

Nucleolus in control of breast tumor heterogeneity

A thesis submitted during 2025 to the University of Hyderabad
in partial fulfilment of the award of a Ph.D. degree in the Department
of Biochemistry

by

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CERTIFICATE

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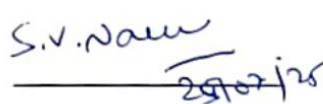
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Sustainable Development Goals (SDG)

Page

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The United Nations Sustainable Development Goal 3 (SDG 3) aims to promote good health and well-being globally, with a specific focus on reducing deaths caused by non-communicable diseases (NCDs) by one-third between 2015 and 2030. Breast cancer is the prominent cause of cancer-related deaths among women and remains an important health challenge. Its complexity and dynamic nature often led to difficulties in treatment, contributing to high mortality rates. Hence, an in-depth understanding of the molecular mechanisms behind breast cancer is essential. This knowledge can guide the development of more effective therapies. The present research aims to explore these molecular aspects in order to improve treatment outcomes and ultimately reduce mortality.

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Dedication

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1 Abbreviations

1.	2'-O-Me	2'-O-Methylation
2.	ARM	Arginine rich motif
3.	c-Myc	Cellular Myc
4.	CBX4	Chromobox4
5.	EMT	Epithelial to mesenchymal transition
6.	FACT	Facilitates Chromatin Transcription
7.	FBL	Fibrillarin
8.	FGF	Fibroblast growth factor
9.	IGF	Insulin like growth factor
10.	IRES	Internal ribosome entry site
11.	OCT4	Octamer-binding Transcription Factor 4
12.	RBFs	Ribosome biogenesis factors
13.	RNF4	RING finger 4
14.	rRNA	Ribosomal RNA
15.	RTLP	Reverse Transcription at Low dNTP concentration
16.	SEMT	Spheroid-induced EMT
17.	SENP	Sentrin/SUMO-specific Protease
18.	SIM	SUMO interaction motif
19.	SIRT7	Sirtuin 7
20.	snoRNP	Small Nucleolar Ribonucleoprotein
21.	SOX2	SRY (Sex-determining Region Y)-Box 2
22.	STUbL	SUMO-Targeted Ubiquitin Ligase
23.	SUMO	Small Ubiquitin-like Modifier
24.	TERT	Telomerase Reverse Transcriptase

25.	TNBC	Triple-Negative Breast Cancer
26.	UBC9	Ubiquitin-Conjugating Enzyme 9
27.	VEGF	Vascular Endothelial Growth Factor
28.	ZEB	Zinc Finger E-box-Binding Homeobox
29.	RNA pol	RNA polymerase
30.	PARIS	Parkin Interacting Substrate
31.	HIF1α	Hypoxia-Inducible Factor 1-alpha
32.	KAP1	KRAB-Associated Protein 1

2 Abstract

Tumor heterogeneity is a characteristic feature of cancer and poses a challenge in developing effective therapeutic strategies. It arises from genetic, epigenetic, and phenotypic variability among tumor cells within the same lesion. A significant contributor to this diversity is cellular plasticity, the ability of cells to shift between different states in response to microenvironmental or intrinsic cues. One well-established example is the epithelial-mesenchymal transition (EMT), a reversible process through which epithelial cells adopt mesenchymal features. EMT aids cancer cells in attaining motility, invasiveness, and resistance to therapy, thereby promoting metastasis and tumor progression. These transitions are not strictly binary; instead, cells can exist in hybrid states, which further enhances intratumoral diversity and contributes to therapy resistance. Given the close relationship between EMT and plasticity, we examined whether nucleolar dynamics, particularly those involving the ribosome biogenesis factor fibrillarin (FBL), could influence these transitions across breast cancer subtypes. We observed that nucleolar size and number differ among subtypes, with triple-negative breast cancer (TNBC) cells exhibiting larger and more numerous nucleoli compared to other types. This increase correlates with elevated rRNA synthesis and 2'-O-methylation, both of which are known to support EMT. We further identified that FBL undergoes SUMOylation, a post-translational modification that changes during spheroid-induced EMT. Interestingly, CBX4, an E3 SUMO ligase, and RNF4, an E3 Ubiquitin ligase, regulate this modification, impacting FBL's localisation and potentially linking nucleolar activity to cellular plasticity. These findings suggest that variations in FBL function and modification may contribute to phenotypic flexibility in breast cancer, thereby fuelling tumor heterogeneity.

3 Introduction

3.1 The structure, function, and development of the mammary gland

The mammary gland is a distinctive characteristic of mammals, responsible for producing milk to nourish their offspring (Peaker, 2002). These glands develop from apocrine sweat glands that arise from the epidermis (Hovey *et al.*, 2002). In adults, mammary glands consist of both glandular and stromal connective tissue. The glandular component comprises 15 to 20 lobes and lobules, which contain anywhere from 10 to 100 alveoli (Philipp *et al.*, 1991). The ducts from the alveoli merge to form a central milk duct that extends through the nipple, where it opens to secrete milk (Moffat & Going, 1996).

Morphologically, the mammary gland is composed of epithelial cells that form the ductal network and stromal connective tissue made up of adipocytes that surround the epithelial structure. Additionally, there are fibroblasts that support hematopoietic cells, endothelial cells that make up blood vessels, nerve cells and immune cells like mast cells and macrophages. The epithelial layer has two distinct components: the inner luminal epithelium, which encircles the lumen and differentiates into lobules and alveoli, and the basal epithelium, which consists of myoepithelial cells along with stem and progenitor cells (Watson & Khaled, 2008; Muschler & Streuli, 2010). Surrounding the epithelium is the basement membrane, which serves as a barrier between it and the surrounding stroma (Streuli, 2003).

The mammary glands undergo significant physiological changes throughout a female's life, progressing through three main stages: embryonic, pubertal, and reproductive (Macias & Hinck, 2012). In the embryonic stage, mesenchymal tissue beneath the mammary line initiates signals that lead to the differentiation of mammary epithelial cells (Cunha *et al.*, 1995). This results in a rudimentary structure that eventually develops into a primitive branched ductal tree, which integrates into a fat pad (Cowin & Wysolmerski, 2010). After birth, the mammary gland

remains inactive until puberty begins. From birth until puberty, isometric growth occurs in a hormonally independent fashion (Macias & Hinck, 2012).

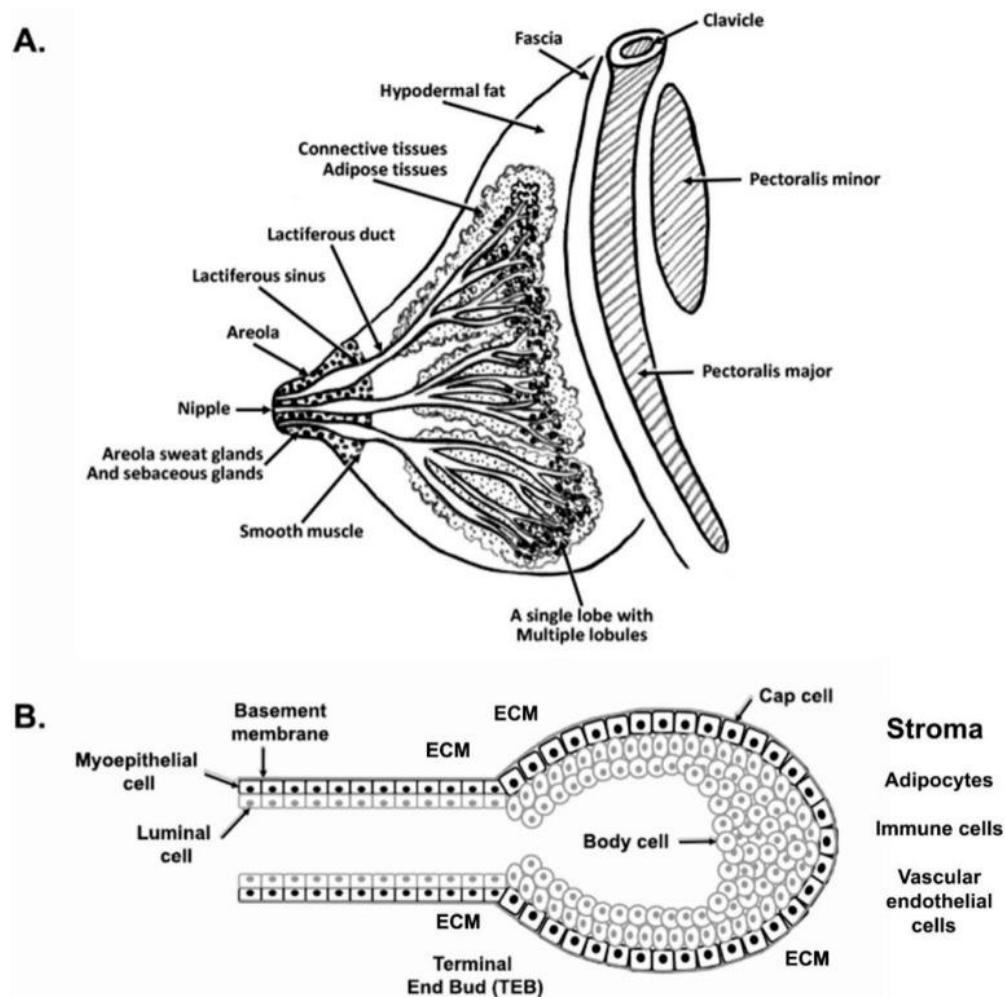


Figure 1 : Mammary gland. **A)** A schematic representation of the anatomy of the mammary gland **(B)** A schematic representation of the terminal end bud (TEB) showing luminal cells surrounded by myoepithelial cells, a fibrous connective tissue and adipose tissue. *Source: (Biswas et al., 2022).*

With the onset of puberty, the elongation and branching of the mammary ducts commence under the influence of hormones from the hypothalamic-pituitary-ovarian axis, leading to allometric growth (Kleinberg & Ruan, 2008; Macias & Hinck, 2012; Sternlicht, 2005). During pregnancy, hormones such as progesterone and prolactin trigger a process known as tubule-alveologenesis, essential for the synthesis and secretion of milk (Haslam, 1989; Lydon *et al.*, 1995; Das & Vonderhaar, 1997). In lactating mammary glands, blood vessels expand to

facilitate milk production by providing essential macromolecules (Djonov *et al.*, 2001). Following weaning, the mammary glands undergo remodelling through involution, during which apoptosis of epithelial cells helps return the glands to their pre-pregnancy state (Monks *et al.*, 2005, 2008).

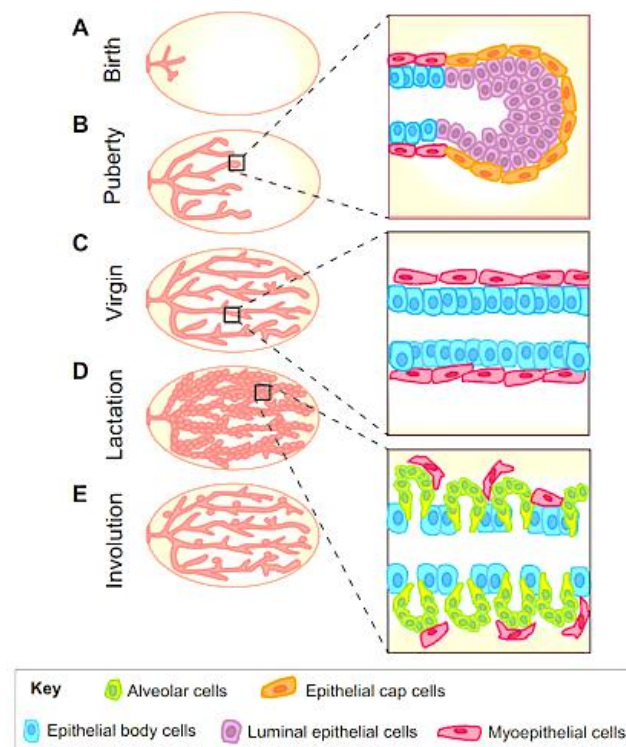


Figure 2: The multistage development of the mammary gland after birth. (A) At birth, the gland consists of a basic ductal structure with limited branching. (B) Puberty triggers significant growth, with ducts extending into the fat pad, led by specialized structures called terminal end buds (TEBs), which contain organized epithelial cells. (C) In adult virgin glands, a more elaborate ductal network is established, composed of inner luminal epithelial cells and outer myoepithelial cells. (D) During pregnancy and lactation, alveolar units differentiate and expand to produce and secrete milk. (E) Following weaning, the gland undergoes involution, involving cell loss and tissue remodelling to return to a resting state. *Source: (Inman et al., 2015).*

3.2 The prevalence of breast cancer in women is significant

In certain cases, mutations accumulate in the mammary epithelium, resulting in uncontrolled cell divisions of epithelial cells that can spread to other parts of the body, ultimately leading to the development of breast cancer. It is the prominent cause of death in

women worldwide. According to statistics from the World Health Organization (WHO), approximately 2.3 million women were detected with breast cancer globally in 2022, with 670,000 women succumbing to the disease. It is the most common cancer in women across 157 of the 185 countries surveyed. Since 2008, the frequency of breast cancer has risen by 20%, while mortality rates have increased by 14%.

In India, it is also the most commonly diagnosed cancer among women, comprising about 28.2% of all female cancer cases, with an estimated 216,108 reported cases in 2022 (Sathishkumar *et al.*, 2022). Additionally, over the span of 26 years, from 1990 to 2016, India witnessed a 39.1% rise in the age-adjusted incidence rate of female breast cancer, a pattern consistent across every state (Dhillon *et al.*, 2018). The SURVCAN-3 study (Cancer Survival in Countries in Transition), released in 2023, reported that the median 3-year survival rate for breast cancer patients was 68% in India (Soerjomataram *et al.*, 2023). Also, according to the CONCORD-3 study (2010–2014), India has a 5-year breast cancer survival rate of 66.1% (Allemani *et al.*, 2018).

3.3 Hallmarks of cancer

Cancer is a multistep process, where cancer cells acquire various capabilities throughout their development, known as hallmarks, as described by Hanahan and Weinberg (Hanahan, 2022; Hanahan & Weinberg, 2000, 2011).

3.3.1 Sustained proliferation signalling

One of the primary hallmarks of cancer is the abnormal and unchecked proliferation of cells. These cells harbour somatic mutations that lead to the constitutive activation of signalling pathways, which primarily promote cell proliferation through growth factors (Davies & Samuels, 2010; Yuan & Cantley, 2008). Additionally, cancer cells often show a dysregulation

of negative feedback mechanisms that would normally diminish the suppressive effects of signalling, thereby facilitating their uncontrolled proliferation (Amit *et al.*, 2007; Cabrita & Christofori, 2008; Wertz & Dixit, 2010). However, some research studies suggest that the overactivation of oncogenic signalling can activate the cells' intrinsic defence mechanisms, like apoptosis and senescence (Collado & Serrano, 2010; Evan & d'Adda Di Fagagna, 2009; Lowe *et al.*, 2004). Consequently, cancer cells must strike a balance between mitogenic signalling and these defence mechanisms; alternatively, they may choose to deactivate them altogether.

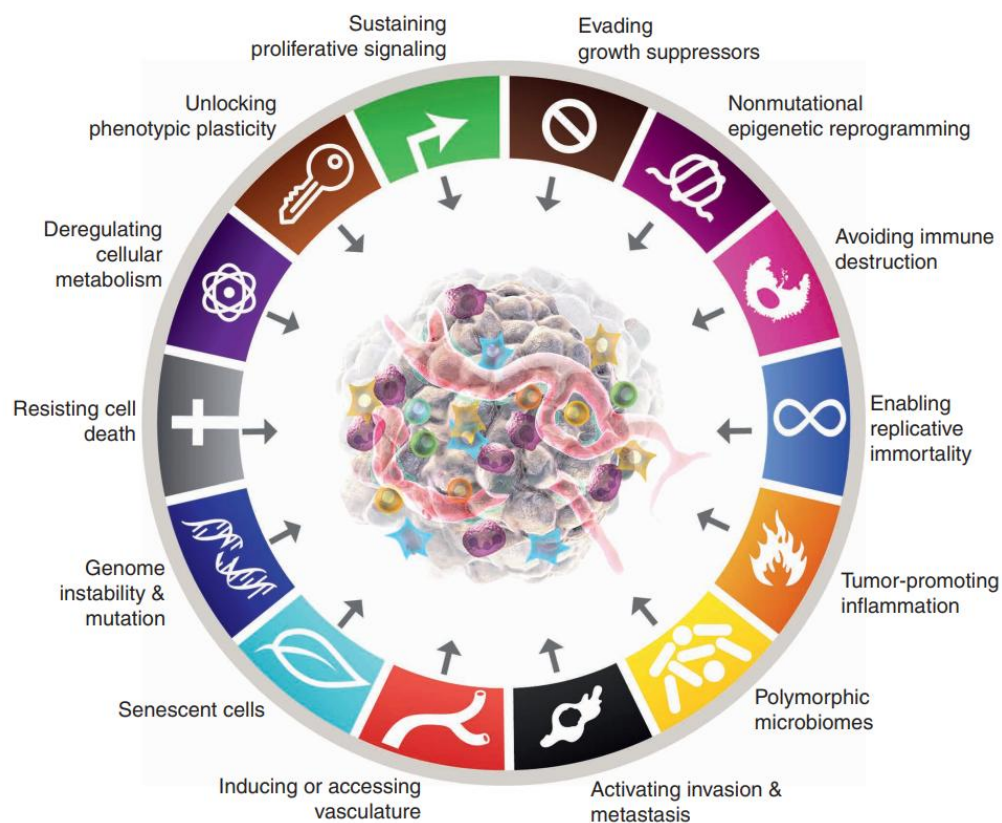


Figure 3: The key biological characteristics and supportive factors that drive cancer progression. This schematic illustrates the fundamental hallmarks of cancer, including sustained cell proliferation, resistance to growth suppression, evasion of immune destruction, resistance to cell death, and limitless replicative potential. Additionally, it highlights enabling characteristics such as genome instability and non-mutational epigenetic changes, as well as promoting factors like inflammation, angiogenesis, metabolic reprogramming, cellular plasticity, and interactions with the tumor microenvironment, such as the microbiome and senescent cells. *Source: (Hanahan, 2022).*

3.3.2 Evading growth suppressors

In normal cells, tumor suppressors play an essential role in negatively regulating abnormal proliferation. However, cancer cells circumvent this regulation by inactivating tumor suppressor genes. The two primary tumor suppressors frequently deregulated in cancer are p53 and pRb, which are crucial for regulating cell proliferation, senescence, and apoptosis (Sherr & McCormick, 2002). Additionally, contact inhibition typically serves as a barrier to uncontrolled cell growth; normal cells halt division upon cell-to-cell contact, maintaining tissue homeostasis through tumor suppressors. In contrast, cancer cells lose this capacity and continue to proliferate unchecked (Hanahan & Weinberg, 2011). Furthermore, the redirection of TGF β signalling from proliferation suppression to epithelial-mesenchymal transition (EMT) can also enhance the malignancy of cancer cells (Bierie & Moses, 2006; Ikushima & Miyazono, 2010).

3.3.3 Resisting cell death

The DNA damage in the cells usually triggers apoptosis by activating p53, but cancer cells overcome this by inactivating it. Multiple antiapoptotic pathways are activated in the cancer cells (Adams & Cory, 2007; Lowe *et al.*, 2004). They utilize multiple other pathways to overcome cell death, not only due to apoptosis. The induction of autophagy usually occurs during cellular stresses such as nutrient deficiency. Similarly, the cancer cells also utilize this pathway to survive nutrient starvation (White & DiPaola, 2009). Notably, chemotherapy, radiation therapy and nutrient starvation promote tumor progression by enhancing autophagy (Amaravadi & Thompson, 2007). Necrosis is yet another pathway exploited by them for their advantage (Zong & Thompson, 2006; Galluzzi & Kroemer, 2008). In contrast to apoptosis and autophagy, proinflammatory cytokines are released into the surrounding tissue during necrosis. As a result, inflammatory cells are recruited to the tissue, which promotes tumor progression by enhancing angiogenesis, cell proliferation and invasion. Furthermore, necrosis can stimulate the neighboring cells to proliferate, promoting neoplasia (Grivennikov *et al.*, 2010).

3.3.4 Enabling replicative immortality

Unlike normal cells, which have a limited capacity for replication, cancer cells attain immortality by overcoming critical proliferation barriers such as senescence and crisis (cell death). The length of telomeres is essential for enabling unlimited replication potential; when telomeres shorten, it can trigger senescence and crisis (Blasco, 2005; Shay & Wright, 2000). Telomerase, a type of DNA polymerase, plays a vital role in maintaining telomere length. While telomerase is absent in non-immortalized cells, its high expression is detected in human cancer cells and immortalized cells. Conversely, the absence of telomerase function in the primitive stages of tumor development fosters neoplasia through genomic alterations, including deletions and amplifications of chromosomal segments (Hanahan & Weinberg, 2011). Furthermore, the non-canonical functions of telomerase, particularly the telomerase subunit TERT, can contribute to tumor progression by promoting cell proliferation, evading apoptosis, and facilitating DNA damage repair (H. J. Kang *et al.*, 2004; Masutomi *et al.*, 2005).

3.3.5 Senescent cells

Senescence is generally induced in response to various environmental stresses, such as nutrient deprivation, dysregulation of cellular signalling, and damage to DNA, organelles, and the overall cellular infrastructure (Birch & Gil, 2020; Gorgoulis *et al.*, 2019). Although it has long been acknowledged that senescence acts as a protective mechanism against cancer, recent studies indicate that senescent cells may also contribute to tumor development through what is known as the senescence-associated secretory phenotype (SASP) by secreting bioactive molecules such as chemokines, cytokines and proteases in a paracrine fashion (Lee & Schmitt, 2019; B. Wang *et al.*, 2020).

3.3.6 Unlocking cellular plasticity

Tissue formation occurs hierarchically, beginning with stem cells that undergo multiple divisions to generate terminally differentiated cells essential for maintaining homeostasis. In a healthy physiological context, these differentiated cells cease to proliferate and act as protective mechanisms against the development of cancer. However, cancer cells can employ various strategies to evade this terminal differentiation, thereby promoting their progression (Hanahan, 2022). For instance, a nascent cancer cell may emerge from a normal cell and revert to a more primitive, progenitor-like state, a process known as *dedifferentiation*. Likewise, cancer can originate from stem cells that completely suppress their differentiation potential, referred to as *blocked differentiation*. Furthermore, cells that were initially committed to a specific differentiation pathway may transition to an alternative state to facilitate tumor progression, a phenomenon known as *trans differentiation* (Drapkin & Minna, 2020; Hanahan, 2022).

3.3.7 Reprogramming energy metabolism

The biomolecule glucose undergoes glycolysis to generate pyruvate, which is subsequently converted to generate energy through oxidative phosphorylation in the mitochondria. In contrast, cancer cells modify this metabolic pathway and rely exclusively on glycolysis for their energy requirements, even under oxygen rich conditions. This process is referred to as the Warburg effect (Warburg, 1956b, 1956a). The energy glycolysis produces is roughly 18 times less than that derived from mitochondrial oxidative phosphorylation. Cancer cells increase glucose uptake by enhancing glucose transporter proteins, such as GLUT1, to compensate for this reduced energy output (DeBerardinis *et al.*, 2008; Hsu & Sabatini, 2008; Jones & Thompson, 2009). Moreover, the intermediates generated during glycolysis serve as precursors for the biosynthesis of nucleosides and amino acids, supporting the formation of organelles necessary for generating new cells (Potter, 1958; Vander Heiden *et al.*, 2009).

3.3.8 Evading immune destruction

The immune system is crucial in monitoring and eliminating cancerous cells and tissues throughout the body. Research indicates that immune-compromised mice develop tumors more frequently than their immunocompetent counterparts, underscoring the importance of a functional immune system in combating cancer. Cancer cells, however, have developed multiple strategies to escape recognition and removal by the immune system and acquire a malignant phenotype. One common strategy employed by them is the inactivation of infiltrating cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (R. Kim *et al.*, 2007; Teng *et al.*, 2008). This can occur through the expression of immune suppressors like TGF β , which dampens the immune response (L. Yang *et al.*, 2010). Additionally, cancer cells can recruit other inflammatory cells, such as regulatory T cells (T reg cells) and myeloid-derived suppressor cells (MDSCs), which further dampen the T-lymphocytes' cytotoxic activity (Mougiakakos *et al.*, 2010; Ostrand-Rosenberg & Sinha, 2009). These tactics allow cancer cells to create an immunosuppressive microenvironment that enables their survival and proliferation.

3.3.9 Inducing angiogenesis

Vasculature plays a crucial role in supplying nutrients and oxygen to tissues while removing metabolic wastes and carbon dioxide from the body. Vasculogenesis and angiogenesis are crucial processes that typically take place during embryonic development but usually become quiescent afterwards. However, transient activation of angiogenesis can occur during wound healing and also throughout the female reproductive cycle. In contrast, in cancer, angiogenesis is often constitutively activated, facilitating tumor progression (Hanahan & Folkman, 1996). The regulation of angiogenesis involves both inducers and endogenous inhibitors. One of the most well-known inducers of angiogenesis is vascular endothelial growth factor A (VEGFA), while inhibitors include thrombospondin-1 (TSP1), fragments of plasmin,

and type 18 collagen (Nyberg *et al.*, 2005; Ribatti, 2009). The expression of the VEGF gene is typically induced during hypoxic conditions or in response to oncogenic signalling. Additionally, VEGFs are found in the extracellular matrix and are released during the activity of matrix metalloproteinases (MMPs). Fibroblast growth factors (FGFs) also contribute to promoting angiogenesis. New studies have also emphasized the role of bone marrow-derived factors in stimulating angiogenesis (Hanahan & Weinberg, 2011).

3.3.10 Activating invasion and metastasis

Cancer cells typically transition from neoplasia to a malignant state, which is characterized by their ability to invade nearby tissues and metastasize to distant sites. During this process, changes occur in the shape of the cells, along with altered interactions with the surrounding stroma and extracellular matrix. Notably, the expression of cell adhesion molecules that help maintain cell-cell contacts and adhesion to the matrix decreases, while that of molecules that facilitate cell migration increases (Hanahan & Weinberg, 2011).

The sequence of invasion and metastasis is known as the *invasion-metastatic cascade*. In the initial phase, cancer cells invade the surrounding tissue, followed by intravasation into the bloodstream and lymphatic system. They then move through these systems and extravasate into remote tissues, leading to the formation of micro metastases, which can grow into macroscopic tumors through a process called colonization (Fidler, 2003; Talmadge & Fidler, 2010).

To invade and spread, cancer cells activate developmental programs like the EMT. This EMT can be activated in stable or transient ways, resulting in a range of EMT phenotypes. Major transcription factors such as Snail, Slug, Twist, and Zeb1/2, which are responsible for executing EMT during embryogenesis, are also utilized by cancer cells. These factors regulate overlapping target genes and modulate each other's activity. The activation of these

transcription factors leads to several cellular changes, such as loss of adherens junctions, enhanced resistance to apoptosis, increased motility, and increased expression of matrix-degrading enzymes (Micalizzi *et al.*, 2010; J. Yang & Weinberg, 2008). Typically, the interaction between cancer cells and the surrounding stroma triggers the activation of these transcription factors. For instance, CCL5 secretion by mesenchymal stem cells, along with metalloproteinases and cysteine cathepsin proteases from macrophages, can enhance invasive behaviour (Karnoub & Weinberg, 2007).

Moreover, cancer cells employ various modes of migration, including mesenchymal migration, collective invasion, and amoeboid invasion. In collective invasion, cancer cells move en masse into adjacent tissues (Friedl & Wolf, 2008), while in amoeboid invasion, they navigate through the interstices of the extracellular matrix (Sabeh *et al.*, 2009). Inflammatory cells contribute matrix-degrading enzymes and other factors that further promote invasion.

Colonization is a challenging endeavor for cancer cells spawning from primary tumors, as these cells often struggle to establish tumors. They may enter a dormant state, potentially remaining inactive for decades before developing into tumors. This dormancy is influenced by suppressive factors from the primary tumors, anti-growth signals present in normal tissue ECM, or the tumor-suppressing capabilities of the immune system (Aguirre-Ghiso, 2007; Barkan *et al.*, 2010). Additionally, autophagy induction may also lead to tumor shrinkage and promote dormancy (Kenific *et al.*, 2010). Despite these challenges, cancer cells manage to disseminate and successfully colonize. The intricate molecular mechanisms underlying these processes are still being elucidated.

3.3.11 Non-mutational epigenetic reprogramming

Mutations in genomic DNA are pivotal to the development of cancer; however, the reprogramming of the cancer genome is also significantly influenced by epigenetic alterations.

For instance, in a hypoxic microenvironment, cancer cells reduce the activity of TET methylases, leading to modifications in the methylome (Thienpont *et al.*, 2016). Furthermore, during periods of nutrient deprivation resulting from reduced vascularization, translational control may be disrupted, promoting a malignant phenotype (Gameiro & Struhl, 2018).

The transcription factor ZEB1, which is associated with the EM, enhances the expression of the histone methyltransferase SETD1B, establishing a positive feedback loop that sustains its own expression (Lindner *et al.*, 2020). Similarly, the transcription factor Snail1 promotes the expression of chromatin modifiers that help maintain the tumor phenotype (Javaid *et al.*, 2013). Additionally, paracrine signalling from soluble factors in the tumor extracellular matrix (ECM) further facilitates EMT.

Moreover, accessory cells within the tumor microenvironment, such as cancer-associated fibroblasts, immune cells, endothelial cells, and pericytes, do not typically undergo genomic alterations. Instead, these cells exhibit epigenetic reprogramming, which significantly contributes to tumor progression (Hanahan & Coussens, 2012).

3.3.12 Polymorphic microbiomes

The symbiotic relationship between the microbiome and human tissues has long been recognized, with the ecosystem created by these microbiomes significantly influencing health and disease (Dzutsev *et al.*, 2017). The toxins produced by bacteria can damage DNA, disrupt the mechanisms that maintain genome integrity, and indirectly hinder DNA replication and repair processes. Additionally, certain bacteria can stimulate cellular proliferation by mimicking ligands (Dzutsev *et al.*, 2017).

Butyrate-producing bacteria can induce complex physiological effects and lead to the production of senescent epithelial and fibroblastic cells (Okumura *et al.*, 2021). Moreover, the

microbiota can affect the host's immune response, contributing to tumorigenesis (Hanahan, 2022).

Enabling characteristics

3.3.13 Genomic instability and mutation

Cancer cells display deficiencies in genome maintenance and repair mechanisms, as well as instability in gene copy number and nucleotide sequences. These recurring genetic alterations contribute to tumor pathogenesis, providing cancer cells with a selective advantage that facilitates tumor progression. This genomic instability ultimately leads to the acquisition of key hallmarks of cancer (Hanahan & Weinberg, 2011).

3.3.14 Tumor-promoting inflammation

Inflammatory processes within tumors support various cancer hallmarks by supplying bioactive molecules, including growth and survival factors, pro-angiogenic signals, and enzymes that remodel the extracellular matrix, thereby enhancing angiogenesis, invasion, metastasis, and the initiation of epithelial-mesenchymal transition (EMT). Additionally, immune cells in the tumor microenvironment produce reactive oxygen species (ROS), which can cause genetic mutations (Hanahan & Weinberg, 2011).

3.4 Tumor heterogeneity

Our understanding of cancer has advanced significantly, and treatment strategies have improved; however, addressing cancer remains complex due to its heterogeneous nature (Dagogo-Jack & Shaw, 2018). The characteristics of cancer tissue reflect the diverse traits of various cancer cell types rather than those of a single cell. Therefore, it is essential to understand cancer tissue and explore the underlying causes of this heterogeneity to develop more effective treatment strategies. Treatment approaches should typically involve a

multidimensional strategy tailored to the complexity of the disease. The variation in cell types complicates treatment because a single approach is unlikely to be effective for all patients. When therapies target the predominant clones within a tumor, this can lead to the emergence of drug-resistant clones, resulting in tumor recurrence and relapse. Thus, therapeutic resistance contributes to both recurrence and metastasis (Dagogo-Jack & Shaw, 2018).

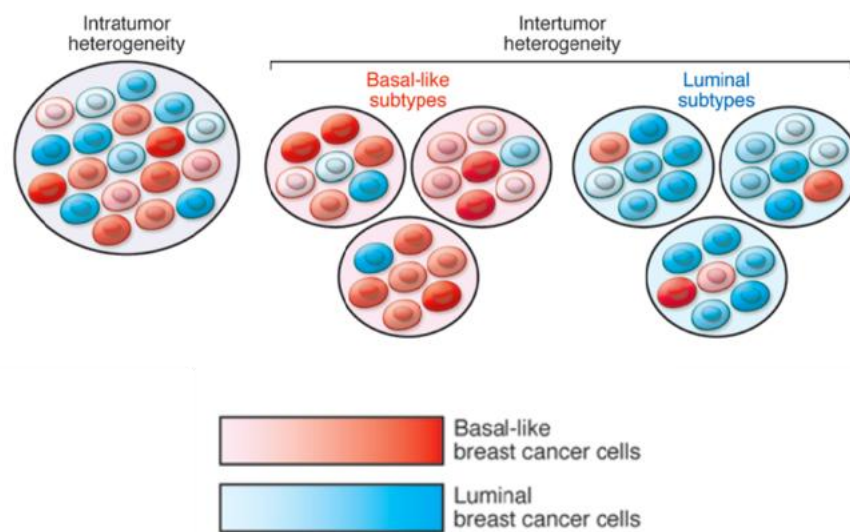


Figure 4: Intratumor and intertumor heterogeneity in breast cancer subtypes. The figure illustrates two levels of heterogeneity observed in breast cancer. On the left, intratumor heterogeneity is shown by the coexistence of both basal-like (red) and luminal (blue) breast cancer cell types within a single tumor. On the right, intertumor heterogeneity is represented by tumors composed predominantly of either basal-like or luminal subtypes, highlighting the variability between different tumors. *Source: modified from (Polyak, 2011).*

The histopathologic analysis and genomic and transcriptomic profiling of primary breast cancer classifies it into five intrinsic subtypes: Luminal A, Luminal B, HER2-enriched, Basal-like, and Claudin-low subtypes. This variation in tumor types from one patient to another is referred to as intertumoral heterogeneity (Fumagalli & Barberis, 2021).

3.4.1 Intratumor heterogeneity

In contrast, intra-tumoral heterogeneity refers to the differences observed within the cells of a single tumor and between its various metastases (Fumagalli & Barberis, 2021; Marusyk & Polyak, 2010).

3.4.1.1 Temporal heterogeneity

Cancer is a dynamic and constantly evolving disease that follows the Darwinian model of evolution, where the fittest clones survive (Nowell, 1976). DNA sequencing analysis reveals the presence of distinct clones organized in a phylogenetic tree. The accumulation of mutations in cancer cells and changes in cancer-related genes lead to genetic diversity. As selective pressures shift, subclones develop over time, a phenomenon known as temporal heterogeneity (Lüönd *et al.*, 2021).

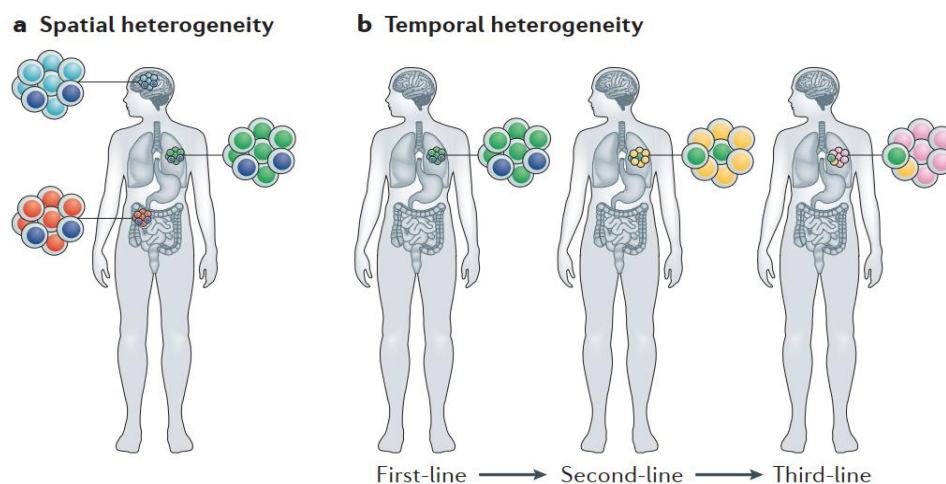


Figure 5: Spatial and temporal heterogeneity in cancer. (A) Spatial heterogeneity refers to the presence of genetically or phenotypically distinct cancer cell populations located in different regions of the body at the same time. Tumor subclones can evolve independently in separate metastatic sites. (B) Temporal heterogeneity represents how tumor composition can change over time, especially in response to therapies. As treatment progresses from first-line to third-line, selective pressure leads to the emergence of new resistant subclones, altering the tumor landscape. *Source: (Dagogo-Jack and Shaw, 2018).*

Several genetic profiling studies have identified four different modes of cancer evolution: late seeding from either a single or multiple subclones, a parallel evolution model

with early spread, simple linear evolution, and a multiclonal invasion model. Regardless of the mode of evolution, the emergence of subclones from cancer cells worsens the prognosis. Given these dynamics, current treatment plans may soon become inadequate (Fumagalli & Barberis, 2021; Lüönd *et al.*, 2021).

3.4.1.2 Spatial heterogeneity

Tumor tissue consists of subclones of different regions of the same tumor or tumors of the different metastases. Uneven distribution of subclones in different sites is called spatial heterogeneity (Fumagalli & Barberis, 2021; Lüönd *et al.*, 2021).

3.4.2 Sources of tumor heterogeneity

The **intrinsic factors**, such as cell autonomous properties including differentiation state and mutations and the **extrinsic factors**, such as tumor microenvironment, can act as sources of tumor heterogeneity.

3.4.2.1 Tumor microenvironment

The tumor microenvironment (TME) is not homogeneous and consists of different kinds of cells, such as fibroblasts, immune cells, pericytes, extracellular matrix, adipocytes, along with epithelial cells. The interactions between tumor cells through physical interactions or by paracrine signalling contribute to tumor heterogeneity. Further interestingly, there occurs a cooperativity within these cells rather than competition, promoting tumor progression (Lüönd *et al.*, 2021; Turashvili & Brogi, 2017).

3.4.2.2 Differentiation state of the cell of origin

Every mammary epithelial cell has a distinct molecular profile that results in a unique differentiation state. A specific tumor-initiating genetic event leads to a particular phenotype.

Depending on the initial genetic event, numerous phenotypes can develop from a single cell of origin (Turashvili & Brogi, 2017).

3.4.2.3 Cancer stem cells and cellular plasticity

According to the cancer stem cells theory, the cancer stem cells are responsible for the maintenance and progression of tumor as these cells possess self-renewing and differentiation potential (Reya *et al.*, 2001). It is believed that tumor cells originate from cancer stem cells that differentiate abnormally, leading to a diverse cell type within the tumor. These cancer stem cells can derive from the differentiation of the cancer cells. Further, the cellular transitions between progenitor cell like, luminal and basal cells are also observed (Gupta *et al.*, 2019). According to the cellular plasticity model, cancer cells exhibit a varying degree of stemness. The microenvironmental stimuli and stochastic gene expression changes in cancer cells can promote the phenotypic changes in the cancer cells (Marusyk & Polyak, 2010; Turashvili & Brogi, 2017). Epithelial to Mesenchymal transition is the best-studied example of cellular plasticity.

3.4.2.3.1 Epithelial to Mesenchymal transition

During EMT, the epithelial cells lose their polarity, morphology, and cell-cell adhesion, adopting quasi-mesenchymal properties. This process promotes the acquisition of stem-like features, such as self-renewal and differentiation potential. It is characterized by the downregulation of epithelial marker genes and also the upregulation of mesenchymal marker genes (Famta *et al.*, 2025; Dongre & Weinberg, 2019). However, it is important to note that EMT is not a binary switch; cancer cells rarely achieve a complete transition to a fully mesenchymal state. Instead, epithelial cells undergoing EMT display a combination of characteristics from both epithelial and mesenchymal cells, leading to a diverse range of phenotypes along the EMT spectrum (Famta *et al.*, 2025; Saitoh, 2018).

3.4.2.3.1.1 Transcription factors regulate EMT

3.4.2.3.1.1.1 Snail

Snail proteins, specifically Snail and Slug, are zinc finger transcription factors that bind to E-box elements in DNA sequences (Lamouille *et al.*, 2014). The binding of these snail proteins leads to the recruitment of polycomb repressive complexes, which result in DNA methylation and the establishment of repressive histone marks on target proteins (Y. Lin *et al.*, 2014). This includes junction proteins in epithelial cells such as E-cadherin, Occludin, and ZO-1, etc. Additionally, Snail proteins can induce activation marks on histones for certain target proteins, including mesenchymal proteins like N-Cadherin and MMP9. Recent studies have also indicated that post-transcriptional and translational regulation of these proteins is becoming an important area of research (Z. Huang *et al.*, 2022).

3.4.2.3.1.1.2 Twist

Twist family proteins, specifically Twist 1 and Twist 2, are basic helix-loop-helix transcription factors (Q. Qin *et al.*, 2012). Similar to the Snail proteins, Twist proteins repress the expression of epithelial proteins like E-cadherin while enhancing the expression of mesenchymal markers, like Fibronectin, N-Cadherin, and Vimentin (Z. Huang *et al.*, 2022). In addition to its association with the PRC2 complex, Twist1 can recruit additional epigenetic modifiers, such as SET8, the NuRD repressive complex, and components of the PRC1 repressive complex, promoting the process of EMT (F. Yang *et al.*, 2012).

3.4.2.3.1.1.3 Zeb1/2

ZEB is a transcription factor that represses epithelial markers such as E-cadherin, ZO-1, and Claudin, etc. (Z. Huang *et al.*, 2022). The ZEB protein family consists of ZEB1 and ZEB2. ZEB1 interacts with the p300 protein, promoting transcription (Postigo, 2003). It also binds to proteins in the SWI/SNF chromatin remodelling complex, suppressing the expression

of E-cadherin (Sánchez-Tilló *et al.*, 2010). In contrast, binding of ZEB2 to CtBP represses the expression of its target genes (Postigo, 2003). Both ZEB1 and ZEB2 can recruit the NuRD complex, which promotes EMT (Z. Huang *et al.*, 2022).

3.4.2.3.1.2 Signalling pathways activated during EMT

Tumor stroma promotes EMT by activating various signalling pathways.

3.4.2.3.1.2.1 TGF β signalling pathway

The TGF β pathway is stimulated by ligands such as TGF β isoforms, activins, and bone morphogenetic proteins (BMPs). When these proteins bind to TGF β receptors, they trigger the formation of a SMAD complex. This trimeric SMAD complex then moves to the nucleus, promoting the EMT by repressing the expression of E-cadherin, while simultaneously promoting mesenchymal protein synthesis of N-cadherin, vimentin, and fibronectin (J. Xu *et al.*, 2009). During this process, the transcription factors Twist, Slug, Snail, and Zeb1 are also increased. These transcription factors further enhance the expression of TGF β 1 receptors through a positive feedback mechanism (Dongre & Weinberg, 2019).

3.4.2.3.1.2.2 WNT signalling pathway

The Wnt pathway can be activated via both canonical and non-canonical mechanisms. When the canonical Wnt pathway is activated, Wnt ligands bind to Wnt Frizzled receptors, leading to nuclear translocation of β -catenin. In healthy epithelial cells, β -catenin is part of a complex with E-cadherin at the adherens junctions. However, when epithelial properties are lost, β -catenin is released from this complex and trans locates to the nucleus. This activation of the Wnt pathway also increases the expression of transcription factors associated with EMT. Specifically, β -catenin directly binds to the promoters of ZEB1, Twist, and Slug, promoting their expression. Additionally, the activation of the non-canonical Wnt pathway through protein kinase C results in an increased expression of snail and vimentin, along with the

downregulation of E-cadherin (Dongre & Weinberg, 2019; Z. Huang *et al.*, 2022; Zhan *et al.*, 2017).

3.4.2.3.1.2.3 Notch signalling pathway

The binding of Jagged and Delta-like ligands to NOTCH receptor isoforms leads to the cleavage of the intracellular NOTCH fragment (Bray, 2016). This fragment then translocates to the nucleus, where it promotes the expression of Snail and Slug, ultimately downregulating E-cadherin expression (Dongre & Weinberg, 2019).

3.4.2.3.1.2.4 Hedgehog signalling pathway

The binding of hedgehog ligands to the Patched receptor activates the hedgehog signalling pathway. This activation leads to the release of inhibition on Smoothened, which subsequently activates the Gli1/2 complex (Briscoe & Thérond, 2013). Once activated, the Gli1/2 complex then moves to the nucleus, promoting EMT by increasing the expression of N-cadherin, Snail, and Vimentin, while repressing E-cadherin (Z. Huang *et al.*, 2022).

3.5 Multicellular spheroids

Multicellular spheroids are self-assembled aggregates of cancer cells that represent the initial stages of tumor growth and occur during avascular phases of tumor development. Culturing cells in spheroid form enhances the expression of stemness genes and promotes multi-differentiation among these cells. This method can also induce the EMT, and furthermore, the formation of spheroids can modify gene expression patterns. Spheroids arise from the aggregation or clonal growth of cancer cells.

3.5.1 Mechanism of spheroid formation

The formation of a spheroid is a multi-step process mediated by cell adhesion molecules like E-cadherin and integrins. Initially, the single cells in suspension form loose aggregates due

to the interaction between long-chain ECM fibres and integrin molecules on the cell surface (R.-Z. Lin *et al.*, 2006). This stimulates the increased expression and accumulation of E-cadherin molecules on the surface of the cell. The strong homophilic interactions between these E-cadherin molecules result in the compaction of spheroids (R.-Z. Lin *et al.*, 2006). The accumulation of β -catenin complex by E-cadherin molecules might facilitate the signal transduction during spheroid formation (Shimazui *et al.*, 2004). Further, the integrin molecules facilitate signal transduction, which leads to cytoskeletal rearrangement. Actin filaments are involved in the mechanical transduction of extracellular cues through integrin molecules on the cell surface, which is essential for spheroid formation. Cytoskeleton provides mechanical integrity, rearrangement, as the actin filaments, as stress fibres, get localised along the periphery and maintain the structural stability of cells. Microtubule polymerisation is essential for spheroid formation (R. Lin & Chang, 2008; R.-Z. Lin *et al.*, 2006; Tzanakakis *et al.*, 2001).

The diffusion limit for small molecules, like oxygen, is approximately 150-200 μm . When the size of a compact spheroid surpasses this threshold, a hypoxic microenvironment emerges, accompanied by the accumulation of metabolic byproducts in the core. This condition results in the stratification of spheroids into three distinct zones: a central necrotic zone, a viable quiescent zone, and an outer proliferative zone of cells (R. Lin & Chang, 2008). Histological examination of the spheroids generated using different cell lines showed that spheroids contain the outer viable layer of cells and the central degenerative or necrotic cells. The doubling time of cells grown in the spheroids is increased for the majority of the cell lines (Carlsson *et al.*, 1983).

The spheroid formation rate depends not only on the expression of the adhesion molecules but also on the initial seeding density of the cells and the doubling time of the cells. Many cells have the capability to form spheroids (Carlsson *et al.*, 1983). The initial high-

density seeding (10^4 - 10^5 cells/ml) gave round spheroids compared to the low-density seeding (10^2 - 10^4 cells/ml). The size of the spheroid depends on the cell line, and the formation of a spheroid is cell-type dependent. Several cells were used to understand the formation of the spheroid. The breast cancer lines, such as BT474, T47D, MCF7, SKBr3, MDA-MB-361, and MDA-MB-435S, are known to form spheroids. Whilst E-cadherin is essential for the tight packing of BT474, T47D, MCF7, SKBr3, and MDA-MB-361 cell lines. N-cadherin in MDA-MB-435S cells or by Integrin β 1 and ECM collagen in MDA-MB-231 and SKBr3 play a pivotal role (Ivascu & Kubbies, 2007). The spheroids represent the avascular stages of solid tumor and micro metastases (Froehlich *et al.*, 2016). The transcriptomic analysis shows that spheroids exhibit an increase in expression of EMT-related genes (Y. P. Kang *et al.*, 2019). Cells grown in spheroids show an increase in the expression of EMT markers (Essid *et al.*, 2018). Cells grown as spheroids also exhibit an increase in stemness, EMT markers and multidrug resistance (Gheytauchi *et al.*, 2021). The levels of the E-Cadherin increased unexpectedly (Gheytauchi *et al.*, 2021).

3.5.2 Molecular characterization of spheroids cultured in a liquid overlay culture

The liquid overlay method of spheroid generation is well-established and cost-effective. Here, we have summarized the molecular characteristics of multicell spheroids generated in low-adhesive dishes such as poly-HEMA, ultra-low attachment, or agar-coated dishes.

The proliferation of cells in MCS is generally lower than that of monolayer cells (Folkman & Moscona, 1978; Ghosh *et al.*, 2005; Steadman *et al.*, 2008; Nazemroaya *et al.*, 2017). However, spheroids demonstrate more prominent angiogenesis and invasive potential. For instance, spheroids generated from adipose stem cells (ASCs) exhibited enhanced expression of HIF1 α and angiogenic growth factor mRNAs such as CD31, CD144, FGF-2,

Flk1, FGFR1, FGFR2, IGF2, MCP-1, PDGF-A, TIE2, VEGF-A, VEGF-C, vWF and more significant invasive potential (J.-H. Kim *et al.*, 2015). In another example, NA8 melanoma spheroids exhibited heightened expression of chemokines such as CXCL1, CXCL2 and CXCL3, IL8, CCL20 and proangiogenic proteins such as angiopoietin-like 4 (ANG-4), hypoxia-inducible protein 2 (HIG2) and CD54 (Ghosh *et al.*, 2005). Transcriptomic profiling of HTR8/SVneo EVT spheroids also showed elevated expression of angiogenesis-related mRNAs such as HIF1 α , VEGFA, VEGFC, and MMP9 (Wong *et al.*, 2019). Additionally, spheroids generated through Wharton's jelly mesenchymal stem cells (WJ-MSCs) exhibit increased angiogenic IDO, IL-10, LIF, ANG1, and VEGF mRNA expression (Thakur *et al.*, 2022).

The anchorage-independent cell survival is a prerequisite for the formation of spheroids. In FaDu spheroids, a cell line derived from head and neck squamous cell carcinoma (HNSCC), activation of MAPK/ERK signalling through EGFR promotes anchorage-independent cell survival (Braunholz *et al.*, 2016). Similarly, in A549 spheroids, SOX2 promotes anchorage-independent survival by activating the AKT pathway (Choe *et al.*, 2018). SKOV3 ovarian cancer cells spheroids showed resistance to anoikis by an increase in expression of methyltransferase EZH2 (Dai *et al.*, 2021). Furthermore, higher anti-apoptotic Bcl-2 protein and lower expression of proapoptotic Bax protein in adipose stem cells (ASCs) promote cell survival (J.-H. Kim *et al.*, 2015).

Spheroids exhibit enhanced cancer stem cell-like phenotype and multilineage differentiation potential. Esophageal cancer cells (KYSE70 and TE1) spheroids show an increase in SOX2, KLF4, and ALDH1A (Yue *et al.*, 2015). A549, NSCLC spheroids, demonstrated elevated expression of the pluripotency-associated transcription factors such as OCT4, SOX2, KLF4, c-MYC and NANOG (Choe *et al.*, 2018). Prostate cancer spheres derived

from LNCap, VCap and DU145 cells showed a notable increase in cancer stem cell characteristics, marked by CD44, CD133 and NANOG (Gao *et al.*, 2018). MCF7 breast cancer cell spheres exhibit an enhanced stem cell phenotype characterized by CD44⁺/CD24^{low} expression (Guttilla *et al.*, 2012). Hepatocellular carcinoma cell spheroids formed from Huh7 cells revealed more significant expression of stemness-related genes, including ATP-binding cassette sub-family G member 2 (ABCG2), EPCAM, NANOG, PROM-1 (CD133), SOX9, and THY1 (CD90) (Ma *et al.*, 2019). Wharton's jelly mesenchymal cells (WJ-MSCs) also form spheroids that show heightened expression of pluripotent markers, such as NANOG, OCT4, SOX2, and contribute to their multi-lineage differentiation potential (Thakur *et al.*, 2022). hMSCs, isolated from the vascular wall, form spheroids characterised by stemness and mesenchymal markers, including CD44, CD90, CD105, CD166, Notch-1 and Runx-1, alongside an increase in the expression of OCT4 and NANOG, was detected (Valente *et al.*, 2022). HeLa cells cultured as spheroids exhibit elevated expression of stemness markers OCT4, SOX2 and ALDH1, enabling multipotent differentiation (L. Wang *et al.*, 2014).

The cells in MCS also demonstrate cellular plasticity and can undergo epithelial-to-mesenchymal transition (EMT). For example, spheroids of esophageal cancer cells, KYSE70 and TE1, exhibit a decrease in differentiation marker CK4, while the expression levels of EMT markers, such as Fibronectin, N-Cadherin and Vimentin, increased (Yue *et al.*, 2015). Similarly, the spheroids of A549 cells show elevated levels of EMT markers, including N-cadherin, Snail and ZEB1 (Choe *et al.*, 2018). Spheroids formed from human retinal pigment epithelium cells (hRPE) undergo dedifferentiation or trans differentiation, enriching retinal progenitor cells (Nazemroaya *et al.*, 2017). Prolonged culture of MCF7 spheroids resulted in EMT characterized by increased Snail1, Slug, ZEB1, ZEB2 and AXL and increased IL6 levels. These spheroids also showed a reduction in epithelial markers ER α , E-Cadherin, GATA3, and Claudin 3 and an increase in mesenchymal markers such as Vimentin, EGFR, α 6 integrins and

moesin (Guttilla *et al.*, 2012). HTR8/SVneo EVT cell spheroids showed an increase in both upstream EMT markers, including TGF β 1, TGF β 2 and WNT5A, as well as downstream EMT markers such as integrin (ITG)A2, ITGA5, ITGB1, and Vimentin (VIM) mRNA levels. Interestingly, Occludin mRNA levels decreased while E-cadherin and zona occludins mRNA levels increased, which is crucial for spheroid formation despite its contrast with EMT. Additionally, tight junction markers Claudin1 and 4 mRNA levels also increased (Wong *et al.*, 2019). Hela cells in spheroids have also exhibited potential for adipogenic differentiation (L. Wang *et al.*, 2014). While prostate spheres contained a lesser number of differentiated cells (Gao *et al.*, 2018).

3.6 Functional role of the nucleolus in breast cancer

The nucleolus is a subnuclear entity formed around the nucleolar organising regions, NORs (clusters of the rDNA repeats) of the acrocentric chromosomes 13,14,15, 21 and 22 (Pederson, 2011). The first report of the abnormalities in nucleolar morphology in cancer cells was reported by Pianese G, an Italian scientist, in 1896. The size and number of the nucleoli increase prominently in cancer, and this increase is associated with the malignancy (Montanaro *et al.*, 2008). Furthermore, the triple-negative breast cancer cells have more nucleoli than non-TNBC (Weeks *et al.*, 2021). The function of the nucleolus is the synthesis of the ribosomes, which is a complex multistep process involving the synthesis of ribosomal RNA (rRNA), its processing and chemical modification, as well as the export and assembly of ribosomal subunits into the cytoplasm to form functional ribosomes (Orsolio *et al.*, 2016).

The human cell consists of nearly 100-500 copies of rDNA repeats (Hori *et al.*, 2021), and nearly half of the rDNA repeats are transcriptionally active, while the remaining half is transcriptionally inactive. The inactive rDNA repeats are associated with the repressive chromatin marks, while the active rDNA repeats are present in open chromatin regions

accessible for transcription by RNA polymerase I (McStay & Grummt, 2008). The RNA Pol I synthesizes the 47S precursor rRNA in association with transcription factors, upstream binding factor (UBF), and selectivity factor (SL1) (Bywater *et al.*, 2013). It then undergoes chemical modifications and processing into 18S rRNA of the small subunit and 28S and 5.8S rRNA of the large subunit. The 5S rRNA is synthesised by RNA pol III in the nucleolus and is transported to the nucleolus (Lewis & Tollervey, 2000). Meanwhile, ribosomal proteins, which are synthesised by RNA polymerase II in the cytoplasm, are imported to the nucleolus to assemble with the rRNA (Ferreira-Cerca *et al.*, 2005).

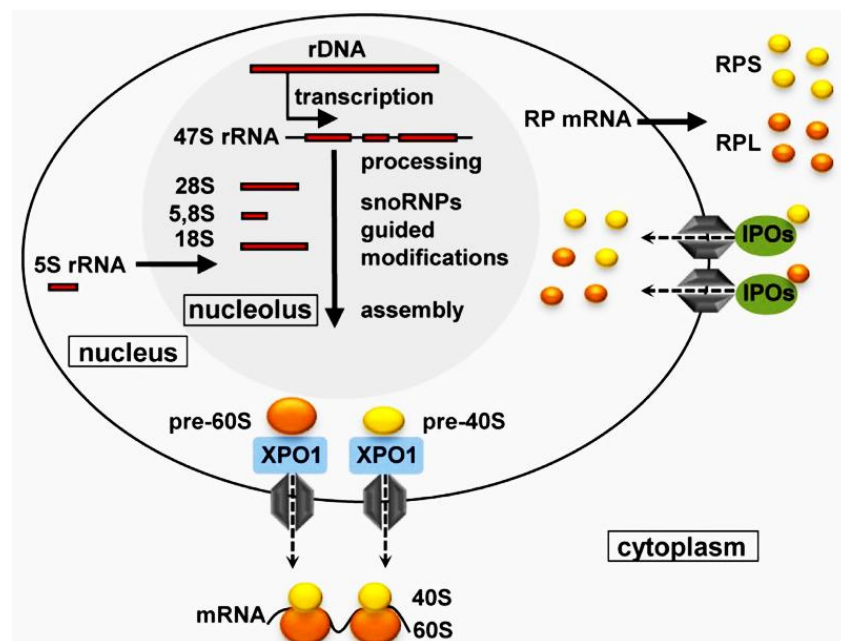


Figure 6: Overview of ribosome biogenesis and export from the nucleus to the cytoplasm. Ribosome production begins in the nucleolus with transcription of rDNA (ribosomal DNA) into 47S precursor rRNA, which is processed into the 28S, 18S, and 5.8S rRNA components. The 5S rRNA is transcribed separately. These rRNA components undergo modifications guided by small nucleolar RNPs (snoRNPs) and are assembled with ribosomal proteins (RPs), which are produced in the cytoplasm and imported into the nucleus via importins (IPOs). The resulting pre-60S and pre-40S ribosomal subunits are then exported to the cytoplasm through exportin 1 (XPO1). In the cytoplasm, the mature 60S and 40S subunits assemble on mRNA to initiate protein translation. *Source: (Orsolic et al., 2016).*

More than 200 non-ribosomal proteins play a crucial role in the processing, modification, and formation of mature ribosomes (Orsolic *et al.*, 2016). These proteins are known as ribosome biogenesis factors (RBFs), which temporarily associate with ribosomal RNA (rRNA)

but do not become incorporated into the mature ribosomes. RBFs not only assist in these modifications but also ensure the proper ribosomal protein assembly and the correct folding of ribosomal particles. Many of these factors have been extensively studied in yeast and have been found to be conserved in humans (Dörner *et al.*, 2023).

Factors involved in rDNA transcription	RNA Polumerase 1 subunits	POLR2E,POLR2F, POLR2H, POLR2L, POLR2K, POLR1C, POLR1D, POLR1A, POLR1B, POLR1F, POLR1H, POLR1E, POLR1G
	PIC formation/promoter escape	TBP, UBF, RRN3, TAF1C, TAF1B, TAF1A,TAF1D ,TAF12, H3C1/H3, H4C1/H4, TOP1, TOP2A, CK2A1, CK2A2, CK2N
	Elongation	SPT4H, SPT5H, PAF1, CTR9, WDR61, RTF1, LEO1, SUPT16H, SSRP1
	Termination	TTF1
	rDNA organisation	CTCF, MYC, MAX, TCOF1, TCOF1, NOLC1 BRD2 BRD4, KAT7, JADE3, LYAR
Factors acting both in rRNA transcription and early processing steps		SIRT7, FBL, UTP4, WDR43/UTP5, HEATR1/UTP10, UTP15, UTP17/WDR75
Factors involved in	snoRNP components	FBL, NOP56, NOP58, SNU13, U3-55K, DKC, NHP2, NOP10, GAR1

rRNA modification	Stand-alone enzymes	TSR3, EMG1, NAT10, WBSCR22, DIMT1, NML/RRP8, NSUN5, NOP2, ZCCHC4, METTL5, TRMT112
Factors involved in pre-rRNA processing		UTP23, UTP24, NOB1, RMRP, POP1, RPP38, RPP29, POP5, RPP25, RPP20, RPP14, RPP30, RPP21, RPP40, LAS1L, XRN2, NOL12, DIS3, EXOSC10, EXOSC1, EXOSC2, EXOSC3, EXOSC4, EXOSC5, EXOSC6, EXOSC7, EXOSC8, EXOSC9, MTR4, PAPD5, PAPD7, ZCCHC7, ZCCHC8, MPP6, C1D, RBM7, REXO5, REXO2, REXO1, Ccr4, PARN, TUT4, TUT7, ISG20L2, ERI1
Chaperones of ribosomal proteins		GRWD1, HEATR3, AAMP, BCCIP, PDCD2, NPM, AK/CINAP, AROS, TSR2
Subcomplexes of small subunit processome and early assembly factors		UTP4/CIRH1A, UTP5/WDR43, NOL11, UTP10/HEATR1, UTP15, UTP17/WDR75, MYBBP1A, PWP2, UTP6, UTP12/WDR3, TBL3, UTP18, WDR36, DDX21, NOP2, FBL, NOP56, NOP58, SNU13/15.5 K, U3-55K/U3IP2, MPHOSPH10, IMP3, IMP4, RRP7A, NOL6, CK2A1, CK2A2, CK2N, CK2N, RRP36, WDR46, UTP14A, WDSOF1, RCL1, BMS1, ENP1/BYSL, NOP14, NOC4L, EMG1, AATF,

	NGDN, NOL10, XRN2, NKRF, DHX15, RSL1D1, UTP11, NOL7, TDIF2, UTP3, UTP24, ESF1, ABT1, DDX49, DDX10, DDX47, SRFBP1, PDCD11, KRR1, DDX52, DEF/C1orf107, KRI1, UTP23, RBM19, DDX48/EIF4A3, NOM1, CMS1, NOP9, UTP20, NAT10, C1orf131, PNO1/DIM2, RRP12, NOB1, DHX37, NML/RRP8, FAM207A/C21orf70, MYBBP1A, WBSCR22, NIP7, FTSJ3
Factors involved in nucleoplasmic steps of pre-40S maturation	TSR1, NOB1, CKId/CSNK1D, CKIe/CSNK1E, RIOK1/RIO1, RIOK2/RIO2, LTV1, PARN, FAM207A/C21orf70
Factors involved in nuclear export of ribosomal pre-particles	XPO1/CRM1, PNO1/DIM2, RIOK2, LTV1, PDCD2L, RANBP3, FAM207A/C21orf70, RRP12, NXF1, NXT1, NMD3, PA2G4/EBP1, ZNF593, SRSF1, RAE1, XPO5
Factors involved in cytoplasmic pre-40S maturation	ENP1/BYSL, LTV1, TSR1, DIMT1, RRP12, PNO1/DIM2, NOB1, CKI-d/CSNK1D, CKI- e/CSNK1E, RIOK1/RIO1, RIOK2/RIO2, RIOK3, DHX15, SON, PINX1, HBS1, PELO, ABCE1, EIF5B, USP16, LRRC47, EIF1AD
Factors involved in nucleolar steps of maturation of 60S	PDCD11, CEBPZ, NOC2L/NIR, URB1, URB2, DDX51, DDX5, DDX31, DDX24, WDR74,

	RPF1, MAK16, RRP1, RBM28, PUM3, RRP15, PPAN, SURF6, METTL18, PES1, BOP1, WDR12, DDX27, MKI67IP, RLP7, PWP1, RBM34, DDX18, BRX1, EBNA1BP2, NIP7, NOP2, RRS1, BXDC1, DDX55, PAK1IP1, DDX54, GNL3, RLP24/RSL24D1, EIF6, GTPBP4, NSA2, NLE1, MYBBP1A, NOC3L, GNL2, NOP16, GLTSCR2/NOP53, FTSJ3, MRT4/MRTO4, N6AMT1, LLPH, ZNF593, PA2G4/EBP1, NVL2, MDN1, LAS1L, NOL9
Factors involved in nucleoplasmic steps of pre-60S maturation	CCDC86, SDAD1, PELP1, TEX10, WDR18, MDN1, SENP3, NF45/ILF2, NF90/ILF3, NMD3
Factors involved in cytoplasmic pre-60S maturation	SPATA5, SPATA5L, CINP, C1ORF109, RLP24, RSL24D1, ZNF593, GNL3, LLPH, TMA16, NSA2, GTPBP4/NOG1, PA2G4/EBP1, ZNF622, DNAJC21, HSPA1A, HSPA1B, MRT4/MRTO4, DUSP12, EFL1/ EFTUD1, SBDS, EIF6, LSG1, AAMP, NMD3
Other factors involved in ribosome biogenesis	NPM, NCL, DDX1, HECTD1, USP36, UBR5, VPRBP

#* Combined and adapted from multiple tables in: Dörner, K., Ruggeri, C., Zemp, I., & Kutay, U. (2023). Ribosome biogenesis factors—from names to functions. The EMBO Journal, 42(7), e112699. <https://doi.org/10.15252/emj.2022112699> Licensed under CC BY-NC-ND 4.0.

3.6.1 Nucleolar processes are involved in promoting cellular plasticity

Recent discoveries emphasize the importance of ribosome biogenesis in cell fate changes, such as the epithelial-to-mesenchymal transition process (Prakash *et al.*, 2019). Furthermore, epi transcriptomic chemical modifications of rRNA molecules could also bring about cell fate changes and stemness. One such well-studied epi transcriptomic modification is the ribose modification at the 2' position by adding a methyl group (2'-O-methylation or 2'-O-Me) (F. Zhou *et al.*, 2023; Häfner *et al.*, 2023; Munholland & Nazar, 1987).

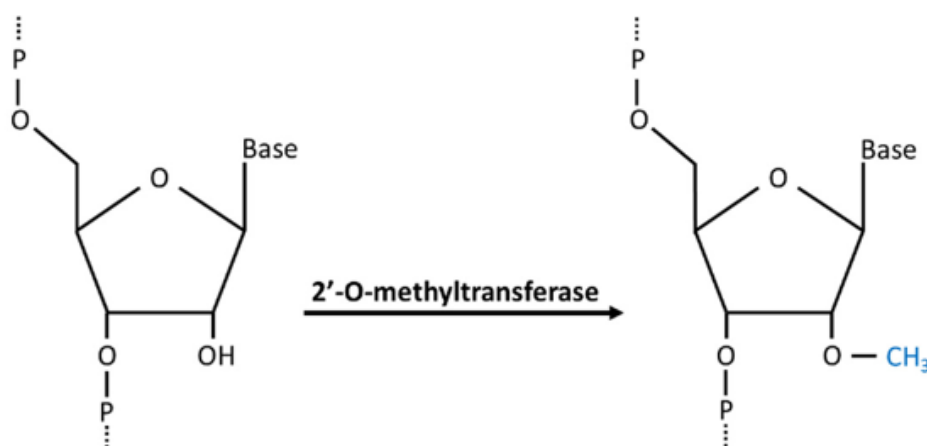


Figure 7: 2'-O-methylation of the ribose sugar by 2'-O-methyltransferase. This schematic illustrates the enzymatic methylation of the ribose 2'-hydroxyl group in an RNA nucleotide. The enzyme 2'-O-methyltransferase catalyses the transfer of a methyl group ($-\text{CH}_3$) to the 2'-OH position of the ribose sugar, resulting in a 2'-O-methylated nucleotide. *Source: (Jaafar et al., 2021).*

3.6.2 Mapping and characterisation of 2'-O-Me

The chemical properties of the modified bases are exploited for mapping the sites of 2'-O-Me of rRNA. These include resistance to alkaline hydrolysis, enzymatic cleavage, periodate oxidation, and steric hindrance through reverse transcription (K. I. Zhou *et al.*, 2024). Using these chemical properties, various high-throughput sequencing-based approaches are exploited to identify 2'-O-Me sites of rRNA, such as RiboMeth-seq (Krogh *et al.*, 2016; Marchand *et al.*, 2016), RibOxi-seq (Y. Zhu *et al.*, 2017), and 2OMe-seq (Incarnato *et al.*, 2017). Among these

high-throughput methods available for identifying 2'-O-Me sites, RiboMeth-seq stands out as a highly effective and valuable technique. The low-throughput method, RTLP, has also been used to map 2'-O-Me in the rRNA (Belin *et al.*, 2009; Z.-W. Dong *et al.*, 2012).

The RiboMeth-seq analysis of rRNA extracted from HCT116 and HeLa cell lines has identified 106 sites on rRNA that can undergo 2'-O-methylation. Out of these, 65 sites are present on 28S rRNA, 39 sites on 18S rRNA, and 2 sites on 5.8S rRNA (Krogh *et al.*, 2016; Sharma *et al.*, 2017). In another study involving HeLa cells, 113 2'-O-Me sites in human rRNA were identified (42 in 18S, 69 in 28S and 2 in 5.8S) (Jansson *et al.*, 2021). New sites are, however, being found and added to this. All the identified sites can undergo either complete or partial methylation (Hebras *et al.*, 2020; Ramachandran *et al.*, 2020). The data indicated that full methylation occurred at about two-thirds of the sites, whereas the rest demonstrated partial methylation patterns, indicating the existence of ribosome heterogeneity (Krogh *et al.*, 2016; Sharma *et al.*, 2017; Marcel *et al.*, 2020). Among the identified sites, those that show no variation are classified as constitutive sites, while the others are categorised as variable or vulnerable sites of the rRNA (Erales *et al.*, 2017; Krogh *et al.*, 2016; Marcel *et al.*, 2020). The vulnerable sites are located outside the functionally important sites of the ribosome and can undergo regulation during normal physiological and pathological conditions (Sharma *et al.*, 2017). Furthermore, 2'-O-Me is characterised as tissue (Nazar *et al.*, 1975) and cell-type-specific. The comparison of rRNA methylation in HeLa and HCT116 cell lines showed nearly identical yet certain differences in the fraction of 2'-O-Me, suggesting a cell-type-specific methylation pattern (Krogh *et al.*, 2016). 2'-O-Me contributes to the cell type-specific transcription and translation (F. Zhou *et al.*, 2023). A comparison of 2'-O-Me of HeLa and mouse ESCs using another high-throughput technique named 2OMe-seq, has shown that it is nearly identical, suggesting that it is conserved. However, species-specific differences in sites were observed (Incarnato *et al.*, 2017).

3.6.3 The nucleolar snoRNP complex is involved in the rRNA 2'-O-Me

In eukaryotes, the methylation of ribosomal RNA at the 2'-O position of ribose sugar is carried out by a small nucleolar ribonucleoprotein (snoRNP) complex that assembles within Cajal bodies. This assembly involves several ribosome biogenesis factors (proteins), such as FBL, 15.5K, NOP58, and NOP56, which are incorporated into the snoRNA, resulting in the formation of the snoRNP particle (Rodriguez-Corona *et al.*, 2015). Human C/D box snoRNAs, typically 70 to 120 nucleotides long, are transcribed from the intronic sequences of protein-coding genes. These snoRNAs contain two conserved sequences known as the C box and D box, with sequences RUGAUGA and CUGA, respectively. Each of these sequences is duplicated, creating C' and D', which are crucial for directing the methylation process. The complementary sequence within the snoRNA targets the rRNA for methylation specifically. In the snoRNP structure, the catalytic site of FBL is located opposite the nucleotide designated for methylation. FBL uses S-adenosyl methionine (SAM) as the methyl donor during this biochemical process. The dimers NOP56 and NOP58 provide structural support, and the 15.5 K protein stabilises the kink (Monaco *et al.*, 2018; Rodriguez-Corona *et al.*, 2015).

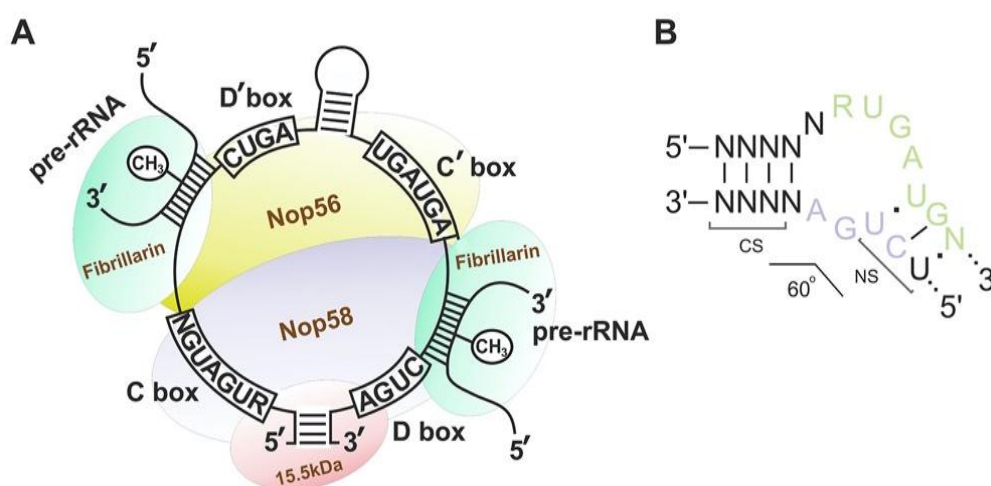


Figure 8: Structure and function of C/D box small nucleolar ribonucleoproteins (snoRNPs) in RNA methylation. (A) Schematic representation of a C/D box snoRNP complex involved in pre-rRNA 2'-O-methylation. The snoRNA contains conserved C (RUGAUGA) and D (CUGA) box motifs as well as less

conserved C' and D' boxes. Proteins such as fibrillarin, Nop56, Nop58, and 15.5 kDa associate with the snoRNA to form the active complex. Fibrillarin catalyzes the methylation reaction, guided by base pairing between the snoRNA and the target rRNA. **(B)** Base pairing interaction between snoRNA and rRNA at the methylation site. The methylated nucleotide (NS) in the rRNA is located 5 nucleotides upstream of the D or D' box in the snoRNA. *Source: (Filippova et al., 2019)*

3.6.4 Structure and function of FBL

The human Fibrillarin is a 321-amino-acid protein made up of two key domains. The N-terminal region, spanning amino acids 8 to 80, is rich in glycine and arginine residues, often referred to as the GAR domain. This is followed by a central methyltransferase domain that extends from amino acid 133 to 306. Within this region, there's a segment likely involved in RNA binding (133–222), and toward the end, an α -helical structure is found between residues 274 and 306 (X. Zhang *et al.*, 2024). Fibrillarin is highly conserved across species, especially within its methyltransferase domain, which shares strong structural and sequence similarity in both eukaryotes and archaea (Aris & Blobel, 1991; Rodriguez-Corona *et al.*, 2015; X. Zhang *et al.*, 2024) and is involved in rRNA processing, rRNA methylation and ribosome assembly (Tollervey *et al.*, 1993).

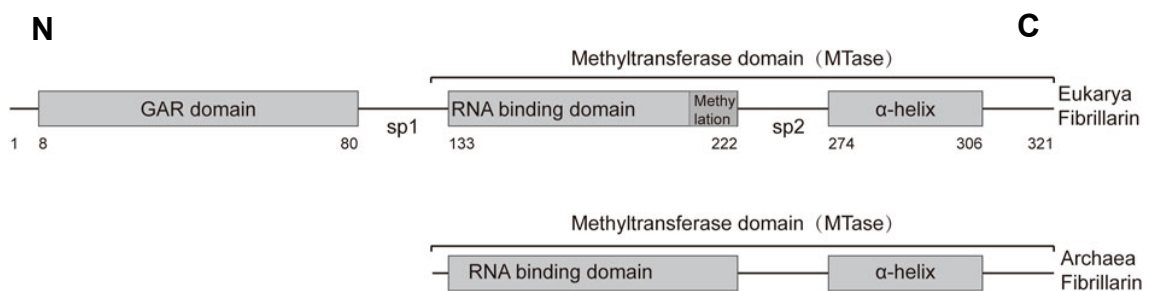


Figure 9: Comparative domain organisation of fibrillarin in eukaryotes and archaea. The schematic illustrates the structural domains of fibrillarin proteins from eukaryotic and archaeal species. In eukaryotes, fibrillarin comprises an N-terminal glycine–arginine-rich (GAR) domain, followed by a central RNA-binding domain and a methyltransferase (MTase) domain, which includes a conserved methylation catalytic site and an α -helix. Archaeal fibrillarin, in contrast, lacks the GAR domain but retains the RNA-binding and MTase domains, highlighting structural conservation essential for catalytic function (N- N terminal, C-C terminal, Number of amino acids = 321). *Source: (Zhang et al., 2024).*

3.6.4.1 Role of FBL in chromatin regulation

FBL catalyses glutamine methylation of histone H2A at glutamine 104 (Q104) (H2AQ104me), a mark linked to active rDNA transcription. This methylation blocks the binding of the chromatin remodeller FACT. As a result, the deposition of the nucleosomes on the rDNA locus is reduced during rDNA transcription, and rRNA synthesis is enhanced (Tessarz *et al.*, 2014). During mitosis, FBL is localised to the chromosomal periphery, resulting in reduced interaction with the SIRT7 deacetylase. Consequently, FBL exists in a hyperacetylated form. Because of this, FBL is unable to methylate the H2A Q104. This leads to the accumulation of nucleosomes and halts ribosome synthesis during mitosis. As cells exit mitosis, FBL is deacetylated by SIRT7, which increases the levels of H2AQ104me and resumes rRNA synthesis (Iyer-Bierhoff *et al.*, 2018). On the other hand, when FBL associates with polycomb protein (CBX4), which is associated with transcriptional repression and heterochromatin (Ren *et al.*, 2019).

3.6.4.2 FBL promotes cancer phenotype

FBL is frequently overexpressed in various cancers and plays a crucial role in tumor biology. High levels of FBL have been reported in acute myeloid leukaemia (AML) stem cells (F. Zhou *et al.*, 2023), mouse embryonic stem cells, and pluripotent cells (Watanabe-Susaki *et al.*, 2014). Ectopic expression of FBL enhances pluripotency markers such as Oct4, Nanog, and SSEA-1, and its methyltransferase activity is critical for maintaining stem cell identity. Conversely, FBL depletion induces differentiation, characterised by increased expression of lineage-specific markers like Fgf5, Flk1, and Brachyury, along with a notable rise in neuronal progenitor markers Pax6 and Nestin. Depletion of FBL particularly affects 18S rRNA processing, leading to reduced rRNA methylation and delayed maturation. Complete knockdown results in apoptosis, while partial knockdown triggers differentiation, both mediated through the p53 pathway (Watanabe-Susaki *et al.*, 2014). Interestingly, during

bacterial infection, FBL levels are downregulated, which correlates with enhanced phagosome maturation and increased production of anti-inflammatory cytokines, ultimately supporting host resistance and cell survival (Tiku *et al.*, 2018).

In breast cancer, FBL upregulation is observed, especially in cases with mutated p53 (Marcel *et al.*, 2013). Its overexpression in MCF7 cells promotes proliferation, anchorage-independent growth, and resistance to chemotherapy (Marcel *et al.*, 2013). Notably, the interaction between FBL and YBX1 through FBL's GAR domain activates BRCA1 transcription and enhances homologous recombination, contributing to chemoresistance (Marcel *et al.*, 2013; Sun *et al.*, 2023). Targeting FBL sensitizes cancer cells to chemotherapeutic agents, indicating it as a therapeutic target (El Hassouni *et al.*, 2019). FBL is also upregulated in mammary tumors, with its expression positively regulated by c-Myc (Su *et al.*, 2014). Two independent studies have shown high FBL expression in prostate cancer samples, both at the protein and mRNA levels (Koh *et al.*, 2011; Su *et al.*, 2014). Moreover, FBL levels are elevated in metastatic prostate cancer in comparison with normal epithelium, and their depletion reduces cell proliferation, rRNA synthesis, nucleolar number, and tumour-forming capacity (Koh *et al.*, 2011; Su *et al.*, 2014).

In hepatocellular carcinoma (HCC), increased FBL levels correlate with larger tumor size and advanced clinical stage. Patients with elevated FBL expression have significantly shorter overall and disease-free survival times, with its upregulation attributed to promoter demethylation rather than gene mutation (J. Zhang *et al.*, 2020). Although overexpression is often linked to tumor progression, recent findings suggest that even low FBL expression may serve as a prognostic marker, indicating its dual role in cancer development (Nguyen Van Long *et al.*, 2022). Elevated expression of FBL has also been observed in lung and colorectal cancers (J. Yang *et al.*, 2022), where it is associated with poor patient outcomes. Silencing FBL in these

cancers reduces colony formation and proliferation, independent of its enzymatic function (Sun *et al.*, 2023). Collectively, these studies highlight FBL as a critical regulator of tumorigenesis and a promising target for cancer therapy.

3.6.4.3 Role of FBL in rRNA 2'-O-Methylation and IRES-mediated translation regulation

Depletion of FBL results in a global decrease in rRNA methylation. However, the decrease in methylation at certain sites was more significant than at others, indicating FBL-induced decrease in 2'-O-Me or rRNA occurs in a site-specific manner (Erales *et al.*, 2017). The functional domains in the A and P sites, inter-subunit bridges, peptide exit channel, but peptidyl transferase centre and the decoding centre did not show any alteration upon the FBL knockdown (Erales *et al.*, 2017). Its depletion results in the translational alteration of certain genes. FBL knockdown results in a decrease in the IRES-mediated translation initiation (Basu *et al.*, 2011; Erales *et al.*, 2017). However, depletion of FBL increased the IRES-mediated expression of p53 mRNA (Su *et al.*, 2014). When the levels of the FBL were high due to p53 KD, the IRES activity of IGF1R, c-MYC, FGF1, FGF2, and VEGFA was high (Marcel *et al.*, 2013). Conversely, FBL depletion decreased the expression from FGF1 and IGF1R IRES, but expression from VEGFA was unaltered (Erales *et al.*, 2017). In another study, when the FBL was silenced, IRES-mediated expression of VEGFA and VEGFC was reduced (Metge *et al.*, 2021).

FBL interacts with the polycomb group protein, EZH2. EZH2 interacts with the RNA-binding domain of the FBL and regulates the 2'-O-Me independent of its lysine methyl transferase activity. Depletion of EZH2 decreased 2'-O-Me in a site-specific manner and reduced the IRES-mediated translation; Therefore, EZH2 regulates IRES-mediated translation initiation by interacting with FBL (Yi *et al.*, 2021). Interestingly, silencing of EZH2 doesn't

alter the expression of FBL (Yi *et al.*, 2021). EZH2 also has a role in assembling box C/D snoRNPs and seems to function at both the transcriptional and translational levels, again without relying on its methyltransferase activity (Yi *et al.*, 2021).

3.7 Role of SUMOylation in ribosome biogenesis

Recent studies highlight the role of post-translational modification, SUMOylation, in regulating nucleolar processes. SUMOylation is an evolutionarily conserved pathway that influences various cellular mechanisms, including ribosome biogenesis. The SUMO pathway is a reversible, multi-step process. SUMO proteins are synthesised as precursor molecules and are subsequently cleaved by SUMO-specific proteases (SENPs) to produce mature SUMO moieties. The SAE1/SAE2, SUMO-activating heterodimer enzyme, activates the mature SUMO moiety (Geiss-Friedlander & Melchior, 2007). This activated SUMO is then transferred to a cysteine residue of the UBC9, a SUMO conjugating enzyme, which ultimately transfers it to the substrate's consensus lysine residue (ψ KxE) through the action of E3 SUMO ligases (Geiss-Friedlander & Melchior, 2007). Additionally, SUMO deconjugating enzymes, such as sentrin-specific proteases (SENPs), remove the SUMO moiety from the substrate (Geiss-Friedlander & Melchior, 2007). Furthermore, E3 ubiquitin ligases that target SUMO proteins for ubiquitination also regulate the SUMOylation status of these targets (Tatham *et al.*, 2008).

Genome-wide binding studies have shown that SUMO is largely recruited across pro-growth factor genes, ribosomal protein (RP) genes, and rDNA. The SUMO pathway has been demonstrated to exert both positive and negative regulation on rRNA synthesis (Y. Yang *et al.*, 2024). SUMOylation promotes the transcription of ribosomal protein genes by recruiting the transcriptional machinery (Chymkowitch *et al.*, 2015). Moreover, the deSUMOylation of Tof2 and Net1 by Ulp2 (a sentrin-specific protease in yeast) enhances the silencing of rDNA (Gillies *et al.*, 2016; J. Liang *et al.*, 2017), with Ulp2 depletion leading to increased expression of

rRNA, snoRNAs, ribosomal protein genes, and essential factors for ribosome synthesis (Ryu *et al.*, 2018) in yeast. Conversely, disruption of the SUMO pathway has also been shown to promote rRNA synthesis (Luo *et al.*, 2020); for instance, UBC9-mediated inhibition of SUMOylation stimulates rRNA synthesis (Neyret-Kahn *et al.*, 2013). SUMOylation decreases rDNA transcription by suppressing the expression of transcription factors, UBF and cMYC (Peng *et al.*, 2019).

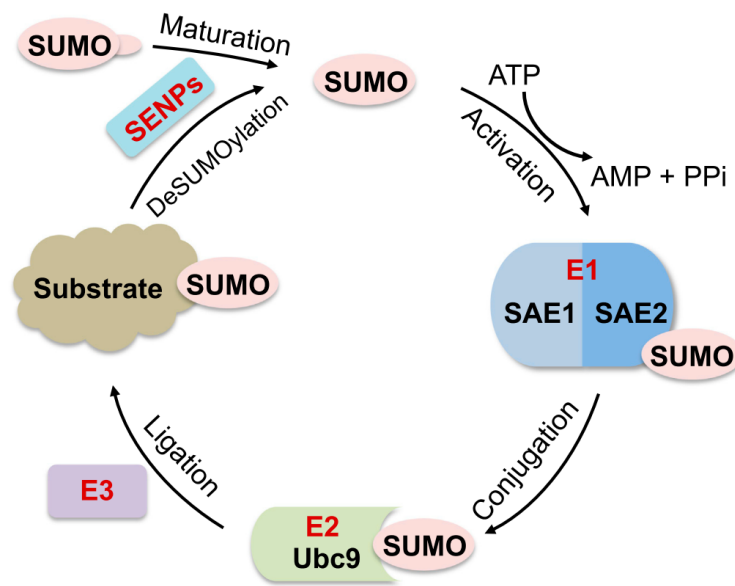


Figure 10: Overview of the SUMOylation and deSUMOylation cycle. The process begins with the maturation of SUMO (Small Ubiquitin-like Modifier) proteins by SENP proteases, which expose a conserved glycine residue necessary for conjugation. Mature SUMO is activated in an ATP-dependent manner by the E1 enzyme complex (comprising SAE1 and SAE2), forming a thioester bond. SUMO is then subsequently transferred to the E2 conjugating enzyme Ubc9. In the presence of E3 ligases, SUMO is covalently attached to target substrates, typically at lysine residues. The modification alters substrate properties such as localization, activity, or stability. SUMO can be removed from substrates by SENPs, completing the reversible cycle. *Source: (Y. Yang et al., 2024).*

Additionally, several nucleolar proteins, such as NPM, Nucleolin, and LAS1L, which are crucial for rRNA synthesis, have been identified as targets of SUMOylation. The proteins in the snoRNP complex, including NOP58, DKC1, and Nhp2, essential for rRNA modifications and processing, have also been found to be SUMOylation targets (Westman *et*

et al., 2010; Westman & Lamond, 2011). Moreover, proteins in the Rixosome and RNA degradation complex (including LAS1L, MDN1, NOL9, PELP1, SENP3, TEX10, WDR18), which are critical for ribosome biogenesis, are similarly targeted for SUMOylation (Y. Yang *et al.*, 2024). On the other hand, the actions of deSUMOylases maintain the SUMOylation status of these proteins. For instance, NPM, LAS1L, and PELP1, through deSUMOylation by SENP3, facilitate rRNA maturation, nucleolar export, and partitioning from the nucleolus to the nucleoplasm (Y. Yang *et al.*, 2024). Together, the proper balance between SUMOylation and deSUMOylation status of RBFs is crucial for efficient ribosome synthesis.

3.8 RNF4 is an E3 ubiquitin ligase for SUMOylated proteins

3.8.1 The structure and function of RNF4

SUMO-targeted ubiquitin ligase (STUbl), RNF4, is an E3 ubiquitin ligase and is conserved in yeast and humans. The yeast complementation studies have identified that the depletion of RNF4 results in the accumulation of SUMOylated proteins (Kosoy *et al.*, 2007). The RING dimerization of RNF4 is crucial for its E3 ubiquitin ligase activity and occurs in a manner that is dependent on the substrate (Liew *et al.*, 2010; Plechanovová *et al.*, 2011, 2012; Rojas-Fernandez *et al.*, 2014). RNF4 is capable of both monoubiquitination and polyubiquitination, enabling it to either stabilize or degrade target proteins (DiBello *et al.*, 2016). This flexibility stems from RNF4's ability to bind to various types of E2 enzymes. Consequently, RNF4 can form multiple types of linkages, including K11, K33, K78, and K63, each of which can play either a stabilizing or destabilizing role (Branigan *et al.*, 2015; Thomas *et al.*, 2016).



Figure 11: Domain structure of human RNF4 (hRNF4). The schematic represents the domain organization of hRNF4, a SUMO-targeted ubiquitin ligase. The SIMs (SUMO Interaction Motifs) are shown as blue bars, located near the N-terminus. The ARM (Arginine-rich Motif) region is depicted as a green box. The RING (Really Interesting New Gene) domain, crucial for ubiquitin ligase activity, is highlighted in yellow. (N- N terminal, C-C terminal, Number of amino acids =190).

3.8.1.1 SUMO interaction motif (SIM)

RNF4 is capable of recognizing both multi-SUMOylated (Aguilar-Martinez *et al.*, 2016) and poly-SUMOylated proteins through its SIMs (Lallemand-Breitenbach *et al.*, 2008; Tatham *et al.*, 2008; Weisshaar *et al.*, 2008). It comprises four SIMs, designated SIM1, SIM2, SIM3, and SIM4. The combination of SIM2 and SIM3 exhibits a strong affinity for SUMO chains and can effectively bind to a chain consisting of two SUMO molecules. SIM4 demonstrates its effectiveness when the chain is extended to four SUMOs. In contrast, SIM1 has a minimal role in SUMO binding, resulting in three primarily functional SIMs. Among these, SIM2 and SIM3 are the principal contributors to binding with the SUMO moiety (Aguilar-Martinez *et al.*, 2016; Keusekotten *et al.*, 2014; Kötter *et al.*, 2020; Kung *et al.*, 2014; Y. Xu *et al.*, 2014).

3.8.1.2 Arginine-rich motif (ARM)

RNF4, distinguished by its arginine-rich motif, specifically recognizes phosphorylated proteins. This phosphorylation-dependent interaction enables RNF4 to promote the ubiquitination and stabilization of its protein targets (Kuo *et al.*, 2014a; Thomas *et al.*, 2016). Furthermore, when both phosphorylation and SUMOylation are involved in substrate recognition, the AR motif enhances substrate specificity and facilitates the SUMO-dependent ubiquitination and subsequent degradation of target proteins (Kuo *et al.*, 2014b).

3.8.2 RNF4 is a tumor promoter

The tumor-promoting role of RNF4 in cancer is gaining attention, with studies highlighting that high mRNA levels of RNF4 are associated with poor patient survival. RNF4 has also been implicated in various other types of cancer, along with breast cancer (Thomas *et al.*, 2016), Colorectal cancer (J. Chen *et al.*, 2024; Wu *et al.*, 2025), Cholangiocarcinoma (J. Huang *et al.*, 2022), Hepatocellular carcinoma (Lv *et al.*, 2022), and Melanoma (Avitan-Hersh *et al.*, 2020). Its elevated expression is not limited to carcinomas; RNF4 is also found at higher levels in several sarcomas, such as Osteosarcoma, Ewing sarcoma, and Liposarcoma (Novak *et al.*, 2022).

3.9 Polycomb group protein (PcG), CBX4, is a tumor promoter

Polycomb group proteins are involved in maintaining the cellular identity and are required for normal differentiation (Van Wijnen *et al.*, 2021). Several interaction studies identified that polycomb group proteins exist as polycomb repressive complexes, PRC1 and PRC2. The recruitment of PRC2 (EZH, EED, SUZ12) to Histone H3 leads to the formation of the repressive H3K27me3 mark, which acts as a docking site for the PRC1 complex. The core protein of PRC1 includes one of the members of the RING1 family (RING1a-RING1b), PCGF family (PCGF1-PCGF6), HPH family (HPH1-HPH3), and CBX family (CBX2, CBX4, CBX6, CBX7, and CBX8) (Di Croce & Helin, 2013; Van Wijnen *et al.*, 2021).

3.9.1 Functional role of CBX4

The primary role of CBX family proteins is to recognise the repressive H3K27me3 mark and recruit the PRC1 complex through their chromodomain. These proteins feature a conserved Polycomb repressor (PcR) box that acts as the binding site for RING1a/b, facilitating the assembly of the PRC1 complex (Di Croce & Helin, 2013). Notably, among the CBX family members, CBX4 is distinct due to its additional SUMO ligase activity, which arises from

unique SUMO interaction motifs (SIM) within its structure (Kagey *et al.*, 2003). Consequently, CBX4 exhibits a diverse array of functions through both its chromodomain and SUMO ligase activities. Moreover, CBX4 can interact with other proteins independently of its chromodomain and SUMO ligase functions. Apart from the N-terminal chromodomain and the C-terminal polycomb repressor box (Pc box), CBX4 has four conserved motifs, namely, Cx4.1, Cx4.2, Cx4.3, and Cx4.4 and the Cx4.2 motif contains a SUMO binding site. It also has a SUMOylation site for modification by SUMO and an ATHL hook essential in binding to DNA (Senthilkumar & Mishra, 2009).

3.9.2 CBX4 is a tumor promoter

Emerging studies suggest that CBX4 is involved in regulating various processes, including proliferation, differentiation, senescence, inflammation, and metastasis. The deregulated expression of CBX4 in cancer cells promotes uncontrolled cell proliferation and metastasis. CBX4 is significantly overexpressed in breast cancer, particularly within the luminal A and B subtypes (Park *et al.*, 2024). Additionally, elevated levels of CBX4 are observed in various malignancies, including hepatocellular carcinoma (Mei *et al.*, 2014; B. Wang *et al.*, 2013), osteosarcoma (J. Yang *et al.*, 2016), lung cancer (Hu *et al.*, 2020), gastric cancer (Pan *et al.*, 2021), clear cell renal cell carcinoma (Jiang *et al.*, 2020), esophageal cancer (J. Hou *et al.*, 2022; H. Zhu *et al.*, 2022), and prostate cancer (X. Xu *et al.*, 2024). These increased levels are frequently associated with poorer patient survival rates, enhanced metastasis, elevated angiogenesis, and tumor resistance. Conversely, in the context of colorectal cancer, CBX4 functions as a tumor suppressor, inhibiting tumor progression (X. Wang *et al.*, 2016), suggesting a context dependent role in cancer.

4 Rationale and hypothesis

Breast cancer is not a single disease but a group of biologically distinct subtypes, each with different molecular features and clinical behaviours. Among these, TNBC is known for its aggressive nature and poor prognosis, while luminal subtypes are generally more differentiated and associated with better outcomes. TNBC cells often display mesenchymal-like traits, while luminal cells tend to maintain epithelial characteristics. These differences reflect varying degrees of cellular plasticity, the ability of cells to shift between states, which has a prerequisite role in tumor development and therapeutic resistance.

On the other hand, nucleolar changes, such as an increase in number or size, have long been used as markers of high metabolic activity and tumor aggressiveness. Despite this, the link between nucleolar architecture and cancer cell plasticity remains underexplored. Given the emerging idea that the nucleolus does more than just ribosome production, that it may influence gene expression and chromatin structure, we wanted to investigate whether nucleolar differences exist across breast cancer subtypes with varying plasticity.

Our hypothesis was that since TNBC and luminal subtypes differ in their mesenchymal and epithelial traits, respectively, the nucleolus might reflect or even contribute to this difference. Studying nucleolar morphology and activity across subtypes could help us understand whether the nucleolus plays a role in maintaining or shifting cell identity. This could open up new ways to think about how nucleolar function is linked to tumor behaviour and plasticity.

5 Objectives

1. To study the correlation between nucleolar activity and cellular plasticity in the breast cancer model.
2. To explore the underlying mechanisms responsible for the distribution of nucleolar proteins and their function in modulating cellular plasticity.

6 Materials and Methods

6.1 Cell culture

The MCF7 and MDA-MB-231 breast cancer cell lines were obtained from the ATCC, USA, a certified biological resource centre. The BT474 cell line was a generous gift from Prof. Annapoorni Rangarajan, Indian Institute of Science (IISc), Bengaluru. The SKBr3, BT549, and HEK293T cell lines were obtained from the National Centre for Cell Science (NCCS) in Pune. The SUM159PT cell line was a kind gift from Prof. Reinhard Depping, University of Lubeck, Germany. All cell lines were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM), added with 10% fetal bovine serum (FBS), as well as penicillin (100 U/mL) and streptomycin (100 µg/mL). The cells were cultured at 37°C in a humidified atmosphere comprising 5% CO₂.

6.2 Cell viability assay

Cell viability was assessed using the MTT assay. Briefly, cells were seeded in a 96-well plate at a density of 5×10^3 cells/well in 100 µL of complete medium and allowed to adhere overnight. The next day, cells were treated with various concentrations of CX-5461 or vehicle control (DMSO) for 24 h. After treatment, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 3–4 hours at 37°C. Following incubation, the medium was carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Biorad). The absorbance values were normalized to the untreated control group to calculate per cent cell viability. The data were then log-transformed and fitted using a non-linear regression model in GraphPad Prism to generate dose–response curves. The half-maximal inhibitory concentration (IC₅₀) was calculated from the fitted curve and plotted as a bar graph. All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD).

6.3 Poly HEMA coating

To prepare the poly-HEMA (poly-2-hydroxyethyl methacrylate) solution, 1.2 grams of polymer was weighed and added to 40 millilitres of 96% ethanol. The mixture was gently rotated overnight at room temperature to ensure thorough dissolution. The following day, the solution was centrifuged at $400 \times g$ for 30 minutes to remove any undissolved residue. The supernatant solution was then passed through a $0.22 \mu\text{m}$ filter to achieve clarity and sterility. For plate coating, appropriate volumes of the filtered solution were dispensed onto tissue culture dishes inside a biosafety cabinet: 1.5 mL for 60 mm dishes, 3.3 mL for 10 cm dishes, 0.6 mL per well for six-well plates, and 0.1 mL per well for 96-well plates. The coated plates were left at room temperature overnight to allow complete evaporation of ethanol. Once dry, plates were rinsed with sterile PBS before being used for cell culture. Coated plates were either used immediately or stored in a sterile environment for future experiments.

6.4 Multicellular spheroid generation

BT474, SKBr3, MDA-MB-231 and SUM159PT lines were cultured on poly-HEMA-coated culture plates to facilitate non-adherent growth. Cells were maintained in high-glucose DMEM supplemented with 10% FBS, 0.5% methylcellulose, penicillin (100 U/mL), and streptomycin (100 $\mu\text{g/mL}$). Cultures were then incubated at 37°C in a humidified environment provided with 5% CO_2 . The addition of methylcellulose increased medium viscosity, aiding in the formation and maintenance of spheroidal structures over the designated culture period.

6.5 Immunoblotting

Proteins were extracted from all cell lines by lysing the harvested cells using either RIPA buffer (composed of 50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium

deoxycholate, 0.1% SDS, and 20 μ M N-ethyl maleimide) or NP-40 lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, and 20 μ M N-ethyl maleimide). Both buffers were supplemented with protease and phosphatase inhibitors to prevent protein degradation and dephosphorylation. The lysates were then resolved on 10–12% SDS-polyacrylamide gels, and immunoblotting was carried out using specific antibodies as listed in **Table 1**.

6.6 Quantitative real-time polymerase chain reaction

Total RNA was isolated using TRIzol reagent, following the standard procedure provided by the manufacturer (RNA isoplus, Takara, Japan, Cat.#9108/9109). The concentration and purity of the RNA were measured using a Nanodrop spectrophotometer. For first-strand cDNA synthesis, the Prime Script RT reagent Kit (Takara, Japan, Cat. #6110A) was used. 1 μ g of total RNA was combined with 1 μ L of oligo(dT) or Random hexamer primers in a final volume of 10 μ L. This mixture was incubated at 65°C for 5 minutes to facilitate primer annealing, then immediately cooled on ice. The reaction was completed by adding 4 μ L of 5 $\times$ Prime Script Buffer, 1 μ L of Prime Script RT Enzyme Mix I, and 4 μ L of RNase-free water, making up a total volume of 20 μ L. The cDNA synthesis was performed at 30°C for 10 minutes, followed by 60 min at 42°C and the reaction was terminated by heating at 70°C for 15 minutes. The synthesized cDNA was then diluted 1:4 with nuclease-free water and stored at –20°C until further use in quantitative PCR. Primers used in the amplification steps are detailed in **Tables 2 and 3**, and all quantitative data were processed using GraphPad Prism software.

6.7 EMT Assay

To assess the occurrence of EMT, the cells were cultured as both a monolayer in a standard cell culture dish and as spheroids in polyHEMA low adhesion dishes for various time

points as indicated. An equal amount of protein lysates of the cell were subjected to comparative immunoblotting analysis with the antibodies listed in **Table 1**.

6.8 Stemness Assay

To assess the stemness characteristics, the cells were cultured as both a monolayer in a standard cell culture dish and as spheroids in polyHEMA low-adhesion dishes for an indicated number of days. The expression of the stemness genes (listed in **Table 2**) in spheroid cells relative to the monolayer cells was assessed using qPCR.

6.9 Dual luciferase rDNA promoter assay

To examine promoter activity, cells were cultured in 60 mm dishes and for each dish, 2 μ g of a Firefly luciferase reporter plasmid containing the rDNA promoter (kind gift from Dr Ratna Vadlamudi, UT-HSC, San Antonio, USA) and 200 ng of a Renilla luciferase plasmid (as a normalization control) were transfected. The plasmid DNA was first diluted in 250 μ L of medium free from serum. In a separate tube, the appropriate amount of transfection reagent, Lipofectamine 2000, was also diluted in 250 μ L of serum-free medium. After incubating both mixtures for 5 minutes, they were combined and left at room temperature for 15–20 minutes to allow complex formation. This complex mixture was then added in a dropwise manner to the cells. Forty-eight hours after transfection, the cells were gently washed once with cold PBS and then lysed in 300 μ L of cell lysis buffer. The lysates were collected into microcentrifuge tubes and centrifuged briefly at high speed to remove debris. For the luciferase assay, 20 μ L of each cleared lysate was transferred to a 96-well white plate. First, firefly luciferase activity was measured by adding 100 μ L of firefly substrate and recording luminescence. Immediately after, 100 μ L of Renilla substrate was added to the same well, and the second luminescence reading was taken. Firefly luciferase values were normalised to Renilla luciferase values to correct for

variability in transfection efficiency. All experiments were carried out in triplicate, and the average values were used for further analysis.

6.10 rRNA methylation assay

Ribose methylation at selected 11 sites within the 5.8S, 18S, and 28S rRNA was assessed using a modified reverse transcription-qPCR approach as previously described (Belin *et al.*, 2009). This technique takes advantage of the observation that reverse transcriptase is blocked by 2'-O-methyl groups at low concentrations of dNTPs, while this inhibition is overcome at higher dNTP levels. Total RNA was extracted using acid-guanidinium-phenol-based reagents according to standard protocols. For each reaction, 1000 ng of total RNA was reverse transcribed using 200 units of RNase H-minus, M-MLV reverse transcriptase, 40 units of a ribonuclease inhibitor, and 1 μ M of site-specific reverse primers targeting regions upstream of known or predicted methylation sites. Two parallel reactions were performed for each target—one with a low dNTP concentration (10 μ M) and the other with a high concentration (1 mM). The RT reactions were carried out at 30 °C for 10 minutes to facilitate primer binding and enzyme activity, followed by 60 minutes at 42 °C for cDNA synthesis. The reactions were terminated by heating at 70 °C for 15 minutes. Quantitative PCR was performed using a SYBR Green-based detection system on a real-time thermal cycler. The relative methylation level at each site was determined using the equation $2^{(CT\ low - CT\ high)}$, where CT low and CT high represent the threshold cycles from reactions carried out at low and high dNTP concentrations, respectively. This ratio reflects the extent of RT inhibition caused by ribose methylation. All primer sequences used in this analysis are listed in **Table 5**.

6.11 Lentivirus-mediated gene silencing (Knockdown)

Stable knockdown of FBL, RNF4, or CBX4 was achieved in BT474 or HEK293T cells using lentiviral transduction. The lentivirus was produced by transfecting the pLKO-shFBL, pLKO-shRNF4, and pLKO-shCBX4 vectors along with lentiviral packaging plasmids into HEK293T cells, as previously described. Following this, the selection was performed using Puromycin at a concentration of 0.5-2 $\mu\text{g/mL}$.

6.12 Molecular cloning

The coding sequence of the FBL gene was amplified from HeLa cDNA using primers that included restriction sites for *XhoI* and *BamHI*. The PCR product (amplicon) and the pEGFP-C1 vector were both digested with the respective enzymes and purified using a gel extraction kit. The insert was then ligated into the vector using T4 DNA ligase, and the ligation mix was incubated overnight at 16°C. Following ligation, the plasmid was transformed into *E. coli* (DH5 α) competent cells using the standard heat-shock method. Transformed colonies were selected on LB agar plates supplemented with kanamycin. Positive clones were cultured, and plasmid DNA was extracted using a miniprep kit. Successful cloning was then confirmed by restriction digestion and verified by Sanger sequencing.

6.13 SUMOylation site prediction

SUMOylation sites within the FBL protein were predicted using multiple bioinformatics tools to ensure accuracy and confidence. The complete amino acid sequence of FBL was analysed using three web-based platforms: SUMOgo, SUMOplot, and JASSA. Each tool generated a list of potential modification sites along with associated prediction scores. Sites that were consistently identified across all three tools were considered the most reliable and were selected for further evaluation.

6.14 Site-directed mutagenesis

A site-directed mutagenesis approach was employed to substitute a lysine residue with arginine in the FBL gene. Primers incorporating the desired base change were designed with the mutation positioned centrally and synthesized by GCC Biotech, India. PCR amplification was carried out using the pEGFPc1-FBL plasmid as a template along with Phusion high-fidelity DNA polymerase to ensure accuracy. Following amplification, the reaction mixture was treated with *DpnI*, at 37 °C for 1 hour to digest the methylated parental plasmid. The resulting DNA was introduced into *E. coli* (DH5 α) competent cells via transformation. Transformed bacteria were plated on LB agar containing kanamycin for selection. Colonies were cultured, and plasmid DNA was isolated using a miniprep procedure. The presence of the intended mutation was verified by Sanger sequencing.

6.15 Bright-field imaging and determination of the rate of spheroid formation

BT474, MDA-MB-231, SUM159PT, and SKBr3 lines were grown in poly-HEMA-coated low-adhesion dishes for the required time periods to encourage spheroid development. Spheroids formed during this incubation were observed under an Olympus bright-field microscope with a 10X objective. To evaluate spheroid morphology, circularity was quantified using ImageJ, and mean values along with standard deviations were calculated in Microsoft Excel. Graphical plots and statistical tests were carried out using GraphPad Prism.

6.16 Nucleolus isolation and mass spectrometry

Nucleoli were isolated from cultured cells based on a modified version of the protocol originally established by the Lamond laboratory. Briefly, cells from four T75 flasks at approximately 80% confluence were harvested by trypsinization and pelleted by centrifugation

at 1000 rpm for 4 minutes. The resulting cell pellet was washed once in cold 1X PBS and centrifuged again under the same conditions. Cells were then resuspended in 5 mL of ice-cold hypotonic lysis buffer (Buffer A: 10 mM HEPES, pH 7.9; 1.5 mM MgCl₂; 10 mM KCl; supplemented with protease inhibitors) and allowed to swell on ice for 5 minutes. The suspension was transferred to a 7 mL Dounce homogenizer and mechanically disrupted by 10 strokes each with pestle A and pestle B to ensure effective lysis. The homogenate was centrifuged at 1000 rpm for 5 minutes at 4°C, and the supernatant was collected as the cytoplasmic fraction. The nuclear pellet was resuspended in 3 mL of S1 buffer (0.25 M sucrose, 10 mM MgCl₂, with protease inhibitors), then carefully layered over 3 mL of S2 buffer (0.35 M sucrose, 0.5 mM MgCl₂, with protease inhibitors) and centrifuged at 2500 rpm for 5 minutes at 4°C. After discarding the supernatant, the pellet was resuspended in 3 mL of S2 buffer and subjected to sonication (6 pulses of 10 seconds each, at 40% amplitude, with 10-second cooling intervals on ice). The sonicated material was gently layered over 3 mL of S3 buffer (0.88 M sucrose, 0.5 mM MgCl₂, with protease inhibitors) and centrifuged at 3500 rpm for 10 minutes at 4°C to separate the nucleoplasmic fraction (supernatant). The nucleolar pellet was washed with 500 µL of S2 buffer and centrifuged at 3500 rpm for 5 minutes at 4°C. Finally, the pellet was resuspended in 100 µL of S2 buffer and stored at –80°C until further analysis. To verify the quality and integrity of the fractionation, western blotting was performed on the cytoplasmic, nucleoplasmic, and nucleolar extracts. The quantitative data thus obtained is subjected to pathway analysis using the <https://reactome.org/> server. The bar graphs were plotted using GraphPad Prism software.

6.17 Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were cut into 4 µm thickness and mounted on charged slides. Slides were first deparaffinized by immersion in xylene and

rehydrated through a descending ethanol gradient (xylene and ethanol (1:1)), 100%, 95%, and 70%) followed by distilled water. Next, antigen retrieval was performed by immersing the slides in 10 mM sodium citrate buffer (pH 6.0) and heating at 95–100 °C for 20–30 minutes, followed by a gradual cool-down to room temperature. Slides were rinsed in phosphate-buffered saline (PBS) before proceeding. For immunostaining, the PolyDetector HRP/DAB Detection System (BioSB, Cat# BSB-0230) with slight modifications was used according to the manufacturer's instructions. The endogenous peroxidase activity was prevented by incubating the sections with the provided peroxide block for 5 minutes. After washing, sections were incubated with the appropriately diluted primary antibody overnight at room temperature. Following primary antibody incubation, the slides were treated with the PolyDetector HRP secondary reagent for 30 minutes. Signal development was achieved by applying the DAB chromogen solution until optimal staining was observed under a microscope (typically 3–10 minutes). The slides were then counterstained with haematoxylin, rinsed, dehydrated, and mounted with a permanent mounting medium. Stained sections were imaged using a brightfield microscope. Staining intensity and positive cell percentage were quantified using FIJI/ImageJ software for further analysis.

6.18 Co-Immunoprecipitation (Co-IP)

Cell lysates were prepared using either RIPA or NP-40 lysis buffer, depending on the downstream application. Protein concentrations were determined using the Lowry assay with a compatible DC protein quantification reagent set. For each immunoprecipitation reaction, approximately 1–3 mg of total protein was incubated with the appropriate primary antibody at 4 °C overnight with gentle rotation at 7 rpm to facilitate antigen–antibody interaction. The next day, the antibody-bound lysates were incubated with Protein A/G PLUS-agarose beads to capture the immune complexes. This mixture was rotated for a few hours at 4 °C. The beads

were then washed 3–4 times with cold lysis buffer on a rotator (10 rpm, 10 minutes per wash) to remove non-specifically bound proteins. After each wash, samples were centrifuged at 1200 rpm for 5 minutes at 4 °C to pellet the beads. Following the final wash, the bead-bound complexes were resuspended in 20 µL of 2X SDS loading dye, boiled, and subjected to SDS-PAGE followed by western blotting using specific primary antibodies as detailed in the corresponding antibody **Table 1**.

6.19 Immunofluorescence

Cells were rinsed with PBS and fixed using 4% paraformaldehyde (PFA) at room temperature for 20 minutes to preserve cellular structure. After fixation, permeabilisation was carried out with 0.5% Triton X-100 in PBS for 10 minutes to allow antibody access to intracellular targets. The cells were then incubated in a blocking solution containing 3% bovine serum albumin (BSA) in TBST for one hour to reduce nonspecific antibody binding. Primary antibody incubation was performed overnight at 4°C. The following day, excess antibody was removed by sequential washing—10 minutes each in TBS, TBST, and TBS. Cells were then incubated with a fluorophore-tagged secondary antibody and washed again in the same sequence to eliminate unbound antibody. Nuclear staining was performed using DAPI, and coverslips were sealed with nail polish to avoid drying. Representative images from 4–5 randomly selected fields were captured using a confocal microscope (Leica TCS SP8 STED, Germany) equipped with a 63× oil immersion lens. Image analysis was performed using ImageJ software.

6.20 Breast cancer patient data analysis

The protein expression analysis in breast cancer patients was conducted by extracting data from the cBioPortal or the TNM plot, and standard deviations were calculated. We utilized

the Kaplan-Meier method for survival analysis, obtaining the necessary data from the KM Plotter. Additionally, mutation and copy-number variation analyses were performed using data from TCGA. Statistical evaluations and plotting of the compiled datasets were performed using GraphPad Prism.

6.21 Statistical analysis

Statistical comparisons between experimental groups were performed using either t-tests or one-way/two-way ANOVA, depending on the experimental design. For comparisons involving two groups, paired or unpaired t-tests were applied as appropriate. For comparisons involving more than two groups, multiple comparison corrections were applied using Sidak's, Tukey's, or Dunnett's tests, as appropriate. Results were considered statistically significant when p-values were less than 0.05. Asterisks indicate significance levels: * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$ and ns for non-significant.

6.22 Tables

6.22.1 Table 1: List of antibodies					
S NO	Antibody	Host & Isotype	Company	Catalogue No	Dilution
1.	FBL	Mouse (IgG1)	Novus	#NB300-269	1:1000
2.	FBL	Rabbit (IgG)	CST	#2639	1:1000
3.	RNF4	Rabbit (IgG)	Proteintech	#17810-1-AP	1:1000
4.	CBX4	Rabbit (IgG)	CST	#30559	1:1000
5.	SUMO 2/3	Mouse (IgG2b)	Abcam	#ab81371	1:1000
6.	GFP	Rabbit (IgG)	Merck-Sigma	#G1544	1:10000
7.	c-Myc	Rabbit (IgG)	CST	#18583	1:1000

8.	Snail	Rabbit (IgG)	CST	#3879	1:1000
9.	Slug	Rabbit (IgG)	CST	#9585	1:1000
10.	β catenin	Rabbit (IgG)	CST	#8480	1:1000
11.	β actin	Mouse IgG1	Sigma	#A3854	1:25000
12.	Secondary Rabbit	Mouse (IgG)	SCBT	#SC2357	1:10000
13.	Secondary Mouse	Goat (IgG)	Jackson laboratory	#115-035-146	1:10000
14.	Normal Rabbit	Rabbit IgG	CST	#2729	1 μ g
15.	Normal mouse	Mouse IgG	SCBT	#SC2025	1 μ g
16.	Alexaflor 488	Anti-Rabbit	Invitrogen	#A11008	1:1000
17.	Alexaflor 546	Anti-Rabbit	Invitrogen	#A11010	1:1000
18.	Alexaflor 488	Anti-Mouse	Invitrogen	#	1:1000
19.	Alexaflor 546	Anti-Mouse	Invitrogen	#A11003	1:1000

6.22.2 Table 2: List of primers for stemness phenotype

S.No	Gene	Primer	Primer sequence 5'-3'	Product size
1.	OCT4	Forward	AGTGAGAGGCAACCTGGAGA	107
		Reverse	ACACTCGGACCACATCCTTC	
2.	SOX2	Forward	AACCCCAAGATGCACAACCTC	100
		Reverse	GCTTAGCCTCGTCGATGAAC	
3.	cMYC	Forward	TTCTCTCCGTCCTCGGATTCTC	203
		Reverse	GAAGGTGATCCAGACTCTGACCT	
4.	FBL	Forward	CCTGGGGAATCAGTTTATGG	75
		Reverse	CCAGGCTCGGTACTCAATTT	
5.	RNF4	Forward	GTCAGTTGTCCCATCTGCATGG	109

		Reverse	AATCACGGAGGCACTGGCTACA	
6.	β -Actin	Forward	AGCCATGTACGTAGCCATCC	240
		Reverse	CTCTCAGCTGTGGTGGTGAA	

6.22.3 Table 3: List of primers for ribosome biogenesis

S.No	rRNA transcript	Primer	Primer sequence 5'-3'	Product size
1.	45S	Forward	GAACGGTGGTGTGTCGTT	130
		Reverse	GCGTCTCGTCTCGTCTCACT	
2.	5.8S	Forward	ACTCGGCTCGTGCGTC	136
		Reverse	GCGACGCTCAGACAGG	
3.	18S	Forward	GATGGTAGTCGCCGTGCC	110
		Reverse	GCCTGCTGCCTTCCTTGG	
4.	28S	Forward	GAGGTAAACGGGTGGGGTC	113
		Reverse	GGGGTCGGGAGGAACGG	

6.22.4 Table 4: List of primers used for cloning and site directed mutagenesis

S.No	Cloning / SDM	Primer	Primer sequence 5'-3'
1.	pEGFPc1-FBL	Forward	ATCGCTCGAGATATGAAGCCAGGATTCAG
		Reverse	ATATGGATCCTCAGTTCTTCACCTTGGG
2.	FBL-K102R	Forward	TGTCGAGGAAGGGAAGATGCACTG
		Reverse	CAGTGCATCTTCCCTTCCTCGACA

3.	FBL-K109R	Forward	CTGGTCACCAGGAACCTGGTCCCT
		Reverse	AGGGACCAGGTTCTTGGTGACCAGT
4.	FBL-K131R	Forward	GGAGATGACAGAATTGAGTACCGA
		Reverse	TCGGTACTCAATTCTGTCATCTCC
5.	FBL-K143R	Forward	TTCCGCTCCAGGCTAGCAGCAGCAAT
		Reverse	ATTGCTGCTGCTAGCCTGGAGCGGAA
6.	FBL-K158R	Forward	ATCCACATCAGACCGGGGGCTAAG
		Reverse	CTTAGCCCCCGGTCTGATGTGGAT

6.22.5 Table 5: List of primers used in rRNA methylation assay

S.No	Methylation site	Primer	Primer sequence 5'-3'	Product size
1.	5.8S-124	Forward	AGCTGCGAGAATTAATGTGAA	60
		Reverse	AAGTGCGTTCGAAGTGTCG	
2.	18S-37	Forward	ACCTGGTTGATCCTGCCAGT	72
		Reverse	GGCCGTGCGTACTTAGACAT	
3.	18S-1489	Forward	CACCCGAGATTGAGCAATAA	81
		Reverse	CGCTGAGCCAGTCAGTGTAG	
4.	18S-1713	Forward	GTCCCTGCCCTTGTACACAC	54
		Reverse	CACTAAACCATCCAATCGGTA	
5.	18S-1803	Forward	GCGGAGCGCTGAGAAGAC	89
		Reverse	GATCCTTCCGCAGGTTAC	
6.	28S-390	Forward	CCGTAAGGGAAAGTTGAAAAG	78
		Reverse	CCCCACCCGTTTACCTCTTA	

7.	28S-1612	Forward	CGGTCCTGACGTGCAAAT	84
		Reverse	TCGGAGGGAACCAGCTACTA	
8.	28S-1858	Forward	GTGGGCCCACTTGGTAAGC	91
		Reverse	TTTCTGGGGTCTGATGAGC	
9.	28S-2848	Forward	AGGTAAGGGAAGTCGGCAAG	62
		Reverse	CAGCCCTTAGAGCCAATCCT	
10.	28S-4198	Forward	GCGGTACACCTGTCAAACG	69
		Reverse	CACGGGAGGTTTCTGTCCT	
11.	28S-4436	Forward	CGCTTTTTGACCTTCGATGT	51
		Reverse	GCGAATTCTGCTTCCAATGA	

6.22.6 Table: 6 List of Reagents

S.No	Reagents	Catalogue No	Company
1.	High Glucose DMEM (Dulbecco's modified eagle medium)	11965118	ThermoFisher Scientific, USA
2.	PBS (Phosphate Buffered Saline)	10010049	ThermoFisher Scientific, USA
3.	FBS (Fetal Bovine serum)	10270106	ThermoFisher Scientific, USA
4.	Antibiotic-Antimycotic (100X)	15240-062	ThermoFisher Scientific, USA
5.	Trypsin	25200072	ThermoFisher Scientific, USA
6.	poly(HEMA) (poly(2 hydroxyethylmethacrylate))	P3932	Sigma Aldrich, USA
7.	SYBR Green	RR420A	Takara, Japan
8.	Lipofectamine 2000	11668019	ThermoFisher Scientific, USA

9.	Opti-MEM (Optimal Minimal Essential Media)	31985070	ThermoFisher Scientific, USA
10.	Protein A/G beads	SC-2003	SantaCruz Biotechnology, USA
11.	2D08	505156	Sigma Aldrich, USA
12.	CX-5461	A8337	APExBio, USA
13.	DAPI (4',6-Diamidino-2-phenylindole)	ab104139	Abcam, USA
14.	PFA (Para formaldehyde)	TCL119	Himedia, India
15.	(MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	M6494	Thermofisher Scientific, USA

7 Results

7.1 Objective 1: To study the correlation between nucleolar activity and cellular plasticity in the breast cancer model

7.1.1 The nucleolar prominence varies in different breast cancer subtypes

The increase in size and number (prominence) of the nucleoli in breast cancer tissues is well established (Helpap, 1989). We next sought to determine whether the nucleolar prominence varies across different breast cancer subtypes. To address this, we compared the number of nucleoli in breast cancer cells across representative cell lines corresponding to ER-positive (BT474, MCF7), HER2-positive (SKBr3), and TNBC (MDA-MB-231) cell lines by immunofluorescence, and used fibrillarin (FBL) as a nucleolar marker for this analysis. These results show that the prominence of the nucleoli significantly varies in different breast cancer subtypes, with MDA-MB-231 showing the highest prominence, followed by SKBr3, MCF7, and BT474 at the least (**Figure 12A and 12B**). To substantiate our findings further, we conducted an immunohistochemistry analysis of ER⁺ and TNBC tissues obtained from breast cancer patients. Our observations indicated that the prominence of nucleolar marker FBL significantly increases in TNBC tissues compared to ER⁺ tissues (**Figure 12C and 12D**).

Since enhanced nucleolar prominence is associated with elevated rRNA synthesis and ribosome biogenesis (Montanaro *et al.*, 2008), and TNBC cells exhibited greater nucleolar prominence than non-TNBC cells, we hypothesised that rRNA synthesis might be elevated in TNBC cells. To test this, we treated TNBC and non-TNBC breast cancer cell lines with CX-5461, the RNA pol I inhibitor, and assessed cell viability using the MTT assay. IC₅₀ values were calculated for each cell line to evaluate their sensitivity to rRNA synthesis inhibition. The results showed that TNBC cell lines, MDA-MB-231 and BT549, had higher IC₅₀ (1537 nM and 1014 nM, respectively) values than ER⁺ cell lines, MCF7 and BT474 (766.6 nM and 457.1 nM, respectively), indicating reduced sensitivity to CX-5461 (**Figure 12E and 12F**). These

findings suggest that TNBC cells may have higher basal rRNA synthesis activity and that nucleolar number may positively correlate with rRNA synthesis levels across breast cancer subtypes.

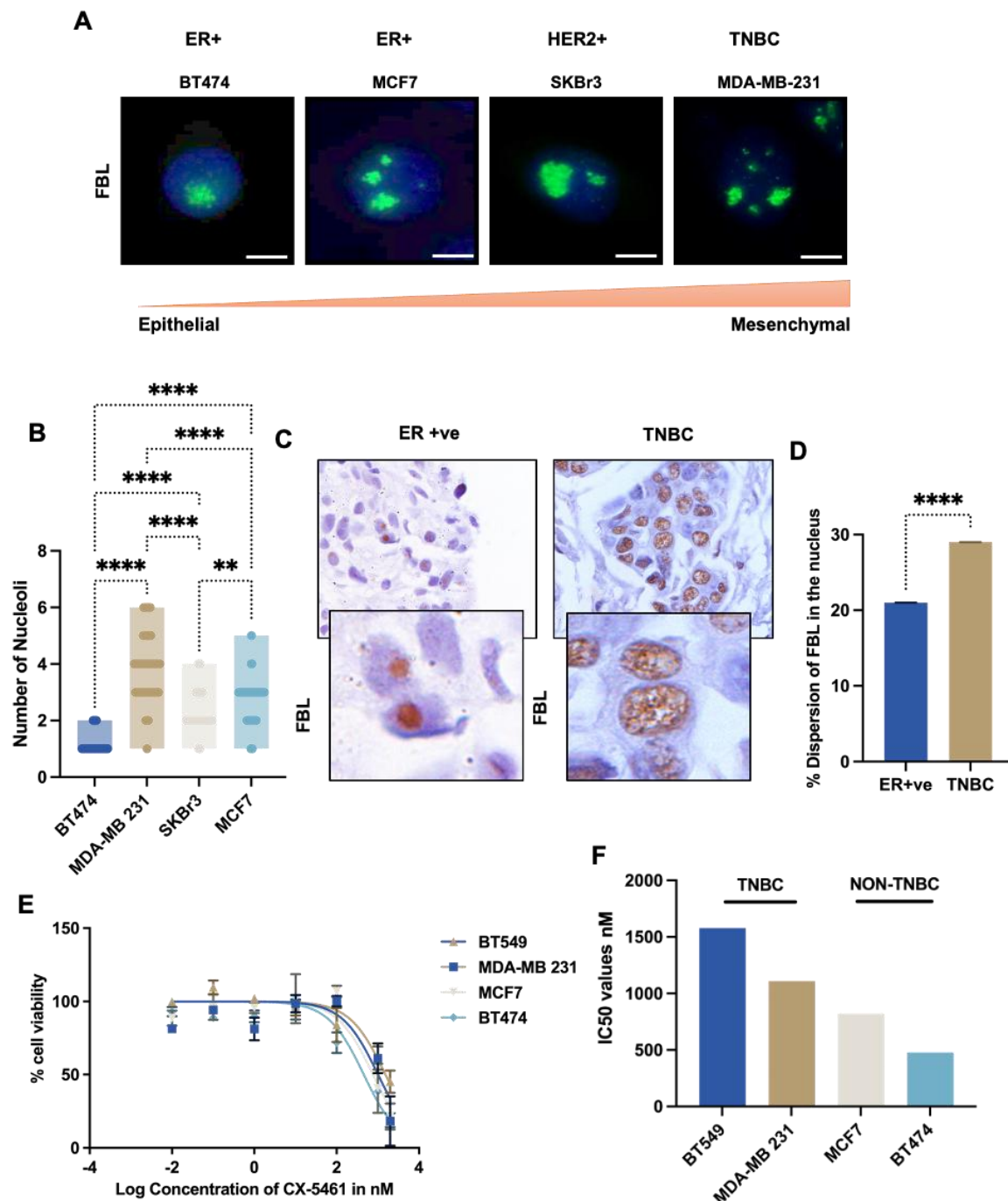


Figure 12: Nucleolar prominence, FBL expression, and sensitivity to rRNA synthesis inhibition in breast cancer models. (A) Representative immunofluorescence images (Green-FBL, Blue-DAPI, Nucleus, Scale bar represents 10 μ m) (B) Quantification of nucleolar prominence in breast cancer cell lines representing distinct molecular subtypes. (C and D) Immunohistochemical staining and quantification of

the nucleolar protein FBL in breast cancer tissue samples. FBL expression is elevated in TNBC cases relative to ER-positive tumors. **(E and F)** Analysis of cell viability following treatment with CX-5461, the RNA polymerase I inhibitor. Cells were exposed to increasing concentrations of the drug, and viability was analysed using the MTT assay. IC₅₀ values were derived for TNBC cell lines (MDA-MB-231 and BT549) and non-TNBC cell lines (BT474 and MCF7), revealing reduced sensitivity to CX-5461 in TNBC cells. Statistical comparisons were performed using one-way ANOVA (Tukey's multiple comparison test); significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$.

7.1.2 Multicellular spheroids exhibit EMT

Since TNBC displays mesenchymal-like characteristics in contrast to epithelial-like ER-positive cells, which exhibit epithelial characteristics (Blick *et al.*, 2008), we hypothesised a role for rRNA synthesis in promoting epithelial-mesenchymal transition (EMT). To investigate this, we employed a poly-HEMA-based multicellular spheroid culture system, a well-established model system to study cellular plasticity and EMT (Im *et al.*, 2015; M.-M. Dong, 2012; Steadman *et al.*, 2008).

To determine which breast cancer cell lines have enhanced spheroid-forming ability, we cultured BT474, SUM159PT, SKBr3, and MDA-MB-231 cells under both standard monolayer conditions and in low-attachment plates coated with poly-HEMA for a defined period. Spheroid formation was evaluated by measuring the circularity index over time. Based on the slope of circularity progression, BT474 (ER+ve) and SUM159PT (TNBC) cells formed spheroids at a faster rate compared to SKBr3 and MDA-MB-231 cells, indicating a higher efficiency in 3D structure formation (**Figure 13A–C**).

Next, to confirm that spheroids formed in poly-HEMA-based low attachment dishes exhibit EMT, we cultured the cell lines BT474 and SUM159PT in both spheroid and monolayer conditions for 48 hours. We then performed immunoblotting analysis to assess the EMT-promoting transcription factors Snail and Slug and β -catenin levels. These results indicated that the levels of Snail and Slug were increased in Spheroids of BT474 and SUM159PT; however, the levels of β -catenin were inconsistent during spheroid formation (**Figures 13D and 13E**).

Furthermore, qRT-PCR analysis of stemness genes SOX2, c-MYC, and OCT4 showed a significant increase in their expression in spheroid culture in comparison to monolayer cultures. We therefore termed this process Spheroid-induced EMT (SEMT) (**Figure 13F**).

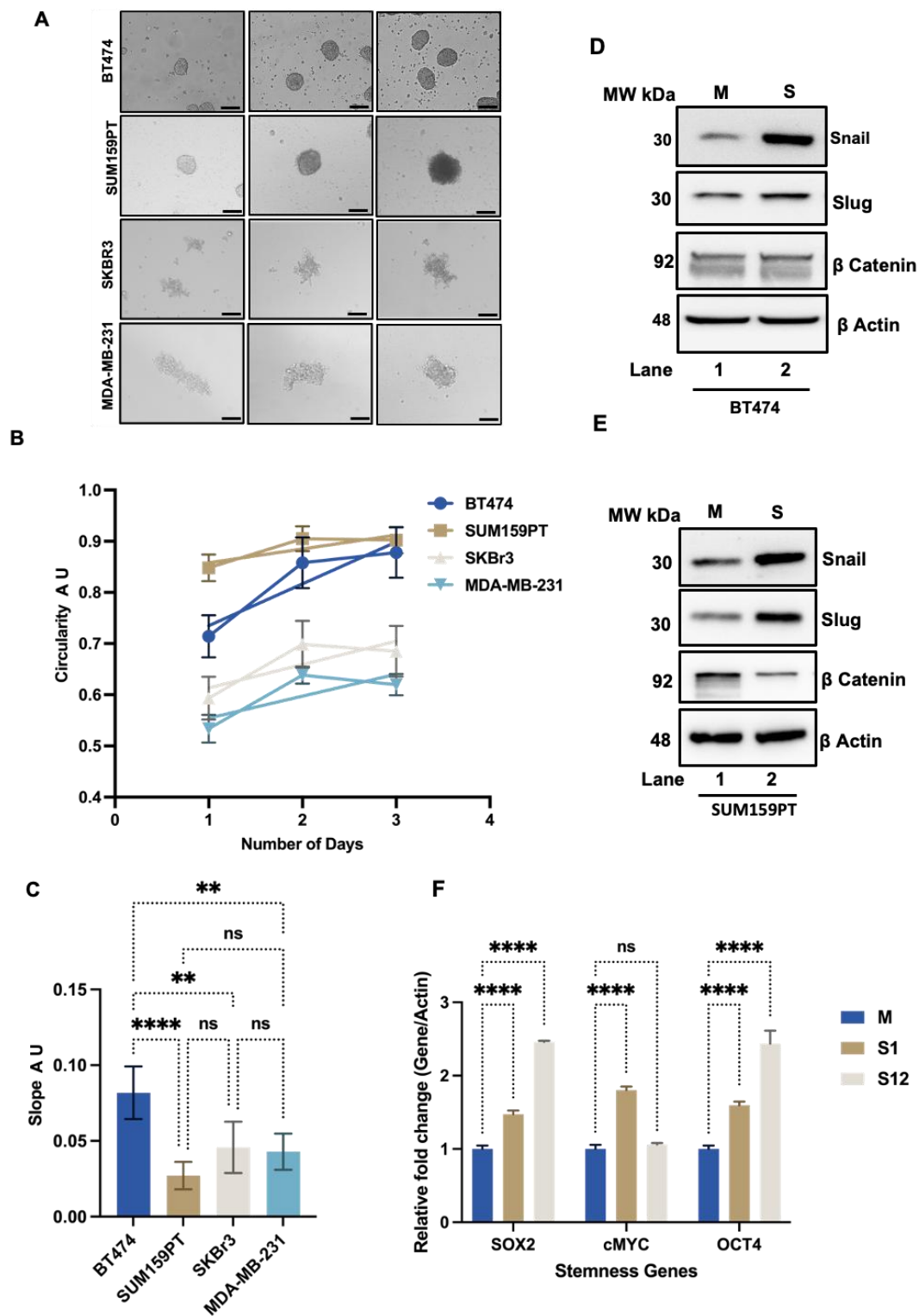


Figure 13: Multicellular spheroids derived from breast cancer cells exhibit features of epithelial-to-mesenchymal transition (EMT) and stemness. (A) Representative images of spheroids formed by BT474, SUM159PT, SKBr3, and MDA-MB-231 cell lines cultured under non-adherent conditions. The Scale bar represents 100 μ m. (B) Quantitative comparison of spheroid formation rate across the four cell lines, based on changes in circularity over time. (C) Graph showing the calculated slope of spheroid formation for each cell line. BT474 cells demonstrated the steepest slope, indicating a faster and more efficient spheroid formation. A.U.-Arbitrary units (D and E) Western blot analysis showing protein expression levels of EMT-related transcription factors Snail, Slug, and β -catenin. β -actin was used as a loading control. Increased levels of Snail and Slug were observed across multiple cell lines. (F) Quantitative PCR analysis showing elevated expression of stemness-associated genes, including SOX2, c-Myc, and OCT4, in spheroid cultures. Statistical comparisons were performed using Sidak's two-way ANOVA test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns-non-significant. (S1, S12 represent spheroids at days 1 and 12).

7.1.3 rRNA synthesis and 2'-O-Me are sensitive to spheroid-induced EMT

To investigate if the nucleolar functions are altered during SEMT, we checked the two major nucleolar processes: rRNA synthesis and 2'-O-Me of rRNA due to their role in promoting cellular transitions (Häfner *et al.*, 2023; Prakash *et al.*, 2019). To determine if rRNA transcription is altered during spheroid formation, we quantified the levels of rRNA transcripts from MCF7 and BT474 by qPCR using the specific primers for rRNA transcripts. Compared to the monolayer cultures, spheroids showed a significant upregulation in the expression of 45S, 28S, 18S, and 5.8S rRNA transcripts (**Figure 14A and 14B**). Next, we analysed rDNA promoter activity by performing a dual luciferase reporter assay in BT474 cells. Cells were co-transfected with the Firefly luciferase construct driven by rDNA promoter and Renilla luciferase plasmid as an internal control. The luciferase activity was measured, and expression was normalised to Renilla luciferase expression. Spheroid cells of BT474 showed higher rDNA promoter activity than monolayer cells. The similar results were observed in non-breast cancer cell line HEK293T. These results suggest that rRNA synthesis is elevated during spheroid cultures. (**Figure 14C and 14D**). We next examined the rRNA 2'-O-Me by performing a site-specific qPCR-based approach to evaluate the methylation of rRNA of 11 different sites in the functionally important domains of rRNA in two different cell lines. The extent of the methylation ratio was determined for each site. One (28S-4198) out of the 11 sites showed distinct 2'-O-Me during SEMT (**Figure 14E and 14F**).

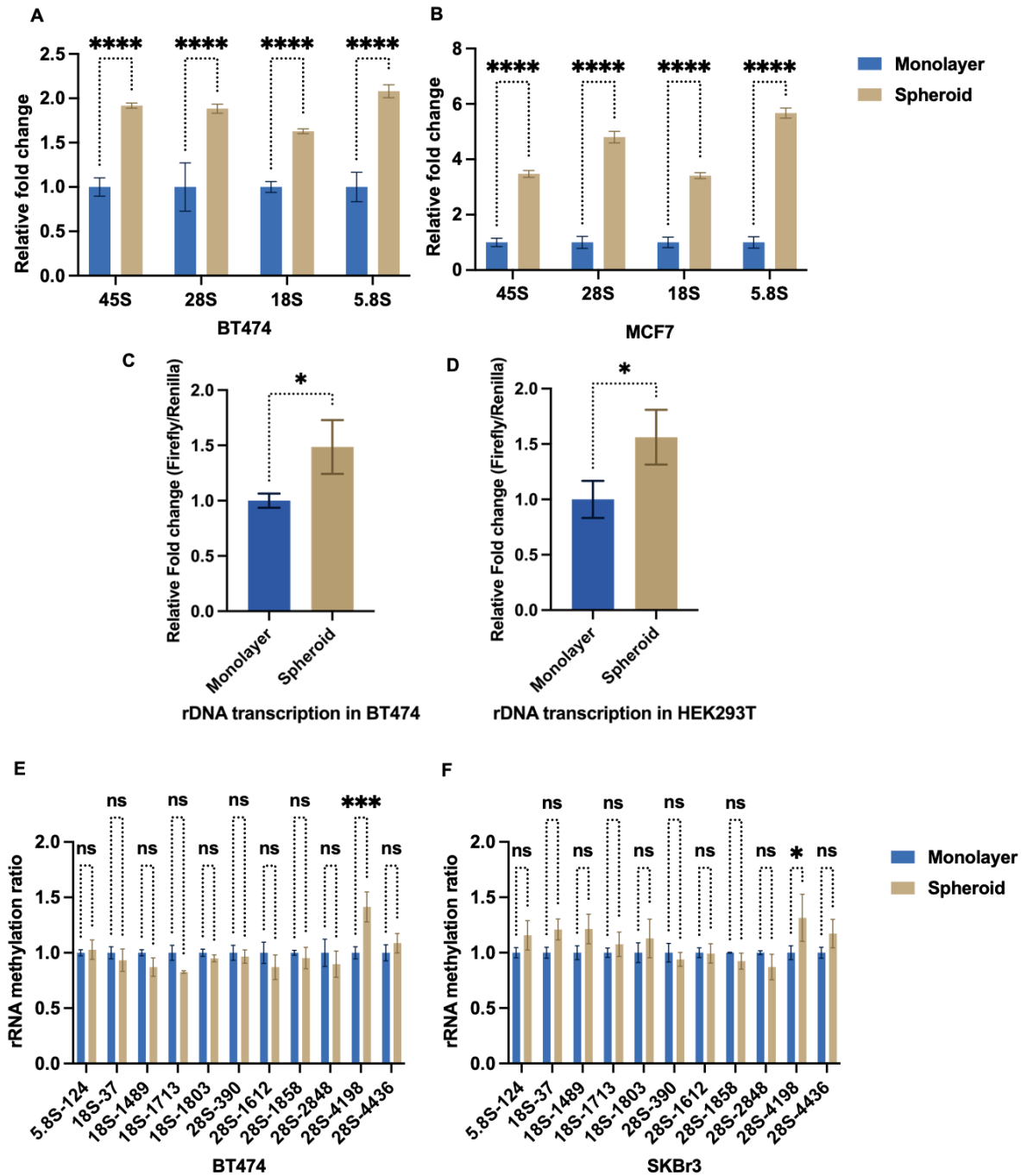


Figure 14: Spheroid-induced EMT enhances nucleolar activity. (A) BT474 cells grown under spheroid conditions show elevated rRNA transcript levels, as measured by quantitative PCR. (B) A similar increase in rRNA transcripts is observed in MCF7 cells cultured as spheroids. (C and D) Dual-luciferase assays reveal increased rDNA promoter activity in both HEK293T and BT474 cells following spheroid culture. (E and F) Site-specific 2'-O-methylation analysis of rRNA demonstrates a significant rise in methylation at position 28S-4198 in BT474 and SKBr3 cells under spheroid conditions. Data are presented as mean $\pm$ SEM from a minimum of three independent replicates. Statistical comparisons were performed using Tukey's two-way ANOVA (A and B), Unpaired t-test (C and D), Sidak's two-way ANOVA test (E and F); significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$, ns-non-significant.

7.1.4 Summary of objective 1

We observed significant variation in nucleolar prominence across different breast cancer subtypes using FBL as a nucleolar marker. Among the cell lines studied, luminal-type BT474 and MCF7 cells exhibited an average of two and three nucleoli per cell, respectively. In contrast, HER2-positive SKBr3 cells displayed two large, prominent nucleoli, while basal-like MDA-MB-231 cells exhibited a higher nucleolar count, averaging 5 to 6 nucleoli per cell. Immunohistochemistry analysis further revealed subtype-specific nucleolar features in tissue samples. Estrogen receptor-positive tissues typically showed single, inconspicuous nucleoli, whereas TNBC tissues exhibited a striking redistribution of FBL throughout the nucleoplasm. Functional assays using MTT demonstrated that TNBC cell lines, including BT549 and MDA-MB-231, were more resistant to inhibitors of rRNA synthesis compared to non-TNBC lines such as MCF7 and BT474. This suggests a higher basal rate of rRNA transcription in TNBC subtypes. Additionally, studies in multicellular spheroid models revealed a positive correlation between nucleolar prominence (in terms of both size and number) and rRNA synthesis activity. Taken together, these findings support a positive correlation between the spheroid-induced EMT (SEMT) phenotype and enhanced nucleolar processes, including rRNA synthesis and 2'-O-methylation.

7.2 Objective 2: To explore the underlying mechanisms responsible for the distribution of the nucleolar proteins and their function in modulating cellular plasticity

7.2.1 Nucleolar methyltransferase FBL promotes SEMT

Nucleolar methyltransferase Fibrillarin (FBL) is a key nucleolar protein involved in the 2'-O-Me of rRNA and pre-rRNA processing as a small nucleolar ribonucleolar protein complex (snoRNP) component. Furthermore, FBL is also involved in maintaining heterochromatin of rDNA, thereby epigenetically regulating the ribosome biogenesis (Tessarz *et al.*, 2014). We therefore asked if the alteration of rRNA synthesis and 2'-O-Me observed during SEMT could be attributable to changes in the levels of FBL. First, to examine the FBL's role in breast cancer, we assessed the publicly available datasets using the TNM plot (<https://tnmplot.com>). We observed that the levels of FBL are significantly increased in tumor samples compared to those of normal tissues (**Figure 15A**). To investigate whether FBL expression altered during SEMT, we performed immunoblotting analysis of BT474 cells cultured in monolayer and spheroid conditions for an indicated number of days, and lysates were then subjected to immunoblotting. The study revealed that FBL levels were elevated at 24 and 48 hours of spheroid culture. The densitometry analysis showed a significant increase in levels of the FBL, suggesting that FBL is involved in SEMT (**Figure 15B and 15C**). Furthermore, immunoblotting analysis of FBL showed a similar rise in SKBr3 cells, but MDA-MB-231 cells showed no significant change, probably due to their reduced spheroid-forming capability (**Figure 15E**). Next, to evaluate if the levels of FBL promote SEMT, we performed lentiviral-mediated knock down of FBL in BT474 cells and subjected them to immunoblotting analysis. The results indicated that the KD of FBL reduces the levels of Snail and Slug in both monolayer and spheroid cultures (**Figure 15F**). Together, these results suggest the potential role of FBL during spheroid-induced EMT.

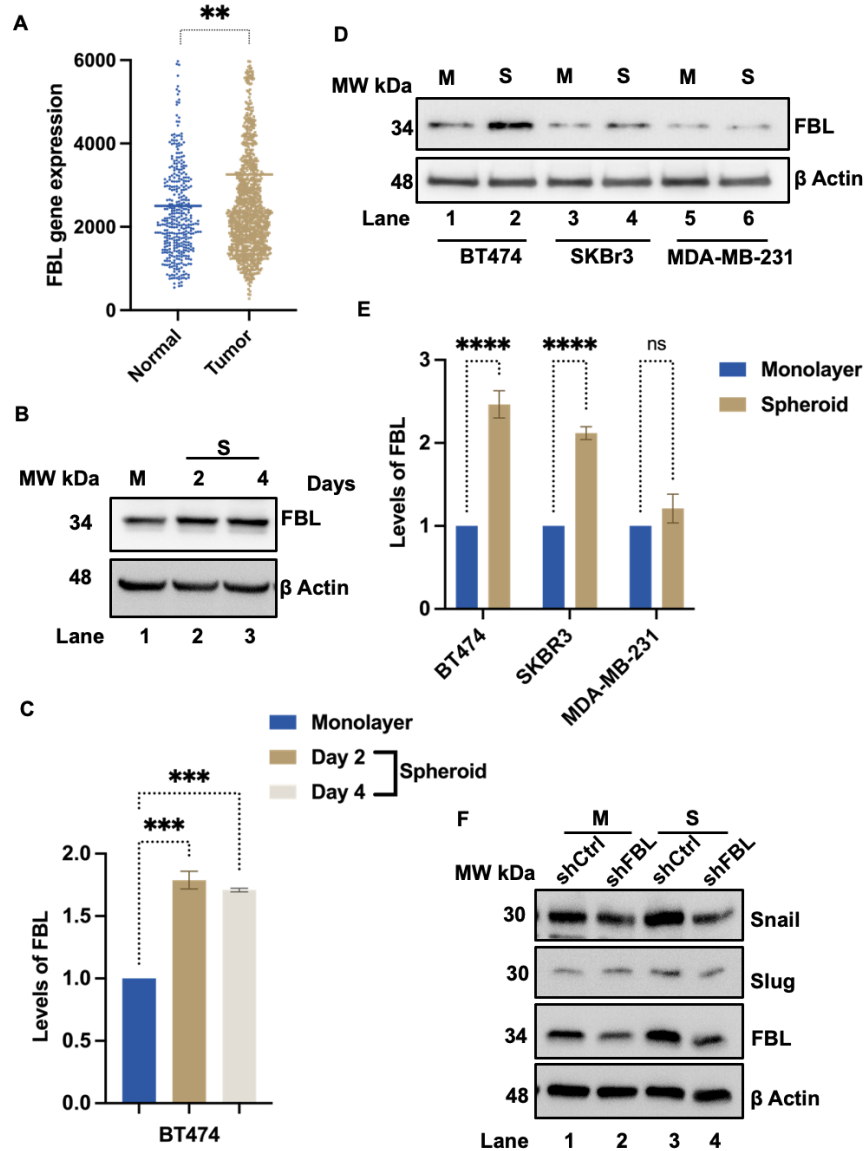


Figure 15: FBL facilitates spheroid-associated epithelial-to-mesenchymal transition (SEMT) **A.** Dot plot from TNMplot.com illustrating a significant increase in FBL expression in breast tumor tissues in comparison to normal tissues. **B.** Immunoblot analysis showing elevated FBL protein levels in spheroid cultures of BT474 cells. **C.** Quantification of FBL expression levels in spheroid vs monolayer cultures of BT474 cells (related to panel B). **D.** Immunoblot showing upregulation of FBL in spheroid cultures of BT474 and SKBr3 cells, but not in MDA-MB-231 cells. **E.** Densitometric analysis representing FBL expression from panel D. **F.** Immunoblot analysis displaying changes in EMT transcription factor expression following knockdown of FBL. Statistical comparisons were performed using t-test, Dunnett's one-way and two-way ANOVA test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns-non-significant.

7.2.2 Elevated levels of FBL are not due to genomic alterations

To determine whether the FBL's regulation in breast cancer is due to genetic alterations, we analyzed the TCGA (The Cancer Genome Atlas) dataset for copy number variations and mutations (**Figure 16A and 16B**). These results suggest no significant changes in the FBL locus, indicating that elevated protein levels of FBL are not due to genomic alterations.

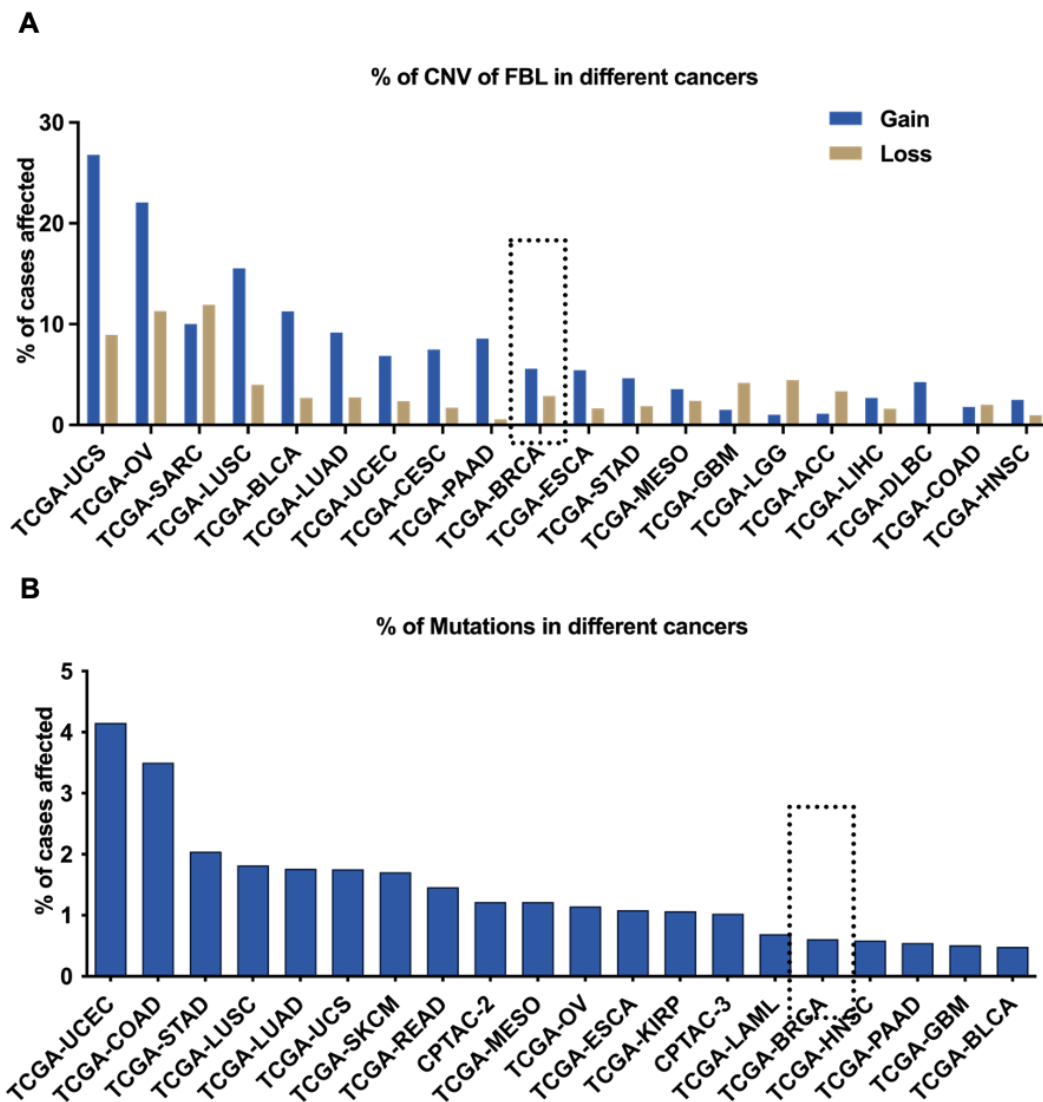


Figure 16: FBL exhibits a low frequency of genetic alterations in breast cancer. (A) Bar graph depicting copy number variation data for the FBL gene in breast cancer, based on TCGA analysis. The results show that gains or losses in FBL copy number are uncommon across the cohort. **(B)** Bar graph illustrating the percentage of breast cancer cases with FBL gene mutations, using TCGA data. The mutation rate is minimal, indicating that genetic alterations in FBL are rare events in breast cancer.

7.2.3 FBL is a substrate for SUMOylation

Unexpectedly, western blotting analysis revealed high molecular weight bands of FBL. These high-molecular-weight bands were prominent under monolayer conditions and were notably reduced in spheroid cultures. The densitometry analysis of immunoblots demonstrated a significant decrease in the ratio of modified forms to unmodified forms of FBL in spheroid cultures compared to monolayer conditions (**Figure 17A and 17B**). A similar significant reduction in the ratio of modified to unmodified forms of FBL was observed in TNBC, MDA-MB-231 cells, compared to ER+ve BT474 cells, likely because spheroids reflect characteristics of TNBC tumors (**Figure 17C and 17D**). This shift in the band pattern suggests that spheroid-induced changes in the protein migration profile likely reflect the modification state of the FBL.

FBL has previously been reported to undergo post-translational modifications (PTMs), acetylation (Iyer-Bierhoff *et al.*, 2018) and methylation (Shubina *et al.*, 2020); however, the molecular weight shift does not correspond to the shift observed in our immunoblot. We, therefore, hypothesized that FBL might undergo alternative post-translational modifications. Since protein SUMOylation is a nuclear-enriched post-translational modification (Z. Chen *et al.*, 2024), we hypothesized that FBL might undergo post-translational modification by SUMO.

To verify that FBL is a SUMO substrate, we performed co-immunoprecipitation with an anti-SUMO 2/3 antibody and immunoblotting analysis with an anti-FBL antibody. These results reveal the presence of a high molecular weight band at 70 kDa in the IP sample but not in the control samples (**Figure 17E**). To further validate our findings, we performed reverse immunoprecipitation with anti-FBL antibody and western blotting with SUMO 2/3 antibody in monolayer and spheroid cultures. Consistent with the previous Co-IP results, we observed a

high molecular weight band of FBL at 70 kDa, and notably, the levels of this band decreased in spheroid cultures (**Figure 17F**).

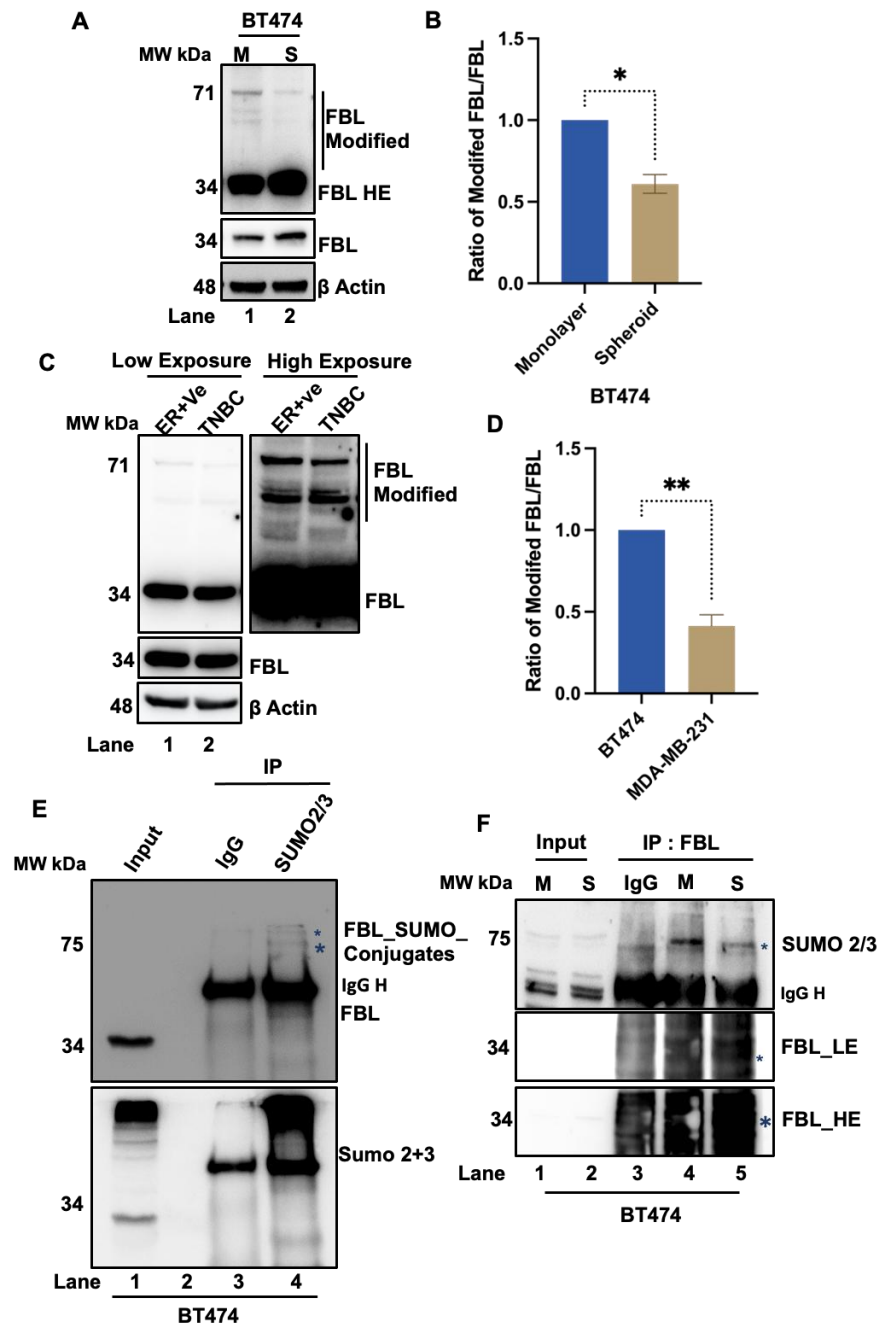


Figure 17: Post-translational modification of FBL and its SUMOylation profile. **A.** Immunoblot showing the presence of modified and unmodified forms of FBL in both spheroid and monolayer cultures of BT474 cells. HE, High exposure and LE, Low exposure. **B.** Quantification showing the ratio of modified to unmodified FBL in BT474 spheroid and monolayer cultures. **C.** Immunoblot comparing modified forms of

FBL between the ER-positive cell line BT474 and the triple-negative breast cancer (TNBC) cell line MDA-MB-231. **D.** Densitometric analysis showing the ratio of modified to unmodified FBL in BT474 and MDA-MB-231 cell lines. **E.** Co-immunoprecipitation indicating SUMOylation of FBL. **F.** Coimmunoprecipitation showing SUMOylation levels of FBL in monolayer versus spheroid cultures. IgG H, Heavy chain. Statistical comparisons were performed using t-test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$.

After identifying that FBL undergoes SUMOylation, we next sought to determine the site of SUMOylation in FBL. To this end, we used prediction software such as JASSA, SUMOgo and SUMO-Plot (**Figure 18A-C**) and performed the multiple sequence alignment (ClustalW) of FBL (**Figure 19A**). Although multiple conserved SUMOylation sites were identified using MSA and SUMO prediction software, two sites (K102 and K131) were consistently predicted across all software with relatively high confidence scores. Furthermore, high-throughput studies also predicted these two sites as potential SUMOylation sites of FBL. Hence, we selected these sites for further experimental validation.

Next, we generated the point mutations of FBL by substituting the Lysine residue (K102 and K131) with Arginine (K102R and K131R) at these predicted sites. The cells were then transfected with wild-type or point mutant constructs of FBL and subjected to immunoprecipitation analysis with anti-SUMO 2/3 antibody, followed by immunoblotting with anti-GFP antibody. The immunoblot analysis revealed a loss of the modified form of FBL (~100 kDa) for the K131 construct compared to wildtype or K102R, suggesting the K131 site as a potential site for SUMOylation of FBL (**Figure 19B**).

A JASSA

3/3/2020

[JASSA] Joined Advanced SUMOylation site and SIM Analyser

[-] Results

Results for putatifs SUMO site

Results for putatifs SIM [PSmax=38.183 | Cut-off=0.269]

Position K	Sequence	Best PS	Consensus direct				Consensus Inverted				Position site	Sequence	Type	a/s stretch	PS	DB Hit
			Type	PSd	DB Hit	Type	PSi	DB Hit								
K102	RHEGVFICRGKEDALVTKNLV	None	Weak	Consensus	None	None	None	None	None	AA 210-213	NLAKKRTNIIIPVIEDARHPH	SIM Type β	[N](SIM)[N]	0.272		
K121	LVPGESVYGEKRVISIEGDDK	None	None	None	None	1	None	None	None	AA 230-233	KYRMLIAMVDVIFADVAQPD	SIM Type α	[N](SIM)[N]	0.665		
K131	KRVISIEGDDKIEYRAWNPFR	Low	None	None	None	None	Strong consensus inv	Low	None							
K143	EYRAWNPFRSKLAAAILGGVD	None	None	None	None	None	None	None	1	AA 261-264	FLRNGGHFVISIFANCIDST	SIM Type β	[N](SIM)[N]	0.752		
K162	VDQIHIKPGAKVLYLGAASGT	None	None	None	None	1	None	None	None	AA 309-312	PYERDHAVVVGVRPPPKVK	SIM Type β	[N](SIM)[N]	0.673		
K265	RNGGHFVISIKANCIDSTASA	None	None	None	None	None	None	None	1							
K284	SAEAVFASEVKKMQQENMKPQ	Low	None	None	None	None	None	Low	2							
K318	VVVGVRPPPKVKN-----	None	None	None	None	None	None	None	1							
K320	VGVRPPPKVKN-----	None	None	None	None	None	None	None	2							

B SUMOgo

(Score >= 0.5 for SUMOylation site, Score < 0.5 for non-SUMOylation site)	
Position	Confidence Score
71	0.511482
205	0.49061
98	0.477699
42	0.347036
224	0.251466
232	0.244724
102	0.241186
260	0.230736
49	0.219215
258	0.201595
24	0.195469
162	0.186099
225	0.169314
83	0.16763
145	0.14752
61	0.117298
146	0.111622

C SUMOplot

Protein ID: sp P22087.2 FBRL_HUMAN							
Defintion:	RecName: Full=rRNA 2'-O-methyltransferase fibrillarin; AltName: Full=34 kDa nucleolar scleroderma antigen; AltName: Full=Histone-glutamine methyltransferase						
Length:	321 aa						
1MKPGFSPRGGFGFGRGGFGDRGGRGGRGFGGGGRGGGGFRGRGRGGGGG 51GGGGGGGGRRGGGFHSGGNRGRGRGKRGNGQSGKNMVEPHRHEGVFICR 101GKEDALVTKNLVPGESVYGEKRVSISEGDDKIEYRAWNPFSKLAAAILG 151GVDQIHIKPGAKVLYLGAASGTTVSHVSDIVGPDGLVYAVEFSHRSGRDL 201INLAKKRTNIPVIEDARHPHKYRMLIAMVDVIFADVAPQDQTRIVALNA 251HTFLRNGGHFVISIKANCIDSTASAEAVFASEVKKMQQENMKPQEQLTLE 301PYERDHAVVGVYRPPPKVKN Motifs with high probability Motifs with low probability Overlapping Motifs							
No.	Pos.	Group	Score	No.	Pos.	Group	Score
1	K158	VDQIH IKPG AKVLY	0.77	4	K131	ISEGD DKIE YRAWN	0.5
2	K102	VFICR GKED ALVTK	0.67	5	K77	GRGRG GKRG NQSGK	0.5
3	K2	MKPG FSPRG	0.63				

Figure 18: Identification and prediction of SUMOylation sites in FBL A, B and C. In silico prediction of SUMOylation sites in FBL using JASSA, SUMOgo, and SUMOplot tools, indicating potential modification hotspots.

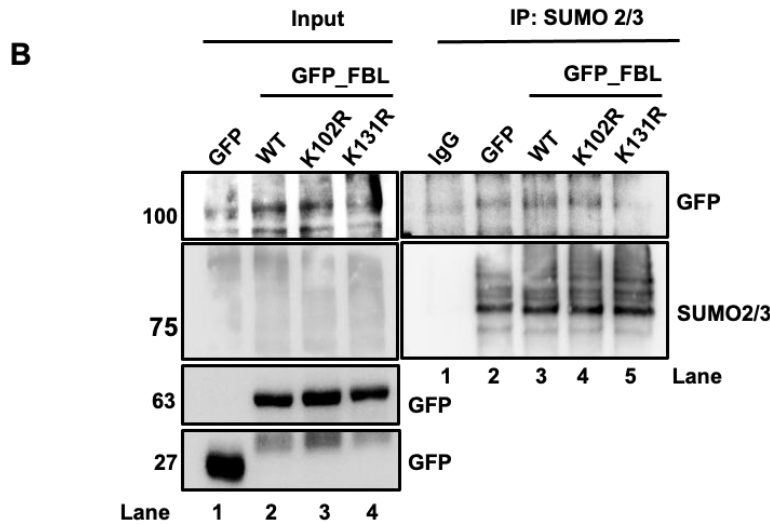
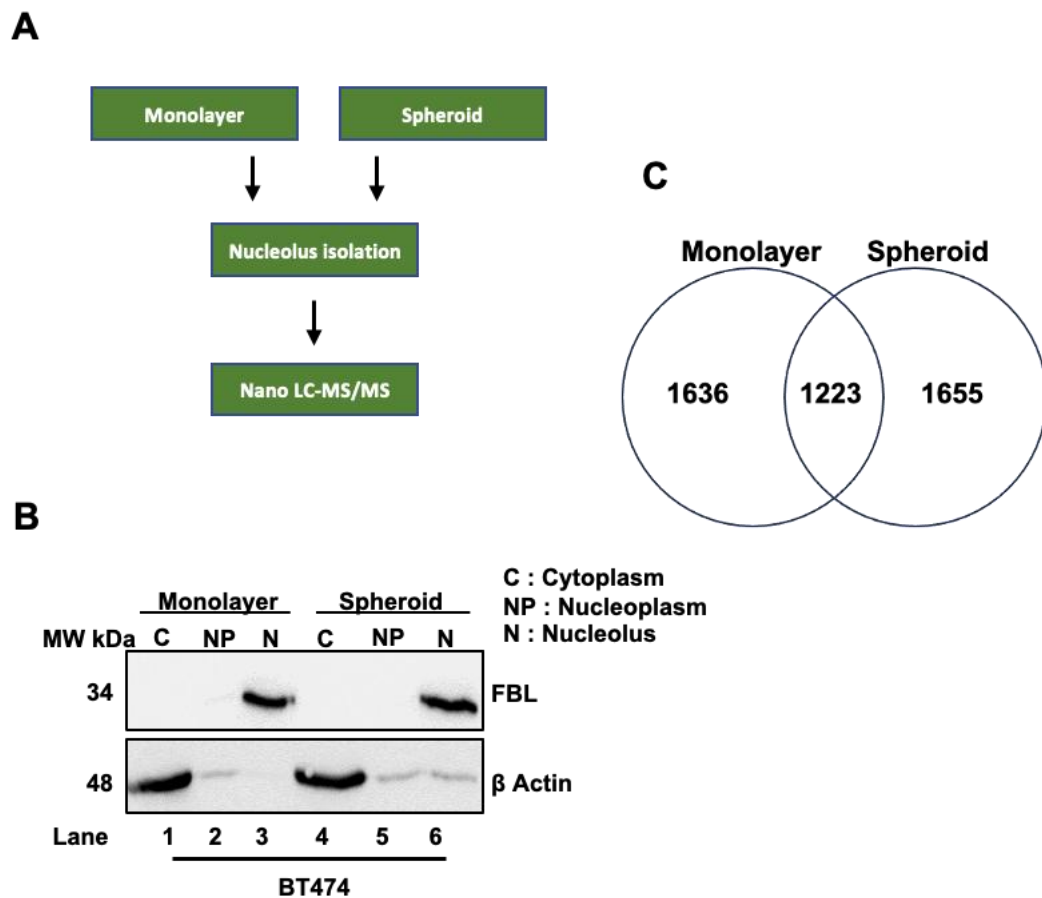


Figure 19: Identification and prediction of SUMOylation sites in FBL (A) Multiple sequence alignment highlighting conserved SUMOylation motifs across FBL orthologs in different species. **(B)** Co-immunoprecipitation identifying the specific lysine residue(s) in FBL undergoing SUMOylation.

7.2.4 CBX4 promotes SUMOylation of FBL

To further explore the regulation of SUMOylation of FBL, we isolated nucleoli from BT474 cells cultured under monolayer and spheroid conditions and subjected them to Mass Spectrometry after verifying their purity by immunoblotting (**Figure 20A-C**). Comparative proteome analysis revealed that E3 SUMO ligase CBX4 (31.9643% coverage) is enriched in the nucleoli of monolayer conditions but not in the nucleoli of spheroid cells (**Figure 20D**). Interestingly, CBX4 has been previously reported to regulate rDNA's heterochromatin and

transcriptional regulation by interacting with FBL in normal mesenchymal stem cells (Ren *et al.*, 2019). However, its role as an E3 SUMO ligase of FBL has been unexplored. To address whether CBX4 is the potential E3 SUMO ligase of FBL, we first performed immunoprecipitation with an anti-CBX4 antibody from BT474 cells, followed by western blotting with an anti-FBL antibody. These results suggest that FBL co-precipitates with CBX4 (**Figure 21A**). Furthermore, confocal microscopy analysis showed that CBX4 colocalizes with FBL (**Figure 21B and C**). Next, we performed lentivirus-mediated knockdown of CBX4 in BT474 cells, followed by immunoprecipitation using anti-SUMO 2/3 antibody. Immunoblotting with FBL has shown a significant reduction of SUMOylated FBL upon depletion of CBX4 in comparison with control cells (**Figure 21D**). Together, these results indicate that CBX4 promotes SUMOylation of FBL and is a probable E3 SUMO ligase of it.



D

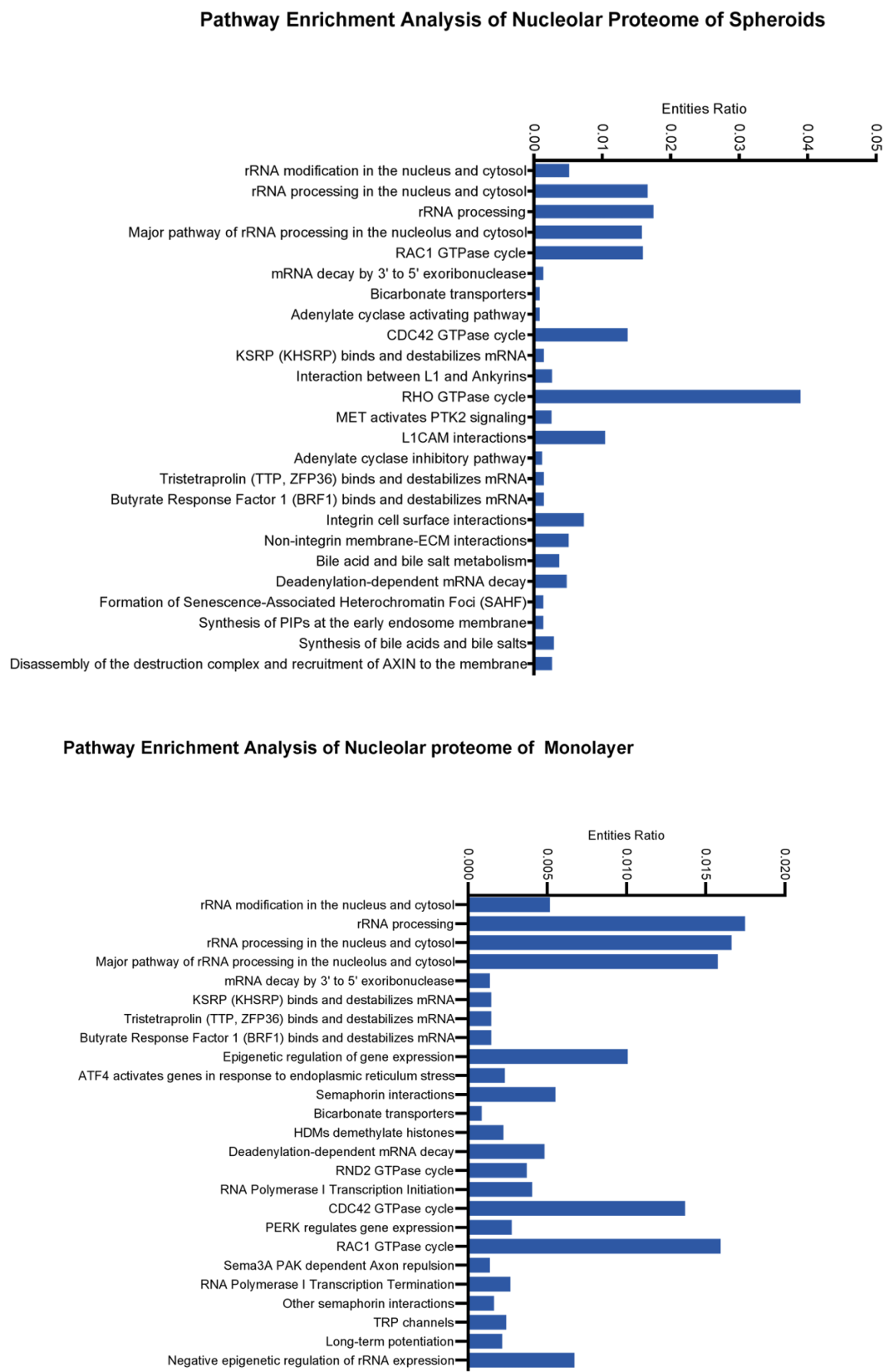


Figure 20: Nucleolar isolation and proteomic profiling from monolayer and spheroid cultures **A.** Schematic representation of the workflow used for nucleolar isolation from cultured cells. **B.** Immunoblot analysis confirming successful enrichment of FBL in the nucleolar fraction. **C.** Venn diagram illustrating

the overlap and unique nucleolar proteins identified in monolayer versus spheroid cultures. **D.** Pathway enrichment analysis of the nucleolar proteome, comparing functional categories between monolayer and spheroid conditions.

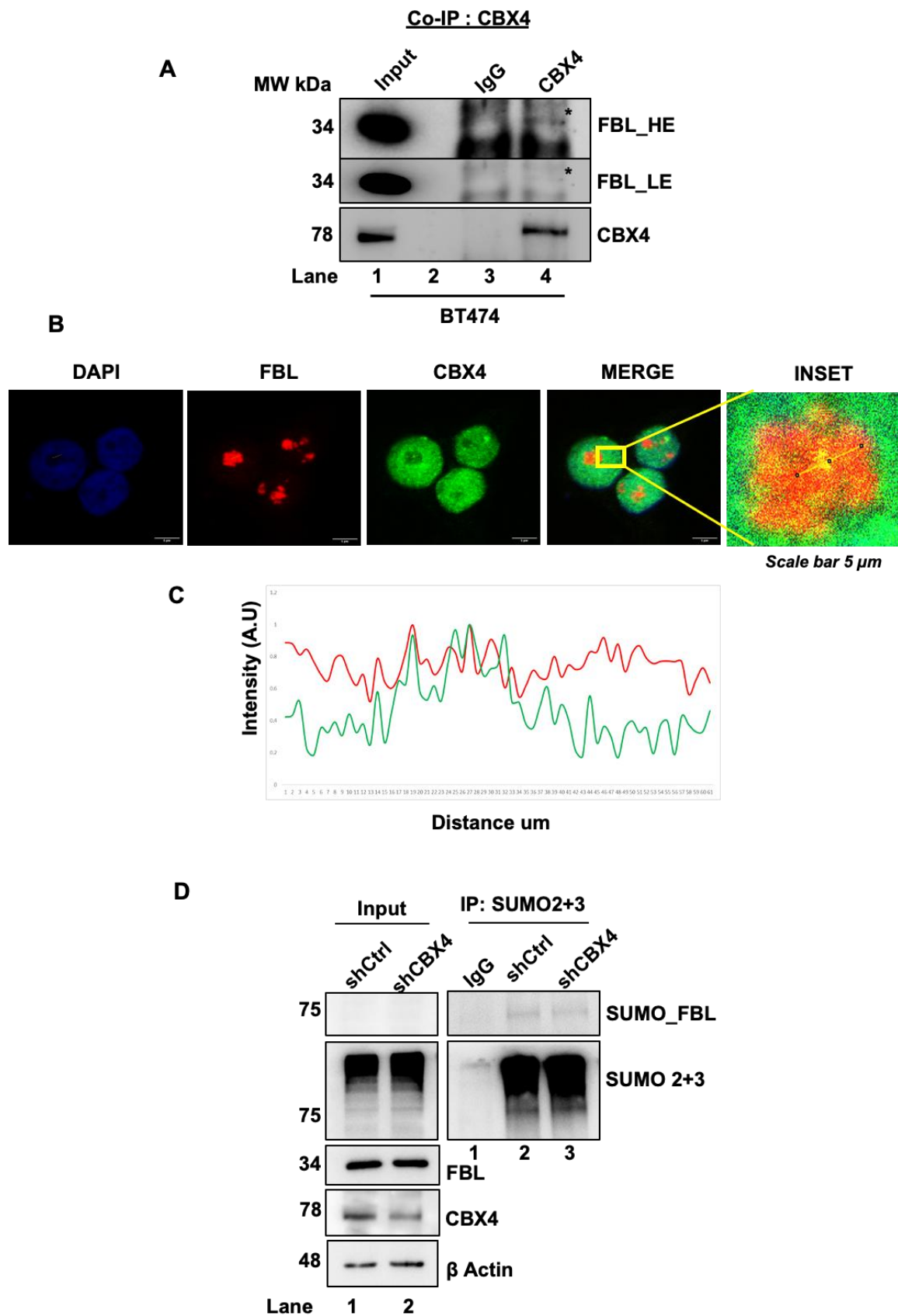


Figure 21: Interaction and SUMOylation regulation of FBL by CBX4. **A.** Co-immunoprecipitation assay confirming the physical interaction between CBX4 and FBL. **B.** Confocal microscopy images showing the subcellular co-localisation of CBX4 and FBL in cells. **C.** Line scan profile analysis demonstrating the overlap of CBX4 and FBL fluorescence signals, indicating co-localisation. **D.** A Co-immunoprecipitation revealing reduced SUMOylation of FBL following CBX4 knockdown.

7.2.5 RNF4 regulates the SUMOylation state of FBL

To investigate the effect of SUMOylation on FBL, BT474 cells were exposed to SUMO inhibitor 2D08, an inhibitor that inhibits SUMOylation by blocking transfer of SUMO from UBC9 to substrate, at a concentration of 30 μ M for the indicated time points. Interestingly, inhibiting SUMOylation led to an increase in levels of FBL in both monolayer and spheroid cultures. A further prominent increase in the levels of FBL was observed in spheroid cultures (**Figure 22A**). These results indicate that SUMOylation promotes the degradation of the FBL. Given the RNF4's role in targeting the SUMOylated proteins for ubiquitination (Lallemand-Breitenbach *et al.*, 2008; Tatham *et al.*, 2008; Weisshaar *et al.*, 2008), we asked if SUMOylated FBL is a target for RNF4. To address this, we first performed the lentiviral-mediated knockdown of RNF4 in BT474 cells. The western blotting analysis of whole cell lysates has revealed a significant accumulation of a slower migrating band of FBL at 70 kDa in RNF4-depleted cells compared to that of control cells (**Figure 22B-D**).

To further examine if the accumulated slower migrating band in RNF4-depleted BT474 cells is a SUMOylated FBL, we performed immunoprecipitation with an anti-SUMO2/3 antibody, followed by immunoblotting with an anti-FBL antibody. The immunoblotting analysis revealed the significant accumulation of SUMOylated FBL upon depletion of RNF4 in comparison with control cells (**Figure 22E**). These results indicate that RNF4 promotes the degradation of the SUMOylated FBL. Next, to determine if RNF4 interacts with SUMOylated FBL, we performed immunoprecipitation of RNF4 in both Monolayer and spheroid cultures of BT474. The immunoblotting analysis with anti-FBL antibody revealed that RNF4 co-immunoprecipitated with SUMOylated FBL in Monolayer conditions compared to Spheroid

cultures (**Figure 23A**). Further, RNF4 colocalises with FBL (**Figure 23B and C**). These results suggest that RNF4 interacts specifically with SUMOylated FBL.

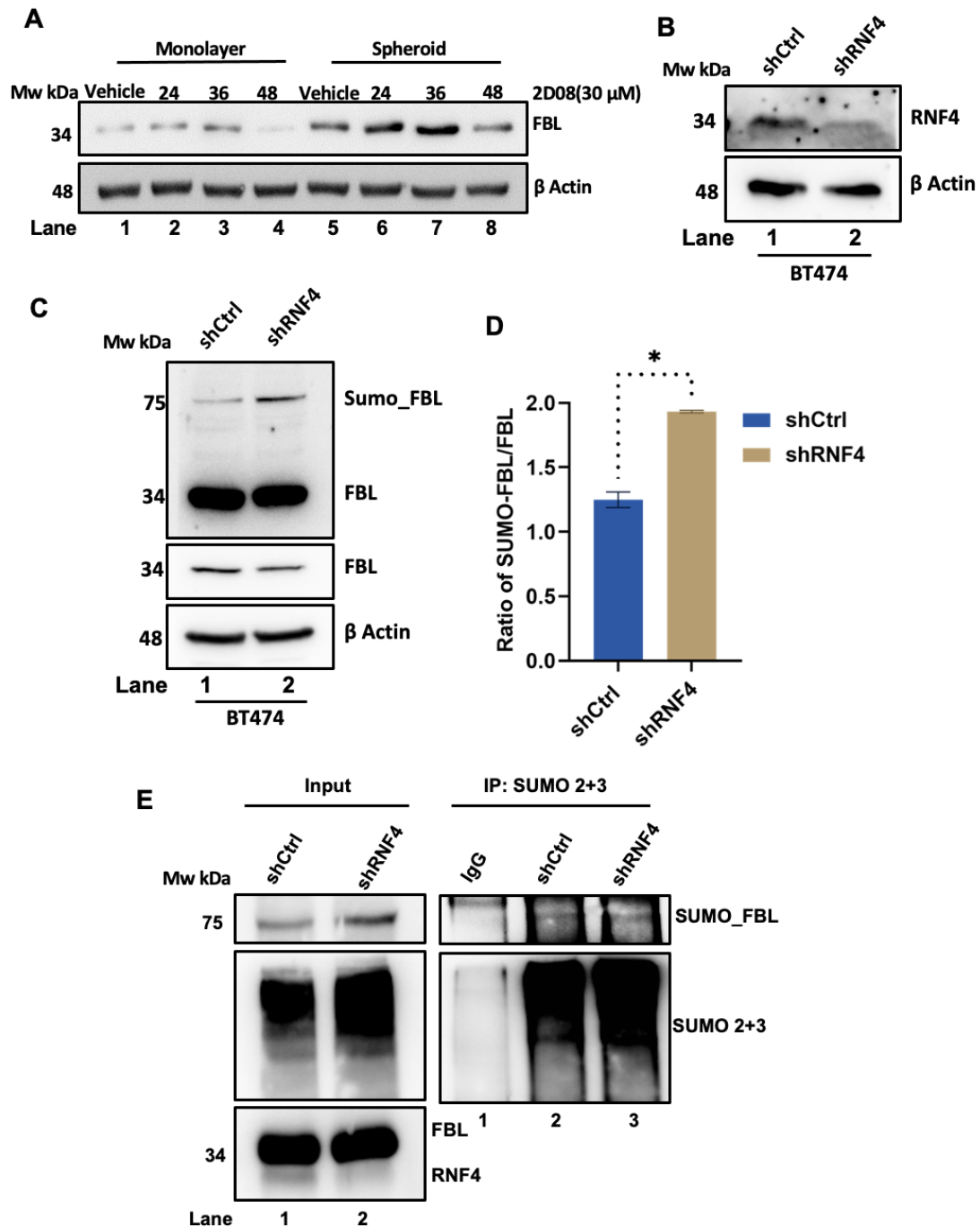


Figure 22: RNF4 regulates the SUMOylation and stability of FBL. Immunoblot analysis showing elevated levels of FBL upon pharmacological inhibition of SUMOylation. **B.** Immunoblot confirming the knockdown efficiency of RNF4. **C.** Immunoblot showing increased accumulation of SUMOylated FBL following RNF4 depletion. **D.** Bar graph quantifying the ratio of SUMOylated FBL to total FBL. **E.** Co-immunoprecipitation demonstrating enhanced accumulation of SUMO-FBL upon RNF4 knockdown.

Statistical comparisons were performed using t-test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

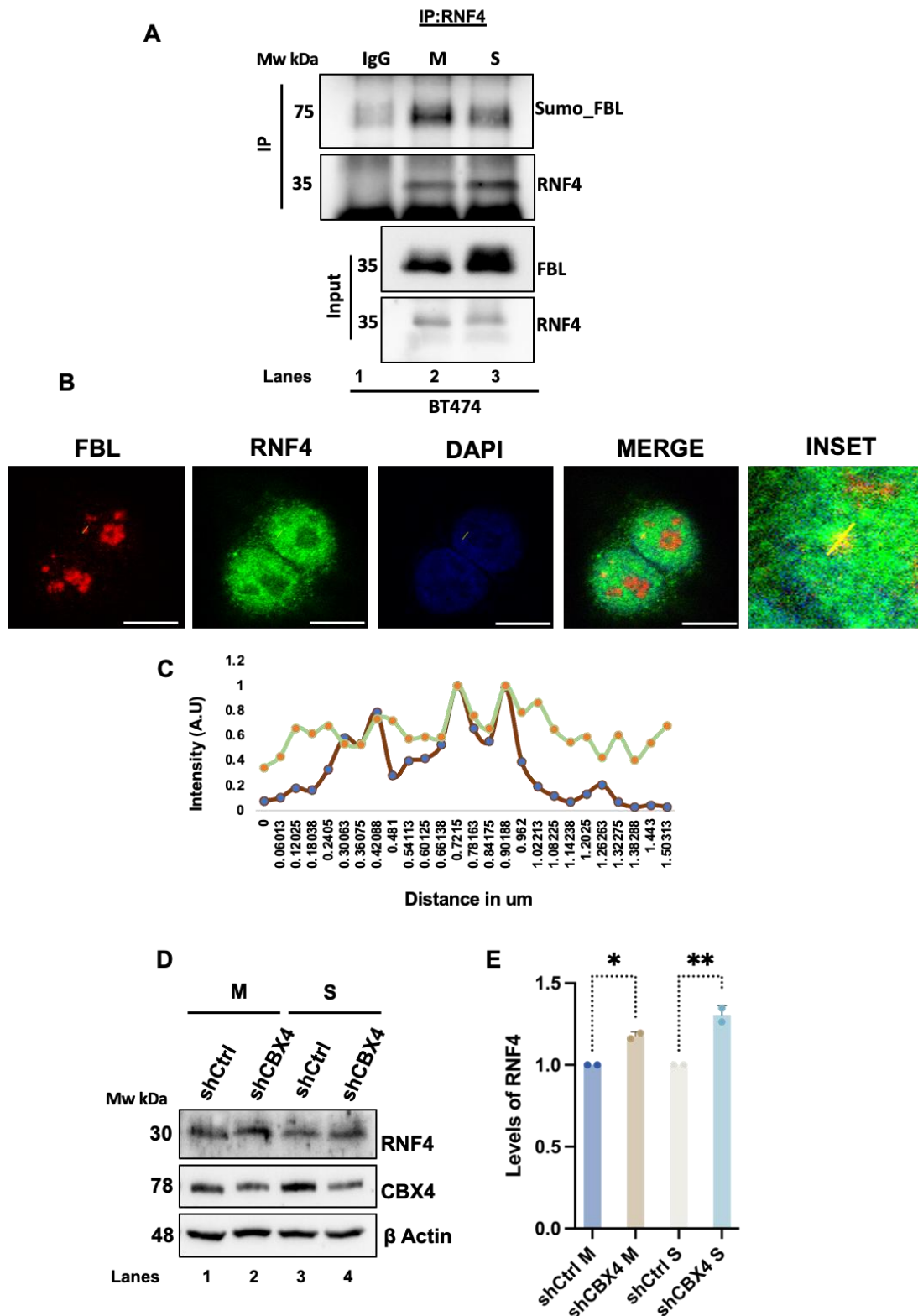


Figure 23: RNF4 is responsive to CBX4-mediated SUMOylation. **A.** Co-immunoprecipitation assay confirming the interaction between RNF4 and SUMOylated FBL. The scale bar represents 20 μm . **B.** Confocal microscopy images depicting the co-localisation of RNF4 and FBL in cells. **C.** Line profile analysis

supporting the spatial overlap between RNF4 and FBL signals. **D.** Immunoblot illustrating the influence of RNF4 on CBX4-mediated SUMOylation of FBL. **E.** Quantitative analysis of RNF4 expression levels upon CBX4 knockdown. Statistical comparisons were performed using Tukey's two-way ANOVA test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$.

Given that protein SUMOylation enhances the dimerization and activation of RNF4, leading to its autoubiquitination (Rojas-Fernandez *et al.*, 2014), we proposed that SUMOylation of proteins mediated by CBX4 could trigger autoubiquitination. We examined whether RNF4 responds to CBX4-mediated SUMOylation and performed lentiviral knockdown of CBX4 in BT474 cells. The immunoblotting analysis employing anti-RNF4 antibodies demonstrated that the levels of RNF4 were elevated upon CBX4 KD in both monolayer and spheroid cultures (**Figure 23D and E**). These results suggest that CBX4-mediated SUMOylation could possibly promote the autoubiquitination of RNF4.

7.2.6 SUMOylation of FBL regulates its nucleoplasmic distribution

We next asked whether the SUMOylation of FBL regulates its function. Interestingly, while characterising the expression and localisation of the wildtype (GFP-FBL) and point mutant (GFP-FBL K131R) of FBL, we observed that the K131R point mutant of FBL exhibits a significant redistribution of the mutant throughout the nucleoplasm. In contrast, the wild type is restricted to the nucleolus (**Figure 24A and B**). Since CBX4 promotes the SUMOylation of FBL and, conversely, RNF4 suppresses the SUMOylation of FBL, we hypothesised that depletion of CBX4 and RNF4 might regulate the similar redistribution of FBL into the nucleoplasm. To address this, we performed lentiviral-mediated KD of RNF4 and CBX4 in BT474 cells and subjected them to confocal microscopy analysis with anti-FBL or anti-CBX4 or anti-RNF4 antibodies. The depletion of CBX4 has resulted in the significant redistribution of FBL into nucleoplasm (**Figure 24C and D**); conversely, the depletion of RNF4 showed a substantial reduction in the redistribution of FBL into nucleoplasm (**Figure 24E and F**).

Together, these results suggest that SUMOylation of FBL probably regulates the functions of FBL by maintaining its compartmentalisation between the nucleus and nucleolus.

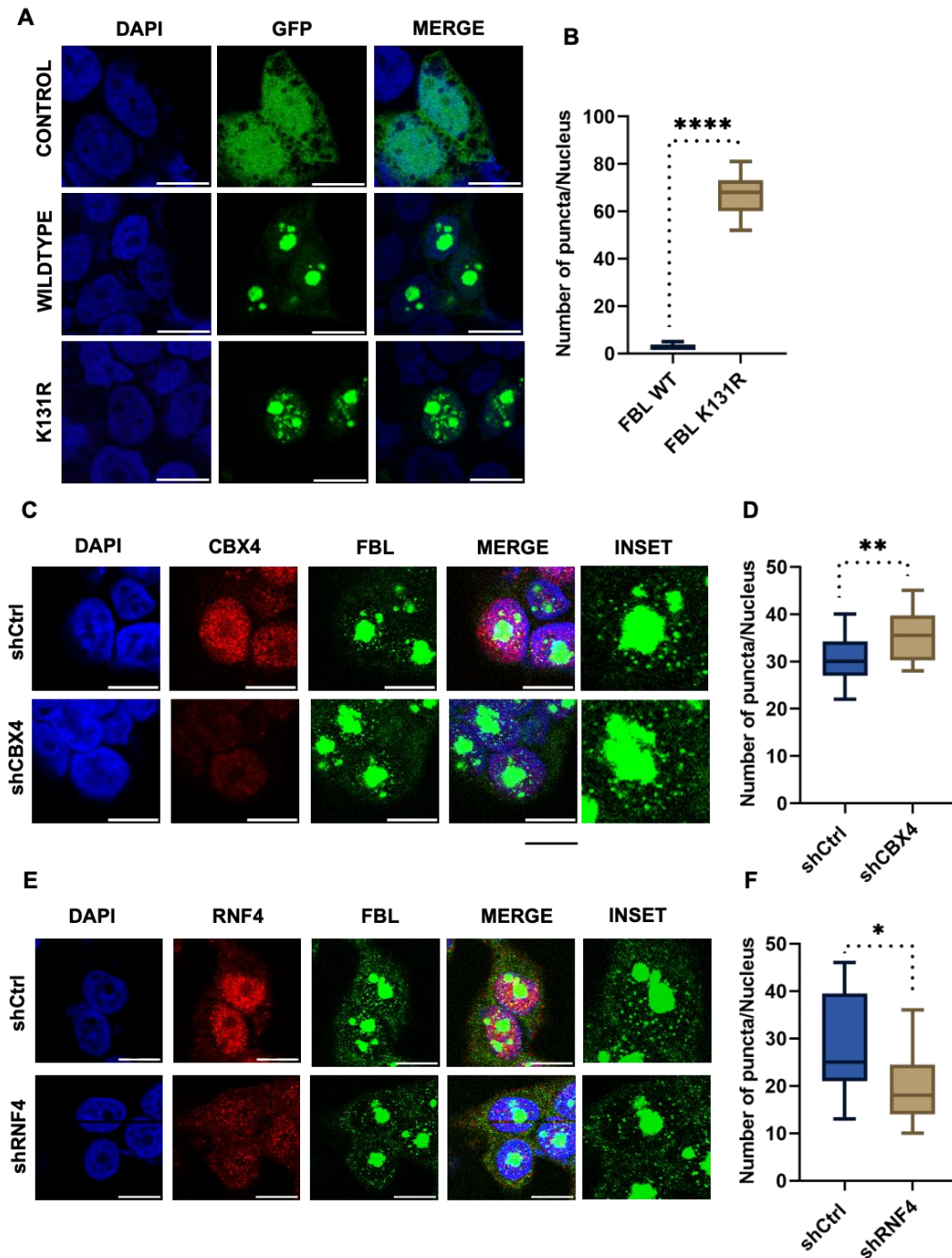


Figure 24: SUMOylation influences the subnuclear localization of FBL between the nucleolus and nucleoplasm. **A.** Confocal microscopy images showing that the SUMOylation-deficient FBL mutant exhibits increased redistribution across the nucleoplasm. **B.** Box-and-whisker plot quantifying the enhanced nucleoplasmic distribution of the SUMO-deficient FBL mutant. **C.** Confocal images depicting increased

nucleoplasmic localization of FBL following CBX4 knockdown. **D.** Box-and-whisker plot showing elevated redistribution of FBL upon CBX4 depletion. **E.** Confocal analysis indicating reduced nucleoplasmic redistribution of FBL upon RNF4 knockdown. **F.** Box-and-whisker plot quantifying the decreased redistribution of FBL after RNF4 depletion. Statistical comparisons were performed using Welch's t-test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The scale bar represents 20 μm .

7.2.7 The deSUMOylation state of FBL correlates with cellular plasticity

We next examined how SUMOylation affects the functions of FBL. Given the role of FBL in rRNA synthesis and rRNA 2'-O-Me, and to draw the correlation between the SUMOylation status of FBL and its function, we performed lentiviral-mediated knockdown of CBX4 in BT474 cells. We assessed the levels of rRNA transcripts using qPCR and the methylation ratio of 11 rRNA methylation sites through site-specific rRNA methylation assays. Interestingly, we observed a significant increase in rRNA transcripts and the methylation ratio of these sites in CBX4-depleted cells in comparison with control cells (**Figure 25A and B**). Subsequently, we assessed the ratio of SUMO-FBL to FBL in both monolayer and spheroid conditions. The ratio of SUMO-FBL to FBL significantly decreases upon CBX4 depletion in monolayer culture. However, no significant change in the ratio is observed in spheroid cultures, likely due to the absence of CBX4 in the nucleolus of spheroid cultures, as indicated by our comparative mass spectrometry analysis (**Figure 25C-E**). We then analysed the expression of transcription factors after determining the ratio of SUMOylation to check its effect on EMT. In line with our earlier findings, we found that a decrease in the SUMOylation ratio upon CBX4 depletion correlates with a significant increase in Snail expression in spheroid cultures. The levels of c-Myc did not increase, possibly due to elevated levels under suspension conditions. However, β -Catenin levels did not show any significant alteration (**Figure 25 F and G**).

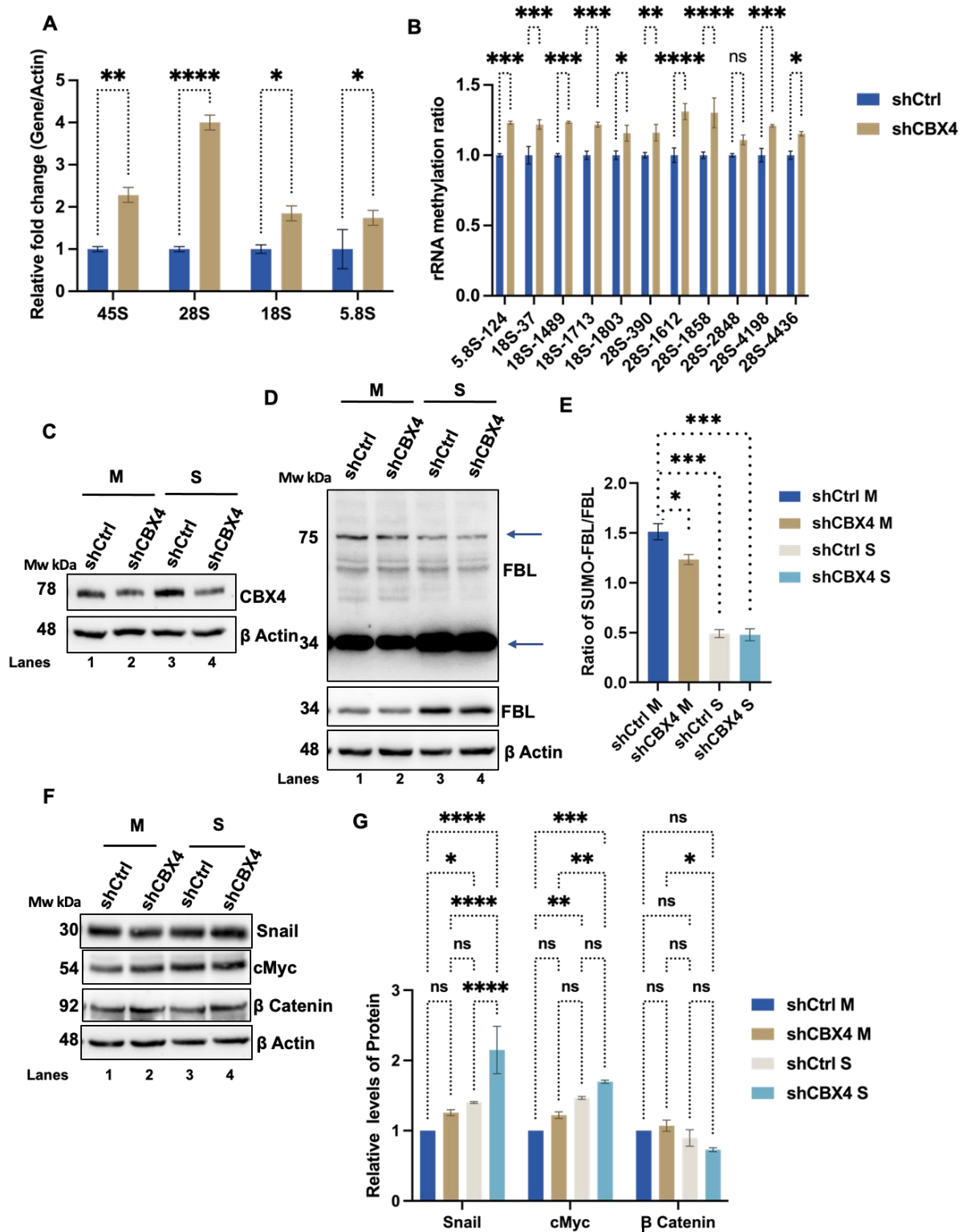


Figure 25: CBX4 modulate FBL modification, rRNA synthesis, and EMT-related transcription. **A.** Bar graph depicting changes in rRNA transcript levels following CBX4 knockdown, as measured by qPCR. **B.** Bar graph illustrating the rRNA methylation ratio upon CBX4 depletion. **C.** Immunoblot analysis confirming CBX4 knockdown under monolayer and spheroid culture conditions. **D.** Immunoblot showing the altered levels of modified FBL following CBX4 knockdown in both monolayer (M) and spheroid cultures (S). Arrow marks denote 70kDa and 34 kDa bands of FBL. **E.** Densitometric analysis quantifying the modified FBL levels post-CBX4 depletion in both conditions. **F.** Immunoblot demonstrating changes in EMT-

associated transcription factors following CBX4 depletion under both monolayer and spheroid conditions. **G.** Densitometric quantification of EMT transcription factor levels shown in (F). Statistical comparisons were performed using Sidak's two-way ANOVA multiple comparison test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns-non significant.

We also conducted lentiviral-mediated knockdown of RNF4 in BT474 cells, measuring rRNA transcript levels via qPCR and the methylation ratio of 11 rRNA methylation sites using a site-specific assay. Notably, RNF4-depleted cells exhibited a significant decrease in rRNA transcript levels and the methylation ratios at these sites when compared to control cells (**Figure 26A and B**). We subsequently investigated the ratio of SUMO-FBL to FBL in both monolayer and spheroid conditions. The ratio of SUMO-FBL to FBL rose significantly with RNF4 depletion in monolayer cultures. However, no significant change was noted in the ratio within spheroid cultures, likely due to the already active RNF4 pathway suggested by our western blot results (**Figure 26C-E**). We then assessed the expression of transcription factors after determining the SUMOylation ratio to evaluate its impact on epithelial-mesenchymal transition (EMT). Unlike CBX4 depletion, we found that an increased SUMOylation ratio following RNF4 depletion correlated with a substantial decrease in Snail expression across both spheroid and monolayer cultures. The levels of c-Myc remained unchanged in monolayer cultures but were significantly altered in spheroid cultures. In contrast, β -Catenin levels did not exhibit significant changes (**Figure 26F and G**).

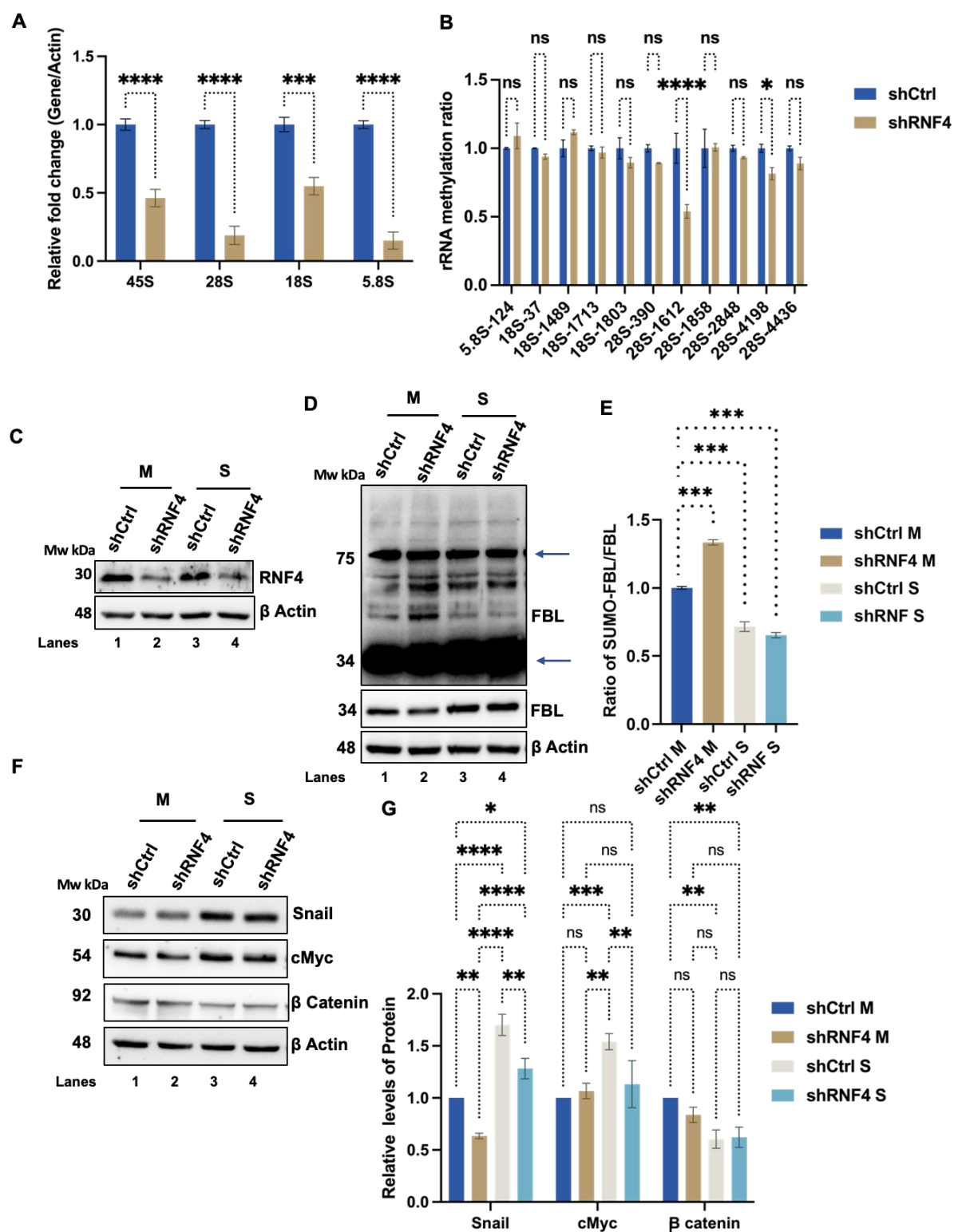


Figure 26: RNF4 modulate FBL modification, rRNA synthesis, and EMT-related transcription. **A.** Bar graph showing rRNA transcript levels upon RNF4 knockdown as assessed by qPCR. **B.** Bar graph depicting rRNA methylation ratio changes following RNF4 depletion. **C.** Immunoblot validating RNF4 knockdown in both monolayer (M) and spheroid (S) cultures. **D.** Immunoblot displaying changes in modified FBL levels after RNF4 knockdown across both culture conditions. Arrow marks denote 70kDa and 34 kDa bands of FBL. **E.** Densitometry analysis quantifying modified FBL levels from panel (D). **F.** Immunoblot assessing EMT-related transcription factor levels following RNF4 knockdown in monolayer (M) and spheroid (S) cultures. **G.** Densitometric

representation of the immunoblot data from panel (F). Statistical comparisons were performed using Sidak's two-way ANOVA multiple comparison test; significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$, ns-non significant.

8 Discussion

Tumor heterogeneity exacerbates the disease and complicates treatment, often resulting in drug resistance and relapse. This heterogeneity arises from various sources, among them, cellular plasticity is also a significant contributor. In cancers, this cellular plasticity is observed as the transition or switching of epithelial phenotype cells to mesenchymal phenotype cells, called as EMT. The molecular mechanisms contributing to the EMT still remain unclear.

The increase in nucleolar size and number in cancers is well known, and this increase reflects the increase in ribosome biogenesis (Orsolic *et al.*, 2016). It is a complicated multi-step process involving the regulation of nucleosome assembly on the rDNA, rRNA transcription, processing, chemical modification, assembly with ribosomal proteins and the export of ribosomal subunits to the cytoplasm. Nearly 200 ribosome biogenesis factors are involved in these processes (Y. Yang *et al.*, 2024).

8.1 Role of ribosome synthesis in cellular plasticity

The activation of oncogenes and the suppression of tumor suppressors lead to increased ribosome biogenesis (Orsolic *et al.*, 2016), which promotes cancer progression (Montanaro *et al.*, 2008). The synthesis of ribosomes occurs in the nucleolus, with each step carefully orchestrated. Dysregulation of any factors involved in ribosome synthesis can result in malignancy. Emerging studies suggest that ribosome biogenesis may induce EMT rather than being a mere consequence of it. Several mechanisms demonstrate how ribosomal RNA synthesis influences EMT (Fernández-Calero *et al.*, 2020; Prakash *et al.*, 2019) (C. Wu *et al.*, 2022), including the downregulation of ribosomal synthesis, which is linked to the upregulation of E-cadherin (Brighenti *et al.*, 2016). Another mechanism involves LARP6, a protein that facilitates the translocation of ribosomal protein mRNA to cell protrusions. This process enhances the local translation of ribosomal proteins and promotes ribosome biogenesis during EMT in breast cancer cells (Dermitt *et al.*, 2020). Furthermore, airway basal cells can induce

hypoxia-mediated EMT by increasing ribosome biogenesis (Y. Hou *et al.*, 2021). In non-small cell lung cancer, suppression of rRNA synthesis via LIX1L has been reported to impair EMT. (M. Li *et al.*, 2023). Additionally, melanoma patients with a gene expression signature associated with ribosomal biogenesis tend to have poorer prognoses (Bakr *et al.*, 2022). Using small molecules, such as CX5461 and BMH21, to inhibit ribosome biogenesis has proven effective in decreasing EMT and reducing chemoresistance (Ban *et al.*, 2024).

8.1.1 Alterations in 2'-O-Me pattern promote cellular plasticity

The 2'-O-Me of rRNA is dynamic and exhibits plasticity in response to various cellular conditions. In human induced pluripotent stem cells (iPSCs) and totipotent human embryonic stem cells (hESCs), the pattern of 2'-O-Me changes during differentiation and in the specification of germ layers. Notably, alterations in 2'-O-Me are also observed during mouse embryonic and postnatal brain development (Häfner *et al.*, 2023). Similarly, changes in the methylation of 5.8S rRNA at the 2'-O position occur during differentiation. For example, when muscle myoblasts differentiate into muscle fibers, an increase in 2'-O-Me is noted (Munholland & Nazar, 1987). Moreover, enhanced 2'-O-Me is associated with the maintenance of leukemic stem cell activity (F. Zhou *et al.*, 2023) and supports self-renewal potential during leukemogenesis (F. Zhou *et al.*, 2017). Additionally, the levels of rRNA 2'-O-Me are considerably higher in adult tissues in comparison with developing tissues, with almost all identified methylation sites exhibiting complete or partial methylation (Hebras *et al.*, 2020). A similar phenomenon is noted in zebrafish development, where adult tissues display fully methylated rRNA sites compared to developing tissues (Ramachandran *et al.*, 2020).

2'-O-Me exhibits tissue specificity, as evidenced in mouse brains (Häfner *et al.*, 2023); however, some studies suggest a lack of tissue-specific methylation in mice (Hebras *et al.*, 2020). Distinct patterns of 2'-O-Me have been identified across various breast cancer subtypes and in comparison to normal breast tissue (Marcel *et al.*, 2020). A study of different subtypes

of diffuse large B-cell lymphoma (DLBCL) indicated reduced rRNA 2'-O-Me levels in the more aggressive activated B-cell-like (ABC) subtype, as opposed to the germinal centre B-cell-like (GCB) subtype (Krogh *et al.*, 2020). Furthermore, variations in 2'-O-Me are found among various subtypes of breast cancer (Marcel *et al.*, 2020). For instance, Arl2-silenced MCF7 cells, which display more aggressive phenotypes, show increased rates of rRNA methylation (Belin *et al.*, 2009). These findings highlight that 2'-O-Me modifications exhibit subtype-specific variation in both breast cancer and hematologic malignancies.

The regulation of 2'-O-Me is also responsive to environmental cues. For example, treating HeLa cells with etoposide induced 2'-O-Me in 28S rRNA at two specific sites, A2388 and G3923 (Burke *et al.*, 2019). Additionally, exposure to hypoxia altered the pattern of 2'-O-Me in MCF10A and T47D cells (Metge *et al.*, 2021). The modulation of oncogenes and tumor suppressor genes can affect 2'-O-Me levels. For instance, changes in p53 levels impact the extent of 2'-O-Me (Marcel *et al.*, 2013; Sharma *et al.*, 2017) and c-Myc (Jansson *et al.*, 2021). An increase in c-Myc expression enhances 2'-O-Me at certain sites while decreasing it at others, such as 28S: U2031 and 28S: U2402 (Jansson *et al.*, 2021). The inactivation of p53 also leads to site-specific changes in the rRNA methylation pattern (Marcel *et al.*, 2013).

The modulation of snoRNA expression or the formation of snoRNP complexes significantly impacts the pattern of 2'-O-Me. This topic has been extensively reviewed (Barros-Silva *et al.*, 2021). RNA-binding proteins such as FMRP interact with snoRNAs and influence 2'-O-Me patterns in hESCs and human neural progenitor cells (hNPCs) (D'Souza *et al.*, 2018). During leukemogenesis, the interaction of AES with DDX21 facilitates the formation of the snoRNP complex, thereby promoting 2'-O-Me (F. Zhou *et al.*, 2017). NPM1 is also involved in promoting snoRNP formation and enhancing 2'-O-Me levels (Nachmani *et al.*, 2019). Additionally, the interaction of a component of snoRNP, FBL, with EZH2 promotes 2'-O-Me by facilitating snoRNP assembly (Yi *et al.*, 2021).

8.1.2 2'-O-Me modulates various cellular processes

Numerous studies underscore the significance of 2'-O-Me modifications in maintaining the conformational stability and structural integrity of rRNA (Monaco *et al.*, 2018; K. I. Zhou *et al.*, 2024). However, the implications of 2'-O-Me on various cellular processes continue to prompt considerable interest as it has a crucial role in various cellular processes, including differentiation, maintaining cell identity and self-renewal potential, stemness, cell growth and proliferation in various physiological and pathological conditions.

Even minor changes in 2'-O-Me, such as at a single site, can significantly affect cellular phenotype. For instance, the loss of methylation at site 28S: U3904 in hESCs (human embryonic stem cells) led to their differentiation towards a neuroectoderm fate by activating the canonical WNT pathway (Häfner *et al.*, 2023). Additionally, a FBL-induced increase in rRNA methylation at site 18S: G1447 in AML was shown to elevate the leukemic stem cell (LSC) phenotype and promote tumor progression (F. Zhou *et al.*, 2023). Lastly, induction of c-Myc led to increased methylation at site 18S C174, resulting in reduced proliferation (Jansson *et al.*, 2021).

The oncoproteins also bring about phenotypic changes by modulating 2'-O-Me. The AML-ETO9a fusion oncoprotein promotes stem cell activity and self-renewal potential by inducing 2'-O-Me, which promotes leukemogenesis (F. Zhou *et al.*, 2017). Further, promoting 2'-O-Me by NPM1 is a prerequisite for cell growth, differentiation, and maintaining hematopoietic stem cells (Nachmani *et al.*, 2019).

2'-O-Me of rRNA influences the translational capability and translational fidelity of the ribosomes. Specifically, IRES-mediated translation is affected by the alteration of 2'-O-Me. For example, in Arl2-silenced MCF7 cells, those exhibiting more aggressive phenotypes showed increased rates of rRNA methylation, a decrease in IRES-mediated translation

initiation, and a reduced translation fidelity (Belin *et al.*, 2009). Furthermore, modulation of FBL activity by RPL13a results in decreased IRES-mediated translation initiation. The loss of 2'-O-Me disrupts 80S ribosomal complex assembly, leading to the reduced expression of several IRES-regulated genes, including c-Myc, p27, p53, SNAT2, CAT1, and c-Src (Basu *et al.*, 2011). In contrast, increased expression of FBL due to p53 knockdown in HCT116 cells resulted in decreased translational fidelity and increased IRES-mediated translational initiation of IGF1R, MYC, FGF1, FGF2, and VEGFA mRNAs (Marcel *et al.*, 2013). Additionally, the depletion of FBL in HeLa cells led to a site-specific alteration in 2'-O-Me, which led to a decrease in IRES-dependent translation of FGF1 and IGF-1R, although translation of VEGFA remained unaffected (Erales *et al.*, 2017). Silencing of EZH2 resulted in a site-specific reduction of 2'-O-Me, which in turn led to reduced IRES-mediated translation, indicating that EZH2 enhances IRES-mediated translation by promoting 2'-O-Me. The IRES activity of mRNA, such as XIAP, c-MYC and IGF1R, is modulated upon EZH2 depletion (Yi *et al.*, 2021). When MCF10A and T47D cells were exposed to hypoxia, the pattern of 2'-O-Me was altered, resulting in the initiation of IRES-mediated translation of VEGF-C mRNA (Metge *et al.*, 2021).

2'-O-Me alters the translation in a codon-dependent manner. The increase in c-Myc-induced methylation at site 18S C174 has been shown to reduce mRNA translation that contains codons, which are rich in AU (Jansson *et al.*, 2021). Furthermore, an increase in rRNA methylation at 18S, induced by FBL, enhances the Leukemic stem cell phenotype and promotes tumor progression by shifting the translation program toward amino acid mRNAs that feature optimal codons (F. Zhou *et al.*, 2023). 2'-O-Me also affects the interactome and regulates translation. The ribosomal interactome changes when 2'-O-Me levels are altered. For example, the binding of the FMRP protein to ribosomes is enhanced when there is a reduction in 28S:U3904 methylation, which may hinder translational elongation (Häfner *et al.*, 2023). In another

instance, reduced binding of eIF1 results in translational infidelity (Khoshnevis *et al.*, 2022). The impact of 2'-O-Me on translation can be both inhibitory and stimulatory. The depletion of FBL in HCT116 cells leads to a global decrease in methylation; however, specific sites are affected more significantly. One such site is in the 18S rRNA (Gm1447), along with 14 specific sites on the 28S rRNA: Am 1326 (1313), Cm 1340 (1327), Gm 1522 (1509), Cm 1881 (1868), Am 2401 (2388), Um 2415 (2402), Am 2787 (2774), Cm 2824 (2811), Cm 3701 (3680), Gm 3744 (3723), Um 3925 (3904), Gm 4042 (4020), Gm 4618 (4588), and Um 4620 (4590) (Sharma *et al.*, 2017). Similarly, the knockdown (KD) of FBL reduced 2'-O-Me at sites 18S G1447 and 28S G2863, as noted in G4560, G4588, and G3723 (F. Zhou *et al.*, 2023).

Current literature suggests that rRNA 2'-O-Me is influenced by changes in the microenvironment. For instance, a hypoxic environment has been shown to impact 2'-O-Me levels (Metge *et al.*, 2021). Consistent with this research, our findings indicate that rRNA 2'-O-Me increases during the formation of multicellular spheroids, which also contain a hypoxic core that promotes this modification. Furthermore, enhanced 2'-O-Methylation promotes cell fate transitions and stemness. FBL is essential for maintaining stemness in mouse embryonic stem cells, and our results support this observation. We found that FBL levels increase during spheroid-induced plasticity.

8.2 SUMOylation status of FBL regulates cellular plasticity

The comprehensive transcriptomic profiling of several breast cancer cell lines categorizes them into luminal (e.g., BT474, MCF7, SKBr3) and basal (e.g., MDA-MB-231, BT549) subtypes, with basal subtypes expressing enhanced EMT-associated transcription factors (Blick *et al.*, 2008). We therefore envisioned that comparing different breast cancer cell lines could delineate the mechanisms driving EMT. Since upregulated ribosome biogenesis is the hallmark of the EMT, we asked if the nucleolar prominence varies among the different

breast cancer subtypes and if it could be a cause of EMT. Using immunofluorescence analysis with FBL as a nucleolar marker we found that the nucleolar prominence differs across various breast cancer subtypes using FBL as a nucleolar marker. Among the studied cell lines, luminal cells BT474 and MCF7 each exhibited an average of 2 and 3 nucleoli, respectively; HER2+ve SKBr3 displayed two large, prominent nucleoli; while basal cells MDA-MB-231 revealed 5 to 6 nucleoli. Moreover, immunohistochemistry analysis indicated that ER+ve tissues had single, inconspicuous nucleoli, contrasting with TNBC tissues, which displayed a redistribution of nucleolar protein FBL throughout the nucleoplasm. Through cell viability assays (MTT), we found that TNBC cell subtypes, including BT549 and MDA-MB-231, demonstrated greater resistance to rRNA synthesis inhibition compared to non-TNBC cell lines such as MCF7 and BT474, indicating a heightened basal level of rRNA synthesis. These results suggested a positive correlation between nucleolar prominence (size and number) and rRNA synthesis.

Three-dimensional multicellular spheroids are a well-established model for studying EMT transitions. Accordingly, we employed this system to explore how breast cancer cell lines undergo these changes. Four lines, BT474, SKBr3, MDA-MB-231 and SUM159PT, were evaluated for their capability to form spheroids. Of these, BT474 showed the most efficient spheroid formation, followed by SUM159PT, SKBr3, and MDA-MB-231. Based on its robust spheroid-generating capacity, we selected BT474 for subsequent detailed analyses. To evaluate if the spheroids formed from these breast cancer cells exhibit cellular plasticity, we conducted an immunoblotting analysis to examine the expression levels of EMT-associated transcription factors, Snail and Slug. Spheroids formed by BT474 and SUM159PT cells revealed increased expression of EMT-related transcription factors, suggesting that these spheroids exhibit an EMT phenotype. Next, we assessed whether nucleolar processes were modified during spheroid-induced EMT. We analysed the levels of rRNA transcripts (45S, 18S, 28S, and 5.8S) using qRT-PCR across two different cell lines. The qPCR results indicated a marked increase

in rRNA transcript levels. We further confirmed the rise in rRNA synthesis by evaluating rDNA promoter activity in both cell lines through a Dual luciferase assay. These findings suggested an increase in rDNA promoter activity, indicating elevated rRNA synthesis. Additionally, we examined the rRNA methylation ratio in the two cell lines using a site-specific rRNA methylation assay as described earlier (Belin *et al.*, 2009). The rRNA methylation ratio for site 28S-4198 significantly increased during spheroid-induced epithelial-mesenchymal transition (SEMT) in BT474 and SKBr3 cells. Overall, these results demonstrated a positive correlation between SEMT and nucleolar processes such as rRNA synthesis and 2'-O-methylation.

Given the established function of ribosome biogenesis factor, FBL, in regulating both rRNA synthesis and early steps of rRNA processing and modification, we assessed FBL protein levels during SEMT using immunoblotting techniques. Our findings indicate an increase in FBL during SEMT, which is positively correlated with enhanced rRNA synthesis and rRNA methylation. Notably, during our immunoblotting, we detected higher molecular weight modified forms of FBL, which decreased during SEMT. A similar decline in the proportion of modified forms to free FBL was seen in TNBC MDA-MB-231 cells in comparison with BT474 cells. This led us to suspect potential post-translational modifications of FBL. However, the previously known modifications, such as acetylation (Iyer-Bierhoff *et al.*, 2018) or methylation (Shubina *et al.*, 2020), did not exhibit the shift evident in our analysis. Given that protein SUMOylation is predominantly enriched in the nucleus (Z. Chen *et al.*, 2024), we hypothesised that FBL could be undergoing this type of modification. Our immunoprecipitation analysis indicated that FBL is indeed a SUMO substrate, with decreased SUMOylation observed during SEMT. The SUMOylated form corresponds to the 70 kDa band previously associated with modified FBL. We identified Lysine 131 as a key SUMO site on FBL.

To investigate the SUMOylation mechanism of FBL, we isolated nucleoli from Monolayer and Spheroid cultures of BT474 and conducted Nano-LC MS/MS analysis. We pinpointed CBX4 as a probable SUMO ligase for FBL, as levels of SUMOylated FBL were reduced following the CBX4 depletion. Additionally, we identified RNF4 as the likely E3 ubiquitin ligase for FBL. Depleting RNF4, an E3 ligase for SUMOylated proteins, caused SUMOylated FBL to accumulate. RNF4 binds to SUMOylated substrates and is subjected to degradation (autoubiquitination). Our immunoblotting revealed that RNF4 levels rise upon CBX4 depletion, suggesting RNF4's responsiveness to CBX4-mediated SUMOylation. We further noted that the SUMOylation-defective mutant of FBL diffuses throughout the nucleoplasm. Similarly, CBX4 depletion promoted the redistribution of FBL, while RNF4 depletion reduced this effect, indicating that SUMOylation of FBL helps maintain its compartmentalization between the nucleus and nucleolus. We then correlated FBL's SUMOylation status with nucleolar functions and EMT. Lentiviral-mediated FBL KD, followed by rescue experiments with the lysine 131 mutant, resulted in higher Snail expression compared to the wild-type protein. Moreover, decreased SUMOylation of FBL following CBX4 KD was associated with increased rRNA synthesis and 2'-O-Me of rRNA, while increased SUMOylation of FBL with RNF4 KD led to diminished rRNA synthesis and 2'-O-Me of rRNA. Collectively, these results imply that FBL undergoes SUMOylation during SEMT and CBX4~RNF4 interplay will balance its expression status.

8.3 SUMOylation modulates ribosome biogenesis

The effect of SUMOylation in the modulation of ribosome biogenesis is recently emerging. Genome-wide binding studies have shown that SUMO is broadly recruited across ribosomal protein genes, rDNA and pro-growth factor genes. The SUMO pathway has been demonstrated to exert both positive and negative regulation on rRNA synthesis (Y. Yang *et al.*, 2024).

SUMOylation promotes the transcription of ribosomal protein genes by recruiting the transcriptional machinery (Chymkowitch *et al.*, 2015). Moreover, the deSUMOylation of Tof2 and Net1 by Ulp2 (a sentrin-specific protease in yeast) enhances the silencing of rDNA (Gillies *et al.*, 2016; J. Liang *et al.*, 2017), with Ulp2 depletion leading to upregulation of snoRNAs, rRNA, ribosomal protein genes, and essential factors for synthesis (Ryu *et al.*, 2018) in yeast. Conversely, disruption of the SUMO pathway has also been shown to promote rRNA synthesis (Luo *et al.*, 2020); for instance, UBC9-mediated inhibition of SUMOylation stimulates rRNA synthesis (Neyret-Kahn *et al.*, 2013). SUMOylation decreases rDNA transcription by reducing the expression of UBF and c-Myc transcription factors (Peng *et al.*, 2019).

Additionally, several nucleolar proteins, such as NPM, Nucleolin, and LAS1L, which are crucial for rRNA synthesis, have been identified as targets of SUMOylation. The proteins in the snoRNP complex, including NOP58, DKC1, and Nhp2, which are essential for rRNA modifications and processing, have also been found to be SUMOylation targets (Westman *et al.*, 2010; Westman & Lamond, 2011). Moreover, proteins in the Rixosome or RNA degradation complex (including LAS1L, MDN1, NOL9, PELP1, SENP3, TEX10, WDR18), which are essential for ribosome biogenesis, are similarly targeted for SUMOylation (Y. Yang *et al.*, 2024). On the other hand, the actions of deSUMOylases maintain the SUMOylation status of these proteins. For instance, NPM, LAS1L, and PELP1, through deSUMOylation by SENP3, facilitate rRNA maturation, nucleolar export, and partitioning from the nucleolus to the nucleoplasm (Y. Yang *et al.*, 2024). Together, the proper balance between SUMOylation and deSUMOylation of RBFs is crucial for efficient ribosome synthesis.

In this study, we identify FBL, a ribosome biogenesis factor, as a novel target of SUMOylation. The SUMOylation of FBL is crucial for its compartmentalisation between the nucleus and the nucleolus. FBL acts as the enzymatic component of the snoRNP particle, which

also includes proteins such as NOP58, NOP56, and 15.5K. While it is already known that the SUMOylation of NOP58 is essential for rRNA modification and processing, our findings reveal a negative correlation between the SUMOylation of FBL and both rRNA synthesis and modification. This suggests that the SUMOylation of ribosome biogenesis factors may have differing effects on their functions.

8.4 Role of CBX4 in EMT

High expression of CBX4 is positively correlated with EMT in hepatocellular carcinoma (HCC) (Wang *et al.*, 2021) and papillary thyroid cancer cells (H. Yu *et al.*, 2024). CBX4 regulates the β -catenin signalling at the transcriptional and translational levels. Depletion of CBX4 in airway epithelial cells reduces β -catenin SUMOylation, which results in its membrane localization and repression of Wnt/ β catenin signalling, indicating that CBX4 activates β -Catenin (Deng *et al.*, 2024). In lung adenocarcinoma cells, depletion of CBX4 reduced the expression of β -catenin and its downstream targets c-Myc and cyclinD1, indicating a positive correlation between these two (Z. Wang *et al.*, 2021). In osteosarcoma cells, a reduction in CBX4 levels decreased the Wnt/ β -catenin pathway (Deng *et al.*, 2024). In gastric cancer cells, enhanced levels of CBX4 are correlated to an increase in Wnt/ β -catenin signalling and HIF1 α pathways (Fang & Pan, 2022). In laryngeal carcinoma, Circular RNA has_circ_PVT1 mediated an increase in CBX4, which is correlated with an increase in Wnt4/ β -catenin signalling (F. Yu *et al.*, 2021). In breast cancer, CBX4 promotes Notch1 signalling by repressing the promoter expression of miR-137 in a mechanism dependent on PRC1 (Zeng *et al.*, 2018). Further, CBX4 is also known to regulate Notch signalling by interacting with LIM domain protein KyoT2, which is a suppressor of Notch signalling (H. Qin *et al.*, 2005). In gastric cancer, high expression of CBX4 is correlated to Notch, Hippo and pluripotency-associated pathways (Pan *et al.*, 2021). SUMOylation of YAP1 by CBX4 abolishes its phosphorylation, leading to YAP1

retention in the nucleus, inactivating the Hippo pathway and preventing cellular senescence (Gu *et al.*, 2024). CBX4 alleviates the transcriptional repression of E-cadherin expression by SUMOylating SIP1 or ZEB2. This modification hinders the recruitment of CtBP to the promoter due to the SUMOylation of SIP1, thus inhibiting EMT (Long *et al.*, 2005). In contrast, the SUMOylation of hTERT by CBX4 facilitates the retention of the hTERT/ZEB1 complex on the E-cadherin promoter, enhancing its repression and ultimately promoting EMT (Sanyal *et al.*, 2020). HIF1 α promotes EMT by promoting the expression of EMT-associated transcription factors. CBX4 promotes HIF1 α -mediated EMT. Interaction of CBX4 with HIF1 α enhances its transcriptional activity in osteosarcoma cells (Z. Zhang *et al.*, 2020). CBX4-mediated SUMOylation of HIF1 α enhances its transcriptional activity and promotes the expression of VEGF in hepatocellular carcinoma (J. Li *et al.*, 2014). Further HIF1 α SUMOylation by CBX4 is essential for differentiation to TH9 cells, which promotes asthma (W. Huang *et al.*, 2023). RNF180-mediated ubiquitination of CBX4 in Osteosarcoma inhibited EMT, concluding that CBX4 is the promoter of EMT (Zhao *et al.*, 2023).

We have identified CBX4 as a potential SUMO ligase for FBL. Previous studies suggest that CBX4 interacts with FBL and maintains nucleolar homeostasis by preserving the heterochromatin state of the nucleolus, thus preventing the senescence of mesenchymal stem cells (MSCs). Depletion of CBX4 in MSCs enhances rRNA biogenesis and promotes cellular senescence (Ren *et al.*, 2019). These findings align with observations, demonstrating that the depletion of CBX4 in BT474 cells also enhances ribosome biogenesis and rRNA methylation. This suggests that CBX4 may act as a negative regulator of rRNA synthesis. Additionally, FBL enhances the transcription of rDNA by preventing the accumulation of nucleosomes in the presence of RNA polymerase I (Tessarz *et al.*, 2014). This raises the possibility that the SUMO-mediated recruitment of FBL by CBX4 may play a role in regulating rRNA synthesis.

Our comparative mass spectrometry analysis of the nucleolar proteome isolated from monolayer and spheroid conditions reveals dynamic localisation patterns of CBX4 SUMOylation targets. hTERT, a known SUMO substrate of CBX4, is consistently detected in the nucleolus of both monolayer and spheroid cells. In contrast, its interacting partner, ZEB1, appears exclusively in the nucleolus of spheroid cells, with 25.44% sequence coverage. Similarly, β -catenin (22.79% coverage) and HIF1 α (22.76% coverage) are confined to the nucleolus of spheroid cells, suggesting a context-dependent nucleolar localization linked to the EMT associated with CBX4 function.

8.5 Role of RNF4 in EMT

RNF4 falls into the category of non-oncogene addiction genes. The genes encoded by these are not tumorigenic but maintain the tumor phenotypes. They are not essential for normal cell survival and can serve as excellent drug targets. RNF4 is an STUBL that mediates the proteasomal degradation of SUMOylated proteins and is also a transcriptional coregulator. Depletion of RNF4 leads to the accumulation of SUMOylated c-Myc in U2OS cells. RNF4 binds to multi-SUMOylated c-Myc and ubiquitinates it, and then subjects it to proteasomal degradation (González-Prieto *et al.*, 2015). RNF4 also promotes the stabilisation of oncoproteins such as β -catenin, c-Myc, c-Jun, and N-ICD, in a phosphorylation-dependent manner with its ARM motif in breast cancer cells. The activation of the Wnt/ β -catenin pathway enhances c-Myc expression. In turn, c-Myc increases RNF4 expression, as it is a direct target of both c-Myc and Wnt/ β -catenin signalling. RNF4 promotes Wnt signalling by inactivating its corepressor, Groucho/TLE, and stabilising the coactivator β -catenin along with its target, c-Myc, through phosphorylation and ubiquitination. The positive feedback loop established by this mechanism contributes to maintaining the phenotype of the cell (Thomas *et al.*, 2016). Similarly, the activation of the Notch signalling pathway stimulates c-Myc expression, which

subsequently boosts RNF4 expression. RNF4 stabilizes N-ICD and c-Myc by ubiquitinating them in a phosphorylation-dependent manner. A positive feedback loop created by this action also upholds the phenotype of the cell (Thomas *et al.*, 2016). Inversely, HES1 degradation by RNF4 in a RASSF1A-dependent manner disrupts the Notch signalling pathway (Papaspapopoulos *et al.*, 2022). In colorectal cancer, RNF4 activates the Wnt β -catenin pathway, and its depletion in colorectal cancer cells reduces the mRNA levels of Snail, Slug, Twist, and Vimentin. In contrast, the E-Cadherin mRNA levels increased, which decreased metastasis (J. Chen *et al.*, 2024) and also Wnt/ β -catenin signalling (Y. Wu *et al.*, 2025).

PARIS functions as a transcription factor that controls rRNA synthesis (H. Kang & Shin, 2015), and the SUMOylation of PARIS has been shown to inhibit rRNA transcription. Additionally, the degradation of SUMOylated PARIS by RNF4 promotes rRNA biogenesis (Nishida & Yamada, 2020). Our results support this notion; the knockdown of RNF4 resulted in reduced rRNA biogenesis and decreased 2'-O-Me. Furthermore, RNF4 has been established as a transcriptional coregulator and can act both as a transcriptional activator and repressor of global transcription. Our findings indicate that RNF4 knockdown led to a decrease in levels of free FBL. This reduction in FBL levels could be due to a decrease in global transcription. Therefore, RNF4 might promote rRNA synthesis by enhancing the expression of key factors at both the transcriptional and translational levels.

8.6 Future directions

Our study has unveiled a crucial regulatory axis involving RNF4, FBL, and CBX4 in the nucleolus. Building on these findings, we propose several avenues for future investigation:

1. Reciprocal Regulation: FBL, c-Myc, and p53: FBL is transcriptionally modulated by c-Myc (activator) and p53 (repressor), with both transcripts containing IRES elements that facilitate cap-independent translation. Literature indicates that low FBL enhances p53 IRES

translation, whereas elevated FBL supports c-Myc IRES translation, mediated through FBL's 2'-O-Me of rRNA. Investigating how SUMOylation of FBL influences this IRES-dependent feedback loop could significantly deepen our understanding of translational control in cellular stress and proliferation contexts.

2. SUMO/Ubiquitin Coordination: CBX4 and RNF4: CBX4 and RNF4 share several substrates—c-Myc, β -catenin, and KAP1 (Trim28), suggesting coordinated regulation through SUMOylation (by CBX4) and ubiquitin-mediated turnover (by RNF4). Exploring how this coordination impacts nucleolar and genomic stability could unearth new layers of post-translational control.

3. β -Catenin's Impact on Nucleolar Structure: β -Catenin is essential for nucleolar maintenance. Its SUMO1-mediated SUMOylation by CBX4 has been shown to trigger its nuclear signalling, while RNF4 promotes β -catenin stability via phosphorylation-dependent ubiquitination. Dissecting how both modifications regulate β -catenin's role in RNA synthesis and nucleolar integrity is a compelling future direction.

4. c-Myc in Ribosomal Biogenesis: c-Myc is a master regulator of rRNA transcription. Its activity is modulated by SUMOylation and phosphorylation-dependent RNF4 ubiquitination. Considering that CBX4 enhances c-Myc via β -catenin/Wnt signalling, and RNF4 stabilizes c-Myc, deciphering how these modifications influence ribosomal biogenesis and cancer progression represents an exciting next step.

5. RNF4's Role in the CBX4–FBL–KAP1 Complex: CBX4, FBL, and KAP1 form a nucleolar complex essential for heterochromatin maintenance. Our identification of FBL as an RNF4 substrate, alongside literature showing KAP1's RNF4 dependency, suggests that RNF4 may disrupt this complex, with broad implications for nucleolar function and epigenetic

regulation. Investigating this interplay could advance our understanding of nucleolar stress responses and breast cancer cell plasticity.

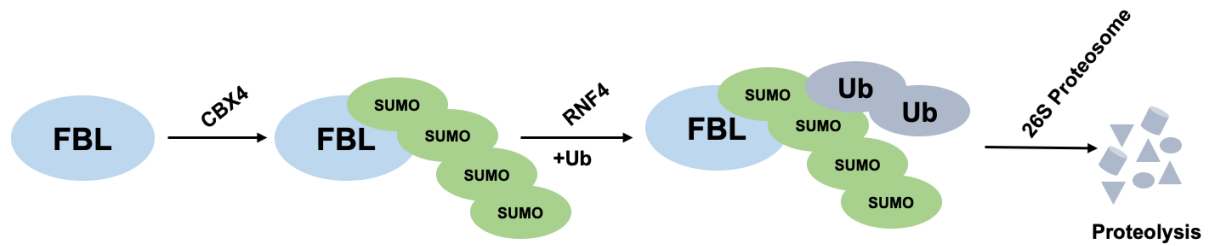
8.7 Limitations

Our findings highlight a previously underexplored aspect of SUMOylation of FBL in SEMT. However, several limitations should be acknowledged. First, while knockdown of RNF4 led to the accumulation of FBL, suggesting a potential regulatory interaction, the underlying mechanism remains unverified, as no ubiquitination assay was performed to determine whether the accumulation resulted from impaired proteasomal degradation or increased stability. Second, because knockdown of FBL caused significant cell death, in vivo validation through animal models was not feasible, limiting the extrapolation of these findings to physiological systems. Third, although enhanced nucleolar size under stress conditions was interpreted as a consequence of increased heterochromatin formation and FBL's known association with heterochromatin, this conclusion remains correlative, as no chromatin immunoprecipitation (ChIP) assay was conducted to directly confirm chromatin binding. Together, these limitations underscore the need for future mechanistic studies using ChIP, proteasomal inhibition assays, and inducible in vivo models to further substantiate and expand upon the findings presented here.

9 Conclusion

We found that the number and size of nucleoli vary among different breast cancer subtypes, with TNBC cells demonstrating a significant increase in the number and size (prominence) of nucleoli compared to non-TNBC cells. A positive correlation exists between the increase in number and size and rRNA synthesis, consistent with previous findings. By utilising a multicellular spheroid model to study epithelial-mesenchymal transition (EMT), we call it SEMT, we observed a positive correlation among rRNA synthesis, 2'-O-methylation, and EMT. The nucleolar protein FBL, which regulates ribosome biogenesis at multiple stages, influences rRNA synthesis by maintaining the heterochromatin structure of ribosomal DNA (rDNA) and regulating rRNA processing and methylation. Here, we found that FBL undergoes post-translational modification through SUMO, and its SUMOylation decreases during spheroid-induced EMT (SEMT). We identified CBX4 as a potential E3 SUMO ligase and STUbL, RNF4 as its E3 ubiquitin ligase, which together maintain the SUMOylation status of FBL, crucial for its compartmentalisation between the nucleus and nucleolus. This varying SUMOylation status of FBL across different cellular phenotypes probably contributes to cellular plasticity (EMT), promoting tumor heterogeneity (**Figure 27**).

A



B

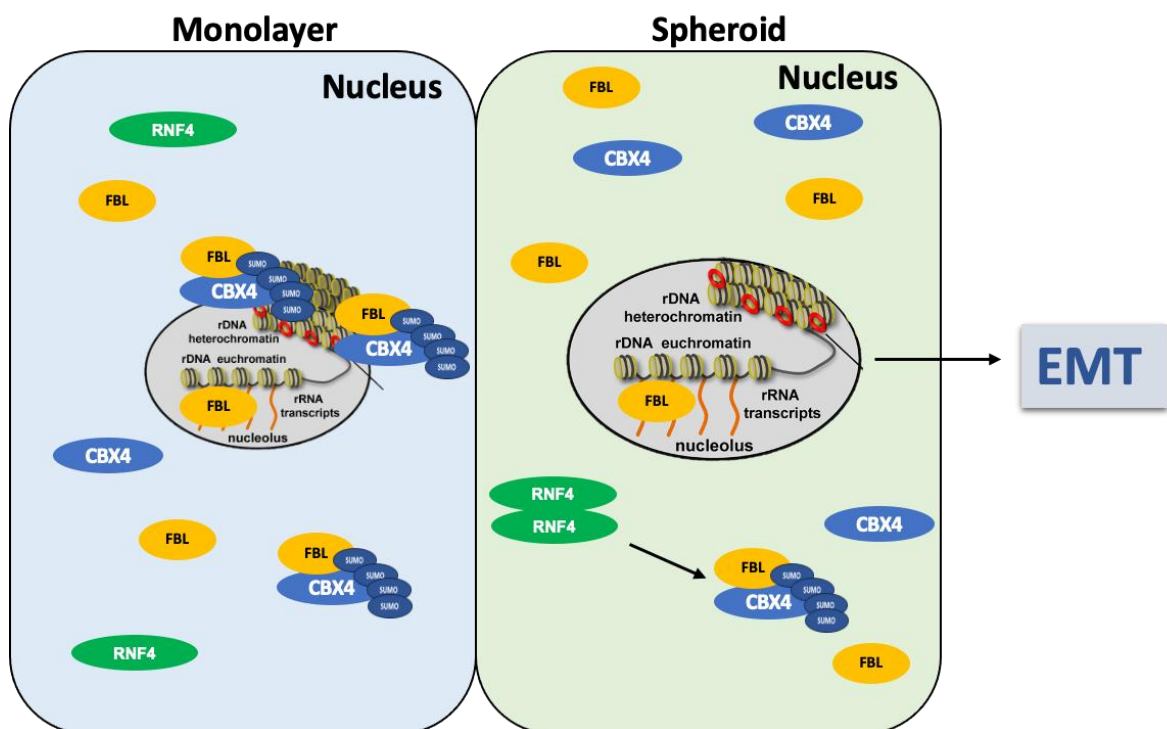


Figure 27 A proposed model illustrating the differential SUMOylation of FBL in monolayer and spheroid conditions and its impact on nucleolar functions. A) This schematic diagram shows the regulation of SUMOylation status of FBL by the E3 SUMO ligase CBX4 and the E3 ubiquitin ligase RNF4. B) In monolayer cells, the interaction between CBX4 and FBL promotes the SUMOylation of FBL, allowing it to localize to the nucleolar heterochromatin, which maintains basal rRNA synthesis. In spheroid cells, the activation of the RNF4 pathway results in the maintenance of the deSUMOylated form of FBL. This reduced SUMOylation of FBL probably inhibits its localization to heterochromatin, ultimately enhancing rRNA synthesis and rRNA methylation and promoting epithelial-mesenchymal transition (EMT). Ub, Ubiquitin.

10 Bibliography

- Adams, J. M., & Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*, 26(9), 1324–1337. <https://doi.org/10.1038/sj.onc.1210220>
- Aguilar-Martinez, E., Guo, B., & Sharrocks, A. D. (2016). RNF4 interacts with multiSUMOylated ETV4. *Wellcome Open Research*, 1, 3. <https://doi.org/10.12688/wellcomeopenres.9935.2>
- Aguirre-Ghiso, J. A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. *Nature Reviews Cancer*, 7(11), 834–846. <https://doi.org/10.1038/nrc2256>
- Allemani, C., Matsuda, T., Di Carlo, V., Harewood, R., Matz, M., Nikšić, M., Bonaventure, A., Valkov, M., Johnson, C. J., Estève, J., Ogunbiyi, O. J., Azevedo E Silva, G., Chen, W.-Q., Eser, S., Engholm, G., Stiller, C. A., Monnereau, A., Woods, R. R., Visser, O., ... Lewis, C. (2018). Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): Analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. *The Lancet*, 391(10125), 1023–1075. [https://doi.org/10.1016/S0140-6736\(17\)33326-3](https://doi.org/10.1016/S0140-6736(17)33326-3)
- Amaravadi, R. K., & Thompson, C. B. (2007). The Roles of Therapy-Induced Autophagy and Necrosis in Cancer Treatment. *Clinical Cancer Research*, 13(24), 7271–7279. <https://doi.org/10.1158/1078-0432.CCR-07-1595>
- Amit, I., Citri, A., Shay, T., Lu, Y., Katz, M., Zhang, F., Tarcic, G., Siwak, D., Lahad, J., Jacob-Hirsch, J., Amariglio, N., Vaisman, N., Segal, E., Rechavi, G., Alon, U., Mills, G. B., Domany, E., & Yarden, Y. (2007). A module of negative feedback regulators defines growth factor signaling. *Nature Genetics*, 39(4), 503–512. <https://doi.org/10.1038/ng1987>
- Aris, J. P., & Blobel, G. (1991). cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proceedings of the National Academy of Sciences*, 88(3), 931–935. <https://doi.org/10.1073/pnas.88.3.931>
- Avitan-Hersh, E., Feng, Y., Oknin Vaisman, A., Abu Ahmad, Y., Zohar, Y., Zhang, T., Lee, J. S., Lazar, I., Sheikh Khalil, S., Feiler, Y., Kluger, H., Kahana, C., Brown, K., Ruppin, E., Ronai, Z. A., & Orian, A. (2020). Regulation of eIF2 α by RNF4 Promotes Melanoma Tumorigenesis and Therapy Resistance. *The Journal of Investigative Dermatology*, 140(12), 2466–2477. <https://doi.org/10.1016/j.jid.2020.04.008>
- Bakr, M. N., Takahashi, H., & Kikuchi, Y. (2022). Analysis of Melanoma Gene Expression Signatures at the Single-Cell Level Uncovers 45-Gene Signature Related to Prognosis. *Biomedicines*, 10(7), 1478. <https://doi.org/10.3390/biomedicines10071478>
- Ban, Y., Zou, Y., Liu, Y., Lee, S., Bednarczyk, R. B., Sheng, J., Cao, Y., Wong, S. T. C., & Gao, D. (2024). Targeting ribosome biogenesis as a novel therapeutic approach to overcome EMT-related chemoresistance in breast cancer. *eLife*, 12, RP89486. <https://doi.org/10.7554/eLife.89486>
- Barkan, D., Green, J. E., & Chambers, A. F. (2010). Extracellular matrix: A gatekeeper in the transition from dormancy to metastatic growth. *European Journal of Cancer*, 46(7), 1181–1188. <https://doi.org/10.1016/j.ejca.2010.02.027>
- Barros-Silva, D., Klavert, J., Jenster, G., Jerónimo, C., Lafontaine, D. L. J., & Martens-Uzunova, E. S. (2021). The role of OncoSnoRNAs and Ribosomal RNA 2'-O-methylation in Cancer. *RNA Biology*, 18(sup1), 61–74. <https://doi.org/10.1080/15476286.2021.1991167>
- Basu, A., Das, P., Chaudhuri, S., Bevilacqua, E., Andrews, J., Barik, S., Hatzoglou, M., Komar, A. A., & Mazumder, B. (2011). Requirement of rRNA Methylation for 80S Ribosome Assembly on a Cohort of Cellular Internal Ribosome Entry Sites. *Molecular and Cellular Biology*, 31(22), 4482–4499. <https://doi.org/10.1128/MCB.05804-11>
- Belin, S., Beghin, A., Solano-González, E., Bezin, L., Brunet-Manquat, S., Textoris, J., Prats, A.-C., Mertani, H. C., Dumontet, C., & Diaz, J.-J. (2009). Dysregulation of ribosome

- biogenesis and translational capacity is associated with tumor progression of human breast cancer cells. *PloS One*, 4(9), e7147. <https://doi.org/10.1371/journal.pone.0007147>
- Bierie, B., & Moses, H. L. (2006). TGF β : The molecular Jekyll and Hyde of cancer. *Nature Reviews Cancer*, 6(7), 506–520. <https://doi.org/10.1038/nrc1926>
- Birch, J., & Gil, J. (2020). Senescence and the SASP: Many therapeutic avenues. *Genes & Development*, 34(23–24), 1565–1576. <https://doi.org/10.1101/gad.343129.120>
- Blasco, M. A. (2005). Telomeres and human disease: Ageing, cancer and beyond. *Nature Reviews Genetics*, 6(8), 611–622. <https://doi.org/10.1038/nrg1656>
- Blick, T., Widodo, E., Hugo, H., Waltham, M., Lenburg, M. E., Neve, R. M., & Thompson, E. W. (2008). Epithelial mesenchymal transition traits in human breast cancer cell lines. *Clinical & Experimental Metastasis*, 25(6), 629–642. <https://doi.org/10.1007/s10585-008-9170-6>
- Branigan, E., Plechanovová, A., Jaffray, E. G., Naismith, J. H., & Hay, R. T. (2015). Structural basis for the RING-catalyzed synthesis of K63-linked ubiquitin chains. *Nature Structural & Molecular Biology*, 22(8), 597–602. <https://doi.org/10.1038/nsmb.3052>
- Braunholz, D., Saki, M., Niehr, F., Öztürk, M., Borràs Puértolas, B., Korschak, R., Budach, V., & Tinhofer, I. (2016). Spheroid Culture of Head and Neck Cancer Cells Reveals an Important Role of EGFR Signalling in Anchorage Independent Survival. *PloS One*, 11(9), e0163149. <https://doi.org/10.1371/journal.pone.0163149>
- Bray, S. J. (2016). Notch signalling in context. *Nature Reviews Molecular Cell Biology*, 17(11), 722–735. <https://doi.org/10.1038/nrm.2016.94>
- Brighenti, E., Giannone, F. A., Fornari, F., Onofrillo, C., Govoni, M., Montanaro, L., Treré, D., & Derenzini, M. (2016). Therapeutic dosages of aspirin counteract the IL-6 induced pro-tumorigenic effects by slowing down the ribosome biogenesis rate. *Oncotarget*, 7(39), 63226–63241. <https://doi.org/10.18632/oncotarget.11441>
- Briscoe, J., & Théron, P. P. (2013). The mechanisms of Hedgehog signalling and its roles in development and disease. *Nature Reviews Molecular Cell Biology*, 14(7), 416–429. <https://doi.org/10.1038/nrm3598>
- Burke, M. F., McLaurin, D. M., Logan, M. K., & Hebert, M. D. (2019). Alteration of 28S rRNA 2'-O-methylation by etoposide correlates with decreased SMN phosphorylation and reduced Droscha levels. *Biology Open*, 8(3), bio041848. <https://doi.org/10.1242/bio.041848>
- Bywater, M. J., Pearson, R. B., McArthur, G. A., & Hannan, R. D. (2013). Dysregulation of the basal RNA polymerase transcription apparatus in cancer. *Nature Reviews Cancer*, 13(5), 299–314. <https://doi.org/10.1038/nrc3496>
- Cabrita, M. A., & Christofori, G. (2008). Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis*, 11(1), 53–62. <https://doi.org/10.1007/s10456-008-9089-1>
- Carlsson, J., Nilsson, K., Westermarck, B., Pontén, J., Sundström, C., Larsson, E., Bergh, J., Pahlman, S., Busch, C., & Collins, V. P. (1983). Formation and growth of multicellular spheroids of human origin. *International Journal of Cancer*, 31(5), 523–533. <https://doi.org/10.1002/ijc.2910310502>
- Chen, J., Li, Z.-Y., Zheng, G., Cao, L., Guo, Y.-M., Lian, Q., Gu, B., & Yue, C.-F. (2024). RNF4 mediated degradation of PDHA1 promotes colorectal cancer metabolism and metastasis. *Npj Precision Oncology*, 8(1), 258. <https://doi.org/10.1038/s41698-024-00724-5>
- Chen, Z., Luo, J., Zhang, Y., Zheng, S., Zhang, H., Huang, Y., Wong, J., & Li, J. (2024). SUMOylation is enriched in the nuclear matrix and required for chromosome

- segregation. *Journal of Biological Chemistry*, 300(1), 105547. <https://doi.org/10.1016/j.jbc.2023.105547>
- Choe, C., Kim, H., Min, S., Park, S., Seo, J., & Roh, S. (2018). SOX2, a stemness gene, induces progression of NSCLC A549 cells toward anchorage-independent growth and chemoresistance to vinblastine. *OncoTargets and Therapy*, Volume 11, 6197–6207. <https://doi.org/10.2147/OTT.S175810>
- Chymkowitch, P., P, A. N., Aanes, H., Koehler, C. J., Thiede, B., Lorenz, S., Meza-Zepeda, L. A., Klungland, A., & Enserink, J. M. (2015). Sumoylation of Rap1 mediates the recruitment of TFIID to promote transcription of ribosomal protein genes. *Genome Research*, 25(6), 897–906. <https://doi.org/10.1101/gr.185793.114>
- Collado, M., & Serrano, M. (2010). Senescence in tumours: Evidence from mice and humans. *Nature Reviews Cancer*, 10(1), 51–57. <https://doi.org/10.1038/nrc2772>
- Cowin, P., & Wysolmerski, J. (2010). Molecular Mechanisms Guiding Embryonic Mammary Gland Development. *Cold Spring Harbor Perspectives in Biology*, 2(6), a003251–a003251. <https://doi.org/10.1101/cshperspect.a003251>
- Cunha, G. R., Young, P., Christov, K., Guzman, R., Nandi, S., Talamantes, F., & Thordarson, G. (1995). Mammary Phenotypic Expression Induced in Epidermal Cells by Embryonic Mammary Mesenchyme. *Cells Tissues Organs*, 152(3), 195–204. <https://doi.org/10.1159/000147698>
- Dagogo-Jack, I., & Shaw, A. T. (2018). Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews Clinical Oncology*, 15(2), 81–94. <https://doi.org/10.1038/nrclinonc.2017.166>
- Dai, Z.-Y., Jin, S.-M., Luo, H.-Q., Leng, H.-L., & Fang, J.-D. (2021). LncRNA HOTAIR regulates anoikis-resistance capacity and spheroid formation of ovarian cancer cells by recruiting EZH2 and influencing H3K27 methylation. *Neoplasma*, 68(03), 509–518. https://doi.org/10.4149/neo_2021_201112N1212
- Das, R., & Vonderhaar, B. K. (1997). [No title found]. *Journal of Mammary Gland Biology and Neoplasia*, 2(1), 29–39. <https://doi.org/10.1023/A:1026369412612>
- Davies, M. A., & Samuels, Y. (2010). Analysis of the genome to personalize therapy for melanoma. *Oncogene*, 29(41), 5545–5555. <https://doi.org/10.1038/onc.2010.323>
- DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., & Thompson, C. B. (2008). The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell Metabolism*, 7(1), 11–20. <https://doi.org/10.1016/j.cmet.2007.10.002>
- Deng, Z., Yuan, J., Ma, B., Zhu, J., Yan, B., Wei, J., Jin, X., Li, J., Zhang, Q., & Ma, B. (2024). Ziyuglycoside II, a triterpene glycoside compound in *Sanguisorbae officinalis* l. Extract, suppresses metastasis in osteosarcoma via CBX4-mediated Wnt/ β -catenin signal pathway. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, 132, 155716. <https://doi.org/10.1016/j.phymed.2024.155716>
- Dermitt, M., Dodel, M., Lee, F. C. Y., Azman, M. S., Schwenzer, H., Jones, J. L., Blagden, S. P., Ule, J., & Mardakheh, F. K. (2020). Subcellular mRNA Localization Regulates Ribosome Biogenesis in Migrating Cells. *Developmental Cell*, 55(3), 298–313.e10. <https://doi.org/10.1016/j.devcel.2020.10.006>
- Dhillon, P. K., Mathur, P., Nandakumar, A., Fitzmaurice, C., Kumar, G. A., Mehrotra, R., Shukla, D. K., Rath, G. K., Gupta, P. C., Swaminathan, R., Thakur, J. S., Dey, S., Allen, C., Badwe, R. A., Dikshit, R., Dhaliwal, R. S., Kaur, T., Katak, A. C., Visweswara, R. N., ... Dandona, L. (2018). The burden of cancers and their variations across the states of India: The Global Burden of Disease Study 1990–2016. *The Lancet Oncology*, 19(10), 1289–1306. [https://doi.org/10.1016/S1470-2045\(18\)30447-9](https://doi.org/10.1016/S1470-2045(18)30447-9)

- Di Croce, L., & Helin, K. (2013). Transcriptional regulation by Polycomb group proteins. *Nature Structural & Molecular Biology*, 20(10), 1147–1155. <https://doi.org/10.1038/nsmb.2669>
- DiBello, A., Datta, A. B., Zhang, X., & Wolberger, C. (2016). Role of E2-RING Interactions in Governing RNF4-Mediated Substrate Ubiquitination. *Journal of Molecular Biology*, 428(23), 4639–4650. <https://doi.org/10.1016/j.jmb.2016.09.018>
- Djonov, V., Andres, A. C., & Ziemiecki, A. (2001). Vascular remodelling during the normal and malignant life cycle of the mammary gland. *Microscopy Research and Technique*, 52(2), 182–189. [https://doi.org/10.1002/1097-0029\(20010115\)52:2<182::AID-JEMT1004>3.0.CO;2-M](https://doi.org/10.1002/1097-0029(20010115)52:2<182::AID-JEMT1004>3.0.CO;2-M)
- Dong, M.-M. (2012). Cancer stem-like side population cells in the human nasopharyngeal carcinoma cell line cne-2 possess epithelial mesenchymal transition properties in association with metastasis. *Oncology Reports*. <https://doi.org/10.3892/or.2012.1781>
- Dong, Z.-W., Shao, P., Diao, L.-T., Zhou, H., Yu, C.-H., & Qu, L.-H. (2012). RTL-P: A sensitive approach for detecting sites of 2'-O-methylation in RNA molecules. *Nucleic Acids Research*, 40(20), e157. <https://doi.org/10.1093/nar/gks698>
- Dongre, A., & Weinberg, R. A. (2019). New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nature Reviews Molecular Cell Biology*, 20(2), 69–84. <https://doi.org/10.1038/s41580-018-0080-4>
- Dörner, K., Ruggeri, C., Zemp, I., & Kutay, U. (2023). Ribosome biogenesis factors—From names to functions. *The EMBO Journal*, 42(7), e112699. <https://doi.org/10.15252/emj.2022112699>
- Dou, Z., Lu, F., Hu, J., Wang, H., Li, B., & Li, X. (2023). MicroRNA-6838-5p suppresses the self-renewal and metastasis of human liver cancer stem cells through downregulating CBX4 expression and inactivating ERK signaling. *Biological Chemistry*, 404(1), 29–39. <https://doi.org/10.1515/hsz-2022-0156>
- Drapkin, B. J., & Minna, J. D. (2020). Studying Lineage Plasticity One Cell at a Time. *Cancer Cell*, 38(2), 150–152. <https://doi.org/10.1016/j.ccell.2020.07.001>
- D'Souza, M. N., Gowda, N. K. C., Tiwari, V., Babu, R. O., Anand, P., Dastidar, S. G., Singh, R., James, O. G., Selvaraj, B., Pal, R., Ramesh, A., Chattarji, S., Chandran, S., Gulyani, A., Palakodeti, D., & Muddashetty, R. S. (2018). FMRP Interacts with C/D Box snoRNA in the Nucleus and Regulates Ribosomal RNA Methylation. *iScience*, 9, 399–411. <https://doi.org/10.1016/j.isci.2018.11.007>
- Dzutsev, A., Badger, J. H., Perez-Chanona, E., Roy, S., Salcedo, R., Smith, C. K., & Trinchieri, G. (2017). Microbes and Cancer. *Annual Review of Immunology*, 35(1), 199–228. <https://doi.org/10.1146/annurev-immunol-051116-052133>
- El Hassouni, B., Sarkisjan, D., Vos, J. C., Giovannetti, E., & Peters, G. J. (2019). Targeting the Ribosome Biogenesis Key Molecule Fibrillarin to Avoid Chemoresistance. *Current Medicinal Chemistry*, 26(33), 6020–6032. <https://doi.org/10.2174/0929867326666181203133332>
- Erales, J., Marchand, V., Panthu, B., Gillot, S., Belin, S., Ghayad, S. E., Garcia, M., Laforêts, F., Marcel, V., Baudin-Baillieu, A., Bertin, P., Couté, Y., Adrait, A., Meyer, M., Therizols, G., Yusupov, M., Namy, O., Ohlmann, T., Motorin, Y., ... Diaz, J.-J. (2017). Evidence for rRNA 2'-O-methylation plasticity: Control of intrinsic translational capabilities of human ribosomes. *Proceedings of the National Academy of Sciences of the United States of America*, 114(49), 12934–12939. <https://doi.org/10.1073/pnas.1707674114>
- Essid, N., Chambard, J. C., & Elgaaïed, A. B. (2018). Induction of epithelial-mesenchymal transition (EMT) and Gli1 expression in head and neck squamous cell carcinoma

- (HNSCC) spheroid cultures. *Bosnian Journal of Basic Medical Sciences*, 18(4), 336–346. <https://doi.org/10.17305/bjbms.2018.3243>
- Evan, G. I., & d’Adda Di Fagagna, F. (2009). Cellular senescence: Hot or what? *Current Opinion in Genetics & Development*, 19(1), 25–31. <https://doi.org/10.1016/j.gde.2008.11.009>
- Famta, P., Shah, S., Dey, B., Kumar, K. C., Bagasariya, D., Vambhurkar, G., Pandey, G., Sharma, A., Srinivasarao, D. A., Kumar, R., Guru, S. K., Raghuvanshi, R. S., & Srivastava, S. (2025). Despicable role of epithelial–mesenchymal transition in breast cancer metastasis: Exhibiting de novo restorative regimens. *Cancer Pathogenesis and Therapy*, 3(1), 30–47. <https://doi.org/10.1016/j.cpt.2024.01.001>
- Fang, X., & Pan, A. (2022). MiR-507 inhibits the progression of gastric carcinoma via targeting CBX4-mediated activation of Wnt/ β -catenin and HIF-1 α pathways. *Clinical and Translational Oncology*, 24(10), 2021–2028. <https://doi.org/10.1007/s12094-022-02862-3>
- Fernández-Calero, T., Davyt, M., Perelmuter, K., Chalar, C., Bampi, G., Persson, H., Tosar, J. P., Hafstað, V., Naya, H., Rovira, C., Bollati-Fogolín, M., Ehrlich, R., Flouriot, G., Ignatova, Z., & Marín, M. (2020). Fine-tuning the metabolic rewiring and adaptation of translational machinery during an epithelial-mesenchymal transition in breast cancer cells. *Cancer & Metabolism*, 8(1), 8. <https://doi.org/10.1186/s40170-020-00216-7>
- Ferreira-Cerca, S., Pöll, G., Gleizes, P.-E., Tschochner, H., & Milkereit, P. (2005). Roles of Eukaryotic Ribosomal Proteins in Maturation and Transport of Pre-18S rRNA and Ribosome Function. *Molecular Cell*, 20(2), 263–275. <https://doi.org/10.1016/j.molcel.2005.09.005>
- Fidler, I. J. (2003). The pathogenesis of cancer metastasis: The “seed and soil” hypothesis revisited. *Nature Reviews Cancer*, 3(6), 453–458. <https://doi.org/10.1038/nrc1098>
- Folkman, J., & Moscona, A. (1978). Role of cell shape in growth control. *Nature*, 273(5661), 345–349. <https://doi.org/10.1038/273345a0>
- Friedl, P., & Wolf, K. (2008). Tube Travel: The Role of Proteases in Individual and Collective Cancer Cell Invasion. *Cancer Research*, 68(18), 7247–7249. <https://doi.org/10.1158/0008-5472.CAN-08-0784>
- Froehlich, K., Haeger, J.-D., Heger, J., Pastuschek, J., Photini, S. M., Yan, Y., Lupp, A., Pfarrer, C., Mrowka, R., Schleußner, E., Markert, U. R., & Schmidt, A. (2016). Generation of Multicellular Breast Cancer Tumor Spheroids: Comparison of Different Protocols. *Journal of Mammary Gland Biology and Neoplasia*, 21(3–4), 89–98. <https://doi.org/10.1007/s10911-016-9359-2>
- Fumagalli, C., & Barberis, M. (2021). Breast Cancer Heterogeneity. *Diagnostics*, 11(9), 1555. <https://doi.org/10.3390/diagnostics11091555>
- Galluzzi, L., & Kroemer, G. (2008). Necroptosis: A Specialized Pathway of Programmed Necrosis. *Cell*, 135(7), 1161–1163. <https://doi.org/10.1016/j.cell.2008.12.004>
- Gameiro, P. A., & Struhl, K. (2018). Nutrient Deprivation Elicits a Transcriptional and Translational Inflammatory Response Coupled to Decreased Protein Synthesis. *Cell Reports*, 24(6), 1415–1424. <https://doi.org/10.1016/j.celrep.2018.07.021>
- Gao, W., Wu, D., Wang, Y., Wang, Z., Zou, C., Dai, Y., Ng, C.-F., Teoh, J. Y.-C., & Chan, F. L. (2018). Development of a novel and economical agar-based non-adherent three-dimensional culture method for enrichment of cancer stem-like cells. *Stem Cell Research & Therapy*, 9(1), 243. <https://doi.org/10.1186/s13287-018-0987-x>
- Geiss-Friedlander, R., & Melchior, F. (2007). Concepts in sumoylation: A decade on. *Nature Reviews Molecular Cell Biology*, 8(12), 947–956. <https://doi.org/10.1038/nrm2293>
- Gheytauchi, E., Naseri, M., Karimi-Busheri, F., Atyabi, F., Mirsharif, E. S., Bozorgmehr, M., Ghods, R., & Madjd, Z. (2021). Morphological and molecular characteristics of

- spheroid formation in HT-29 and Caco-2 colorectal cancer cell lines. *Cancer Cell International*, 21(1), 204. <https://doi.org/10.1186/s12935-021-01898-9>
- Ghosh, S., Spagnoli, G. C., Martin, I., Ploegert, S., Demougin, P., Heberer, M., & Reschner, A. (2005). Three-dimensional culture of melanoma cells profoundly affects gene expression profile: A high density oligonucleotide array study. *Journal of Cellular Physiology*, 204(2), 522–531. <https://doi.org/10.1002/jcp.20320>
- Gillies, J., Hickey, C. M., Su, D., Wu, Z., Peng, J., & Hochstrasser, M. (2016). SUMO Pathway Modulation of Regulatory Protein Binding at the Ribosomal DNA Locus in *Saccharomyces cerevisiae*. *Genetics*, 202(4), 1377–1394. <https://doi.org/10.1534/genetics.116.187252>
- González-Prieto, R., Cuijpers, S. A., Kumar, R., Hendriks, I. A., & Vertegaal, A. C. (2015). C-Myc is targeted to the proteasome for degradation in a SUMOylation-dependent manner, regulated by PIAS1, SENP7 and RNF4. *Cell Cycle (Georgetown, Tex.)*, 14(12), 1859–1872. <https://doi.org/10.1080/15384101.2015.1040965>
- Gorgoulis, V., Adams, P. D., Alimonti, A., Bennett, D. C., Bischof, O., Bishop, C., Campisi, J., Collado, M., Evangelou, K., Ferbeyre, G., Gil, J., Hara, E., Krizhanovsky, V., Jurk, D., Maier, A. B., Narita, M., Niedernhofer, L., Passos, J. F., Robbins, P. D., ... Demaria, M. (2019). Cellular Senescence: Defining a Path Forward. *Cell*, 179(4), 813–827. <https://doi.org/10.1016/j.cell.2019.10.005>
- Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, Inflammation, and Cancer. *Cell*, 140(6), 883–899. <https://doi.org/10.1016/j.cell.2010.01.025>
- Gu, Y., Xu, T., Fang, Y., Shao, J., Hu, T., Wu, X., Shen, H., Xu, Y., Zhang, J., Song, Y., Xia, Y., Shu, Y., & Ma, P. (2024). CBX4 counteracts cellular senescence to desensitize gastric cancer cells to chemotherapy by inducing YAP1 SUMOylation. *Drug Resistance Updates*, 77, 101136. <https://doi.org/10.1016/j.drug.2024.101136>
- Gupta, P. B., Pastushenko, I., Skibinski, A., Blanpain, C., & Kuperwasser, C. (2019). Phenotypic Plasticity: Driver of Cancer Initiation, Progression, and Therapy Resistance. *Cell Stem Cell*, 24(1), 65–78. <https://doi.org/10.1016/j.stem.2018.11.011>
- Guttilla, I. K., Phoenix, K. N., Hong, X., Tirnauer, J. S., Claffey, K. P., & White, B. A. (2012). Prolonged mammosphere culture of MCF-7 cells induces an EMT and repression of the estrogen receptor by microRNAs. *Breast Cancer Research and Treatment*, 132(1), 75–85. <https://doi.org/10.1007/s10549-011-1534-y>
- Häfner, S. J., Jansson, M. D., Altinel, K., Andersen, K. L., Abay-Nørgaard, Z., Ménard, P., Fontenas, M., Sørensen, D. M., Gay, D. M., Arendrup, F. S., Tehler, D., Krogh, N., Nielsen, H., Kraushar, M. L., Kirkeby, A., & Lund, A. H. (2023). Ribosomal RNA 2'-O-methylation dynamics impact cell fate decisions. *Developmental Cell*, 58(17), 1593–1609.e9. <https://doi.org/10.1016/j.devcel.2023.06.007>
- Hanahan, D. (2022). Hallmarks of Cancer: New Dimensions. *Cancer Discovery*, 12(1), 31–46. <https://doi.org/10.1158/2159-8290.CD-21-1059>
- Hanahan, D., & Coussens, L. M. (2012). Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment. *Cancer Cell*, 21(3), 309–322. <https://doi.org/10.1016/j.ccr.2012.02.022>
- Hanahan, D., & Folkman, J. (1996). Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis. *Cell*, 86(3), 353–364. [https://doi.org/10.1016/S0092-8674\(00\)80108-7](https://doi.org/10.1016/S0092-8674(00)80108-7)
- Hanahan, D., & Weinberg, R. A. (2000). The Hallmarks of Cancer. *Cell*, 100(1), 57–70. [https://doi.org/10.1016/S0092-8674\(00\)81683-9](https://doi.org/10.1016/S0092-8674(00)81683-9)
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, 144(5), 646–674. <https://doi.org/10.1016/j.cell.2011.02.013>

- Haslam, S. Z. (1989). The Ontogeny of Mouse Mammary Gland Responsiveness to Ovarian Steroid Hormones*. *Endocrinology*, 125(5), 2766–2772. <https://doi.org/10.1210/endo-125-5-2766>
- Hebras, J., Krogh, N., Marty, V., Nielsen, H., & Cavaillé, J. (2020). Developmental changes of rRNA ribose methylations in the mouse. *RNA Biology*, 17(1), 150–164. <https://doi.org/10.1080/15476286.2019.1670598>
- Helpap, B. (1989). Nucleolar grading of breast cancer. *Virchows Archiv A*, 415(6), 501–508. <https://doi.org/10.1007/BF00718643>
- Hori, Y., Shimamoto, A., & Kobayashi, T. (2021). The human ribosomal DNA array is composed of highly homogenized tandem clusters. *Genome Research*, 31(11), 1971–1982. <https://doi.org/10.1101/gr.275838.121>
- Hou, J., Yang, Y., Gao, H., Ouyang, T., Liu, Q., Ding, R., & Kan, H. (2022). Systematic investigation of the clinical significance and prognostic value of the CBXs in esophageal cancer. *Medicine*, 101(40), e30888. <https://doi.org/10.1097/MD.00000000000030888>
- Hou, Y., Ding, Y., Du, D., Yu, T., Zhou, W., Cui, Y., & Nie, H. (2021). Airway Basal Cells Mediate Hypoxia-Induced EMT by Increasing Ribosome Biogenesis. *Frontiers in Pharmacology*, 12, 783946. <https://doi.org/10.3389/fphar.2021.783946>
- Hovey, R. C., Trott, J. F., & Vonderhaar, B. K. (2002). [No title found]. *Journal of Mammary Gland Biology and Neoplasia*, 7(1), 17–38. <https://doi.org/10.1023/A:1015766322258>
- Hsu, P. P., & Sabatini, D. M. (2008). Cancer Cell Metabolism: Warburg and Beyond. *Cell*, 134(5), 703–707. <https://doi.org/10.1016/j.cell.2008.08.021>
- Hu, C., Zhang, Q., Tang, Q., Zhou, H., Liu, W., Huang, J., Liu, Y., Wang, Q., Zhang, J., Zhou, M., Sheng, F., Lai, W., Tian, J., Li, G., & Zhang, R. (2020). CBX4 promotes the proliferation and metastasis via regulating BMI-1 in lung cancer. *Journal of Cellular and Molecular Medicine*, 24(1), 618–631. <https://doi.org/10.1111/jcmm.14771>
- Huang, J., Yang, W., Jiang, K., Liu, Y., Tan, X., & Luo, J. (2022). RNF4 promotes tumorigenesis, therapy resistance of cholangiocarcinoma and affects cell cycle by regulating the ubiquitination degradation of p27kip1 in the nucleus. *Experimental Cell Research*, 419(1), 113295. <https://doi.org/10.1016/j.yexcr.2022.113295>
- Huang, W., Yu, C., Wu, H., Liang, S., Kang, J., Zhou, Z., Liu, A., & Liu, L. (2023). Cbx4 governs HIF-1 α to involve in Th9 cell differentiation promoting asthma by its SUMO E3 ligase activity. *Biochimica Et Biophysica Acta. Molecular Cell Research*, 1870(7), 119524. <https://doi.org/10.1016/j.bbamcr.2023.119524>
- Huang, Z., Zhang, Z., Zhou, C., Liu, L., & Huang, C. (2022). Epithelial-mesenchymal transition: The history, regulatory mechanism, and cancer therapeutic opportunities. *MedComm*, 3(2), e144. <https://doi.org/10.1002/mco2.144>
- Ikushima, H., & Miyazono, K. (2010). TGF β signalling: A complex web in cancer progression. *Nature Reviews Cancer*, 10(6), 415–424. <https://doi.org/10.1038/nrc2853>
- Im, C.-N., Yun, H. H., Yoo, H. J., Park, M.-J., & Lee, J.-H. (2015). Enhancement of SOX-2 expression and ROS accumulation by culture of A172 glioblastoma cells under non-adherent culture conditions. *Oncology Reports*, 34(2), 920–928. <https://doi.org/10.3892/or.2015.4021>
- Incarnato, D., Anselmi, F., Morandi, E., Neri, F., Maldotti, M., Rapelli, S., Parlato, C., Basile, G., & Oliviero, S. (2017). High-throughput single-base resolution mapping of RNA 2'-O-methylated residues. *Nucleic Acids Research*, 45(3), 1433–1441. <https://doi.org/10.1093/nar/gkw810>
- Ivascu, A., & Kubbies, M. (2007). Diversity of cell-mediated adhesions in breast cancer spheroids. *International Journal of Oncology*, 31(6), 1403–1413.

- Iyer-Bierhoff, A., Krogh, N., Tessarz, P., Ruppert, T., Nielsen, H., & Grummt, I. (2018). SIRT7-Dependent Deacetylation of Fibrillarin Controls Histone H2A Methylation and rRNA Synthesis during the Cell Cycle. *Cell Reports*, 25(11), 2946-2954.e5. <https://doi.org/10.1016/j.celrep.2018.11.051>
- Jansson, M. D., Häfner, S. J., Altinel, K., Tehler, D., Krogh, N., Jakobsen, E., Andersen, J. V., Andersen, K. L., Schoof, E. M., Ménard, P., Nielsen, H., & Lund, A. H. (2021). Regulation of translation by site-specific ribosomal RNA methylation. *Nature Structural & Molecular Biology*, 28(11), 889–899. <https://doi.org/10.1038/s41594-021-00669-4>
- Javaid, S., Zhang, J., Anderssen, E., Black, J. C., Wittner, B. S., Tajima, K., Ting, D. T., Smolen, G. A., Zubrowski, M., Desai, R., Maheswaran, S., Ramaswamy, S., Whetstine, J. R., & Haber, D. A. (2013). Dynamic Chromatin Modification Sustains Epithelial-Mesenchymal Transition following Inducible Expression of Snail-1. *Cell Reports*, 5(6), 1679–1689. <https://doi.org/10.1016/j.celrep.2013.11.034>
- Jiang, N., Niu, G., Pan, Y.-H., Pan, W., Zhang, M.-F., Zhang, C. Z., & Shen, H. (2020). CBX4 transcriptionally suppresses KLF6 via interaction with HDAC1 to exert oncogenic activities in clear cell renal cell carcinoma. *EBioMedicine*, 53, 102692. <https://doi.org/10.1016/j.ebiom.2020.102692>
- Jones, R. G., & Thompson, C. B. (2009). Tumor suppressors and cell metabolism: A recipe for cancer growth. *Genes & Development*, 23(5), 537–548. <https://doi.org/10.1101/gad.1756509>
- Kagey, M. H., Melhuish, T. A., & Wotton, D. (2003). The Polycomb Protein Pc2 Is a SUMO E3. *Cell*, 113(1), 127–137. [https://doi.org/10.1016/s0092-8674\(03\)00159-4](https://doi.org/10.1016/s0092-8674(03)00159-4)
- Kang, H. J., Choi, Y. S., Hong, S.-B., Kim, K.-W., Woo, R.-S., Won, S. J., Kim, E. J., Jeon, H. K., Jo, S.-Y., Kim, T. K., Bachoo, R., Reynolds, I. J., Gwag, B. J., & Lee, H.-W. (2004). Ectopic Expression of the Catalytic Subunit of Telomerase Protects against Brain Injury Resulting from Ischemia and NMDA-Induced Neurotoxicity. *The Journal of Neuroscience*, 24(6), 1280–1287. <https://doi.org/10.1523/JNEUROSCI.4082-03.2004>
- Kang, H., & Shin, J.-H. (2015). Repression of rRNA transcription by PARIS contributes to Parkinson's disease. *Neurobiology of Disease*, 73, 220–228. <https://doi.org/10.1016/j.nbd.2014.10.003>
- Kang, Y. P., Yoon, J.-H., Long, N. P., Koo, G.-B., Noh, H.-J., Oh, S.-J., Lee, S. B., Kim, H. M., Hong, J. Y., Lee, W. J., Lee, S. J., Hong, S.-S., Kwon, S. W., & Kim, Y.-S. (2019). Spheroid-Induced Epithelial-Mesenchymal Transition Provokes Global Alterations of Breast Cancer Lipidome: A Multi-Layered Omics Analysis. *Frontiers in Oncology*, 9, 145. <https://doi.org/10.3389/fonc.2019.00145>
- Karnoub, A. E., & Weinberg, R. A. (2007). Chemokine Networks and Breast Cancer Metastasis. *Breast Disease*, 26(1), 75–85. <https://doi.org/10.3233/BD-2007-26107>
- Kenific, C. M., Thorburn, A., & Debnath, J. (2010). Autophagy and metastasis: Another double-edged sword. *Current Opinion in Cell Biology*, 22(2), 241–245. <https://doi.org/10.1016/j.ceb.2009.10.008>
- Keusekotten, K., Bade, V. N., Meyer-Teschendorf, K., Sriramachandran, A. M., Fischer-Schrader, K., Krause, A., Horst, C., Schwarz, G., Hofmann, K., Dohmen, R. J., & Praefcke, G. J. K. (2014). Multivalent interactions of the SUMO-interaction motifs in RING finger protein 4 determine the specificity for chains of the SUMO. *The Biochemical Journal*, 457(1), 207–214. <https://doi.org/10.1042/BJ20130753>
- Khoshnevis, S., Dreggors-Walker, R. E., Marchand, V., Motorin, Y., & Ghalei, H. (2022). Ribosomal RNA 2'-O-methylations regulate translation by impacting ribosome

- dynamics. *Proceedings of the National Academy of Sciences of the United States of America*, 119(12), e2117334119. <https://doi.org/10.1073/pnas.2117334119>
- Kim, J.-H., Lim, I.-R., Joo, H. J., Choi, S.-C., Choi, J.-H., Cui, L.-H., Im, L., Hong, S. J., & Lim, D.-S. (2015). Sphere formation of adipose stem cell engineered by poly-2-hydroxyethyl methacrylate induces in vitro angiogenesis through fibroblast growth factor 2. *Biochemical and Biophysical Research Communications*, 468(1–2), 372–379. <https://doi.org/10.1016/j.bbrc.2015.10.083>
- Kim, R., Emi, M., & Tanabe, K. (2007). Cancer immunoediting from immune surveillance to immune escape. *Immunology*, 121(1), 1–14. <https://doi.org/10.1111/j.1365-2567.2007.02587.x>
- Kim, Y. S., Keyser, S. G. L., & Schneekloth, J. S. (2014). Synthesis of 2',3',4'-trihydroxyflavone (2-D08), an inhibitor of protein sumoylation. *Bioorganic & Medicinal Chemistry Letters*, 24(4), 1094–1097. <https://doi.org/10.1016/j.bmcl.2014.01.010>
- Kleinberg, D. L., & Ruan, W. (2008). IGF-I, GH, and Sex Steroid Effects in Normal Mammary Gland Development. *Journal of Mammary Gland Biology and Neoplasia*, 13(4), 353–360. <https://doi.org/10.1007/s10911-008-9103-7>
- Koh, C. M., Gurel, B., Sutcliffe, S., Aryee, M. J., Schultz, D., Iwata, T., Uemura, M., Zeller, K. I., Anele, U., Zheng, Q., Hicks, J. L., Nelson, W. G., Dang, C. V., Yegnasubramanian, S., & De Marzo, A. M. (2011). Alterations in Nucleolar Structure and Gene Expression Programs in Prostatic Neoplasia Are Driven by the MYC Oncogene. *The American Journal of Pathology*, 178(4), 1824–1834. <https://doi.org/10.1016/j.ajpath.2010.12.040>
- Kosoy, A., Calonge, T. M., Outwin, E. A., & O'Connell, M. J. (2007). Fission Yeast Rnf4 Homologs Are Required for DNA Repair. *Journal of Biological Chemistry*, 282(28), 20388–20394. <https://doi.org/10.1074/jbc.m702652200>
- Kötter, A., Mootz, H. D., & Heuer, A. (2020). Insights into the Microscopic Structure of RNF4-SIM-SUMO Complexes from MD Simulations. *Biophysical Journal*, 119(8), 1558–1567. <https://doi.org/10.1016/j.bpj.2020.09.003>
- Krogh, N., Asmar, F., Côme, C., Munch-Petersen, H. F., Grønbaek, K., & Nielsen, H. (2020). Profiling of ribose methylations in ribosomal RNA from diffuse large B-cell lymphoma patients for evaluation of ribosomes as drug targets. *NAR Cancer*, 2(4), zcaa035. <https://doi.org/10.1093/narcan/zcaa035>
- Krogh, N., Jansson, M. D., Häfner, S. J., Tehler, D., Birkedal, U., Christensen-Dalsgaard, M., Lund, A. H., & Nielsen, H. (2016). Profiling of 2'-O-Me in human rRNA reveals a subset of fractionally modified positions and provides evidence for ribosome heterogeneity. *Nucleic Acids Research*, 44(16), 7884–7895. <https://doi.org/10.1093/nar/gkw482>
- Kung, C. C.-H., Naik, M. T., Wang, S.-H., Shih, H.-M., Chang, C.-C., Lin, L.-Y., Chen, C.-L., Ma, C., Chang, C.-F., & Huang, T.-H. (2014). Structural analysis of poly-SUMO chain recognition by the RNF4-SIMs domain. *The Biochemical Journal*, 462(1), 53–65. <https://doi.org/10.1042/BJ20140521>
- Kuo, C.-Y., Li, X., Kong, X.-Q., Luo, C., Chang, C.-C., Chung, Y., Shih, H.-M., Li, K. K., & Ann, D. K. (2014a). An arginine-rich motif of ring finger protein 4 (RNF4) oversees the recruitment and degradation of the phosphorylated and SUMOylated Krüppel-associated box domain-associated protein 1 (KAP1)/TRIM28 protein during genotoxic stress. *The Journal of Biological Chemistry*, 289(30), 20757–20772. <https://doi.org/10.1074/jbc.M114.555672>
- Kuo, C.-Y., Li, X., Kong, X.-Q., Luo, C., Chang, C.-C., Chung, Y., Shih, H.-M., Li, K. K., & Ann, D. K. (2014b). An Arginine-rich Motif of Ring Finger Protein 4 (RNF4) Oversees

- the Recruitment and Degradation of the Phosphorylated and SUMOylated Krüppel-associated Box Domain-associated Protein 1 (KAP1)/TRIM28 Protein during Genotoxic Stress. *Journal of Biological Chemistry*, 289(30), 20757–20772. <https://doi.org/10.1074/jbc.M114.555672>
- Lallemant-Breitenbach, V., Jeanne, M., Benhenda, S., Nasr, R., Lei, M., Peres, L., Zhou, J., Zhu, J., Raught, B., & de Thé, H. (2008). Arsenic degrades PML or PML-RAR α through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nature Cell Biology*, 10(5), 547–555. <https://doi.org/10.1038/ncb1717>
- Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial–mesenchymal transition. *Nature Reviews Molecular Cell Biology*, 15(3), 178–196. <https://doi.org/10.1038/nrm3758>
- Lee, S., & Schmitt, C. A. (2019). The dynamic nature of senescence in cancer. *Nature Cell Biology*, 21(1), 94–101. <https://doi.org/10.1038/s41556-018-0249-2>
- Lewis, J. D., & Tollervey, D. (2000). Like Attracts Like: Getting RNA Processing Together in the Nucleus. *Science*, 288(5470), 1385–1389. <https://doi.org/10.1126/science.288.5470.1385>
- Li, J., Xu, Y., Long, X.-D., Wang, W., Jiao, H.-K., Mei, Z., Yin, Q.-Q., Ma, L.-N., Zhou, A.-W., Wang, L.-S., Yao, M., Xia, Q., & Chen, G.-Q. (2014). Cbx4 governs HIF-1 α to potentiate angiogenesis of hepatocellular carcinoma by its SUMO E3 ligase activity. *Cancer Cell*, 25(1), 118–131. <https://doi.org/10.1016/j.ccr.2013.12.008>
- Li, M., Zhang, J., Meng, X., Liu, B., Xie, S. M., Liu, F., Yao, D., Zhang, J., Shen, H., & Xing, L. (2023). Limb expression 1-like protein promotes epithelial-mesenchymal transition and epidermal growth factor receptor-tyrosine kinase inhibitor resistance via nucleolin-mediated ribosomal RNA synthesis in non-small cell lung cancer. *Cancer Science*, 114(4), 1740–1756. <https://doi.org/10.1111/cas.15687>
- Liang, J., Singh, N., Carlson, C. R., Albuquerque, C. P., Corbett, K. D., & Zhou, H. (2017). Recruitment of a SUMO isopeptidase to rDNA stabilizes silencing complexes by opposing SUMO targeted ubiquitin ligase activity. *Genes & Development*, 31(8), 802–815. <https://doi.org/10.1101/gad.296145.117>
- Liang, S., Zhou, Z., Zhou, Z., Liang, J., Lin, W., Zhang, C., Zhou, C., Zhao, H., Meng, X., Zou, F., Yu, C., & Cai, S. (2022). Blockade of CBX4-mediated β -catenin SUMOylation attenuates airway epithelial barrier dysfunction in asthma. *International Immunopharmacology*, 113, 109333. <https://doi.org/10.1016/j.intimp.2022.109333>
- Liew, C. W., Sun, H., Hunter, T., & Day, C. L. (2010). RING domain dimerization is essential for RNF4 function. *The Biochemical Journal*, 431(1), 23–29. <https://doi.org/10.1042/BJ20100957>
- Lin, R., & Chang, H. (2008). Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology Journal*, 3(9–10), 1172–1184. <https://doi.org/10.1002/biot.200700228>
- Lin, R.-Z., Chou, L.-F., Chien, C.-C. M., & Chang, H.-Y. (2006). Dynamic analysis of hepatoma spheroid formation: Roles of E-cadherin and β 1-integrin. *Cell and Tissue Research*, 324(3), 411–422. <https://doi.org/10.1007/s00441-005-0148-2>
- Lin, Y., Dong, C., & Zhou, B. P. (2014). Epigenetic regulation of EMT: The Snail story. *Current Pharmaceutical Design*, 20(11), 1698–1705. <https://doi.org/10.2174/13816128113199990512>
- Lindner, P., Paul, S., Eckstein, M., Hampel, C., Muenzner, J. K., Erlenbach-Wuenssch, K., Ahmed, H. P., Mahadevan, V., Brabletz, T., Hartmann, A., Vera, J., & Schneider-Stock, R. (2020). EMT transcription factor ZEB1 alters the epigenetic landscape of colorectal cancer cells. *Cell Death & Disease*, 11(2), 147. <https://doi.org/10.1038/s41419-020-2340-4>

- Long, J., Zuo, D., & Park, M. (2005). Pc2-mediated Sumoylation of Smad-interacting Protein 1 Attenuates Transcriptional Repression of E-cadherin. *Journal of Biological Chemistry*, 280(42), 35477–35489. <https://doi.org/10.1074/jbc.M504477200>
- Lowe, S. W., Cepero, E., & Evan, G. (2004). Intrinsic tumour suppression. *Nature*, 432(7015), 307–315. <https://doi.org/10.1038/nature03098>
- Luo, Y., Fefelova, E., Ninova, M., Chen, Y.-C. A., & Aravin, A. A. (2020). Repression of interrupted and intact rDNA by the SUMO pathway in *Drosophila melanogaster*. *eLife*, 9, e52416. <https://doi.org/10.7554/eLife.52416>
- Löönd, F., Tiede, S., & Christofori, G. (2021). Breast cancer as an example of tumour heterogeneity and tumour cell plasticity during malignant progression. *British Journal of Cancer*, 125(2), 164–175. <https://doi.org/10.1038/s41416-021-01328-7>
- Lv, B., Pan, Y., Hou, D., Chen, P., Zhang, J., Chu, Y., Li, M., Zeng, Y., Yang, D., & Liu, J. (2022). RNF4 silencing induces cell growth arrest and DNA damage by promoting nuclear targeting of p62 in hepatocellular carcinoma. *Oncogene*, 41(16), 2275–2286. <https://doi.org/10.1038/s41388-022-02247-4>
- Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Shyamala, G., Conneely, O. M., & O'Malley, B. W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & Development*, 9(18), 2266–2278. <https://doi.org/10.1101/gad.9.18.2266>
- Ma, X.-L., Sun, Y.-F., Wang, B.-L., Shen, M.-N., Zhou, Y., Chen, J.-W., Hu, B., Gong, Z.-J., Zhang, X., Cao, Y., Pan, B., Zhou, J., Fan, J., Guo, W., & Yang, X.-R. (2019). Sphere-forming culture enriches liver cancer stem cells and reveals Stearoyl-CoA desaturase 1 as a potential therapeutic target. *BMC Cancer*, 19(1), 760. <https://doi.org/10.1186/s12885-019-5963-z>
- Macias, H., & Hinck, L. (2012). Mammary gland development. *WIREs Developmental Biology*, 1(4), 533–557. <https://doi.org/10.1002/wdev.35>
- Marcel, V., Ghayad, S. E., Belin, S., Therizols, G., Morel, A.-P., Solano-González, E., Vendrell, J. A., Hacot, S., Mertani, H. C., Albaret, M. A., Bourdon, J.-C., Jordan, L., Thompson, A., Tafer, Y., Cong, R., Bouvet, P., Saurin, J.-C., Catez, F., Prats, A.-C., ... Diaz, J.-J. (2013). P53 Acts as a Safeguard of Translational Control by Regulating Fibrillarin and rRNA Methylation in Cancer. *Cancer Cell*, 24(3), 318–330. <https://doi.org/10.1016/j.ccr.2013.08.013>
- Marcel, V., Kielbassa, J., Marchand, V., Natchiar, K. S., Paraquindes, H., Nguyen Van Long, F., Ayadi, L., Bourguignon-Igel, V., Lo Monaco, P., Monchiet, D., Scott, V., Tonon, L., Bray, S. E., Diot, A., Jordan, L. B., Thompson, A. M., Bourdon, J.-C., Dubois, T., André, F., ... Diaz, J.-J. (2020). Ribosomal RNA 2'O-methylation as a novel layer of inter-tumour heterogeneity in breast cancer. *NAR Cancer*, 2(4), zcaa036. <https://doi.org/10.1093/narcan/zcaa036>
- Marchand, V., Blanloeil-Oillo, F., Helm, M., & Motorin, Y. (2016). Illumina-based RiboMethSeq approach for mapping of 2'-O-Me residues in RNA. *Nucleic Acids Research*, 44(16), e135–e135. <https://doi.org/10.1093/nar/gkw547>
- Marusyk, A., & Polyak, K. (2010). Tumor heterogeneity: Causes and consequences. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1805(1), 105–117. <https://doi.org/10.1016/j.bbcan.2009.11.002>
- Masutomi, K., Possemato, R., Wong, J. M. Y., Currier, J. L., Tothova, Z., Manola, J. B., Ganesan, S., Lansdorp, P. M., Collins, K., & Hahn, W. C. (2005). The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proceedings of the National Academy of Sciences*, 102(23), 8222–8227. <https://doi.org/10.1073/pnas.0503095102>

- McStay, B., & Grummt, I. (2008). The Epigenetics of rRNA Genes: From Molecular to Chromosome Biology. *Annual Review of Cell and Developmental Biology*, 24(1), 131–157. <https://doi.org/10.1146/annurev.cellbio.24.110707.175259>
- Mei, Z., Jiao, H., Wang, W., Li, J., Chen, G., & Xu, Y. (2014). Polycomb chromobox 4 enhances migration and pulmonary metastasis of hepatocellular carcinoma cell line MHCC97L. *Science China. Life Sciences*, 57(6), 610–617. <https://doi.org/10.1007/s11427-014-4663-9>
- Metge, B. J., Kammerud, S. C., Pruitt, H. C., Shevde, L. A., & Samant, R. S. (2021). Hypoxia re-programs 2'-O-Me modifications on ribosomal RNA. *iScience*, 24(1), 102010. <https://doi.org/10.1016/j.isci.2020.102010>
- Micalizzi, D. S., Farabaugh, S. M., & Ford, H. L. (2010). Epithelial-Mesenchymal Transition in Cancer: Parallels Between Normal Development and Tumor Progression. *Journal of Mammary Gland Biology and Neoplasia*, 15(2), 117–134. <https://doi.org/10.1007/s10911-010-9178-9>
- Moffat, D. F., & Going, J. J. (1996). Three dimensional anatomy of complete duct systems in human breast: Pathological and developmental implications. *Journal of Clinical Pathology*, 49(1), 48–52. <https://doi.org/10.1136/jcp.49.1.48>
- Monaco, P. L., Marcel, V., Diaz, J.-J., & Catez, F. (2018). 2'-O-Methylation of Ribosomal RNA: Towards an Epitranscriptomic Control of Translation? *Biomolecules*, 8(4), 106. <https://doi.org/10.3390/biom8040106>
- Monks, J., Rosner, D., Jon Geske, F., Lehman, L., Hanson, L., Neville, M. C., & Fadok, V. A. (2005). Epithelial cells as phagocytes: Apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell Death & Differentiation*, 12(2), 107–114. <https://doi.org/10.1038/sj.cdd.4401517>
- Monks, J., Smith-Steinhart, C., Kruk, E. R., Fadok, V. A., & Henson, P. M. (2008). Epithelial Cells Remove Apoptotic Epithelial Cells During Post-Lactation Involution of the Mouse Mammary Gland1. *Biology of Reproduction*, 78(4), 586–594. <https://doi.org/10.1095/biolreprod.107.065045>
- Montanaro, L., Treré, D., & Derenzini, M. (2008). Nucleolus, Ribosomes, and Cancer. *The American Journal of Pathology*, 173(2), 301–310. <https://doi.org/10.2353/ajpath.2008.070752>
- Mougiakakos, D., Choudhury, A., Lladser, A., Kiessling, R., & Johansson, C. C. (2010). Regulatory T Cells in Cancer. In *Advances in Cancer Research* (Vol. 107, pp. 57–117). Elsevier. [https://doi.org/10.1016/S0065-230X\(10\)07003-X](https://doi.org/10.1016/S0065-230X(10)07003-X)
- Munholland, J. M., & Nazar, R. N. (1987). Methylation of ribosomal RNA as a possible factor in cell differentiation. *Cancer Research*, 47(1), 169–172.
- Muschler, J., & Streuli, C. H. (2010). Cell-Matrix Interactions in Mammary Gland Development and Breast Cancer. *Cold Spring Harbor Perspectives in Biology*, 2(10), a003202–a003202. <https://doi.org/10.1101/cshperspect.a003202>
- Nachmani, D., Bothmer, A. H., Grisendi, S., Mele, A., Bothmer, D., Lee, J. D., Monteleone, E., Cheng, K., Zhang, Y., Bester, A. C., Guzzetti, A., Mitchell, C. A., Mendez, L. M., Pozdnyakova, O., Sportoletti, P., Martelli, M.-P., Vulliamy, T. J., Safra, M., Schwartz, S., ... Pandolfi, P. P. (2019). Germline NPM1 mutations lead to altered rRNA 2'-O-methylation and cause dyskeratosis congenita. *Nature Genetics*, 51(10), 1518–1529. <https://doi.org/10.1038/s41588-019-0502-z>
- Nazar, R. N., Sitz, T. O., & Busch, H. (1975). Tissue specific differences in the 2'- O -methylation of eukaryotic 5.8S ribosomal RNA. *FEBS Letters*, 59(1), 83–87. [https://doi.org/10.1016/0014-5793\(75\)80346-2](https://doi.org/10.1016/0014-5793(75)80346-2)
- Nazemroaya, F., Soheili, Z., Samiei, S., Deezagi, A., Ahmadi, H., Davari, M., Heidari, R., Bagheri, A., & Darvishalipour-Astaneh, S. (2017). Induced Retro-Differentiation of

- Human Retinal Pigment Epithelial Cells on PolyHEMA. *Journal of Cellular Biochemistry*, 118(10), 3080–3089. <https://doi.org/10.1002/jcb.26014>
- Neyret-Kahn, H., Benhamed, M., Ye, T., Gras, S. L., Cossec, J.-C., Lapaquette, P., Bischof, O., Ouspenskaia, M., Dasso, M., Seeler, J., Davidson, I., & Dejean, A. (2013). Sumoylation at chromatin governs coordinated repression of a transcriptional program essential for cell growth and proliferation. *Genome Research*, 23(10), 1563–1579. <https://doi.org/10.1101/gr.154872.113>
- Nguyen Van Long, F., Lardy-Cleaud, A., Carène, D., Rossoni, C., Catez, F., Rollet, P., Pion, N., Monchiet, D., Dolbeau, A., Martin, M., Simioni, V., Bray, S., Le Beherec, D., Mosele, F., Bouakka, I., Colombe-Vermorel, A., Odeyer, L., Diot, A., Jordan, L. B., ... Marcel, V. (2022). Low level of Fibrillarin, a ribosome biogenesis factor, is a new independent marker of poor outcome in breast cancer. *BMC Cancer*, 22(1), 526. <https://doi.org/10.1186/s12885-022-09552-x>
- Nishida, T., & Yamada, Y. (2020). RNF4-mediated SUMO-targeted ubiquitination relieves PARIS/ZNF746-mediated transcriptional repression. *Biochemical and Biophysical Research Communications*, 526(1), 110–116. <https://doi.org/10.1016/j.bbrc.2020.03.063>
- Novak, R., Ahmad, Y. A., Timaner, M., Bitman-Lotan, E., Oknin-Vaisman, A., Horwitz, R., Hartmann, O., Reissland, M., Buck, V., Rosenfeldt, M., Nikomarov, D., Diefenbacher, M. E., Shaked, Y., & Orian, A. (2022). RNF4~RGMb~BMP6 axis required for osteogenic differentiation and cancer cell survival. *Cell Death & Disease*, 13(9), 820. <https://doi.org/10.1038/s41419-022-05262-1>
- Nowell, P. C. (1976). The Clonal Evolution of Tumor Cell Populations: Acquired genetic lability permits stepwise selection of variant sublines and underlies tumor progression. *Science*, 194(4260), 23–28. <https://doi.org/10.1126/science.959840>
- Nyberg, P., Xie, L., & Kalluri, R. (2005). Endogenous Inhibitors of Angiogenesis. *Cancer Research*, 65(10), 3967–3979. <https://doi.org/10.1158/0008-5472.CAN-04-2427>
- Okumura, S., Konishi, Y., Narukawa, M., Sugiura, Y., Yoshimoto, S., Arai, Y., Sato, S., Yoshida, Y., Tsuji, S., Uemura, K., Wakita, M., Matsudaira, T., Matsumoto, T., Kawamoto, S., Takahashi, A., Itatani, Y., Miki, H., Takamatsu, M., Obama, K., ... Hara, E. (2021). Gut bacteria identified in colorectal cancer patients promote tumorigenesis via butyrate secretion. *Nature Communications*, 12(1), 5674. <https://doi.org/10.1038/s41467-021-25965-x>
- Orsolic, I., Jurada, D., Pullen, N., Oren, M., Eliopoulos, A. G., & Volarevic, S. (2016). The relationship between the nucleolus and cancer: Current evidence and emerging paradigms. *Seminars in Cancer Biology*, 37–38, 36–50. <https://doi.org/10.1016/j.semcancer.2015.12.004>
- Ostrand-Rosenberg, S., & Sinha, P. (2009). Myeloid-Derived Suppressor Cells: Linking Inflammation and Cancer. *The Journal of Immunology*, 182(8), 4499–4506. <https://doi.org/10.4049/jimmunol.0802740>
- Pan, Y., Li, Q., Cao, Z., & Zhao, S. (2021). The SUMO E3 ligase CBX4 is identified as a poor prognostic marker of gastric cancer through multipronged OMIC analyses. *Genes & Diseases*, 8(6), 827–837. <https://doi.org/10.1016/j.gendis.2020.08.010>
- Papaspyropoulos, A., Angelopoulou, A., Mourkioti, I., Polyzou, A., Pankova, D., Toskas, K., Lanfredini, S., Pantazaki, A. A., Lagopati, N., Kotsinas, A., Evangelou, K., Chronopoulos, E., O'Neill, E., & Gorgoulis, V. (2022). RASSF1A disrupts the NOTCH signaling axis via SNURF/RNF4-mediated ubiquitination of HES1. *EMBO Reports*, 23(2), e51287. <https://doi.org/10.15252/embr.202051287>

- Park, S., Choi, J., Song, J.-K., Jang, B., & Maeng, Y. H. (2024). Subcellular expression pattern and clinical significance of CBX2 and CBX7 in breast cancer subtypes. *Medical Molecular Morphology*, 57(1), 11–22. <https://doi.org/10.1007/s00795-023-00368-7>
- Peaker, M. (2002). [No title found]. *Journal of Mammary Gland Biology and Neoplasia*, 7(3), 347–353. <https://doi.org/10.1023/A:1022860902083>
- Pederson, T. (2011). The Nucleolus. *Cold Spring Harbor Perspectives in Biology*, 3(3), a000638. <https://doi.org/10.1101/cshperspect.a000638>
- Peng, Y., Wang, Z., Wang, Z., Yu, F., Li, J., & Wong, J. (2019). SUMOylation down-regulates rDNA transcription by repressing expression of upstream-binding factor and proto-oncogene c-Myc. *Journal of Biological Chemistry*, 294(50), 19155–19166. <https://doi.org/10.1074/jbc.RA119.010624>
- Philipp, E. E., Setchell, M., & Ginsburg, J. (1991). *Scientific Foundations of Obstetrics and Gynaecology*. Butterworth-Heinemann. https://books.google.co.in/books?id=_tpsAAAAMAAJ
- Plechanovová, A., Jaffray, E. G., McMahon, S. A., Johnson, K. A., Navrátilová, I., Naismith, J. H., & Hay, R. T. (2011). Mechanism of ubiquitylation by dimeric RING ligase RNF4. *Nature Structural & Molecular Biology*, 18(9), 1052–1059. <https://doi.org/10.1038/nsmb.2108>
- Plechanovová, A., Jaffray, E. G., Tatham, M. H., Naismith, J. H., & Hay, R. T. (2012). Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. *Nature*, 489(7414), 115–120. <https://doi.org/10.1038/nature11376>
- Postigo, A. A. (2003). Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. *The EMBO Journal*, 22(10), 2453–2462. <https://doi.org/10.1093/emboj/cdg226>
- Potter, V. R. (1958). The biochemical approach to the cancer problem. *Federation Proceedings*, 17(2), 691–697.
- Prakash, V., Carson, B. B., Feenstra, J. M., Dass, R. A., Sekyrova, P., Hoshino, A., Petersen, J., Guo, Y., Parks, M. M., Kurylo, C. M., Batchelder, J. E., Haller, K., Hashimoto, A., Rundqvist, H., Condeelis, J. S., Allis, C. D., Drygin, D., Nieto, M. A., Andäng, M., ... Vincent, C. T. (2019). Ribosome biogenesis during cell cycle arrest fuels EMT in development and disease. *Nature Communications*, 10(1), 2110. <https://doi.org/10.1038/s41467-019-10100-8>
- Qin, H., Du, D., Zhu, Y., Li, J., Feng, L., Liang, Y., & Han, H. (2005). The PcG protein HPC2 inhibits RBP-J-mediated transcription by interacting with LIM protein KyoT2. *FEBS Letters*, 579(5), 1220–1226. <https://doi.org/10.1016/j.febslet.2005.01.022>
- Qin, Q., Xu, Y., He, T., Qin, C., & Xu, J. (2012). Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. *Cell Research*, 22(1), 90–106. <https://doi.org/10.1038/cr.2011.144>
- Ramachandran, S., Krogh, N., Jørgensen, T. E., Johansen, S. D., Nielsen, H., & Babiak, I. (2020). The shift from early to late types of ribosomes in zebrafish development involves changes at a subset of rRNA 2'-O-Me sites. *RNA*, 26(12), 1919–1934. <https://doi.org/10.1261/rna.076760.120>
- Ren, X., Hu, B., Song, M., Ding, Z., Dang, Y., Liu, Z., Zhang, W., Ji, Q., Ren, R., Ding, J., Chan, P., Jiang, C., Ye, K., Qu, J., Tang, F., & Liu, G.-H. (2019). Maintenance of Nucleolar Homeostasis by CBX4 Alleviates Senescence and Osteoarthritis. *Cell Reports*, 26(13), 3643–3656.e7. <https://doi.org/10.1016/j.celrep.2019.02.088>
- Reya, T., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature*, 414(6859), 105–111. <https://doi.org/10.1038/35102167>
- Ribatti, D. (2009). Endogenous inhibitors of angiogenesis. *Leukemia Research*, 33(5), 638–644. <https://doi.org/10.1016/j.leukres.2008.11.019>

- Rodriguez-Corona, U., Sobol, M., Rodriguez-Zapata, L. C., Hozak, P., & Castano, E. (2015). Fibrillarin from Archaea to human. *Biology of the Cell*, 107(6), 159–174. <https://doi.org/10.1111/boc.201400077>
- Rojas-Fernandez, A., Plechanovová, A., Hattersley, N., Jaffray, E., Tatham, M. H., & Hay, R. T. (2014). SUMO chain-induced dimerization activates RNF4. *Molecular Cell*, 53(6), 880–892. <https://doi.org/10.1016/j.molcel.2014.02.031>
- Ryu, H.-Y., López-Giráldez, F., Knight, J., Hwang, S. S., Renner, C., Kreft, S. G., & Hochstrasser, M. (2018). Distinct adaptive mechanisms drive recovery from aneuploidy caused by loss of the Ulp2 SUMO protease. *Nature Communications*, 9(1), 5417. <https://doi.org/10.1038/s41467-018-07836-0>
- Sabeh, F., Shimizu-Hirota, R., & Weiss, S. J. (2009). Protease-dependent versus -independent cancer cell invasion programs: Three-dimensional amoeboid movement revisited. *Journal of Cell Biology*, 185(1), 11–19. <https://doi.org/10.1083/jcb.200807195>
- Saitoh, M. (2018). Involvement of partial EMT in cancer progression. *The Journal of Biochemistry*, 164(4), 257–264. <https://doi.org/10.1093/jb/mvy047>
- Sánchez-Tilló, E., Lázaro, A., Torrent, R., Cuatrecasas, M., Vaquero, E. C., Castells, A., Engel, P., & Postigo, A. (2010). ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene*, 29(24), 3490–3500. <https://doi.org/10.1038/onc.2010.102>
- Sanyal, S., Mondal, P., Sen, S., Sengupta Bandyopadhyay, S., & Das, C. (2020). SUMO E3 ligase CBX4 regulates hTERT-mediated transcription of CDH1 and promotes breast cancer cell migration and invasion. *The Biochemical Journal*, 477(19), 3803–3818. <https://doi.org/10.1042/BCJ20200359>
- Sathishkumar, K., Chaturvedi, M., Das, P., Stephen, S., & Mathur, P. (2022). Cancer incidence estimates for 2022 & projection for 2025: Result from National Cancer Registry Programme, India. *Indian Journal of Medical Research*, 156(4), 598. https://doi.org/10.4103/ijmr.ijmr_1821_22
- Senthilkumar, R., & Mishra, R. K. (2009). Novel motifs distinguish multiple homologues of Polycomb in vertebrates: Expansion and diversification of the epigenetic toolkit. *BMC Genomics*, 10(1), 549. <https://doi.org/10.1186/1471-2164-10-549>
- Sharma, S., Marchand, V., Motorin, Y., & Lafontaine, D. L. J. (2017). Identification of sites of 2'-O-methylation vulnerability in human ribosomal RNAs by systematic mapping. *Scientific Reports*, 7(1), 11490. <https://doi.org/10.1038/s41598-017-09734-9>
- Shay, J. W., & Wright, W. E. (2000). Hayflick, his limit, and cellular ageing. *Nature Reviews Molecular Cell Biology*, 1(1), 72–76. <https://doi.org/10.1038/35036093>
- Sherr, C. J., & McCormick, F. (2002). The RB and p53 pathways in cancer. *Cancer Cell*, 2(2), 103–112. [https://doi.org/10.1016/S1535-6108\(02\)00102-2](https://doi.org/10.1016/S1535-6108(02)00102-2)
- Shimazui, T., Schalken, J. A., Kawai, K., Kawamoto, R., van Bockhoven, A., Oosterwijk, E., & Akaza, H. (2004). Role of complex cadherins in cell-cell adhesion evaluated by spheroid formation in renal cell carcinoma cell lines. *Oncology Reports*, 11(2), 357–360.
- Shubina, M. Y., Arifulin, E. A., Sorokin, D. V., Sosina, M. A., Tikhomirova, M. A., Serebryakova, M. V., Smirnova, T., Sokolov, S. S., Musinova, Y. R., & Sheval, E. V. (2020). The GAR domain integrates functions that are necessary for the proper localization of fibrillarin (FBL) inside eukaryotic cells. *PeerJ*, 8, e9029. <https://doi.org/10.7717/peerj.9029>
- Soerjomataram, I., Cabasag, C., Bardot, A., Fidler-Benaoudia, M. M., Miranda-Filho, A., Ferlay, J., Parkin, D. M., Ranganathan, R., Piñeros, M., Znaor, A., Mery, L., Joko-Fru, Y. W., Dikshit, R., Sankaranarayanan, R., Swaminathan, R., & Bray, F. (2023). Cancer survival in Africa, central and south America, and Asia (SURVCAN-3): A population-

- based benchmarking study in 32 countries. *The Lancet Oncology*, 24(1), 22–32. [https://doi.org/10.1016/S1470-2045\(22\)00704-5](https://doi.org/10.1016/S1470-2045(22)00704-5)
- Steadman, K., Stein, W. D., Litman, T., Yang, S. X., Abu-Asab, M., Dutcher, S. K., & Bates, S. (2008). PolyHEMA spheroids are an inadequate model for the drug resistance of the intractable solid tumors. *Cell Cycle*, 7(6), 818–829. <https://doi.org/10.4161/cc.7.6.5597>
- Sternlicht, M. D. (2005). Key stages in mammary gland development: The cues that regulate ductal branching morphogenesis. *Breast Cancer Research*, 8(1), 201. <https://doi.org/10.1186/bcr1368>
- Streuli, C. H. (2003). Preface: Cell Adhesion in Mammary Gland Biology and Neoplasia. *Journal of Mammary Gland Biology and Neoplasia*, 8(4), 375–381. <https://doi.org/10.1023/B:JOMG.0000017501.34884.70>
- Su, H., Xu, T., Ganapathy, S., Shadfan, M., Long, M., Huang, T. H.-M., Thompson, I., & Yuan, Z.-M. (2014). Elevated snoRNA biogenesis is essential in breast cancer. *Oncogene*, 33(11), 1348–1358. <https://doi.org/10.1038/onc.2013.89>
- Sun, X., Gao, C., Xu, X., Li, M., Zhao, X., Wang, Y., Wang, Y., Zhang, S., Yan, Z., Liu, X., & Wu, C. (2023). FBL promotes cancer cell resistance to DNA damage and BRCA1 transcription via YBX1. *EMBO Reports*, 24(9), e56230. <https://doi.org/10.15252/embr.202256230>
- Talmadge, J. E., & Fidler, I. J. (2010). AACR Centennial Series: The Biology of Cancer Metastasis: Historical Perspective. *Cancer Research*, 70(14), 5649–5669. <https://doi.org/10.1158/0008-5472.CAN-10-1040>
- Tatham, M. H., Geoffroy, M.-C., Shen, L., Plechanovova, A., Hattersley, N., Jaffray, E. G., Palvimo, J. J., & Hay, R. T. (2008). RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nature Cell Biology*, 10(5), 538–546. <https://doi.org/10.1038/ncb1716>
- Teng, M. W. L., Swann, J. B., Koebel, C. M., Schreiber, R. D., & Smyth, M. J. (2008). Immune-mediated dormancy: An equilibrium with cancer. *Journal of Leukocyte Biology*, 84(4), 988–993. <https://doi.org/10.1189/jlb.1107774>
- Tessarz, P., Santos-Rosa, H., Robson, S. C., Sylvestersen, K. B., Nelson, C. J., Nielsen, M. L., & Kouzarides, T. (2014). Glutamine methylation in histone H2A is an RNA-polymerase-I-dedicated modification. *Nature*, 505(7484), 564–568. <https://doi.org/10.1038/nature12819>
- Thakur, G., Bok, E.-Y., Kim, S.-B., Jo, C.-H., Oh, S.-J., Baek, J.-C., Park, J.-E., Kang, Y.-H., Lee, S.-L., Kumar, R., & Rho, G.-J. (2022). Scaffold-free 3D culturing enhance pluripotency, immunomodulatory factors, and differentiation potential of Wharton's jelly-mesenchymal stem cells. *European Journal of Cell Biology*, 101(3), 151245. <https://doi.org/10.1016/j.ejcb.2022.151245>
- Thienpont, B., Van Dyck, L., & Lambrechts, D. (2016). Tumors smother their epigenome. *Molecular & Cellular Oncology*, 3(6), e1240549. <https://doi.org/10.1080/23723556.2016.1240549>
- Thomas, J. J., Abed, M., Heuberger, J., Novak, R., Zohar, Y., Beltran Lopez, A. P., Trausch-Azar, J. S., Ilagan, M. X. G., Benhamou, D., Dittmar, G., Kopan, R., Birchmeier, W., Schwartz, A. L., & Orian, A. (2016). RNF4-Dependent Oncogene Activation by Protein Stabilization. *Cell Reports*, 16(12), 3388–3400. <https://doi.org/10.1016/j.celrep.2016.08.024>
- Tiku, V., Kew, C., Mehrotra, P., Ganesan, R., Robinson, N., & Antebi, A. (2018). Nucleolar fibrillarin is an evolutionarily conserved regulator of bacterial pathogen resistance. *Nature Communications*, 9(1), 3607. <https://doi.org/10.1038/s41467-018-06051-1>
- Tollervey, D., Lehtonen, H., Jansen, R., Kern, H., & Hurt, E. C. (1993). Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA

- methylation, and ribosome assembly. *Cell*, 72(3), 443–457. [https://doi.org/10.1016/0092-8674\(93\)90120-f](https://doi.org/10.1016/0092-8674(93)90120-f)
- Turashvili, G., & Brogi, E. (2017). Tumor Heterogeneity in Breast Cancer. *Frontiers in Medicine*, 4, 227. <https://doi.org/10.3389/fmed.2017.00227>
- Tzanakakis, E. S., Hansen, L. K., & Hu, W. S. (2001). The role of actin filaments and microtubules in hepatocyte spheroid self-assembly. *Cell Motility and the Cytoskeleton*, 48(3), 175–189. [https://doi.org/10.1002/1097-0169\(200103\)48:3<175::AID-CM1007>3.0.CO;2-2](https://doi.org/10.1002/1097-0169(200103)48:3<175::AID-CM1007>3.0.CO;2-2)
- Valente, S., Ciavarella, C., Hernández-Aguilera, A., Salvador, F., Buzzi, M., Joven, J., & Pasquinelli, G. (2022). Phenotypic, morphological, and metabolic characterization of vascular-spheres from human vascular mesenchymal stem cells. *Microscopy Research and Technique*, 85(2), 447–459. <https://doi.org/10.1002/jemt.23918>
- Van Wijnen, A. J., Bagheri, L., Badreldin, A. A., Larson, A. N., Dudakovic, A., Thaler, R., Paradise, C. R., & Wu, Z. (2021). Biological functions of chromobox (CBX) proteins in stem cell self-renewal, lineage-commitment, cancer and development. *Bone*, 143, 115659. <https://doi.org/10.1016/j.bone.2020.115659>
- Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science*, 324(5930), 1029–1033. <https://doi.org/10.1126/science.1160809>
- Wang, B., Kohli, J., & Demaria, M. (2020). Senescent Cells in Cancer Therapy: Friends or Foes? *Trends in Cancer*, 6(10), 838–857. <https://doi.org/10.1016/j.trecan.2020.05.004>
- Wang, B., Tang, J., Liao, D., Wang, G., Zhang, M., Sang, Y., Cao, J., Wu, Y., Zhang, R., Li, S., Ding, W., Zhang, G., & Kang, T. (2013). Chromobox homolog 4 is correlated with prognosis and tumor cell growth in hepatocellular carcinoma. *Annals of Surgical Oncology*, 20 Suppl 3, S684–692. <https://doi.org/10.1245/s10434-013-3171-7>
- Wang, L., Guo, H., Lin, C., Yang, L., & Wang, X. (2014). Enrichment and characterization of cancer stem-like cells from a cervical cancer cell line. *Molecular Medicine Reports*, 9(6), 2117–2123. <https://doi.org/10.3892/mmr.2014.2063>
- Wang, X., Li, L., Wu, Y., Zhang, R., Zhang, M., Liao, D., Wang, G., Qin, G., Xu, R.-H., & Kang, T. (2016). CBX4 Suppresses Metastasis via Recruitment of HDAC3 to the Runx2 Promoter in Colorectal Carcinoma. *Cancer Research*, 76(24), 7277–7289. <https://doi.org/10.1158/0008-5472.CAN-16-2100>
- Wang, Z., Fang, Z., Chen, G., Liu, B., Xu, J., Li, F., Li, F., Liu, H., Zhang, H., Sun, Y., Tian, G., Chen, H., Xu, G., Zhang, L., Hu, L., & Ji, H. (2021). Chromobox 4 facilitates tumorigenesis of lung adenocarcinoma through the Wnt/ β -catenin pathway. *Neoplasia*, 23(2), 222–233. <https://doi.org/10.1016/j.neo.2020.12.005>
- Warburg, O. (1956a). On respiratory impairment in cancer cells. *Science (New York, N.Y.)*, 124(3215), 269–270.
- Warburg, O. (1956b). On the Origin of Cancer Cells. *Science*, 123(3191), 309–314. <https://doi.org/10.1126/science.123.3191.309>
- Watanabe-Susaki, K., Takada, H., Enomoto, K., Miwata, K., Ishimine, H., Intoh, A., Ohtaka, M., Nakanishi, M., Sugino, H., Asashima, M., & Kurisaki, A. (2014). Biosynthesis of Ribosomal RNA in Nucleoli Regulates Pluripotency and Differentiation Ability of Pluripotent Stem Cells. *Stem Cells*, 32(12), 3099–3111. <https://doi.org/10.1002/stem.1825>
- Watson, C. J., & Khaled, W. T. (2008). Mammary development in the embryo and adult: A journey of morphogenesis and commitment. *Development*, 135(6), 995–1003. <https://doi.org/10.1242/dev.005439>
- Weeks, S. E., Kammerud, S. C., Metge, B. J., AlSheikh, H. A., Schneider, D. A., Chen, D., Wei, S., Mobley, J. A., Ojesina, A. I., Shevde, L. A., & Samant, R. S. (2021). Inhibiting

- β -catenin disables nucleolar functions in triple-negative breast cancer. *Cell Death & Disease*, 12(3), 242. <https://doi.org/10.1038/s41419-021-03531-z>
- Weisshaar, S. R., Keusekotten, K., Krause, A., Horst, C., Springer, H. M., Götttsche, K., Dohmen, R. J., & Praefcke, G. J. K. (2008). Arsenic trioxide stimulates SUMO-2/3 modification leading to RNF4-dependent proteolytic targeting of PML. *FEBS Letters*, 582(21–22), 3174–3178. <https://doi.org/10.1016/j.febslet.2008.08.008>
- Wertz, I. E., & Dixit, V. M. (2010). Regulation of death receptor signaling by the ubiquitin system. *Cell Death & Differentiation*, 17(1), 14–24. <https://doi.org/10.1038/cdd.2009.168>
- Westman, B. J., & Lamond, A. I. (2011). A role for SUMOylation in snoRNP biogenesis revealed by quantitative proteomics. *Nucleus (Austin, Tex.)*, 2(1), 30–37. <https://doi.org/10.4161/nucl.2.1.14437>
- Westman, B. J., Verheggen, C., Hutten, S., Lam, Y. W., Bertrand, E., & Lamond, A. I. (2010). A Proteomic Screen for Nucleolar SUMO Targets Shows SUMOylation Modulates the Function of Nop5/Nop58. *Molecular Cell*, 39(4), 618–631. <https://doi.org/10.1016/j.molcel.2010.07.025>
- White, E., & DiPaola, R. S. (2009). The Double-Edged Sword of Autophagy Modulation in Cancer. *Clinical Cancer Research*, 15(17), 5308–5316. <https://doi.org/10.1158/1078-0432.CCR-07-5023>
- Wong, M. K., Wahed, M., Shawky, S. A., Dvorkin-Gheva, A., & Raha, S. (2019). Transcriptomic and functional analyses of 3D placental extravillous trophoblast spheroids. *Scientific Reports*, 9(1), 12607. <https://doi.org/10.1038/s41598-019-48816-8>
- Wu, C., Xia, D., Wang, D., Wang, S., Sun, Z., Xu, B., & Zhang, D. (2022). TCOF1 coordinates oncogenic activation and rRNA production and promotes tumorigenesis in HCC. *Cancer Science*, 113(2), 553–564. <https://doi.org/10.1111/cas.15242>
- Wu, Y., Li, H., Long, Y., Zhang, Z., Zhang, F., Pan, R., Meng, L., Ma, Z., Wang, K., Zheng, B., Qie, Z., & Gao, W. (2025). Epigenetic Suppression of miR-137 Induces RNF4 Expression, Facilitating Wnt Signaling in Colorectal Cancer. *Molecular Carcinogenesis*, 64(3), 475–489. <https://doi.org/10.1002/mc.23859>
- Xu, J., Lamouille, S., & Derynck, R. (2009). TGF- β -induced epithelial to mesenchymal transition. *Cell Research*, 19(2), 156–172. <https://doi.org/10.1038/cr.2009.5>
- Xu, X., Lai, C., Luo, J., Shi, J., Guo, K., Hu, J., Mulati, Y., Xiao, Y., Kong, D., Liu, C., Huang, J., & Xu, K. (2024). The predictive significance of chromobox family members in prostate cancer in humans. *Cellular Oncology (Dordrecht, Netherlands)*, 47(4), 1315–1331. <https://doi.org/10.1007/s13402-024-00929-7>
- Xu, Y., Plechanovová, A., Simpson, P., Marchant, J., Leidecker, O., Kraatz, S., Hay, R. T., & Matthews, S. J. (2014). Structural insight into SUMO chain recognition and manipulation by the ubiquitin ligase RNF4. *Nature Communications*, 5, 4217. <https://doi.org/10.1038/ncomms5217>
- Yang, F., Sun, L., Li, Q., Han, X., Lei, L., Zhang, H., & Shang, Y. (2012). SET8 promotes epithelial-mesenchymal transition and confers TWIST dual transcriptional activities. *The EMBO Journal*, 31(1), 110–123. <https://doi.org/10.1038/emboj.2011.364>
- Yang, J., Cheng, D., Zhu, B., Zhou, S., Ying, T., & Yang, Q. (2016). Chromobox Homolog 4 is Positively Correlated to Tumor Growth, Survival and Activation of HIF-1 α Signaling in Human Osteosarcoma under Normoxic Condition. *Journal of Cancer*, 7(4), 427–435. <https://doi.org/10.7150/jca.13749>
- Yang, J., Miao, R., Li, Y., Pan, T., Wu, S., Qu, X., & Cui, S. (2022). Atypical chemokine receptor 3 induces colorectal tumorigenesis in mice by promoting β -arrestin-NOLC1-

- fibrillarin-dependent rRNA biogenesis. *Acta Pharmacologica Sinica*, 43(11), 2967–2976. <https://doi.org/10.1038/s41401-022-00901-x>
- Yang, J., & Weinberg, R. A. (2008). Epithelial-Mesenchymal Transition: At the Crossroads of Development and Tumor Metastasis. *Developmental Cell*, 14(6), 818–829. <https://doi.org/10.1016/j.devcel.2008.05.009>
- Yang, L., Pang, Y., & Moses, H. L. (2010). TGF- β and immune cells: An important regulatory axis in the tumor microenvironment and progression. *Trends in Immunology*, 31(6), 220–227. <https://doi.org/10.1016/j.it.2010.04.002>
- Yang, Y., Li, Y., Sears, R. C., Sun, X.-X., & Dai, M.-S. (2024). SUMOylation regulation of ribosome biogenesis: Emerging roles for USP36. *Frontiers in RNA Research*, 2, 1389104. <https://doi.org/10.3389/frnar.2024.1389104>
- Yi, Y., Li, Y., Meng, Q., Li, Q., Li, F., Lu, B., Shen, J., Fazli, L., Zhao, D., Li, C., Jiang, W., Wang, R., Liu, Q., Szczepanski, A., Li, Q., Qin, W., Weiner, A. B., Lotan, T. L., Ji, Z., ... Cao, Q. (2021). A PRC2-independent function for EZH2 in regulating rRNA 2'-O methylation and IRES-dependent translation. *Nature Cell Biology*, 23(4), 341–354. <https://doi.org/10.1038/s41556-021-00653-6>
- Yu, F., Lin, Y., Ai, M.-M., Tan, G.-J., Huang, J.-L., & Zou, Z.-R. (2021). Knockdown of Circular RNA hsa_circ_PVT1 Inhibited Laryngeal Cancer Progression via Preventing wnt4/ β -Catenin Signaling Pathway Activation. *Frontiers in Cell and Developmental Biology*, 9, 658115. <https://doi.org/10.3389/fcell.2021.658115>
- Yu, H., Qu, J., Gou, H., & Zhou, Y. (2024). Formononetin suppresses the malignant progression of papillary thyroid carcinoma depending on downregulation of CBX4. *Experimental and Therapeutic Medicine*, 28(6), 456. <https://doi.org/10.3892/etm.2024.12746>
- Yuan, T. L., & Cantley, L. C. (2008). PI3K pathway alterations in cancer: Variations on a theme. *Oncogene*, 27(41), 5497–5510. <https://doi.org/10.1038/onc.2008.245>
- Yue, D., Zhang, Z., Li, J., Chen, X., Ping, Y., Liu, S., Shi, X., Li, L., Wang, L., Huang, L., Zhang, B., Sun, Y., & Zhang, Y. (2015). Transforming growth factor-beta1 promotes the migration and invasion of sphere-forming stem-like cell subpopulations in esophageal cancer. *Experimental Cell Research*, 336(1), 141–149. <https://doi.org/10.1016/j.yexcr.2015.06.007>
- Zeng, J.-S., Zhang, Z.-D., Pei, L., Bai, Z.-Z., Yang, Y., Yang, H., & Tian, Q.-H. (2018). CBX4 exhibits oncogenic activities in breast cancer via Notch1 signaling. *The International Journal of Biochemistry & Cell Biology*, 95, 1–8. <https://doi.org/10.1016/j.biocel.2017.12.006>
- Zhan, T., Rindtorff, N., & Boutros, M. (2017). Wnt signaling in cancer. *Oncogene*, 36(11), 1461–1473. <https://doi.org/10.1038/onc.2016.304>
- Zhang, J., Yang, G., Li, Q., & Xie, F. (2020). Increased fibrillarin expression is associated with tumor progression and an unfavorable prognosis in hepatocellular carcinoma. *Oncology Letters*, 21(2), 92. <https://doi.org/10.3892/ol.2020.12353>
- Zhang, X., Li, W., Sun, S., & Liu, Y. (2024). Advances in the structure and function of the nucleolar protein fibrillarin. *Frontiers in Cell and Developmental Biology*, 12, 1494631. <https://doi.org/10.3389/fcell.2024.1494631>
- Zhang, Z., Li, N., Wei, X., Chen, B., Zhang, Y., Zhao, Y., Hu, X., & Hou, S. (2020). GRM4 inhibits the proliferation, migration, and invasion of human osteosarcoma cells through interaction with CBX4. *Bioscience, Biotechnology, and Biochemistry*, 84(2), 279–289. <https://doi.org/10.1080/09168451.2019.1673147>
- Zhao, Q., Liu, N., Xu, T., & Song, K. (2023). RING finger gene 180 inhibits osteosarcoma progression through regulating chromobox homolog 4 ubiquitination. *Cell Cycle*, 22(10), 1246–1258. <https://doi.org/10.1080/15384101.2023.2205201>

- Zhou, F., Aroua, N., Liu, Y., Rohde, C., Cheng, J., Wirth, A.-K., Fijalkowska, D., Göllner, S., Lotze, M., Yun, H., Yu, X., Pabst, C., Sauer, T., Oellerich, T., Serve, H., Röllig, C., Bornhäuser, M., Thiede, C., Baldus, C., ... Müller-Tidow, C. (2023). A Dynamic rRNA Ribomethylome Drives Stemness in Acute Myeloid Leukemia. *Cancer Discovery*, 13(2), 332–347. <https://doi.org/10.1158/2159-8290.CD-22-0210>
- Zhou, F., Liu, Y., Rohde, C., Pauli, C., Gerloff, D., Köhn, M., Misiak, D., Bäumer, N., Cui, C., Göllner, S., Oellerich, T., Serve, H., Garcia-Cuellar, M.-P., Slany, R., Maciejewski, J. P., Przychodzen, B., Seliger, B., Klein, H.-U., Bartenhagen, C., ... Müller-Tidow, C. (2017). AML1-ETO requires enhanced C/D box snoRNA/RNP formation to induce self-renewal and leukaemia. *Nature Cell Biology*, 19(7), 844–855. <https://doi.org/10.1038/ncb3563>
- Zhou, K. I., Pecot, C. V., & Holley, C. L. (2024). 2'- O -methylation (Nm) in RNA: Progress, challenges, and future directions. *RNA*, 30(5), 570–582. <https://doi.org/10.1261/rna.079970.124>
- Zhu, H., Chen, H., Chen, G., Liu, M., Chu, L., Gu, Y., Chen, Y., Zhang, C., Qin, Q., Chen, Y., Chen, W., Fan, J., Nie, Y., Chen, J., Wu, S., Sun, X., Zhao, W., & Zhao, K. (2022). CBX4 contributes to radioresistance by regulating autophagic activity in esophageal squamous cell carcinoma. *Annals of Translational Medicine*, 10(18), 959. <https://doi.org/10.21037/atm-21-3630>
- Zhu, Y., Pirnie, S. P., & Carmichael, G. G. (2017). High-throughput and site-specific identification of 2'-O-methylation sites using ribose oxidation sequencing (RibOxi-seq). *RNA (New York, N.Y.)*, 23(8), 1303–1314. <https://doi.org/10.1261/rna.061549.117>
- Zong, W.-X., & Thompson, C. B. (2006). Necrotic death as a cell fate. *Genes & Development*, 20(1), 1–15. <https://doi.org/10.1101/gad.1376506>

11 Conferences and workshops

attended

International Congress of Cell Biology 2018

The Dynamic Cell

from Molecules and Networks to Form and Function

This is to certify that Sruthytha Kalali
has participated in the International Congress of Cell Biology
(ICCB), organized by CSIR-Centre for Cellular & Molecular
Biology (CCMB), Hyderabad, India, during
27th-31st January 2018.



Dr. Vegesna Radha



Dr. Jyotsna Dhawan



Dr. Satyajit Mayor



Organized by CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India



CERTIFICATE OF PARTICIPATION

International Conference on Radiation Research:
Impact on Human Health and Environment (ICRR-HHE 2018)
&
Second Biennial Meeting of Society for Radiation Research (SRR)

Organized by
University of Hyderabad, Prof C.R. Rao Road, Gachibowli, Hyderabad, Telangana 500046
under the aegis of SRR
February 1st – 4th, 2018

This is to certify that.....*Sruchytha Kalali*..... has attended
this pre-conference/ conference and chaired session/ delivered invited lecture/ presented
Oral/Poster.

Dr. Geeta K Vemuganti
Chairman, ICRR-HHE 2018

Dr. Nagraj Huilgol
President, SRR

Dr. B. N. Pandey
Secretary, SRR



December 21 - 23, 2018

XLII ALL INDIA CELL BIOLOGY CONFERENCE
and 2ND International Conference on
TRENDS IN CELL AND MOLECULAR BIOLOGY

CERTIFICATE

This is to certify that Prof./Dr./Mr./Ms. Sanchayita Kalai of University of Hyderabad has participated in the XLII All India Cell Biology Conference.

He/she has presented a paper entitled "_____"
 "_____ in oral/poster session of the conference.

[Signature]

Prof. Pradeep K. Burma
Secretary, ISCB

Angela von Loh

Dr. Angshuman Sarkar
Convener



School of Life Sciences
University of Hyderabad
Hyderabad – 500 046, INDIA



Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms./Mrs. Katali Sruthya
from department of Biochemistry has participated
in BioQuest-2017 held on 12 and 13 October, 2017 at School of Life Sciences, University of
Hyderabad.

Dean
School of Life Sciences

11TH BANGALORE MICROSCOPY COURSE 15-22 SEPTEMBER 2019

Certificate of Participation

This is to certify that **Sruchytha Kalali** of **University of Hyderabad, Hyderabad** has participated in the **11th Bangalore Microscopy Course** during **15th to 22nd September, 2019**, held at **National Centre for Biological Sciences, Bangalore**, jointly organized by the **National Centre for Biological Sciences -TIFR, Institute for Stem Cell Biology and Regenerative Medicine**, and **Centre for Cellular and Molecular Platforms**.


Shreyas Mayur

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WORKSHOP ON TUMOR MICROENVIRONMENT IN CANCER RESEARCH

Organized by

School of Medical Sciences, University of Hyderabad, India

13th November 2018

CERTIFICATE of PARTICIPATION

This is to certify that Dr./Mr./Ms./Shri *K. Suchytha* has
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Research", conducted by School of Medical Sciences, University of Hyderabad.


Dean
School of Medical Sciences,
University of Hyderabad


Prof. Geeta K Vemuganti
School of Medical Sciences,
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Workshop on Animal Models in Cancer Research

Organized by

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24th - 25th October 2017

CERTIFICATE

This is to certify that Dr./Mr./Ms./Smt. *Sruchytha Kalali* has participated
in the workshop on Animal Models in Cancer Research conducted by School of Medical Sciences, University
of Hyderabad.

Prof. Geeta Vemuganti,
Dean, School of Medical Science,
University of Hyderabad

Prof. Sarah E Coupland
Department of Molecular & Clinical Cancer Medicine
University of Liverpool

12 Publications



Potential role of Hematopoietic PBX-Interacting Protein (HPIP) in trophoblast fusion and invasion: Implications in pre-eclampsia pathogenesis

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pre-eclampsia/Furin

ABSTRACT

Pre-eclampsia is a known hypertensive disorder of pregnancy. While abnormal placentation and poor trophoblast invasion into maternal endometrium during blastocyst implantation are primary causes of pre-eclampsia, the underlying mechanisms remain elusive. Hematopoietic PBX-Interacting protein (HPIP) is an estrogen receptor (ER) interacting protein that plays a pivotal role in cell proliferation, migration, and differentiation; however, its role in trophoblast functions is largely unknown. In this study, we used BeWo cells as a model system to investigate trophoblast fusion and syncytialization, focusing on the role of HPIP in regulating these critical aspects of trophoblast functions. Herein, we report that HPIP expression declines during forskolin-induced trophoblast fusion in BeWo cells. In support of these observations, HPIP depletion enhanced forskolin-induced human chorionic gonadotropin- β (β -hCG), ERVWE1, and GCM1 expression, markers for trophoblast fusion. Furthermore, silencing of HPIP decreased cell invasion and epithelial to mesenchymal transition (EMT), a prerequisite for syncytialization in BeWo cells. Functional genomic studies further revealed a regulatory role for HPIP in a subset of gene networks involved in trophoblast fusion and EMT. We also uncovered that HPIP is a proteolytic substrate of furin, which is known to promote trophoblast cell fusion. Clinical data further indicated a significantly lower expression level of HPIP in pre-eclampsia subjects than in normal subjects. These findings imply that HPIP inhibits trophoblast fusion while promoting invasion and EMT, and its downregulation in trophoblasts might have implications for pre-eclampsia development.

1. Introduction

The trophoblasts are the vital components of the placenta and play a pivotal role in fetal development and maternal-fetal interactions. Trophoblasts comprise the outermost layer of the blastocyst, and they are differentiated into two primary subtypes: cytotrophoblasts and syncytiotrophoblasts. Cytotrophoblasts are responsible for proliferation and maintaining the trophoblastic lineage, whereas syncytiotrophoblasts form a multinucleated layer that facilitates hormone production, nutrient uptake, and waste removal [1,2]. During implantation, some

multinucleated syncytia acquire a transiently invasive phenotype and invade the decidualized endometrium. A large body of literature reported that autocrine, paracrine, and juxtacrine interactions mediated by various signaling pathways, including signaling by Forskolin, retinoic acid, transforming growth factor beta (TGF- β), epidermal growth factor (EGF), are known to be active in the trophoblast differentiation and invasion leading to proper implantation [3,4]. However, the precise molecular mechanisms and factors that direct the trophoblast to acquire differentiation and invasive phenotypes are elusive [5,6]. Aberrant trophoblast function has been linked to various pregnancy

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HPIP and RUFY3 are noncanonical guanine nucleotide exchange factors of Rab5 to regulate endocytosis-coupled focal adhesion turnover

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While the role of endocytosis in focal adhesion turnover-coupled cell migration has been established in addition to its conventional role in cellular functions, the molecular regulators and precise molecular mechanisms that underlie this process remain largely unknown. In this study, we report that proto-oncogene hematopoietic PBX-interacting protein (HPIP) localizes to focal adhesions as well as endosomal compartments along with RUN FYVE domain-containing protein 3 (RUFY3) and Rab5, an early endosomal protein. HPIP contains two coiled-coil domains (CC1 and CC2) that are necessary for its association with Rab5 and RUFY3 as CC domain double mutant, that is, mHPIPΔCC1-2 failed to support it. Furthermore, we show that HPIP and RUFY3 activate Rab5 by serving as noncanonical guanine nucleotide exchange factors of Rab5. In support of this, either deletion of coiled-coil domains or silencing of HPIP or RUFY3 impairs Rab5 activation and Rab5-dependent cell migration. Mechanistic studies further revealed that loss of HPIP or RUFY3 expression severely impairs Rab5-mediated focal adhesion disassembly, FAK activation, fibronectin-associated-β1 integrin trafficking, and thus cell migration. Together, this study underscores the importance of HPIP and RUFY3 as noncanonical guanine nucleotide exchange factors of Rab5 and in integrin trafficking and focal adhesion turnover, which implicates in cell migration.

redistribute the pools of integrin from one part of the cell to another (3–5). It has been known that the internalization of integrins *via* clathrin-mediated endocytosis regulates the turnover of focal adhesions (FAs) (6, 7). In this context, focal adhesion kinase (FAK) has been recognized as a key player in FA signaling/turnover and clathrin-mediated endocytosis as FAK-null cells display impaired FA turnover and decreased cell migration (6, 8, 9). While FAK activation is a prerequisite for endosome-mediated FA turnover and cell migration, the molecular regulators of FAK activation and coordinators of this process remain elusive.

Endosomal proteins, such as Rab5 and early endosome antigen 1, are essential players in the generation and propagation of early endosomes during endocytosis (10, 11). While endocytosis is associated with cell migration, in particular, the role of Rab5 in cell adhesion and migration has been pertinent. Several studies revealed that Rab5 regulates cell migration *via* cytoskeletal remodeling that results in Rac1 activation (12–14), β1 integrin internalization and recycling (13), or microtubule (MT)-dependent adhesion disassembly (15). While the current concepts envisage the crucial role of Rab5 in endocytosis-mediated adhesion signaling *via* integrins, the molecular regulators of Rab5 activity and the detailed mechanisms that underlie this process are not fully understood.

Hematopoietic PBX-interacting protein (HPIP) is an oncoprotein with an adaptor function that can integrate various signaling proteins in the regulation of cell migration (16–24). Its overexpression has been reported in various cancers (25). Not only that its overexpression is implicated in the development of osteoarthritis (26). Recent studies revealed a novel signaling axis involving HPIP and Talin in FA turnover and cell migration (27). While there is increasing evidence regarding its role in cell migration, its precise molecular mechanism in FA signaling and cell migration is elusive (27).

In this report, we have characterized HPIP and RUN FYVE domain-containing protein 3 (RUFY3) as noncanonical guanine nucleotide exchange factors (GEFs) of Rab5 that regulate

Endocytosis is an essential cellular process that regulates numerous signaling pathways by controlling cell surface receptors. It is increasingly evident that endocytosis modulates cell adhesion signaling *via* integrins, receptors for extracellular matrix proteins, to control cell migration (1, 2). During cell migration, integrins are internalized from the cell surface *via* endocytosis and recycled back to the plasma membrane to

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Tumor Heterogeneity in Breast Cancer Progression

Yasaswi Gayatri Mishra, Sruchytha Kalali, Ajnas Kizhuvedath, A. Indumathi, Arkaprabha Adhikari, Tanisha, and Bramanandam Manavathi

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Abstract

Cancer is an aberrant growth of cells with dynamic molecular changes as the disease progresses. As a result of these dynamic changes and an increase in cellular plasticity, cancer cells acquire varying molecular signatures as they develop and grow leading to what is now termed as tumor heterogeneity and is one of the leading causes in resistance to therapies. Heterogeneity can show up as

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