Significance levels were denoted as follows: * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

3 RESULTS

3.1 The Role of Nek2A on Centrosome Clustering

3.1.1 Effect of Nek2A Overexpression in the Cells Naturally Harboring Supernumerary Centrosomes

Based on the significant role of Nek2A in controlling the centrosome cycle and separation, we sought its potential role in the regulation of centrosome clustering in cancer cells. To this end, we initially used a mouse neuroblastoma cell line, N1E-115, which is known to harbour supernumerary centrosomes in its entire population and achieve centrosome clustering effectively. N1E-115 cell line has frequently been utilised as a model to study centrosome clustering (David Ring et al., 1982). To verify the cell line characteristic in the laboratory, cells were fixed, and centrosomes were immune-stained using anti γ-Tubulin antibody. DNA was detected by DAPI staining, and microtubules were stained using α-Tubulin (DM1A) antibody. Fluorescent microscopy confirmed that the stock N1E-115 cells harbour supernumerary centrosomes as a whole population (**Figure 3.1 A**).

In order to test the effect of Nek2A overexpression in N1E-115 cells, a lentiviral, tet-inducible overexpression system of Nek2A was generated through subcloning. Next, lentiviral packaging was performed, and the obtained viral particles were transduced to N1E-115 cells. Nek2A overexpression followed by doxycycline induction was confirmed by RT-qPCR (**Figure 3.1 B**). Cells were seeded on coverslips; control group was cultured under tetracycline-free conditions while the test group was treated with doxycycline for 48 hours. Afterwards, cells were fixed, and immunofluorescent staining was performed using anti γ-Tubulin and α-Tubulin antibodies. DNA was stained by DAPI included in the mounting medium. Under fluorescent microscope, metaphase cells were detected based on their DNA, and microtubule conformation. For each sample, around 300 metaphases were examined and stratified into 3 groups: multipolar, bipolar-clustered and bipolar-normal. Representative images of multipolar and bipolar-clustered metaphases observed in N1E-115 cell line are shown in (**Figure 3.1 C**). Bipolar-normal metaphases were recorded in each experiment but excluded from our analysis and graphs since the main interest of this study is to investigate the role of Nek2A kinase on centrosome clustering. Therefore, the cells without extra centrosomes would not contribute to answer the main questions of the present study. Metaphase scoring data showed that overexpression of Nek2A significantly increased formation of multipolar spindles, inhibiting centrosome clustering (**Figure 3.1 D**). To maintain simplicity of data presentation, only the percentage of multipolar metaphases are shown. Since the percentage of bipolar-normal metaphases were excluded, multipolar metaphases and bipolar-clustered metaphases comprise 100 percent.

It is anticipated that a statistically significant increase in the formation of multipolar spindles would negatively impact the cell viability of the cells possessing supernumerary centrosomes since centrosome clustering is essential for the survival. Cell viability assays were performed in N1E-115 cells following 120 hours of Nek2A overexpression. In comparison to control groups, a significant decrease in cell viability was detected when Nek2A was overexpressed (**Figure 3.1 E**). The data supports that expression level or the activity of Nek2A is crucial for the regulation of centrosome clustering and therefore the survival of cancer cells.

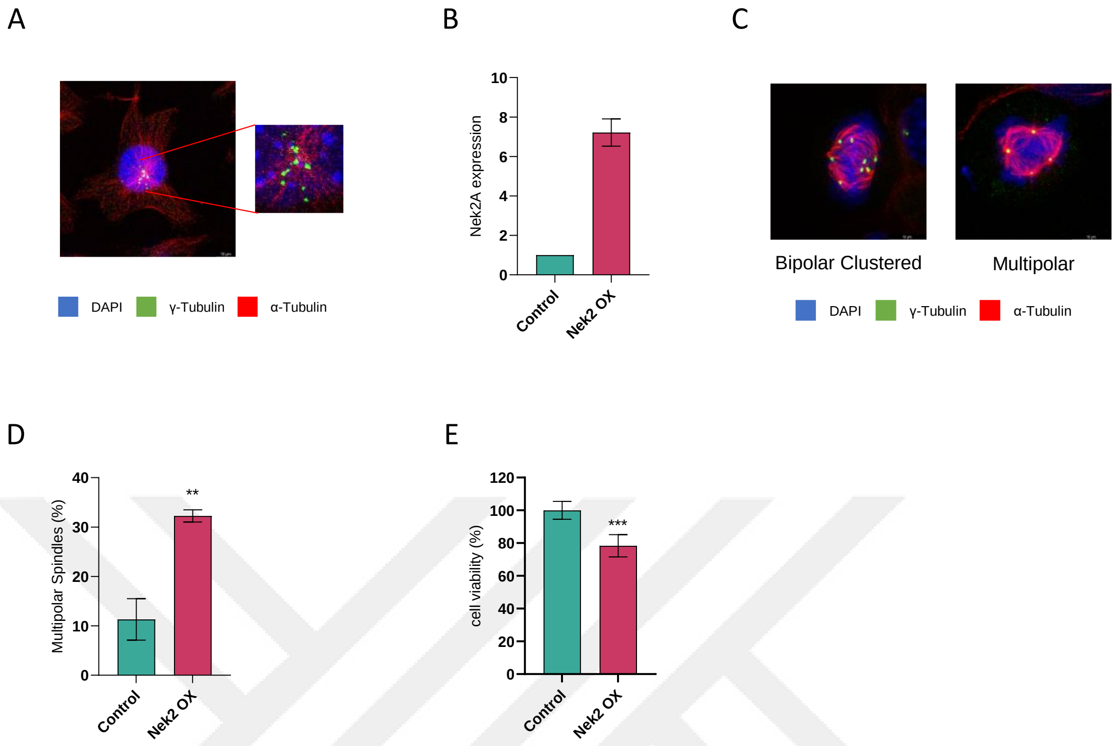

Figure 3.1: The effect of Nek2A overexpression on centrosome clustering and cell viability in mouse neuroblastoma cell line. (A) Immunofluorescence staining shows that N1E-115 cell line harbors supernumerary centrosomes, detected by γ-tubulin. **(B)** RT-qPCR verifying Nek2A overexpression (OX) in N1E-115 cells. **(C)** Immunofluorescence staining to identify multipolar and bipolar-clustered metaphases in N1E-115. **(D)** Metaphase scoring indicates that Nek2A OX significantly increases multipolar spindle pole formation. **(E)** WST-1 cell viability assay showing the decrease in cell viability when Nek2A is overexpressed

To verify the initial findings in human cells, three pancreatic ductal adenocarcinoma (PDAC) cell lines were characterized. Amongst different types of human cancers, pancreatic ductal adenocarcinoma (PDAC) cells have been reported to exhibit higher levels of centrosome amplification (Mittal et al., 2015). RT-qPCR was performed to detect relative expression levels of polo-like kinase 4 (PLK4) and STIL in MIA PaCa-2, Panc1 and SU86.86. High expression levels of PLK4 and STIL are associated with centrosome amplification, and PLK4 overexpression is commonly used as a tool to generate centrosome-amplified cells. Amongst the three PDAC cell lines tested, SU86.86

has the highest level of PLK4 and STIL expressions (**Figure 3.2 A**) and the highest CA (~ 22%), verified by γ-Tubulin staining and cell quantification (**Figure 3.2 B**). Next, SU86.86 cells were transduced to generate dox-inducible Nek2A overexpression, and the expression was verified by RT-qPCR (**Figure 3.2 C**). Likewise, in N1E-115, overexpression of Nek2A significantly induced MPS formation in SU86.86 ($p<0.01$), supporting that Nek2A involves in centrosome clustering (**Figure 3.2 D**). Cell viability assay also confirmed that Nek2A overexpression triggered cell death through unclustering extra centrosomes and inducing multipolar divisions (**Figure 3.2 E**).

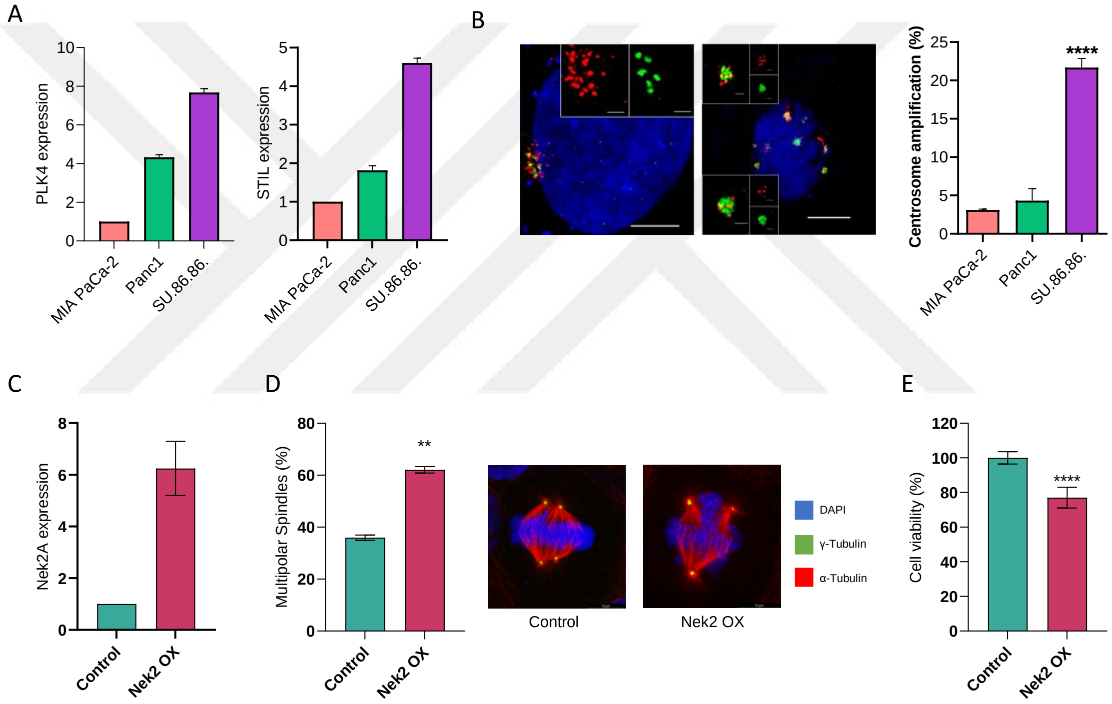

Figure 3.2: The effect of Nek2A overexpression on centrosome clustering and cell viability in human PDAC cell line. (A) RT-qPCR analysis to examine endogenous expression levels of PLK4 and STIL. **(B)** Immunofluorescence staining and quantification of centrosome amplification levels in 3 different PDAC cell lines. Green for γ-Tubulin, red for Centrin2. **(C)** RT-qPCR verifying Nek2A overexpression (OX) in Su86.86 cells. **(D)** Metaphase scoring indicates that Nek2A OX significantly increases multipolar spindle pole formation. **(E)** WST-1 cell viability assay showing the decrease in cell viability when Nek2A is overexpressed.

3.1.2 Effect of Nek2A Overexpression on Centrosome Dispersion

It is frequently observed that supernumerary centrosomes more often appear to be aggregated in close proximity during interphase. Being in close proximity could facilitate an easier, practical way to cluster extra centrosomes into two opposite poles. In addition to Nek2A's un-clustering effect on coalescent centrosomes during mitosis, its potential role in induction of dispersed centrosome arrangement in interphase cells was tested in N1E-115 and SU86.86 cell lines. It was hypothesized that overexpression of Nek2A could interfere centrosome clustering before mitosis, inducing scattering of extra centrosomes and consequently obstruct centrosome clustering.

Cells were seeded on coverslips and fixed after 48 hours of doxycycline-induced overexpression of Nek2A. Immunofluorescent staining was performed using anti γ-Tubulin antibodies to detect the centrosomes under fluorescent microscope. Cells were stratified based on aggregated and dispersed arrangement of supernumerary centrosomes. Nevertheless, Nek2A overexpression did not exhibit any change in the spatial arrangement of extra centrosomes in either N1E-115 or SU86.86 cells, suggesting that Nek2A affects centrosome clustering at the onset of mitosis (**Figure 3.3 A-B)**. Taken together; our data implies that expression levels of Nek2A are an essential factor for centrosome clustering in cancer cells exhibiting supernumerary centrosomes.

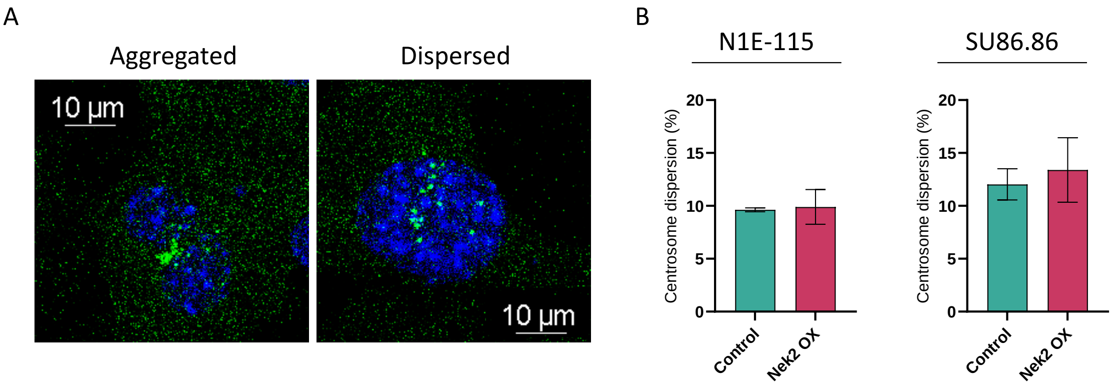

Figure 3.3: The effect of Nek2A overexpression on spatial arrengement of supernumarary centrosomes. (A) Representative images of aggregated and dispersed supernumerary centrosomes observed in interphase cells. Blue for DAPI and green for γ-Tubulin staining. **(B)** Quantification of centrosome dispersion observed in N1E-115 and SU86.86 cell lines.

3.1.3 Application of Centrosome Amplification Methods to Study the Role of Nek2A on Centrosome Clustering

In order to test whether Nek2A can still regulate centrosome clustering in cells with low levels of CA, Nek2A was ectopically overexpressed in MDA-MB-231 and U2OS cells (**Figure 3.4 A)**. MDA-MB-231 cells exhibit ~7% centrosome amplification while U2OS cells have approximately 1% (**Figure 3.4 B**). In contrast to cells with high CA, overexpression of Nek2A did not exhibit any significant reduction of centrosome clustering in neither MDA-MB-231 nor U2OS (**Figure 3.4 C-D**). These results suggest that centrosome un-clustering phenotype driven by Nek2A overexpression becomes clearer when CA levels are higher.

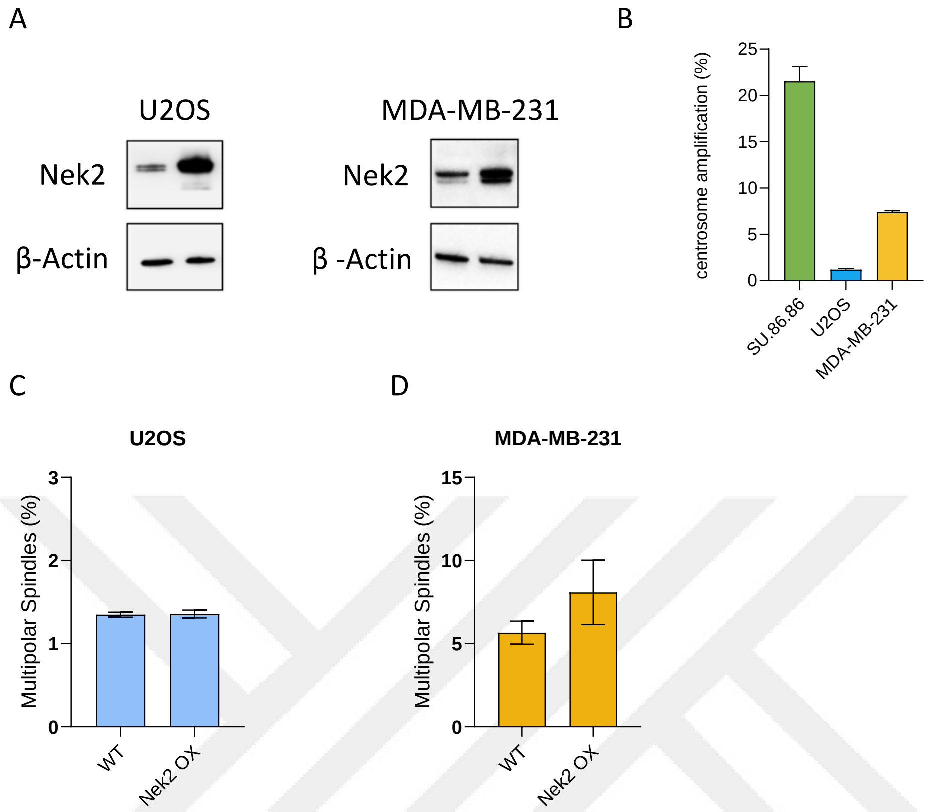

Figure 3.4: The effect of Nek2A overexpression on cell lines with relatively lower CA. (A) Western Blots to verify Nek2A overexpression in U2OS and MDA-MB-231 cell lines. **(B)** Quantification and comparison of centrosome amplification in U2OS and MDA-MB-231 cells. **(C-D)** Metaphase scorings to investigate the effect of Nek2A overexpression on the formation of multipolar spindle poles.

Although a cell line exhibits intrinsically low levels of CA, artificially amplifying centrosomes with several methods, such as Nocodazole treatment, Dihydrocytochalasin B (DCB) treatment and PLK4 or STIL overexpression, is possible. In that case, Nek2A would still be studied as a potential regulator of centrosome clustering. In this study, CA has been primarily induced by two distinct methods: Nocodazole treatment and PLK4 overexpression models alongside in U2OS and MDA-MB-231 cells (**Figure 3.5 A**). Working principles and the experimental setup are depicted in the figure. Nocodazole treatment achieved a three-fold increase in centrosome amplified MDA-MB-231 cell population, while more than ten-fold increase in U2OS cells was obtained. AS the second method, 72 hours of PLK4 overexpression induced CA in up to 40% of total populations in both MDA-MB-231 and U2OS cells (**Figure 3.5 B**). In addition to Nocodazole treatment and PLK4 overexpression methods, CA was successfully induced in U2OS cells using cytokinesis inhibitor DCB and overexpression of STILL (**Figure 3.5 C**).

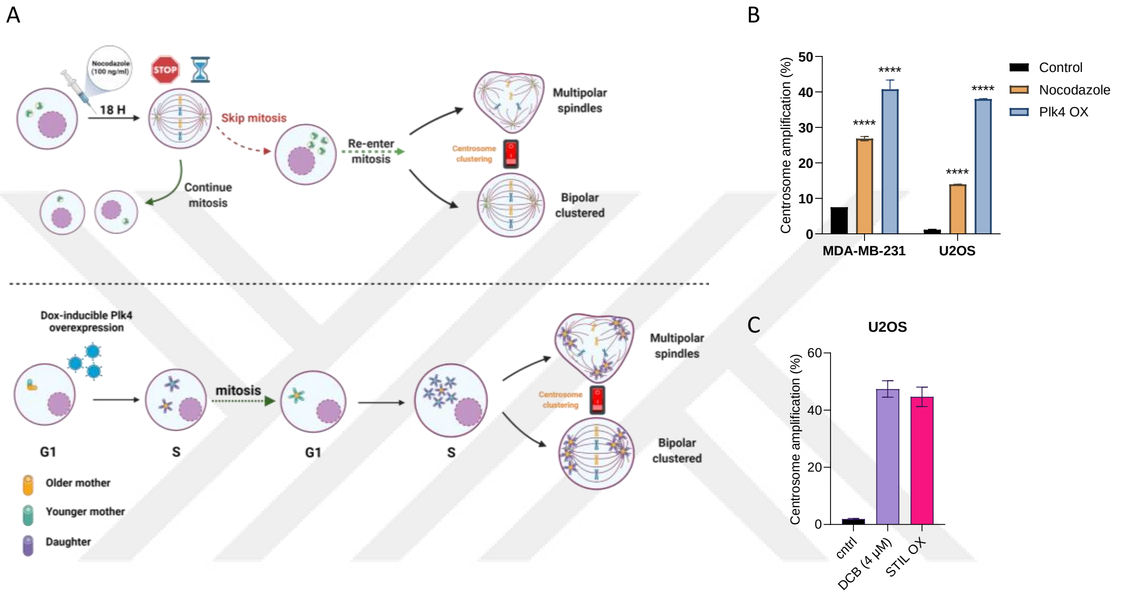

Figure 3.5: Centrosome amplification methdos to study the role of Nek2A. (A) Experimental setups to induce centrosome amplification by nocodazole (upper panel) and overexpression of PLK4 overexpression (lower panel). **(B)** Centrosome amplification levels obtained by nocodazole and PLK4 overexpression models in MDA-MB-231 and U2OS cells. **(C)** Centrosome amplification levels obtained by DCB treatment and STIL overexpression models in MDA-MB-231 and U2OS cells, as alternative methods**.**

3.1.4 Examining the Significance of Expression Levels and Kinase Activity of Nek2A on Centrosome Clustering

Utilizing the centrosome amplification methods discussed above, the importance of the expression levels and kinase activity of Nek2A on centrosome clustering was investigated. As previously described, metaphases were stratified and quantified as bipolar clustered or multipolar. Nek2A overexpression was achieved using the doxycycline-inducible system as previously introduced. Beside overexpression, suppression of Nek2A overexpression was also included in the experimental setup. CRISPR/Cas9 method was utilized to generate Nek2A knock-out monoclonal MDA-MB-231 and U2OS cells. In addition, Nek2A was silenced using siRNA transfections in both cell lines and in both CA models. To address the importance of kinase activity of Nek2A in terms of centrosome clustering, a specific chemical inhibitor JH295 was used. Furthermore, kinase-deficient mutant Nek2A (K37R) (Faragher & Fry, 2003) was generated using site-directed mutagenesis method and overexpressed in both cell lines to provide a dominant-negative phenotype. Expression levels in control, Nek2A overexpression, Nek2A KO and Nek2A RNAi groups were confirmed by Western Blot (**Figure 3.6 A**). Generation of kinase-deficient mutant of Nek2A was verified by Sanger sequencing (**Figure 3.6 B**).

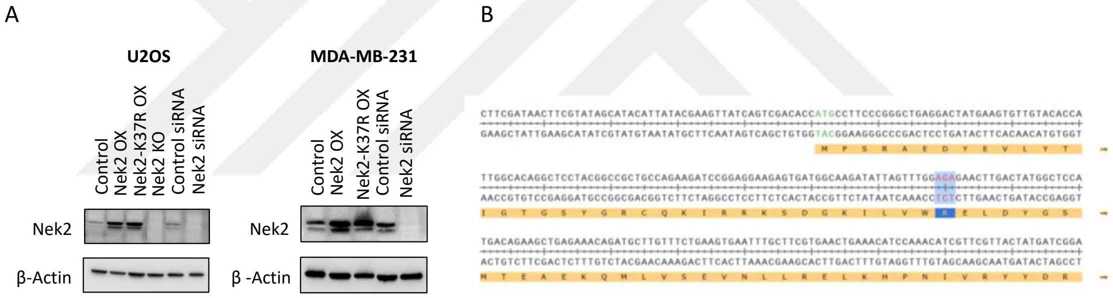

Figure 3.6: Verification of Nek2 expressions and kinase-mutant form. (A) Western Blots verifying the protein levels of Nek2. **(B)** Sanger Sequencing result to validate K37R mutant. Mutation is highlighted with blue and red colors.

Following the induced centrosome amplifications and expression manipulations on Nek2A in MDA-MB-231 and U2OS cells, fixation and γ-Tubulin staining were performed for the analysis of metaphases. As previously observed in N1E-115 and SU86.86 cells, overexpression of Nek2A promoted multipolarity in metaphase, significantly reducing centrosome clustering in both MDA-MB-231 (**Figure 3.7 A**) and U2OS cell lines (**Figure 3.7 B**). In addition, results showed that suppression of Nek2A significantly decreased the percentage of MPS. Furthermore, over-expression of the kinase-dead mutant acted similarly to chemical and transcriptional inhibition of Nek2A, favoring centrosome clustering. Taken together, the data strongly proves that the kinase activity of Nek2A regulates centrosome clustering in cancers cell with CA. Consistent results were obtained using two different cell lines and two distinct methods to amplify centrosomes in vitro. Moreover, two additional methods were applied to induce centrosome amplification and test the effect of Nek2A overexpression and knockdown on centrosome clustering. As an alternative method to Nocodazole treatment, Dihydrocytochalasin B (DCB) treatment was applied to mitotic slippage and result in amplified centrosomes. Overexpression of STIL was used as an alternative route induce centriole over-duplication and consequent CA (**Figure 3.7 C-D**). Supporting and verifying the previous findings, both alternative methods indicated that overexpression of Nek2A significantly increased formation of multipolar spindle poles while suppression of Nek2A expression significantly decreased multipolarity and favored centrosome clustering.

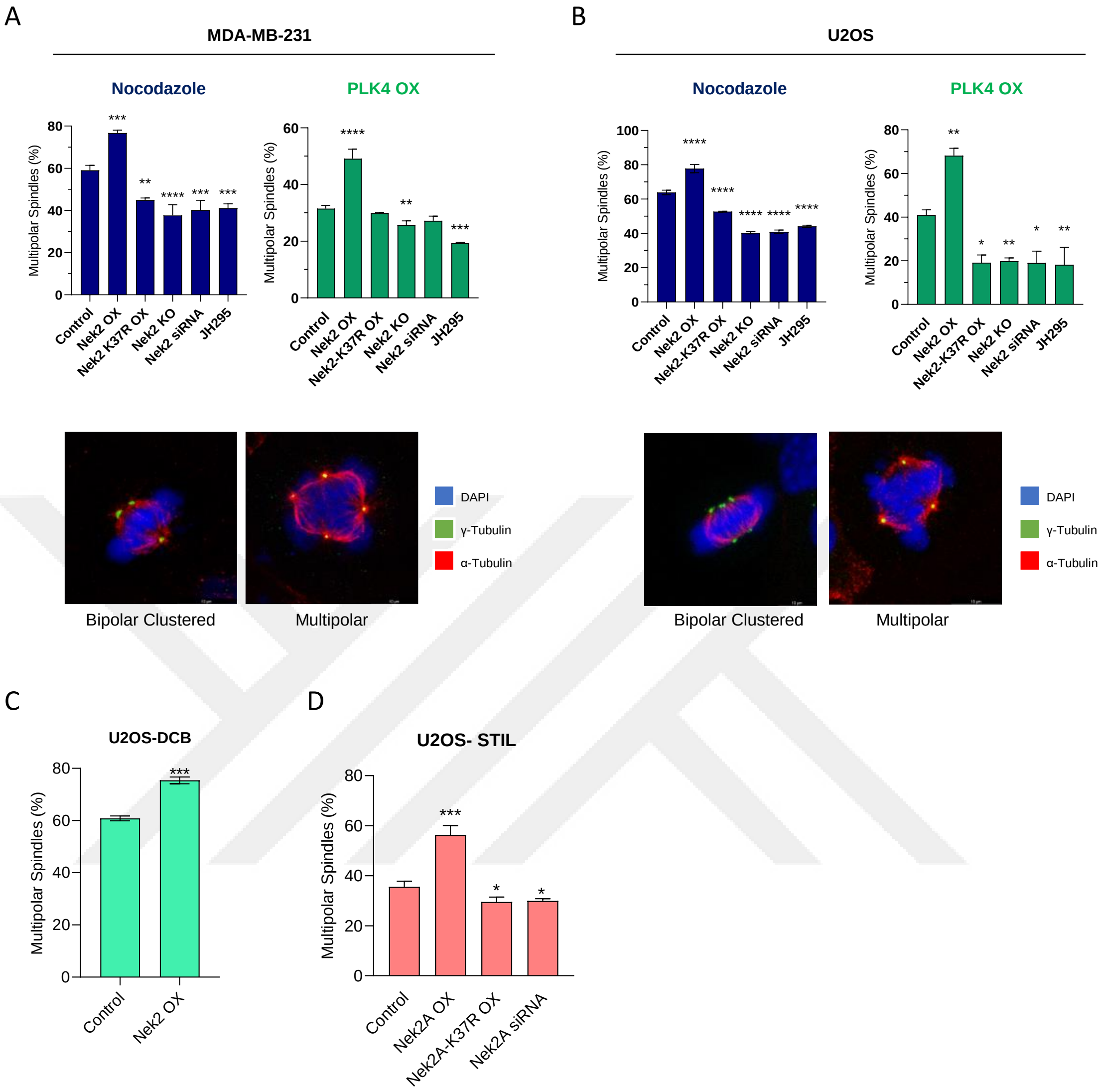

Figure 3.7: Nek2 regulates centrosome clustering in cells which are induced to have amplified centrosomes. (**A**) Percentage multipolarity of MDA-MB-231 cells in nocodazole (top-left), PLK4 (top-right) models and representative images (bottom) showing bipolar clustered and multipolar metaphases. (**B**) Percentage multipolarity of U2OS cells in nocodazole (top-left), PLK4 (top-right) models and representative images (bottom) showing bipolar clustered and multipolar metaphases. (**C-D**) Percentage multipolar spindles observed in DCB and STIL CA models.

3.1.5 Effect of Nek2A on Cell Cycle, Mitotic Index and Metaphase Duration

Nek2A is cell cycle regulated kinase playing important role in the regulation of centrosome cycle. In this part of the present study, it was investigated whether its overexpression or suppression could alter the populations at cell cycle stages, mitotic index, and duration of the metaphase. Nek2A expression was manipulated by overexpression or siRNA mediated knockdown in U2OS cells and cell cycle analysis, immunofluorescent staining, and live-cell imaging was performed at 48 hours. Cell cycle analysis by flow cytometry showed that overexpression of Nek2A does not have a significant effect on percentages of GO/G1, S and G2/M populations in U2OS cells (**Figure 3.8 A**). Mitotic index is defined as the ratio of cells found in mitosis over the total population observed under microscope. Mitotic index of control U2OS cells was compared with Nek2A overexpressing and Nek2A silenced groups. Calculations showed that neither overexpression nor suppression of Nek2A exhibited any significant chance on mitotic index (**Figure 3.8 B**). The impact of Nek2A expression levels on metaphase duration was further tested using live-cell microscopy. Briefly, U2OS cells were labelled with H2B-GFP by lentiviral transduction and seeded to glass-bottom culture dishes to track each cell and record the duration of metaphase. Double thymidine block was applied to synchronize the cells and released prior to live-cell imaging. Results showed that Nek2A overexpression significantly shortened the time spend at metaphase (**Figure 3.8 C**). Although siRNA-mediated knockdown slightly increased metaphase duration, the result did not satisfy statistical significance.

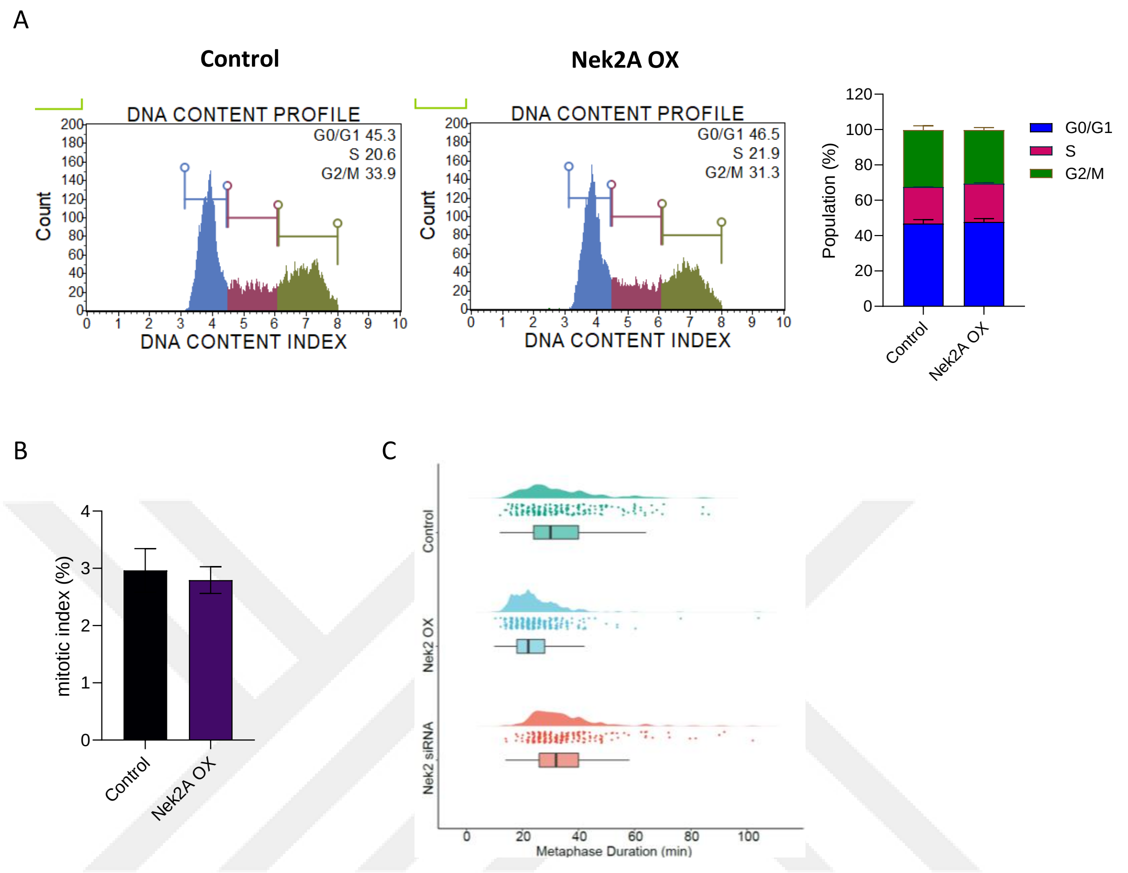

Figure 3.8: The impact of Nek2A expression on cell cycle events. (**A**) flow cytometric analysis indicates that overexpression of Nek2A does not alter cell cycle distribution in U2OS cells. (**B**) Quantification of mitotic index reveals that Nek2A overexpression does not significantly change the fraction of mitotic cells. (**C**) Live-cell imaging data reveals that Nek2A overexpression shortens the duration of metaphase significantlyy, while supression of Nek2A slightly prolongs metaphase.

3.2 Long Term Effects of Nek2A-driven Centrosome Clustering

Centrosome clustering is essential for cells harboring extra centrosomes to survive and proliferate. Overexpression of Nek2A could be a disadvantage to the cells with CA induction. In this part of the present study, *in vitro* and *in vivo* experiments were conducted to investigate whether long-term overexpression of Nek2A would promote depletion of cancer cells with centrosome amplification.

3.2.1 in vitro Competition Assay to Reveal Long Term Effect of Nek2A Overexpression on Centrosome Amplified Cells

Given that Nek2A overexpression impairs centrosome clustering, an in vitro competition assay was conducted to demonstrate that Nek2A-driven multipolar divisions are detrimental to centrosome amplified cells. To this end, Nek2A and PLK4 were overexpressed under dox-inducible promoters in U2OS and MDA-MB-231 cells. Dox-inducible PLK4 and Nek2A co-expressor cells (Dox-Plk4&Nek2A) were tagged with H2B-mCherry and dox-inducible PLK4 cells (Dox-Plk4) were tagged with H2B-eGFP. Next, these cells were mixed and co-cultured within the absence or presence of doxycycline for up to 15 days. Following the incubation periods, the percentages of mCherry tagged cells were quantified using two independent methods, flow cytometry and live-cell imaging (**Figure 3.9 A**). Significant depletions of mCherry-tagged cells, which underwent multipolar divisions due to Nek2A overexpression, were revealed by live cell imaging on day-5 and day-10 in U2OS cells (**Figure 3.9 B**). Consistent with live cell imaging and quantification, flow cytometry data exhibited dramatic depletion of Nek2A over-expressor cells undergoing constant centrosome amplification **(Figure 3.9 C)**.

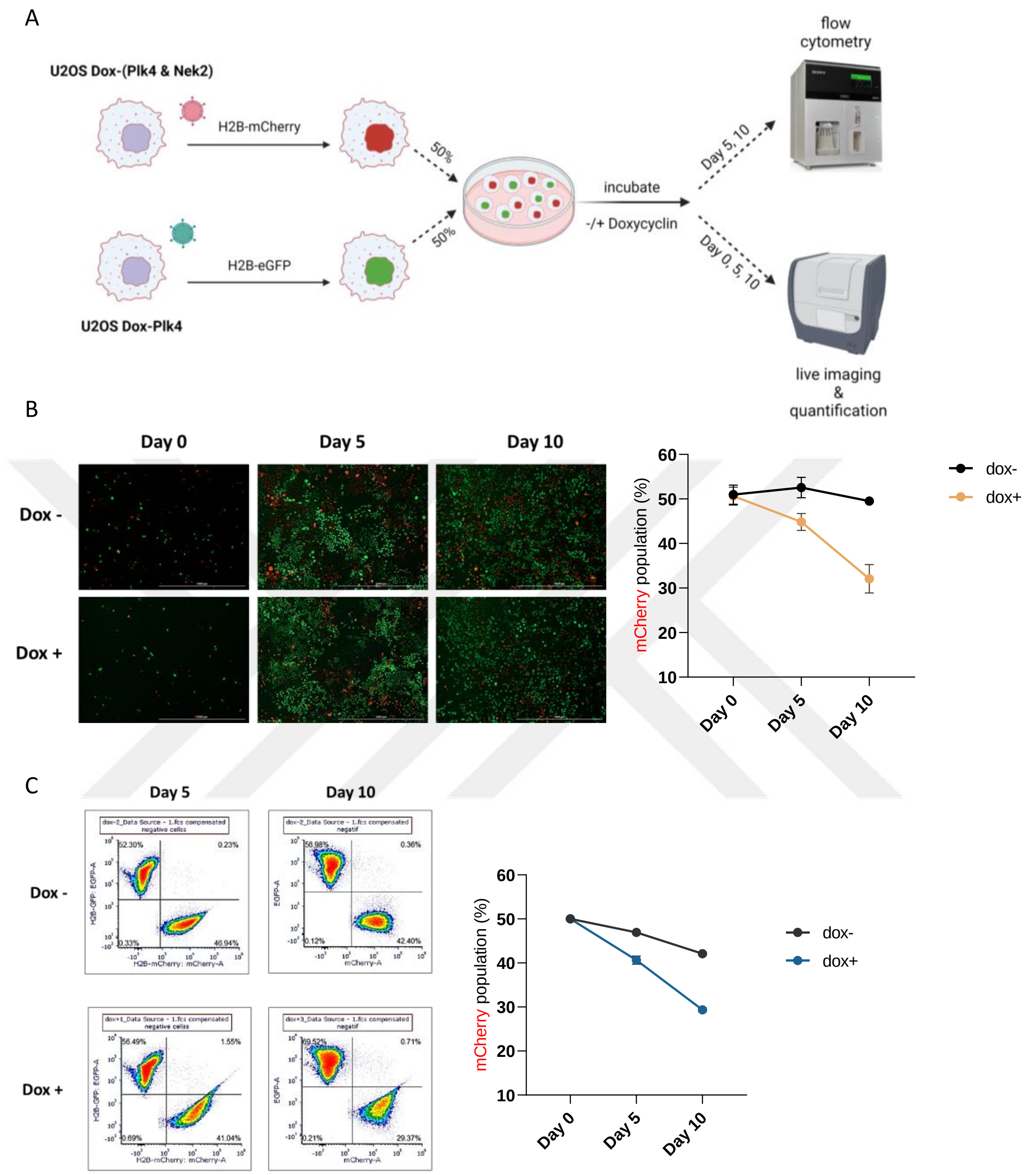

Figure 3.9: Nek2A overexpression results in depletion of cells with PLK4-induced CA. (**A**) Experimental setup of competition assay. (**B**) Represetative images from live-cell imaging and quantification of mcherry-tagged population in U2OS cells. (**C**) Flow cytometry-based aanalysis to verify live-cell imaging results obtained by U2OS cells.

Results were further validated with MDA-MB-231 cells using the same experimental set-up as U2OS (**Figure 3.10 A**). Moreover, the competition assay was conducted using SU86.86(WT)-H2B-eGFP and SU86.86(dox-Nek2A)-H2B-mCherry cells (**Figure 3.10 B**). Cell death and depletion of SU86.86 cells with high CA were confirmed by Nek2A overexpression, consistent with the previous data. The data implies that overexpression of Nek2A is detrimental to the cells with supernumerary centrosomes. Thus, the impairment of centrosome clustering by Nek2A overexpression promoted multipolar divisions and consequent cell death.

Further validation of Nek2A-driven cell death in centrosome amplified cells was performed using cell viability (**Figure 3.10 C**) and Annexin V (**Figure 3.10 D**) in U2OS cells. Data verified that overexpression of Nek2A selectively induced apoptosis and cell death in cells with CA.

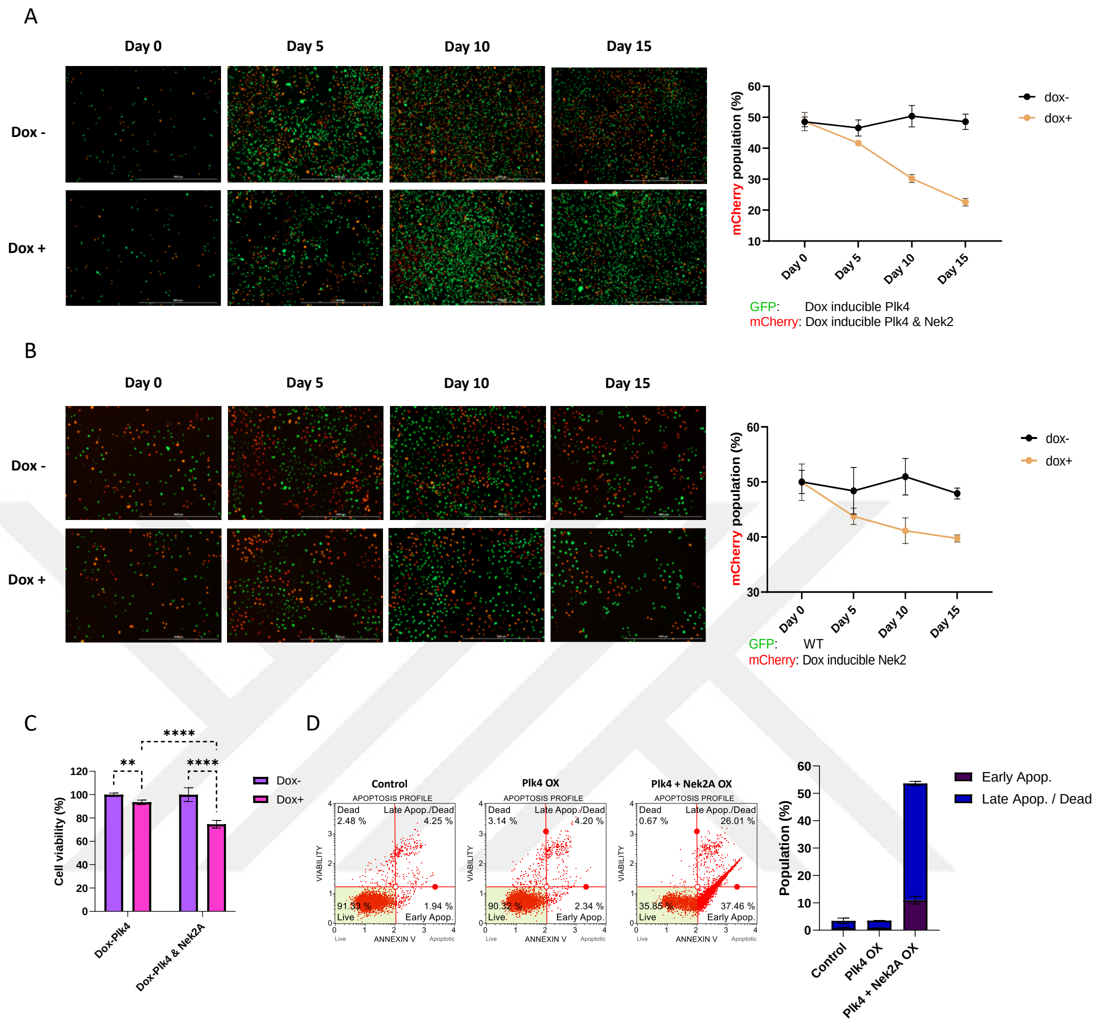

Figure 3.10: Nek2A overexpression results in depletion of cancer cells with CA. (**A**) Representative images of competetion assay and quantification of mcherry-tagged population in MDA-MB-231 cells. (**B**) Represetative images from live-cell imaging and quantification of mcherry-tagged population in SU86.86 cells. (**C**) WST-1 cell viability assay indicating cell death induced by Nek2A OX in centrosome amplified U2OS cells. (**D**) Annexin V assay confirming the apoptosis induced by multipolar metaphases.

3.2.2 in vivo Competition Assay to Reveal Long Term Effect of Nek2A Overexpression on Centrosome Amplified Cells

In addition to *in vitro* investigations, an *in vivo* version of the competition assay was established by subcutaneously implanting equal mixtures of eGFP-tagged U2OS dox(PLK4) and mCherry-tagged U2OS Dox(PLK4&Nek2A) cells (**Figure 3.11 A**). Eight weeks post-injection of the cells, the tumors were collected, sectioned, and prepared for imaging and quantification of mCherry-tagged cell populations in each image taken from different sections of the tumors. Image quantification demonstrated a significant depletion of Nek2A overexpressing cells (mCherry population) with centrosome amplification (**Figure 3.11 B**). In conclusion, the data reveals that multipolar divisions induced by Nek2A overexpression result in cell depletion in both in vitro and in vivo models.

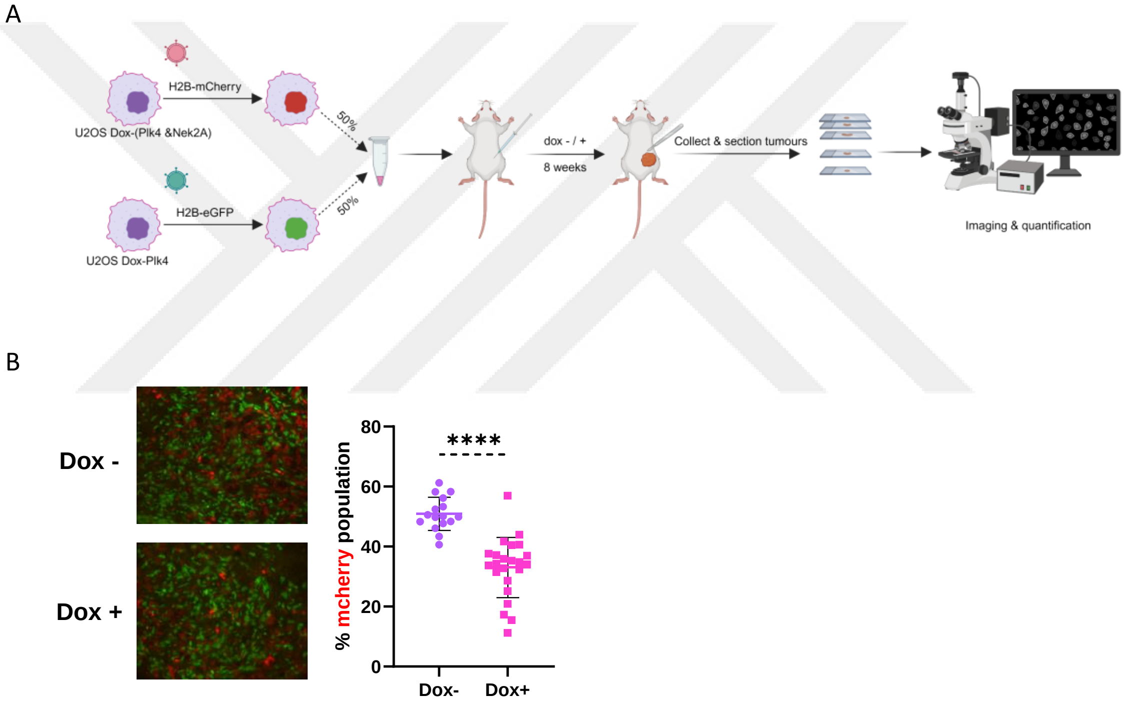

Figure 3.11: Nek2A overexpression results in depletion of cancer cells with CA *in vivo*. (**A**) Experimental setup of *in vivo* competition assay. (**B**) Representative images of tumour tissue slices and quantification of mCherry-tagged U2OS Dox (PLK4 & Nek2) cells.

3.3 Analysis of Known Nek2A Kinase Targets on Centrosome Clustering

3.3.1 Centrosomal Targets of Nek2A Kinase

In the present study, so far, it has been indicated that expression levels of Nek2A are crucial for regulating centrosome clustering during metaphase. Elucidating the molecular mechanism through identifying target proteins of Nek2A is essential to contribute to molecular cancer research. Nek2A kinase has been acknowledged as a regulator of the centrosome cycle by inducing centrosome separation through phosphorylating linker proteins C-Nap1 (CEP250), Rootletin (CROCC) and GAS2L1. Non-centrosomal targets of Nek2A, Hec1 and Trf1 have also been reported to involve in indirect regulation of microtubule attachments could also act as direct downstream targets in Nek2A-driven centrosome unclustering pathway (**Figure 3.12**).

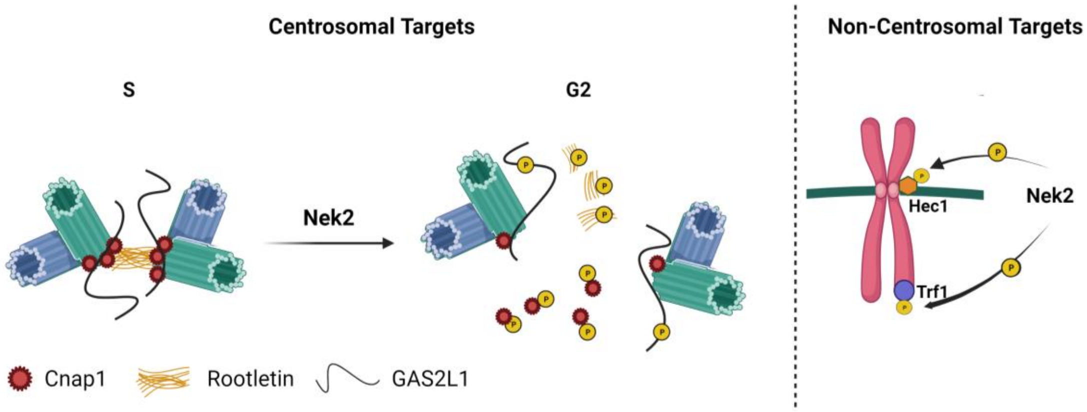

Figure 3.12: Known centrosomal and non-centrosomal targets of Nek2A kinase.

It is primarily hypothesized that Nek2A could involve in centrosome clustering mechanism through phosphorylation of its known centrosomal targets and suppression of these targets would abolish the phenotype observed when Nek2A is overexpressed. In U2OS cells, centrosome amplification (CA) was induced using two independent methods, Nocodazole and Plk4 overexpression, as previously mentioned.

CRISPR/Cas9 technology was utilized to generate monoclonal C-Nap1 knock-out U2OS (dox-Nek2A) cells (**Figure 3.13 A**) and metaphases were scored to investigate the effect on centrosome clustering in combination with doxycycline-inducible Nek2A overexpression. As observed previously, Nek2A overexpression resulted in a significant reduction in the percentage of bipolar clustered metaphases. Intriguingly, C-Nap1 knock-out impaired centrosome clustering in both CA models, independent of Nek2A **(Figure 3.13 B)**. In the nocodazole model of CA, overexpression of Nek2A in C-Nap1 knock-out cells exhibited an extra decrease in centrosome clustering, suggesting that absence of C-Nap1 promotes the formation of multipolar spindles independent of Nek2A activity. Nevertheless, in the Plk4 model, overexpression of Nek2A in C-Nap1 knock-out did not exhibit the additive reduction in bipolar clustered metaphases as observed in the nocodazole model. Since distinct mechanisms to generate centrosome amplified cells were used, phenotypic responses may slightly differ.

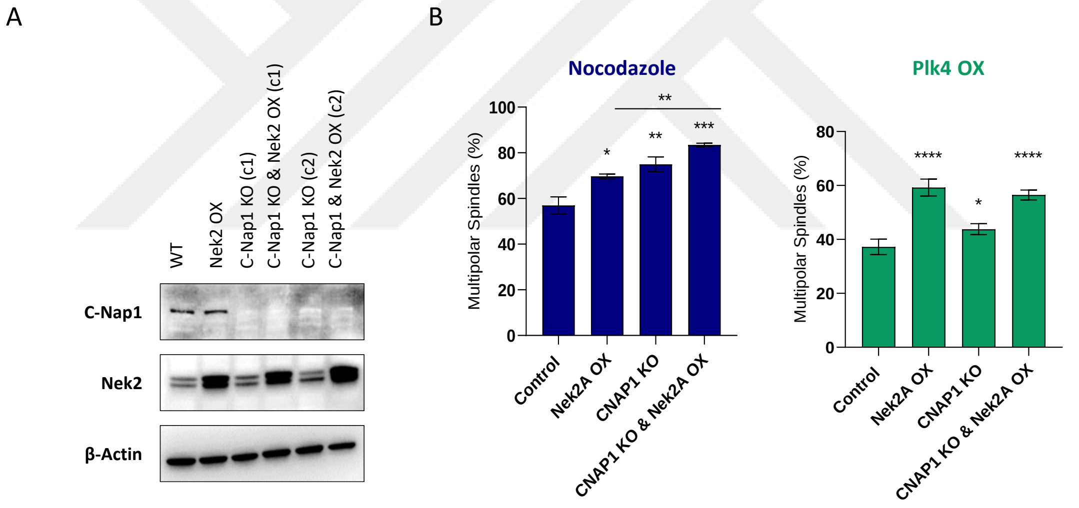

Figure 3.13: Examination of the role of C-Nap1 on Nek2A-regulated centrosome clustering. (**A**) Western Blot verifies CRISPR/Cas) mediated KO of C-Nap1 and overexpression of Nek2A in U2OS cells. (**B**) Percentage multipolar spindles observed and quantified in nocodozole and PLK4 models to examine the role of C-Nap1.

Rootletin is another centriolar linker protein phosphorylated by Nek2A to induce centrosome disengagement. CRISPR/Cas9 technology was utilized to generate monoclonal rootletin knock-out U2OS (dox-Nek2A) cells (**Figure 3.14 A**) and metaphases were scored to investigate the effect on centrosome clustering in combination with doxycycline-inducible Nek2A overexpression. Metaphase scoring of the nocodazole model showed that rootletin knock-out does not affect centrosome clustering, while Nek2A overexpression is still able de-cluster centrosomes and promotes multipolar spindle formation **(Figure 3.14 B)**. Interestingly, rootletin knock-out cells exhibited a significantly increased centrosome clustering ability in Plk4 induced CA. However, overexpression of Nek2A in rootletin knock-out cells decreased centrosome clustering compared to the control group. Moreover, compared to the Nek2A overexpression group, it was noticed that loss of rootletin moderately rescued centrosome clustering ability. The data implies that rootletin may involve centrosome clustering events autonomous of Nek2A kinase.

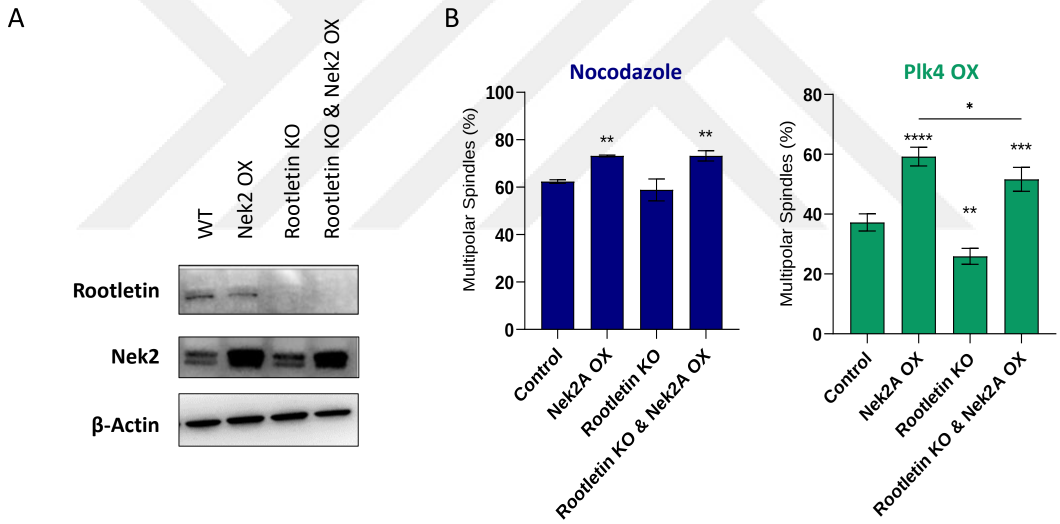

Figure 3.14: Examination of the role of Rootletin on Nek2A-regulated centrosome clustering. (**A**) Western Blot verifies CRISPR/Cas) mediated KO of Rootletin and overexpression of Nek2A in U2OS cells. (**B**) Percentage multipolar spindles observed and quantified in nocodozole and PLK4 models to examine the role of Rootletin.

It was further examined phenotypic outcomes of C-Nap1 and rootletin knock-outs regarding the distance between mature centrosomes at G1/S. It was considered that increased levels of multipolar spindle formations could result from dispersed or early disjointed centrosomes before mitotic entry. Therefore, immunofluorescence staining was performed to label centrosomes using anti-γ-tubulin antibodies and the distance between the centres of γ-tubulin foci were measured by confocal microscopy **(Figure 3.15)**. It was noticed that loss of C-Nap1 caused a significant increase in the median distance between centrosomes, suggesting that centrosome clustering might be disrupted due to dispersion. Rootletin knock-out did not result in a statistically significant increase in centrosome distance.

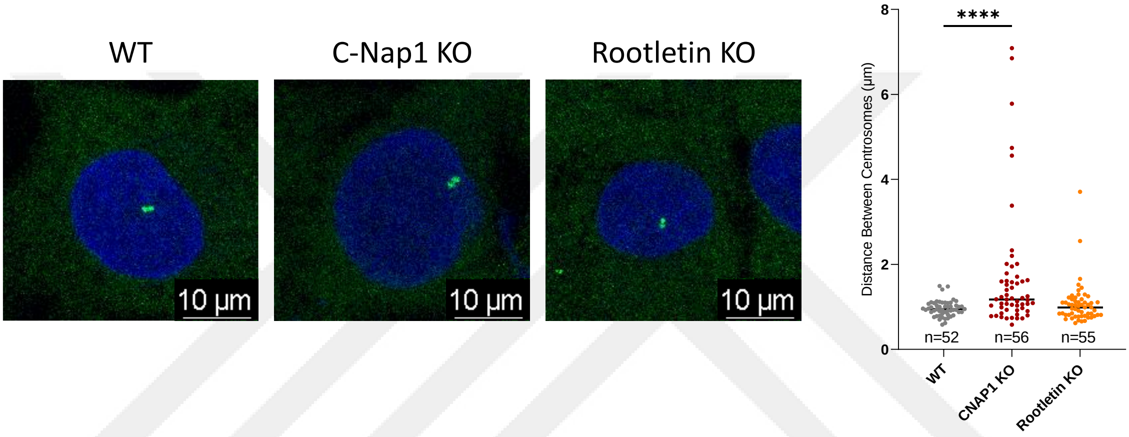

Figure 3.15: Measurement of centrosome distance in C-Nap1 and Rootletin KO cells. Cell were synronized by double-thymidine block and stained with anti γ-Tubulin (green). Centrosome distances were measured manually by using LASX tool provided by Leica.

As another candidate and known centrosomal target of Nek2A, GAS2L1 was examined, which also contributes to centrosome cohesion and is regulated by the kinase activity of Nek2A. GAS2L1 expression was suppressed using siRNA transfection **(Figure 3.16 A)** and its effect on centrosome clustering was tested **(Figure 3.16 B)**. Suppression of GAS2L1 was inefficacious to uncluster extra centrosomes and could not interfere with Nek2A-driven induction of multipolarity in both CA models.

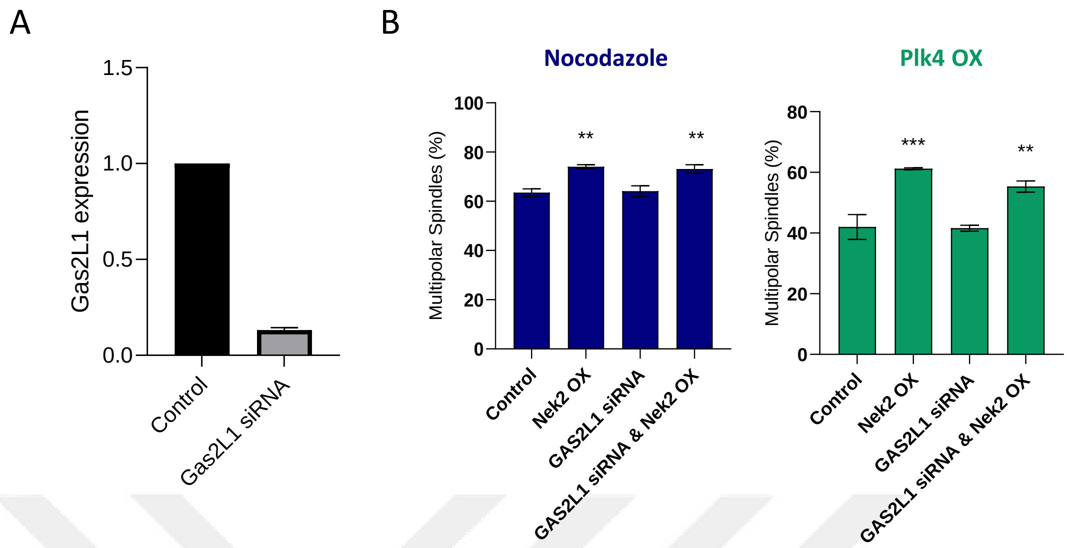

Figure 3.16: Examination of the role of GAS2L1 on Nek2A-regulated centrosome clustering. (**A**) RT-qPCR to verify siRNA-mediated knockdown of GAS2L1 in U2OS cells. (**B**) Percentage multipolar spindles observed and quantified in nocodozole and PLK4 models to examine the role of GAS2L1.

3.3.2 Non-Centrosomal Targets of Nek2A Kinase

In addition to centrosomal targets of Nek2A, two non-centrosomal targets were tested, which could act as downstream targets of the centrosome clustering mechanism regulated by Nek2A activity. TRF1 was silenced using siRNA transfection **(Figure 3.17 A)**. As indicated previously, the importance of TRF1 on centrosome clustering was investigated along with Nek2A **(Figure 3.17 B)**. Results showed that suppression of TRF1 does not inhibit centrosome clustering in the nocodazole CA model and is not able to interfere centrosome unclustering when Nek2A is overexpressed. Intriguingly, in the Plk4 CA model, a significant increase in multipolar metaphases was observed. Overexpression of Nek2A in TRF1 silenced cells still induced multipolar divisions, implying that TRF1 is not essential for the unclustering activity of Nek2A.

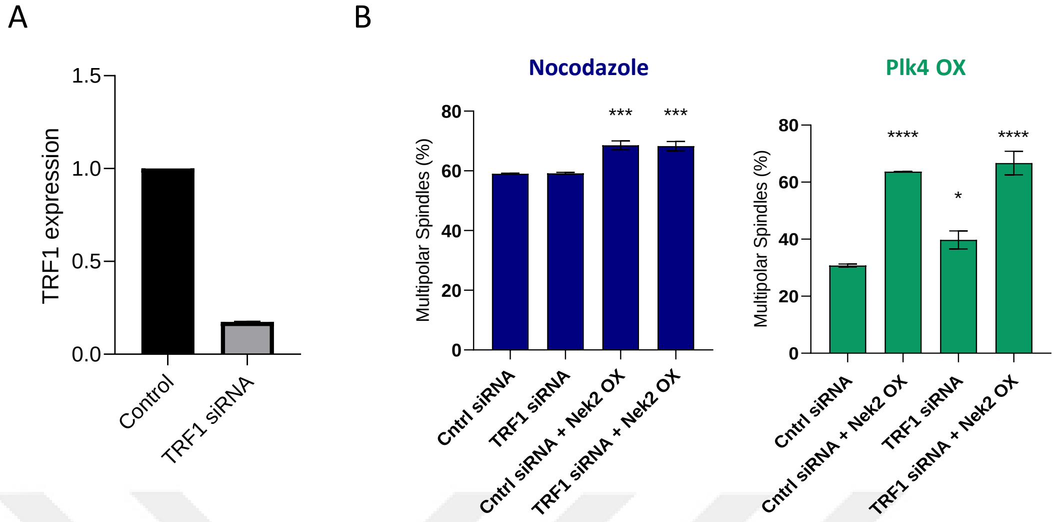

Figure 3.17: Examination of the role of TRF1 on Nek2A-regulated centrosome clustering. (**A**) RT-qPCR to verify siRNA-mediated knockdown of TRF1 in U2OS cells. (**B**) Percentage multipolar spindles observed and quantified in nocodozole and PLK4 models to examine the role of TRF1

Moreover, the contribution of Hec1 on the activity of Nek2A was investigated. U2OS cells were treated with 2.5 µM INH154, a highly potent Nek2A and Hec1 binding inhibitor, centrosome clustering at metaphase was scored. Results in both CA models demonstrated that inhibition of Hec1 and Nek2A interaction does not alter centrosome clustering and ablate the phenotype of Nek2A overexpression at metaphase **(Figure 3.18)**. Inhibition Nek2A and Hec1 interaction using 5 µM INH154 also did not create any significant change in the phenotype. In conclusion, our data suggests that TRF1 and Hec1 do not participate in the underlying molecular mechanism regulated by Nek2A.

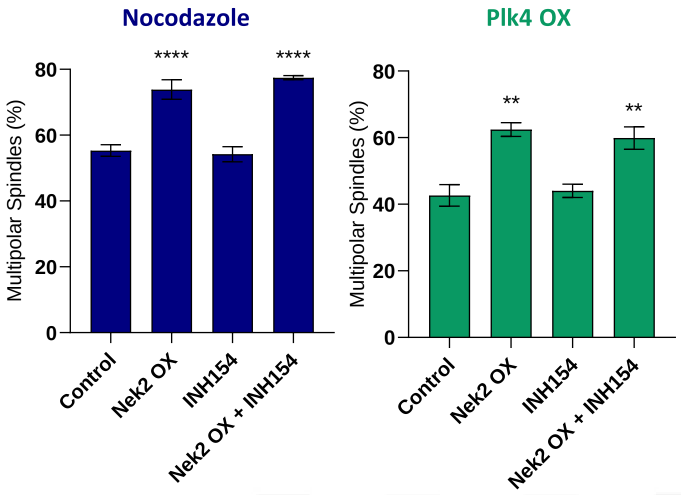

Figure 3.18: Examination of the role of Nek2-Hec1 interaction on Nek2A-regulated centrosome clustering. Percentage multipolar spindles observed and quantified in nocodozole and PLK4 models to examine the role of Nek2-Hec1 interaction using specific inhibitor INH154 (2.5 µM).

3.4 Proximity Labelling to Identify Novel Nek2A Kinase Targets Essential for Centrosome Clustering

Revealing the interaction partners of Nek2A is essential for a better understanding of centrosome clustering in cancer. Thus far, several proteins have been identified as regulators of centrosome clustering. Exploiting the TurboID proximity labelling system and proteomics tools, it was aimed to identify interaction partners of Nek2A to exert its activity on regulation of centrosome clustering.

3.4.1 Establishment, Validation and Preliminary Analysis of TurboID-Nek2A System

Initially, FLAG-TurboID-Nek2A and FLAG-TurboID-Nek2A(K37R) expression plasmids were constructed throught subcloning of WT and kinase-deficient mutant of Nek2A coding DNAs into FLAG-TurboID plasmids to obtain ectopic expression of Nek2A linked with biotin ligase in U2OS cells **(Figure 3.19 A)**. Next, the cellular localization and biotinylated proteins were verified by immunostaining **(Figure 3.19 B)**.

Microscopy images showed that FLAG-TurboID Nek2A localizes to centrosomes as endogenous Nek2A does. Streptavidin staining also confirmed that most of the biotinylated proteins are located in centrosomes, implying that identified and enriched proteins are likely involved in centrosome clustering mechanisms. It was also verified that FLAG-TurboID-Nek2A(K37R) localises to centrosomes, confirming that kinase-dead mutant could provide the identification of interactome similar to wild type version **(Figure 3.19 C)**. Our analysis identified biotinylation of known targets of Nek2A (CROCC & LRRC45), previously reported regulators of centrosome clustering (NUMA1 & KIFC1) and potential novel interaction partners of Nek2A (KIF2C) **(Figure 3.19 D)**

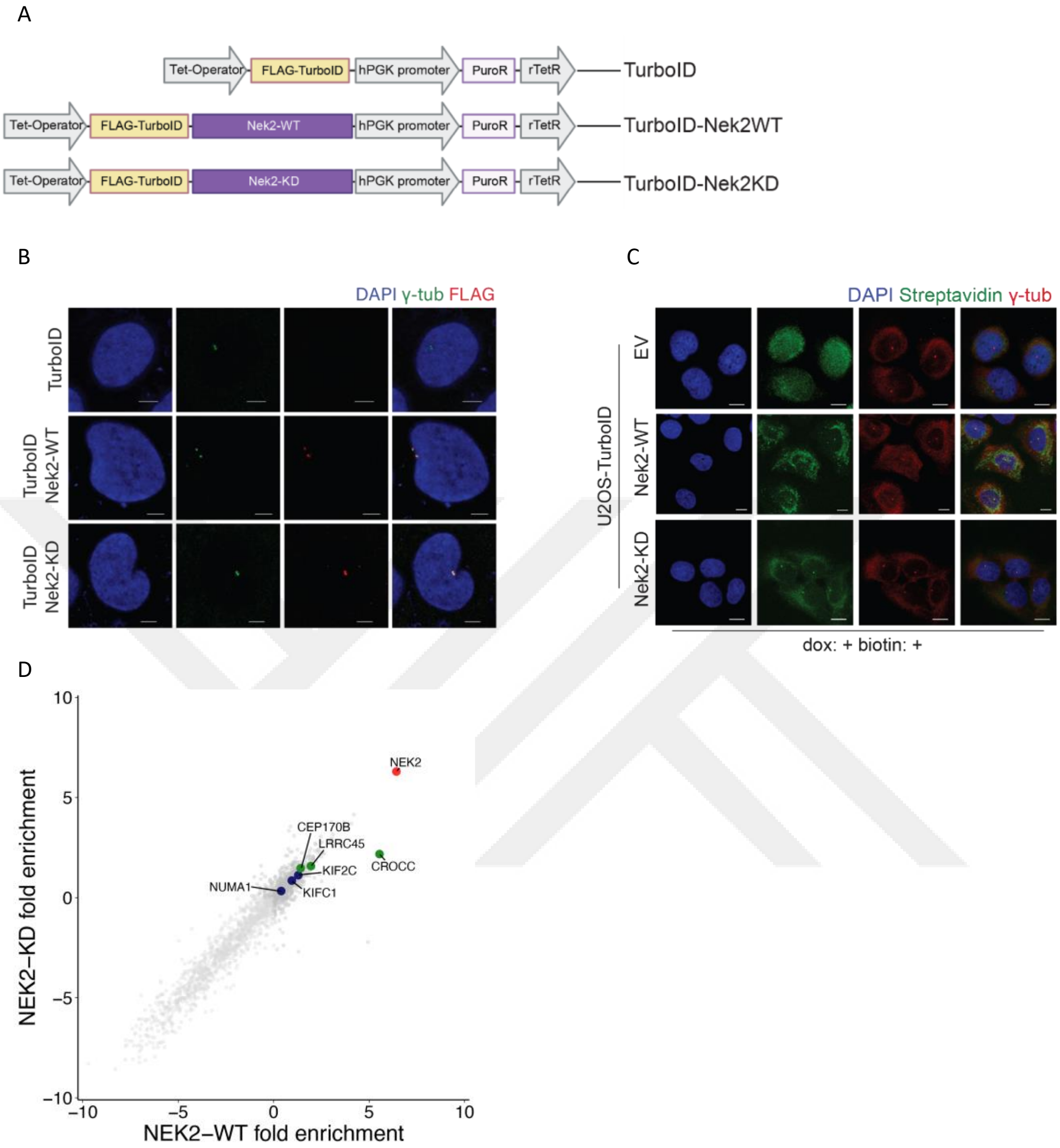

Figure 3.19: Proximity labelling reveals interaction partners of Nek2A. and KIF2C regulating centrosome clustering. (**A**) Turbo-ID proximity labelling system to identify interaction partners of Nek2A. (**B-C**) Cellular localization of TurboID-Nek2A and biotinylated proteins, verified by IF staining. (**D**) Plot showing enriched biotinylated proteins identified by Mass-Spec. Data was generated by MaxQuant LFQ analysis. Targets selected for further analysis were marked with blue colour, known interaction partners of Nek2A was coloured green.

3.4.2 Examination of Previously Described Centrosome Clustering Factors Detected in Close Proximity of Nek2A

Based on the analysis outcome, the presence of previously described centrosome clustering factors, Kifc1 and NuMA led us to investigate a potential interaction collaborative regulation of centrosome clustering along with Nek2A. To test the relationship with Nek2A, NuMA was silenced using siRNA transfection **(Figure 3.20 A)** and Nek2A was overexpressed to assess the dependency of molecular mechanisms regulated by Nek2A activity. As previously reported in literature, silencing NuMA resulted in a significant decrease in multipolar spindle formation. However, overexpression of Nek2A could still induce multipolar division in the absence of NuMA in three different experimental setups, suggesting that Nek2A orchestrates an independent mechanism than NuMA **(Figure 3.20 B)**. Results showed that silencing NuMA did not inhibit the action of Nek2A overexpression but oppositely competed in the formation of multipolar spindle poles during metaphase. Here, the data emphasizes that NuMA and Nek2A regulate centrosome clustering independently and in opposite directions. Alternatively, Nek2A expression was silenced in NuMA overexpressing cells. Overexpression of NuMA resulted in a significant increase of multipolar metaphases despite Nek2A knock-down, and a similar attenuation pattern in centrosome clustering was observed (**Figure 3.20 C**).

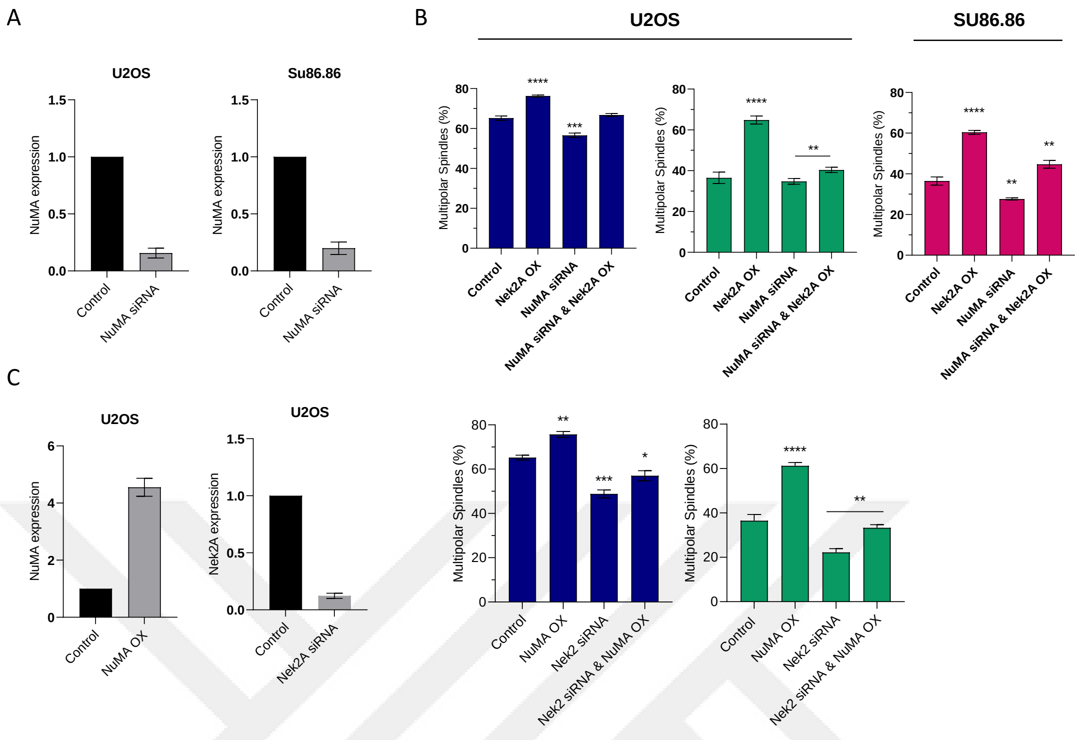

Figure 3.20: The significance of NuMA on the action of Nek2A. (**A**) RT-qPCR analysis to verify knockdown of NuMA in U2OS and SU86.86 cells. (**B-C**) Percentage of multipolar metaphases observed in varying conditions for NuMA. Experiments were performed using nocodazole and Plk4 CA models in U2OS and endogenously extra centrosome harbouring cell line SU86.86.

Furthermore, the prospective interaction and cross-talk between Nek2A and KIFC1, a known centrosome clustering regulator, was examined. Knock-down of KIFC1 impaired centrosome clustering and significantly increased multipolarity. Overexpression of Nek2A in KIFC1 silenced cells could further increase the percentage of multipolar metaphases, suggesting that KIFC1 and Nek2A involve in distinct molecular pathways coordinating centrosome clustering **(Figure 3.21 A)**. Consistently, RNA interference of Nek2A partially improved centrosome clustering in cells lacking KIFC1 expression (**Figure 3.21 B)**.

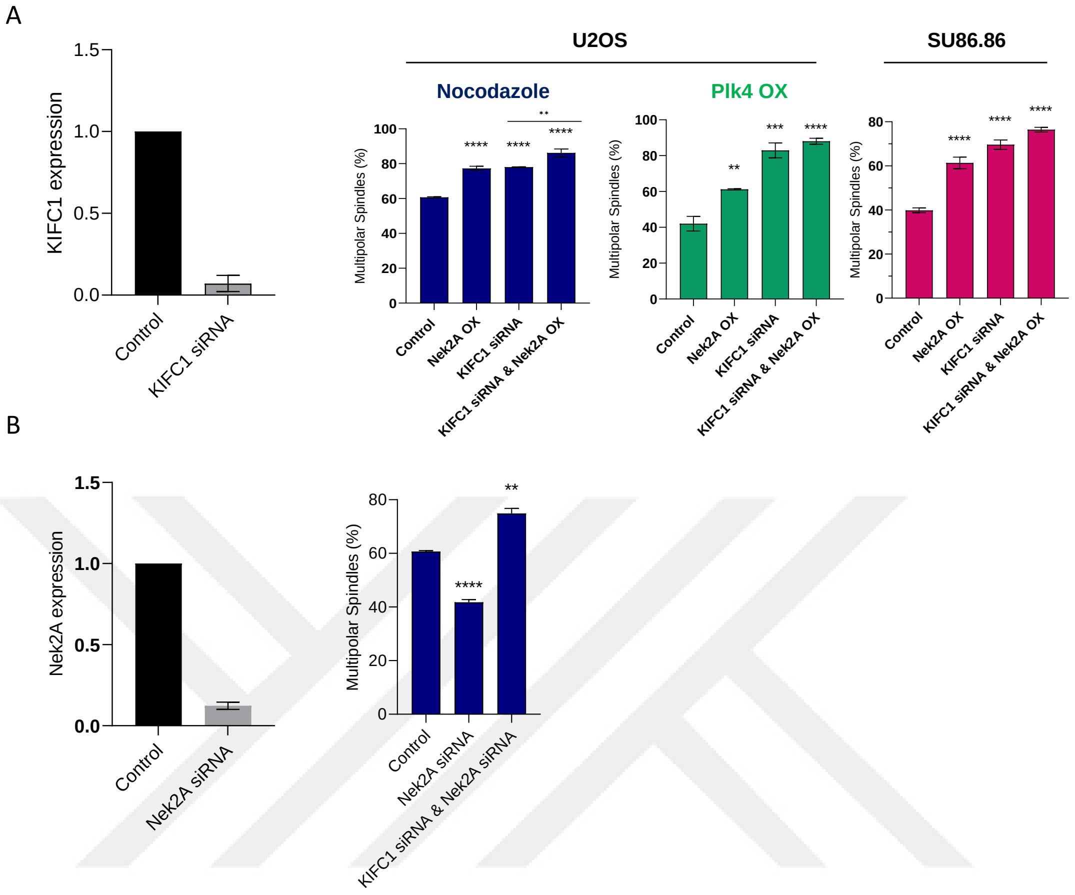

Figure 3.21: The significance of KIFC1 on the action of Nek2A. (**A**) RT-qPCR analysis to verify knockdown of KIFC1 and percent multipolar spindle poles observed in varying conditions. (**B**) RT-qPCR to verify Nek2A knockdown and percentage of multipolar metaphases observed when Nek2A and KIFC1 is silenced in U2OS cells.

3.4.3 Validation of KIF2C as a Novel Interaction Partner of Nek2A

Further evaluation of proximity labelling data led us to investigate the role of KIF2C (kinesin family member 2C) on Nek2A-regulated centrosome clustering mechanism. Intriguingly, cancer patient datasets show that Nek2A and KIF2C expressions are highly correlated (Spearman: 0.75, Pearson: 0.79, R^2: 0.62) **(Figure 3.22 A)**. Positive correlation in terms of mRNA expression and close proximity in these two proteins have promised an interaction which could regulate centrosome clustering. We aimed to verify the direct physical interaction between KIF2C and Nek2A by co-immunoprecipitation **(Figure 3.22 B)**. We ectopically expressed FLAG-tagged Nek2A in U2OS cells were transfected with FLAG-tagged Nek2A. Pull down of Nek2A and interacting proteins was performed by anti-FLAG antibody. Nek2A pulldown resulted in clear co-immunoprecipitation of KIF2C. A second pulldown was also performed using an anti-KIF2C antibody and detected Nek2A as a co-immunoprecipitated protein, verifying the physical interaction. Immunofluorescent staining also confirmed the co-localisation of Nek2A and KIF2C on spindle poles **(Figure 3.22 C)**.

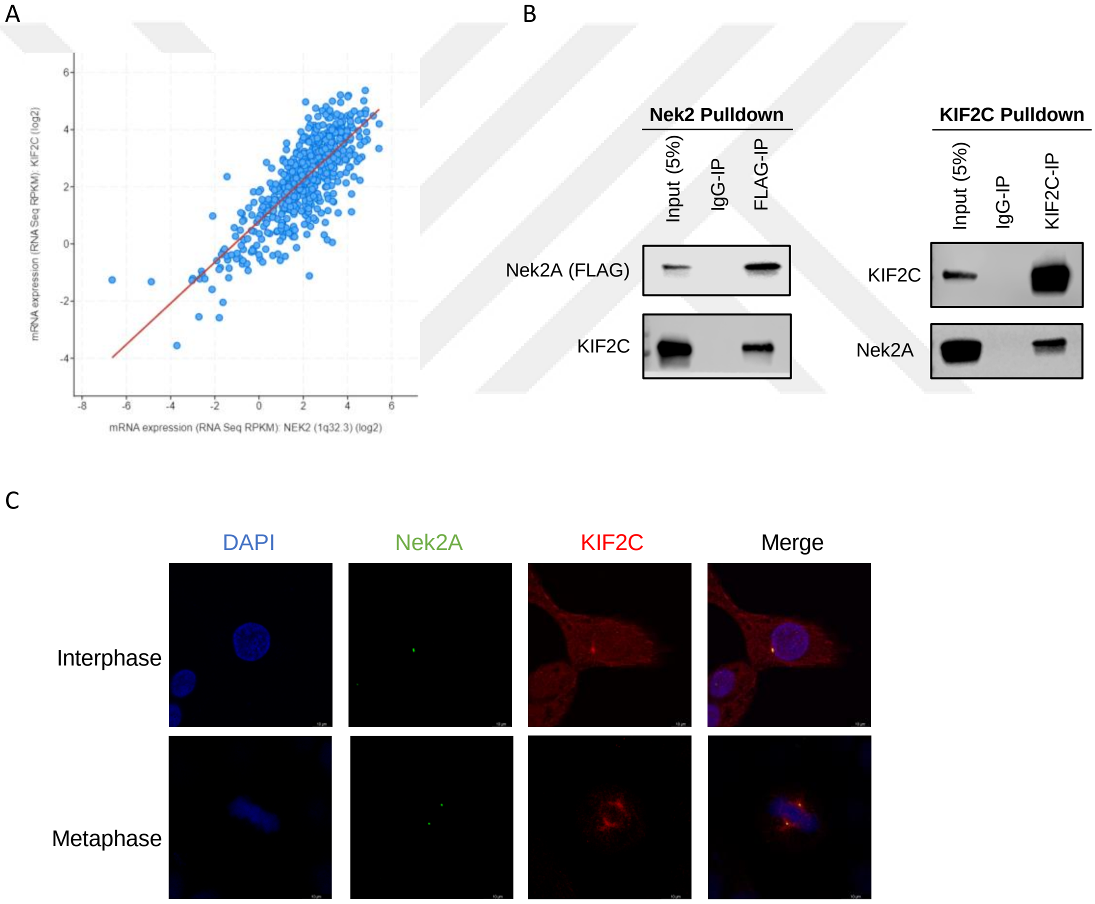

Figure 3.22: Validation of KIF2C as a novel interaction partner of Nek2A. (**A**) Pan-cancer Analysis of Advanced and Metastatic Tumors data retrieved from cBioPortal demonstrates positive correlation between NEK2 and KIF2C expressions. (**B**) Co-IP assay to verify physical interaction between Nek2A and KIF2C. (**C**) IF staining demonstrating the co-localization of Nek2A and KIF2C in centrosomes and spindle poles.

3.4.4 Examining the Role of KIF2C on Centrosome Clustering Mechanism Regulated by Nek2A

In this part of the present study, the effect of KIF2C knock-down in MPS formation was investigated using nocodazole and Plk4 OX models of U2OS and SU86.86 cells **(Figure 3.23 A)**. Suppression of KIF2C significantly decreased the percentage of multipolar metaphases in both models of U2OS and SU86.86 cells. Strikingly, the depletion of KIF2C reverted the action of Nek2A overexpression in terms of centrosome unclustering **(Figure 3.23 B)**. The data suggests that interaction between KIF2C and Nek2A is essential to regulate the clustering of extra centrosomes during metaphase.

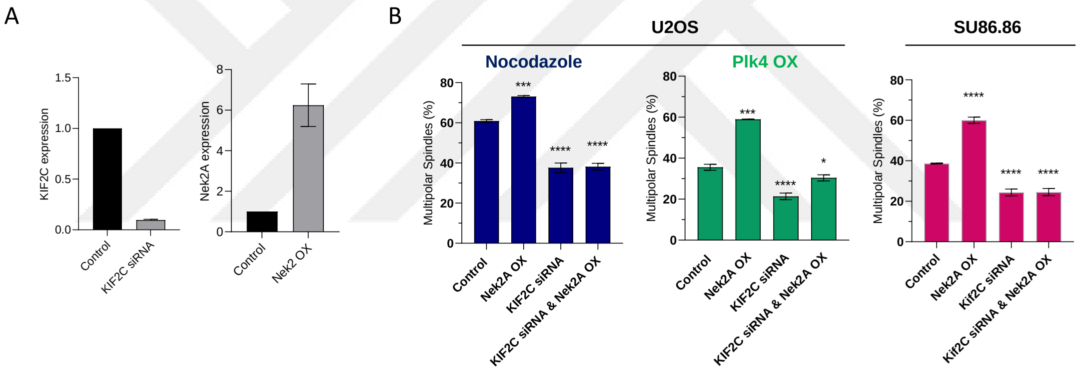

Figure 3.23: **Examining the essentiality of KIF2C for Nek2A to exert its activity on centrosome clustering. (A)** RT-qPCR analysis to verify siRNA-mediated knockdown of KIF2C and overexpression Nek2A. (**B**) Percentage of multipolar spindles observed when KIF2C is silenced.

Further experiments were performed to test whether ablation of KIF2C would inhibit the action of Nek2A overexpression. In order to conduct a competition assay, U2OS cells were engineered to overexpress Nek2A, express KIF2C shRNA and the combination of Nek2A overexpression and KIF2C shRNA. Plk4 was overexpressed to induce CA in all experimental groups **(Figure 3.24 A)**. First, we conducted competition experiments as previously described. Results showed that KIF2C suppression promoted cell viability and proliferation due to a significant decrease in multipolar metaphases **(Figure 3.234 B)**. Nek2A overexpression did not induce multipolar metaphases, hence cell death in cells expressing KIF2C shRNA. The findings were further validated with cell viability **(Figure 3.24 C)** and Annexin-V staining **(Figure 3.24 D)** experiments. Collectively, the data supports that an interaction with KIF2C is required for Nek2A to promote un-clustering of extra centrosomes.

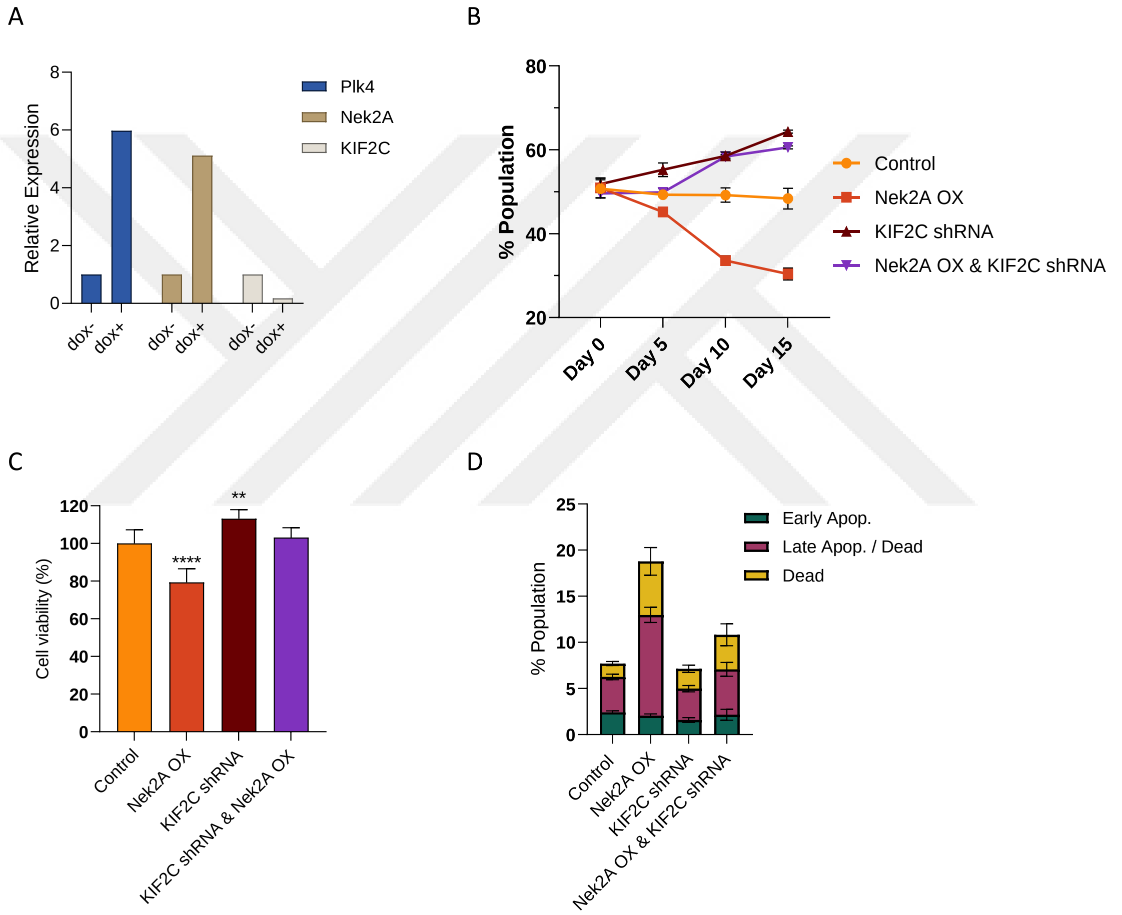

Figure 3.24: **KIF2C is required for Nek2A to exert centrosomal un-clustering activity. (A)** RT-qPCR analysis to verify expression levels of PLK4, Nek2A and KIF2C. (**B**) Competition experiment result displays that suppression of KIF2C promotes cell survival in cells with supernumerary centrosomes. (**C**) WST-1 cell viability assay shows that suppression of KIF2C expression increases cell survival and attenuates the effect of Nek2A overexpression on survival of cells harbouring extra centrosomes induced by PLK4 overexpression. (**D**) Annexin-V staining shows that suppression of KIF2C reverts

apoptotic phenotype in Nek2A overexpressing cells harbouring PLK4-induced extra centrosomes

3.4.5 Localization of KIF2C and Nek2A is Independent

It was investigated whether subcellular localization KIF2C is disturbed when Nek2A is absent. Immunofluorescence staining was performed validate localization of KIF2C in Nek2A knock-out U2OS cells (**Figure 3.25 A**). Results showed that loss of Nek2A does not inhibit the recruitment of KIF2C to the spindle poles and microtubules during metaphase. Similarly, silencing KIF2C expression did not interfere Nek2A localization on spindle poles (**Figure 3.25 B**). Taken together, the results suggest that subcellular localizations of KIF2C and Nek2A are not dependent on the presence of each protein.

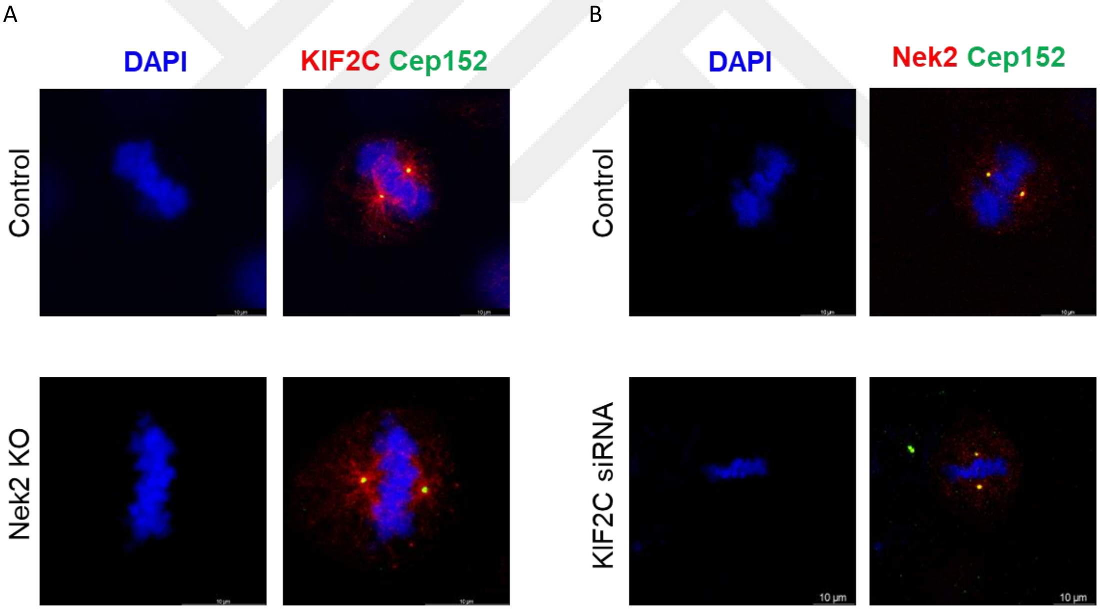

Figure 3.25: Nek2A and KIF2C localizes to spindle poles independently. **(A)** Nek2A knock-out does not affect the localization of KIF2C. (**B**) KIF2C knock-down does not interfere with localization of Nek2A.

3.4.6 in silico Prediction of Potential Phosphorylation Sites on KIF2C by Nek2A

In this study, it is underlined that a physical interaction between Nek2A and KIF2C is essentially important to exert un-clustering phenotype of supernumerary centrosomes and induce cell death. Nek2A is an important mitotic kinase which regulates diversity of cellular process. KIF2C is reported to be phosphorylated and regulated by several mitotic kinases. In the present study, it has also been showed that kinase activity of Nek2A is crucial to induce multipolar spindle pole formation. Thus, it is highly-likely that KIF2C could be a substrate for Nek2A kinase. A preliminary inquisition was performed using the Kinase Library powered by PhosphoSitePlus. Kinase Library serves as an online database tool to predict potential kinases which could phosphorylate given domains (82735 different sites). Analysis, obtained by utilizing this tool, pointed three sites with strong possibilities **(Figure 3.26).** According to predictions, Nek2A kinase exhibits high affinity and selectivity against S111, S187 and S106 positions on KIF2C protein. Even though this data was curated from a reliable and published source (Johnson et al., 2023), wet-lab experiments are required for further validation and identification of exact positions and amino acids phosphorylated by Nek2A.

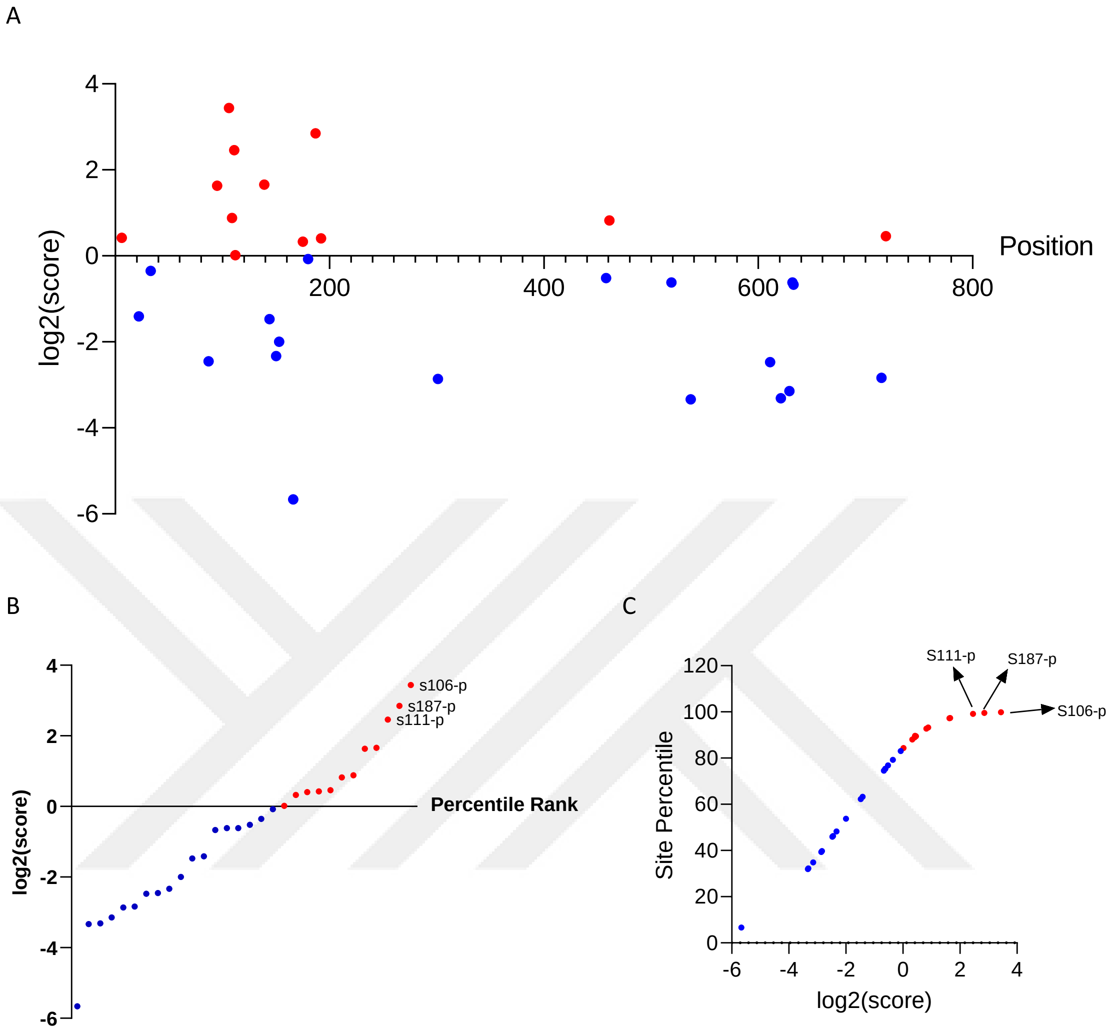

Figure 3.26: ***in silico* kinase prediction reveals potential phosphorylation sites for Nek2A on KIF2C (A)** Plot showing the amino acid positions (S/T) of KIF2C and log2(score) calculated for Nek2A kinase activity. (**B**) Plot for percentile rank and log2(score) indicating top 3 potential targets for Nek2A kinase. (**C**) Plot for site percentile and log2(score) indicating top 3 potential targets for Nek2A kinase. Positions with negative score are colored blue while the positions with positive score are colored red.

4 DISCUSSION

This study aimed to investigate the potential role of Nek2A in the regulation of centrosome clustering in cancer cells. In this present study, our findings indicate that the expression levels of Nek2A play a crucial role in the centrosome clustering process in the cells with supernumerary centrosomes while does not affect the cell viability and proliferation of the cells without centrosome amplification. Thus far, Nek2A has been known to control centrosome separation and regulate kinetochore-spindle attachments and spindle assembly checkpoint (SAC) (Faragher & Fry, 2003; Fry, Meraldi, et al., 1998; Hayward & Fry, 2006). Previous studies have shown that overexpression of Nek2A causes centrosomal aberrations and chromosomal instability (CIN) (Faragher & Fry, 2003; Meraldi & Nigg, 2001). Although Nek2A plays a pivotal role in the centrosome cycle, its potential to regulate the clustering of supernumerary centrosomes has yet to be comprehensively studied. Here we highlight that Nek2A regulates centrosome separation at the mitotic onset and involves centrosome clustering in cancer cells with a unique molecular mechanism. We underlined that there are several methods to induce centrosome amplification in cells and study centrosome clustering (M. Kwon, 2016). In the present study, we utilised three different methods for CA and four different cancer cell lines to verify our data to provide solid proof to indicate that overexpression of Nek2A unclusters extra centrosomes while its suppression or inhibition of kinase activity favours centrosome clustering, regardless of cancer type. To observe the phenotype, the only requirement is extra centrosomes generated naturally or artificially (Nocadozole, DCB, Plk4 overexpression). There exist several methods to induce centrosome amplification in cells. Tubulin-binding agents such as nocodazole disrupt microtubule dynamics and prevent mitosis, arresting the cells at prometaphase (Blajeski et al., 2002). Cells undergoing prolonged arrest at mitosis may exit the cell cycle with duplicated centrosomes and chromosomes. The release of nocodazole provides cells to re-enter the cell cycle and continue mitosis with extra centrosomes. Another widely used method to induce centrosome amplification is overexpressing polo-like kinase 4 (Plk4) (Mijung Kwon, 2016). Plk4 is a master regulator of centriole biogenesis, and its overexpression results in centriole overduplication, thus centrosome amplification (Julia Kleylein-Sohn et al., 2007)

Since centrosome clustering is essential for cancer cell survival and proliferation, a more profound understanding of the molecular mechanisms and identification of regulatory proteins provides invaluable knowledge and the development of novel strategies to treat cancer (Kalkan et al., 2022). We present strong evidence that Nek2A expression levels and kinase activity plays a critical role in the viability of cancer cells with extra centrosomes. Even though Nek2A overexpression has been reported as a prognostic marker and promising target which promotes tumour growth, migration and drug resistance, our findings indicate that the cells harbouring extra centrosomes are prone to undergo apoptosis due to induction of multipolar divisions (Bai et al., 2021; Cusan & Wang, 2022; Toshio Kokuryo et al., 2019; Li et al., 2019; Zhou et al., 2013). Multipolar spindle formations and consequent cell death selectively occur in the cells with extra centrosomes, while the viability of cells without CA remains unaffected.

Centrosomal targets of Nek2A have attracted us to investigate their roles in forming multipolar spindle poles when Nek2A is overexpressed. Nek2A supports maintaining and modulating centrosome architecture in a cell cycle-dependent manner through phosphorylation of centriolar proteins (Fry, 2002). C-Nap1 is a key structural component of the centrosome, required for centrosome cohesion (Mayor et al., 2000). Nek2A controls centrosome separation through phosphorylating C-Nap1 at G2/M transition and inducing its dissociation from mitotic centrosomes (Fry, Mayor, et al., 1998; Mayor et al., 2002). A previous study has shown that loss of C-Nap1 disrupts the spatial organisation of centrosomes and causes an increase in centrosome distance (Panic et al., 2015). More recently, it was reported that cells lacking C-Nap1 undergo multipolar divisions (Theile et al., 2023). Our data also showed that C-Nap1 knock-out significantly increased multipolar metaphases and centrosomal distance. It was also described that KIFC1 inhibition enhanced multipolar spindle formation in C-Nap1 knock-out cells, suggesting that C-Nap1 might be an independent regulator of centrosome clustering (Theile et al., 2023). Similarly, we have found that overexpression of Nek2A in C-Nap1 knock-out cells could further increase multipolar metaphases. Thus, C-Nap1 might have a functional potential to involve in centrosome clustering. Rootletin is another centriolar linker interacting with C-Nap1 and acts as a key component for centrosome cohesion (Yang et al., 2006). Likewise C-Nap1, rootletin is phosphorylated by Nek2A and dissociated from centrosomes at the mitotic onset (Susanne Bahe et al., 2005). Interestingly, there has not been any publication investigating the effect of rootletin on centrosome clustering. In our study, we observed contradictory results between nocodazole and PLK4-induced CA models. Rootletin knock-out decreased multipolar metaphases in the PLK4 model but did not affect nocodazole-treated U2OS cells. The structural differences between nocodazole and PLK4 amplified centrosomes might be the underlying reason for the experimental outcome. As a joint result, overexpression of Nek2A was able to significantly increase multipolarity in cells lacking rootletin, similar to Cnap-1 knock-out cells. GAS2L1 has been recently reported as a centrosomal target of Nek2A (Au et al., 2020). Like C-Nap1 and rootletin, Nek2A phosphorylates GAS2L1 in late G2 and initiates centrosome disjunction (Au et al., 2017). However, GAS2L1 was not associated with centrosome clustering in the literature. Our results indicate that suppression of GAS2L1 does not interfere with centrosome clustering in control cells or Nek2A overexpressors. Collectively, in the present study, we highlight that the unclustering effect of Nek2A overexpression remains unaffected by the absence of its centrosomal targets at the G2/M transition. Here, we suggest that Nek2 might regulate centrosome clustering during mitosis, particularly during metaphase. The clustering of supernumerary centrosomes takes place prior to anaphase, which is controlled by SAC (Godinho & Pellman, 2014; Kwon et al., 2008; Yang et al., 2008). In addition to the targets contributing centrosome architecture, Nek2A interacts and phosphorylates mitotic proteins Hec1, Mad1/2 and CDC20 (Fang & Zhang, 2016). Phosphorylation of Hec1 by Nek2A takes place in mitosis. This phosphorylation is indispensable for accurate kinetochore-microtubule attachments and successful chromosome alignment (Chen et al., 2002b; Du et al., 2008; Wei et al., 2011). Hec1 belongs to the Ndc80 complex regulating microtubule dynamics and kinetochore stability (Ciferri et al., 2007). Silencing Ndc80 complex members inhibits centrosome clustering and causes cells to arrest at metaphase

(Blanka Leber et al., 2010). Instead of silencing Hec1, we used the specific small molecule (INH154) to inhibit Nek2A-Hec1 interaction and phosphorylation of Hec1 by Nek2A (C. M. Hu et al., 2015). Inhibiting the interaction between Nek2A and Hec1 did not affect the centrosome clustering, suggesting that Hec1 is not essential for Nek2A to induce multipolar metaphases. Hec1 is also a key substrate of Aurora B to initialise SAC signalling, which controls the correct attachment of microtubules (Zhu et al., 2013). TRF1 is a negative regulator of telomere elongation, which binds double-stranded telomeric DNA (Walker & Zhu, 2012). Moreover, there is evidence that TRF1 involves the cell cycle and proper segregation of chromosomes, regulating the cohesion of sister chromatids and kinetochore attachments (Ohishi et al., 2014). It was previously reported that Nek2 physically interacts and phosphorylates TRF1 (Prime & Markie, 2005). A previous study demonstrated that overexpression of Nek2A-induced cytokinetic failure in cells with centrosome amplification and knock-down of TRF1 suppressed the action of overexpressed Nek2A (Lee & Gollahon, 2013). This study also showed that overexpression of Nek2A induced centrosome amplification in MCF7 and MDA-MB-231 cells and increased multipolar spindle poles. Notably, this study states that MCF7 cells exhibit high centrosome amplification levels (>30%). MCF7 cells express wild-type and functional p53 (D'Assoro et al., 2008; Kalkan et al., 2022). It is known that impaired p53 causes centrosome amplification. Consistent with the p53 status, MCF7 cells normally exhibit low levels of centrosome amplification (D'Assoro et al., 2008). In our studies, we also noted that they exhibit less than 5% centrosome amplification. Our present study also indicates that overexpression or knock-down of Nek2A did not change the percentage of centrosome amplification in U2OS, MDA-MB-231 and Su86.86 cell lines. In addition, we show that TRF1 is not required for Nek2A to exert its effect on centrosome clustering.

Exploring vital interaction partners of Nek2A to execute its function in centrosome clustering is substantial for understanding the molecular mechanism. TurboID is a robust tool for highly efficient proximity labelling (Branon et al., 2018). Our analysis with Nek2A revealed 20 significantly ($p<0.05$) positive enriched (Fold change > 0) previously known (BioGrid & IntAct databases) Nek2 interaction partners. Nek2 was the most enriched protein in both of the WT and KD pulldowns. Given that kinase function does not alter the intracellular localization of Nek2, enrichment of target proteins should have been similar in both groups. As expected, enrichment results of WT and KD were highly correlated.

In the present study, we highlighted that the centrosome uncustering activity of Nek2A is not influenced by previously described clustering regulators NuMA and KifC1, even though we found them within the labelling radius of Nek2A-TurboID. We also tested the physical interaction between NuMA and Nek2A utilising a co-IP experiment. Nevertheless, NuMA did not co-immunoprecipitate with Nek2A. Albeit TurboID has higher activity than its relatives due to its larger labelling radius (~35 nm), not each biotinylated peptide actually represents a physical interaction (May et al., 2020). NuMA facilitates the bipolarization of spindles, and its function is regulated by phosphorylation (Chinen et al., 2020; Zeng, 2000). Depletion of NuMA suppresses the formation of multipolar spindle poles, while its overexpression disrupts centrosome clustering and increases the percentage of multipolar metaphases (Quintyne et al., 2005). In this study, we provide evidence that, despite they are located in close proximity, Nek2A and NuMA do not physically interact and regulate centrosome clustering independently.

Kinesin-like protein KIFC1, also known as HSET, is a crucial player of centrosome clustering in cancer cells and a promising target for therapeutic approaches (Kwon et al., 2008; Ciorsdaidh A Watts et al., 2013; Wei Zhang et al., 2016). Knock-down or inhibition of KIFC1 results in multipolar divisions, hence cell death (Kwon et al., 2008). KIFC1 is activated through phosphorylation and promotes centrosome clustering (Guangjian Fan et al., 2021). In this study, we indicated that Nek2A overexpression additively increases the formation of multipolar spindles in the absence of KIFC1. In addition, co-suppression of Nek2A and KIFC1 by RNAi partially rescued centrosome clustering, suggesting that each molecule control centrosome clustering event independently. Recently, it was reported that the expression of KIFC1 exhibits a significantly positive correlation with Nek2A expression (Mittal et al., 2016). This is evidence that Nek2A and KIFC1 are competitors in the centrosome clustering / de-clustering battle and an internal balancing mechanism since overexpression of both proteins induces opposite phenotypes.

KIF2C, mitotic centromere-associated kinesin (MCAK), is a popular member of the kinesin-13 family and regulates microtubule dynamics through its depolymerase activity (Ems-McClung & Walczak, 2010; Ritter et al., 2016). Despite being one of the best-character members of the kinesin-13 family, KIF2C has not been deeply investigated in the context of centrosome clustering in human cancer. In a recent study defining the role and importance of KIF2C in cell migration invasion, it was shallowly mentioned that both knock-down and overexpression of KIF2C resulted in an increase of multipolar metaphases in HeLa cells (Moon et al., 2021). This data implies that KIF2C activity should be fine-tuned by regulatory proteins orchestrating mitosis. The activity of KIF2C is regulated through several different mechanisms, which involve mitotic kinases and phosphatases (Sanhaji et al., 2011). Temporal and spatial activation and deactivation of KIF2C are crucial for maintaining the microtubule dynamics. Aurora B phosphorylates and inhibits the activity of KIF2C while positively regulating its localisation to centromeres (Andrews et al., 2004; Lan et al., 2004). Localisation of KIF2C is also directed through phosphorylation by Aurora A (Zhang et al., 2008). Phosphorylation by Cdk1 releases KIF2C from centrosomes and attenuates the microtubule depolymerising activity (Sanhaji et al., 2010). KIF2C is also a substrate of Plk1 at multiple sites, regulating temporal and spatial activity at different stages of mitosis (Zhang et al., 2010). Plk1 also phosphorylates Nek2A to promote centrosome splitting at the G2-M transition (Mbom et al., 2014; Zhang et al., 2005).

Moreover, Cdk1 indirectly promotes the activation of Nek2A to induce centrosome splitting at the onset of mitosis (O'regan et al., 2007). Taken together, Nek2A and KIF2C may be harmoniously regulated in cancer cells with centrosome amplification to balance centrosome clustering. Intriguingly, there have yet to be any publications indicating the interaction and co-localisation between Nek2A and KIF2C. Here, we first time assign a novel function to KIF2C and provide evidence that it interacts with Nek2A, confirmed by proximity labelling, co-staining and co-IP. Several examples underline that centrosome clustering relies on the organisation of microtubule dynamics by motor and microtubule bundling proteins (Blanka Leber et al., 2010; Quintyne et al., 2005; Ryota Uehara et al., 2009). Therefore, it is not surprising that an interaction between a crucial mitotic kinase and a kinesin to moderate clustering of supernumerary centrosomes during mitosis.

In the present study, we identified an essential interaction between KIF2C and Nek2A to regulate centrosome clustering in cancer cells (**Figure 4.1**). The interaction is most likely a kinase-substrate interaction since we have shown that overexpression of kinase-dead mutant of Nek2A acted as dominant-negative and implied that kinase activity is required to exhibit phenotypical outcome. Further studies are required to reveal the details of KIF2C-Nek2A interaction.

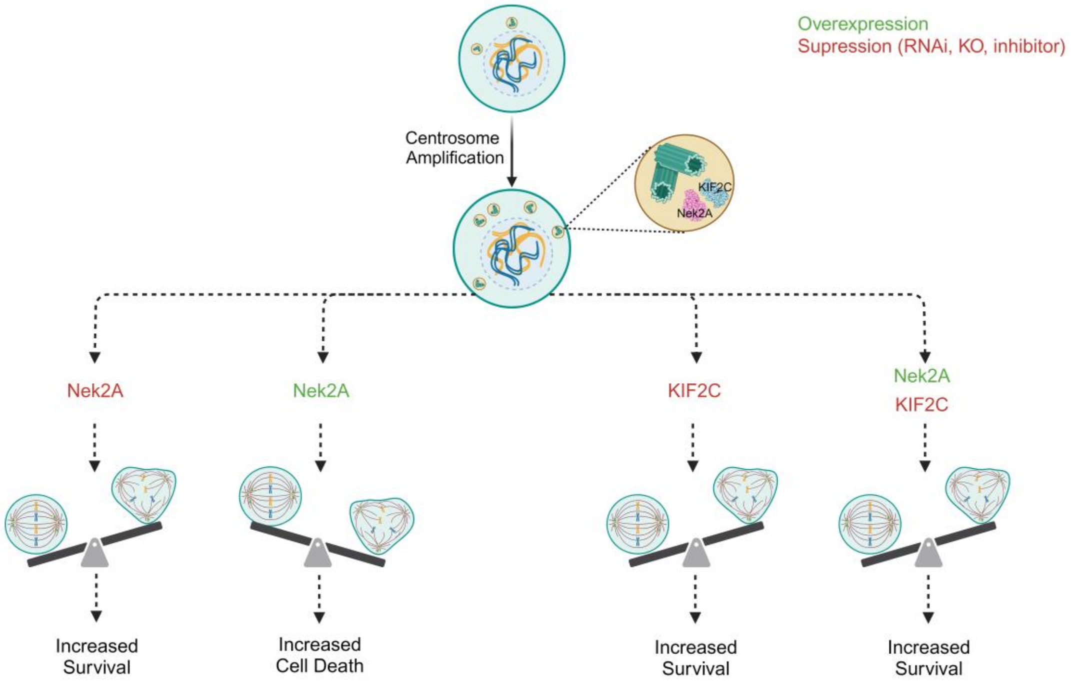

Figure 4.1: Schematic representation of the data demonstrating that Nek2A and KIF2C interaction in centrosomes and spindle poles regulate centrosome clustering and affect cancer cell survival.

In conclusion, this study sheds light on the pivotal role of Nek2A in regulating centrosome clustering in cancer cells with supernumerary centrosomes. Understanding the molecular mechanisms underlying this process may provide valuable insights into novel therapeutic strategies for selectively targeting cancer cells and controlling centrosome-related genomic instability in cancer. Further investigations into the complex interplay between Nek2A, KIF2C, and other potential interactors will undoubtedly deepen our understanding of centrosome clustering regulation and its implications in cancer biology.

A well-established association exists between the occurrence of centrosome amplification and the progression of cancer. Since these abnormalities are not typically found in normal cells, the notion of specifically targeting cells with extra centrosomes holds significant promise for selectively eliminating cancerous cells. Consequently, gaining insights into the underlying molecular processes responsible for centrosome amplification, clustering, or deactivation, as well as comprehending their connections to the advancement of cancer, may open up novel avenues for therapeutic interventions.

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