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Yunfeng Bai
Purdue University

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Sophie Lelievre, Chair	Ann Kirchmaier
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Approved by: Laurie Jaeger 3/3/2015

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ABBREVIATIONS

3C	Chromosome Conformation Capture
3D	three-dimension/dimensional
ANOVA	ANalysis Of VAriance
BM	Basement Membrane
CDS1	CDP-DAG synthease 1
Co-IP	Co-immunoprecipitation
ChIP	Chromatin Immunoprecipitation
CSK	CytoSKeleton
CSK-PI	CSK buffer containing PI
CT	Carboxyl-Terminus
DAPI	4', 6-DiAmidino-2-PhenylIndole dihydrochloride:hydrate
DCIS	Ductal Carcinoma *in situ*
DFC	Dense Fibrillar Component
DTT	DiThioThreitol
ECL	Enhanced ChemiLuminescence
EGF	Epidermal Growth Factor
EGTA	Ethylene Glycol-bis[-aminoethyl ether]-N,N,N',N'-Tetraacetic Acid
FA	Fatty Acid
FCs	Fibrillar Centers
FITC	Fluorescein IsoThioCyanate
GC	Granular Component

H14 medium	DMEM/F12 medium containing additives
HMT-3522	Human Mammary Tumor progression series 3522
LCIS	Luminal Carcinoma *in situ*
IGS	Intergenic Spacer
IF	ImmunoFluorescent labeling
JAM	Junctional Adhesion Molecule
kDa	kilo Dalton
MARs	Matrix Attachment Regions
mRNA	messenger RNA
NDHII	Nuclear DNA Helicase II
NES	Nuclear Export Sequence
NLS	Nuclear Localization Signal
nm	nanometer
NO38	NucleOlar protein 38
Nopp140	Nucleolar phosphoprotein 140 kDa
NPM	NucleoPhosMin
NT	amiNo-Terminus
NuMA	Nuclear-Mitotic Apparatus protein
NuMA-CT	NuMA Carboxyl-Terminus
PBS	Phosphate Buffered Saline
PBS-glycine	PBS containing 50mM glycine
pcv	packed cell volume
PI	Protease- and phosphatase-Inhibitors
PML	Pro Myelocytic Leukemia
PSP 1	ParaSpeckle Protein 1
rDNA	ribosomal DNA

rRNA	ribosomal RNA
RanGTP	Ran Guanosine Tri-Phosphate
RAR	Retinoic Acid Receptor
RCC1	Regulator of Chromosome Condensation 1
rDNA	ribosomal DNA
RNase A	RiboNuclease A
RNPs	RiboNucleoprotein Particles
rRNA	ribosomal RNA
SDS	Sodium DodecylSulfate
snoRNPs	small nucleolar RiboNucleoprotein Particles
snRNPs	small nuclear RiboNucleoprotein Particles
TBST	Tris Buffered Saline Tween
TJ	Tight Junction
UBF	Upstream Binding Factor
v/v	volume/volume
w/v	weight/volume
ZO	Zonula Ocluden

ABSTRACT

Bai, Yunfeng, Ph.D., Purdue University, May 2015. Functional Study of Mammary Epithelial Cell Architecture. Major Professor: Sophie Lelièvre.

Cell organization confers cellular identity and guides cellular functions. Here, the cell organization is defined as the global arrangement of the functional complexes, which are composed of proteins, nucleic acids, lipids, or the combinations of any of them, in the cells. These functional complexes are arranged in a spatial and temporal manner, which closely reflects and regulates cellular activities.Targeting two proteins that act as central nodes in the nucleus, the nuclear mitotic apparatus protein, NuMA, and in the cytoplasm, the CDP-diacylglycerol synthase 1, CDS1, I have studied their influences on two features of the cell architecture, namely the nucleolus and the polarity axis, that play a major role in the homeostasis of mammary epithelial cells and in cancerous development. The nucleolus is an essential nuclear body with functions in ribosome biosynthesis, stress perception, and cell cycle control. Basoapical polarity is the signature structure of the differentiated epithelial cells and is functionally related to cell proliferation and survival. By modulating the expression of NuMA or of CDS1 and assessing the resulting impact on the nucleolus or the tissue polarity axis, respectively, my goal was to further the understanding of the regulation and role of these architectural features in cell phenotypes. I have shown in my major project that NuMA is involved in the control of nucleolar architecture and rDNA transcription. I also showed in my minor project that CDS1 partially restores basal polarity depending on signaling in aggressive MDA-MB-213 cancer cells. I have placed these studies focused on cellular architecture in the context of cancer prevention, from its onset to progression.

CHAPTER 1: INTRODUCTION

1.1. Primary prevention of breast cancer

According to the estimation from the American Cancer Society (American Cancer Society Inc., 2014), about 232,670 new cases of invasive breast cancer and 62,570 extra cases of in situ breast cancer will be diagnosed in American women in 2014. Among the patients diagnosed in 2014, approximately 40,000 will die from breast cancer that same year. Worldwide, about 1.67 million new cases of breast cancer were predicted in 2012 and half-a-million of lives were to be lost (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). Considering these diagnosed patients are mothers, wives, daughters, and sisters, millions of people's lives have been or will be greatly affected not only physically but also psychologically and economically. Beyond the physical and psychological suffering, which is difficult to quantify, the estimated global economic loss from breast cancer was $88 billion in 2008, representing 0.15% of the world's gross domestic product of that year (American Cancer Society and LIVESTRONG, 2010). This economic loss does not even include the direct medical cost of the treatment of breast cancer.

As a devastating disease it still is, our society already has accomplished great strides during the long fight with breast cancer. Statistical data from the National Center for Health Statistics and Centers for the Disease Control and Prevention show that

mortality from breast cancer has steadily decreased annually by 1.8% in white, 1.6% in African American, 1.7% in Hispanics, and 1% in Asians/Pacific Islanders during 2001-2010 in the United States; it is unchanged in American Indians/Alaska Natives (American Cancer Society Inc., 2014). The dropdown of the mortality is attributed to improved treatment and widely applied screening. The overall breast cancer incidence has kept increasing until remained stable since 2004 in the USA, but unfortunately, the rising trend is not changed in most developing countries (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx). With no doubt, decreased mortality is partially due to the improved treatment as well as cancer screening that have attracted most funding resources. Treatment and screening are exclusively dealing with tumors already formed in the patients' body; therefore, these efforts could not affect breast cancer incidence, and the same large number of people still have to suffer from the disease. Primary prevention is the only way to reduce the incidence and the great damage from the disease because it is targeting the reduction of breast cancer occurrence.

Is breast cancer preventable and if so, how? Although the underlying mechanisms of cancer initiation remain largely unknown, existing evidence strongly supports the idea that breast cancer can be prevented to some extent. Studies on native Asians or Asian-Americans (Ziegler, Hoover et al. 1993) have connected the substantial increased risk of breast cancer with the adoption of western lifestyles. The results clearly suggested that the potential risk factors encompass more than the long-known genetic predisposition and carcinogens. Encouragingly for primary prevention research, these lifestyle-related risk factors are controllable, which will provide methodology for practice. To this date, many

risk factors have been identified and are categorized as non-modifiable factors (gender, age, personal and family history of breast cancer, and personal physical conditions) and modifiable factors including both lifestyle-related factors (postmenopausal hormone use, obesity, physical activity, diet, alcohol, tobacco, and oral contraceptive use) and other factors (exposure to radiation, specific chemicals, environmental pollution, and specific occupations). More detailed studies about the relative risk for each factor have been reviewed (Colditz and Bohlke 2014). It should be noted that primary prevention strategies can be designed for application not only to modifiable risk factors but also to some non-modifiable factors. For example, a study showed that treatment with Tamoxifen reduced the risk of Estrogen Receptor positive (ER+) invasive and in situ breast cancer by 42% in women with family history of breast cancer, and thus considered at increased risk for breast cancer (Fisher, Costantino et al. 1998). Prophylactic mastectomy is also considered as a particular primary prevention strategy for women with genetic *BRCA1* or *BRCA2* mutation. Ideally, the strategies of primary prevention should be simple, painless, and less expensive than detection and treatment. With an enhanced understanding of the risk factors in the future, personalized design of primary prevention strategies is expected to play an increasingly important role in the battle against breast cancer.

Yet, one awkward situation that the primary prevention of breast cancer field has to face is that most essential but non-modifiable risk factors, such as aging, being female, and family history of breast cancer, seem to be unpreventable at present. Furthermore, for most of modifiable lifestyle-related risk factors, like alcohol use, the predicted preventive

efficacy can be only expected in populations rather than the individual cases. These issues are actually addressing the same fundamental aspect of the current state of primary prevention research, i.e. our large knowledge gap between the risks and true cancer occurrence. Rome wasn't built in a day, so wasn't the aggressive cancer. A series of errors and abnormal activities have to accumulate to transit a normal cell into a cancer cell and logically, these mistakes should be accompanied with morphological alterations occurring at different levels during tumorigenesis. Cancer is a disease arising from cells that have lost proliferation control. Therefore, understanding the molecular and cellular activities that promote cell proliferation and the related morphological changes at molecular, cellular, and tissue levels will help filling the knowledge gap between risk and cancer initiation. In addition, characterization of the morphological abnormalities of the cells and tissue in pre-cancer and progression stages will also benefit early diagnosis of breast cancer. The mostly applied method for breast cancer screening at present is mammography, which enables the detection of tumor at median size 1.0 – 1.5 cm (Guth, Huang et al. 2008). Better characterization of pathological alterations in early stage cancer nodules will improve the accuracy of cancer diagnosis and provide valuable information for the appropriate treatments of these small tumors.

To achieve these goals we need a better understanding of the cell architecture. The concept of cell architecture here is defined as the characteristic arrangement of cellular complexes based on their function cell-wide under normal physiological condition. The cellular functional complexes can be any entities, comprising proteins, nucleic acids, lipids or their combinations, carrying out certain biological functions and

displaying a distinct ‘geographic’ pattern in a spatial and temporal manner. To be more specific, mammary epithelial cells in the normal breast tissue always display a typical architecture conveyed by cell polarity and nuclear organization, which defines their identity and functions to be different from other types of cells (Lelievre 2010; Grafton, Wang et al. 2011).

1.2. Mammary epithelial polarity as a cell quiescence controlling machinery

It is a well-accepted rule that structure determines function in the inorganic world as well as in organic chemistry; however, in living organisms, this rule is compromised by the evolution. In fact, after long-term natural selection over random genetic mutations, the relationship between structure and function in modern organisms is accustomed to the mode of mutual adaptation. The interdependent nature of structure and function in the body allows for any change on one side to influence the other side, which can be evidenced by the diseased organs, tissues, and cells in the body.

Human breast consists of mammary glands, where breast cancer occurs, surrounded by fatty and connective tissues. The mammary gland is a highly organized tissue composed of two basic units, namely the acinus and the duct. The acinus is a sphere-like structure with a single continuous layer of luminal epithelial cells that secrete milk proteins during the lactation period and a discontinuous layer of myoepithelial cells lining the luminal cells against the stroma to expel milk into the duct. Through a small duct called terminal duct, several acini are connected to larger ducts branched to other ducts also terminated by groups of acini to form a lobular structure similar to a bunch of

grapes. There are about 8-15 of such ductal systems or lobes in each breast that each open via an enlarged duct at the nipple.

Depending on where the lesion grows, breast epithelial proliferative diseases are mainly classified into two types, lobular and ductal. Based on whether the basement membrane is infiltrated or not, breast epithelial proliferative diseases are categorized as pre-invasive and invasive stages. Accordingly, the morphological characteristics for each type of lesion have been well documented and are used for clinical diagnosis. But as mentioned above, the earlier the disease stage, the less we know. Hyperplasia is considered to happen prior to carcinoma *in situ*, both of which are pre-invasive stages. Histological analysis of hyperplasias has revealed that there is a disruption of normal mammary gland architecture. The most prominent change is the replacement of the single layer of luminal epithelium by a multilayered epithelium due to the increased number of cells in the lumen. Studies also suggested a slight increase of risk of invasive carcinoma from both usual hyperplasia and atypical hyperplasia (Elston and Ellis 1991).

It is not known how hyperplasia initiates, yet a clear clue is that the lesion sites contain more cells than in normal conditions, which likely implies somewhat of a loss of proliferation control in a portion of the cell populations. With the exception of pregnancy and lactation, most mammary epithelial cells maintain quiescence. The resting normal epithelium displays the unique tissue architecture, namely basal-apical polarity. It is defined by the location of tight junctions (TJ) at the apical side of cell-cell contacts against the lumen, while at the basal side exemplified by cell-extracellular matrix contacts α6β4 integrin dimers accumulate against the basement membrane. Basal polarity

functions in anchorage and signal exchange between cells and their surrounding extracellular matrix. Impaired basal polarity will induce cell anoikis (Eble and Haier 2006). The classical function for apical polarity is to block the diffusion of small molecules, such as proteins and fat, along the cell membrane of luminal cells (Krug, Schulzke et al. 2014). But lately studies also uncovered its role in cell homeostasis maintenance and proliferation control.

Tight junctions seal two adjacent epithelial cells at the apical pole through the interaction of three transmembrane proteins claudins, occludins, and junctional adhesion molecule (JAM) produced by each neighboring cell. The appropriate localization, assembly, and function of TJ are supported by many other interacting protein complexes under the cell membrane and, together, they are referred as the apical junctional complex (AJP) (Lelievre 2010). Currently identified members that regulate TJ formation are Crumbs3/Pals-1/PATJ complex, PAR-6/PAR-3/aPKC/Cdc42 complex, and Scrib/DLg/Lgl. Zonula Ocluden (ZO)-1/ ZO-2/ZO-3 proteins that provide the scaffold of TJ and mediate the connection of TJ to cytoskeletal F-actin. More information on AJP regulators and their functions can be found in a recent review (Van Itallie and Anderson 2014).

Studies in *Drosophila* indicate that tight junctions form after adherens junctions (Lelievre 2010). Unfortunately the tight junction feature is difficult to reproduce in cell culture. Among numerous human mammary epithelial cell lines that still maintain adherens junctions as well as basal polarity, the non-neoplastic HMT-3522 S1 cell line is one of the very few models that recapitulate apical tight junctions as indicated by staining

for tight junction markers ZO-1, ZO-2, Par3, and Pasl-1 under three dimensional (3D) cell culture conditions (Plachot, Chaboub et al. 2009). It seems that the architecture of apical polarity is extremely sensitive to environmental/cell functional changes compared to lateral and basal structures of polarity. Indeed, the fragility of apical polarity is understandable given the involvement of the ever-increasing number of proteins identified at the APJ. Accordingly, it is not surprising to witness the profound influence that the disruption of apical polarity has on cellular activities when the interactions among different APJ proteins go awry. Work from our laboratory has demonstrated that acini produced in 3D culture to display impaired apical polarity contain cells primed to enter the cell cycle, as indicated by the expression of the cell cycle marker Ki67 upon stimulation, compared to cells contained in acini with intact apical polarity (Chandramouly, Abad et al. 2007). We have hypothesized that apical polarity is a barrier for functionally differentiated mammary epithelial cells to enter the cell cycle upon environmental stimuli. The effect of apical polarity loss to facilitate proliferation could be caused by different mechanisms. The intact AJP is an apparatus composed of proteins important for transcription activity, epigenetic modification, and signal transduction (Gonzalez-Mariscal, Dominguez-Calderon et al. 2014). Thus, the presence of apical polarity possibly acts as a symbol of functional balance among various signaling pathways.

The functional complexity of apical polarity is further evidenced by a microarray study on acini produced in 3D culture with apical polarity disrupted through two different methods conducted by our laboratory. An overlap of thirty genes with transcriptional

changes upon apical polarity disruption has been identified, with genes belonging to distinct groups based on their functions including transcription factors, epigenetic modifiers, and signal transducers. Of particularly interest, a gene *CDS1* involved in lipid metabolism was also identified (Cardenas JM, Ph.D. Thesis, 2012).

1.3. Lipid metabolism in cancer cells and the potential influence on polarity

CDS1 was identified in our earlier work to be down-regulated upon the disruption of apical polarity in a 3D cell culture model mimicking the differentiated mammary epithelium. Its protein product is the cytidine diphosphate-diacylglycerol (CDP-DAG) synthetase 1 responsible for the production of phosphatidylinositols (PI) from phosphatidic acid (PA) (Lykidis, Jackson et al. 1997). The CDP-DAG pathway possesses a unique position and essential functions in the whole lipid metabolism network. The *de novo* synthesis of PA, the upstream substrate of CDS1, uses glucose-6-phosphate and fatty acid (FA) as substrates; thus it connects the glycolysis pathway and the FA synthesis pathway (Baenke, Peck et al. 2013). The CDP-DAG pathway also generates PI, phosphatidylglycerol (PG), and cardiolipin downstream products, suggesting that it mediates multiple biological functions. By all means, CDS1 should be an ideal example to study the influence of the metabolism, especially lipid metabolism, on tissue architecture and cancer development.

Deregulated cellular metabolism in cancer cells has gained more and more attention in recent years thanks to the implementation of several high throughput techniques, like chromatin immunoprecipitation followed by sequencing (ChIP-seq) and liquid chromatography/mass spectrometry (LC-MS). Because of its indispensable role in

supporting rapid cell proliferation, energetic metabolism reprogramming has been considered as an emerging hallmark of cancer (Hanahan and Weinberg 2011). Perhaps the most seminal observation linking metabolism to cancer is the "Warburg effect" proposed in 1920s and revealing that glucose is mainly consumed in a low-ATP-generating way, namely aerobic glycolysis, in cancer cells compared to the much more efficient oxidative phosphorylation in differentiated cells (Warburg 1956; Gatenby and Gillies 2004). Noteworthy, the switch to aerobic glycolysis was also discovered in some types of non-neoplastic proliferating cells in recent studies (Vander Heiden, Cantley et al. 2009). The reason for cancer cells metabolizing glucose primarily through aerobic glycolysis remains elusive, but it is believed to generate more intermediates to meet the needs for the synthesis of macromolecules, like nucleic acid and lipids, in rapidly dividing cells. The cause-or-consequence debate regarding altered metabolism in cancer development lasted until the identification of cancer-associated metabolic enzymes that include tumor suppressor genes fumarate hydratase (FH), succinate dehydrogenase (SDH), and oncogene isocitrate dehydrogenase (IDH) (Losman and Kaelin 2013), as well as the most recent finding of histone deacetylase SIRT6 that controls cancerous metabolism activation (Sebastian, Zwaans et al. 2012). These studies have strongly supported the driver role of dysfunctional metabolism in tumorigenesis.

Together with major glucose metabolic pathways switched to aerobic glycolysis in cancer, *de novo* fatty acid (FA) biosynthesis is also recognized in many cancer tissues as the hallmark of dysfunctional lipid metabolism (Menendez and Lupu 2007). In healthy adult organisms and most tissues (except for liver, adipose tissue and lactating breast), *de*

novo FA biosynthesis shuts down or is only maintained at basal level because cell fatty acids are mostly acquired from the bloodstream; however, such biosynthesis is reactivated in many cancer cells even if enough circulating FAs are present in the microenvironment (Menendez and Lupu 2007). Again, the reason for FA metabolism network redirection is arguably to meet the dividing demands from cancer cells, but that is apparently not the only explanation (Hopperton, Duncan et al. 2014).

Lipids refer to a large group of compounds with diverse structures; roughly, they can be considered as the collection of fatty acids, their derivatives and substances related biosynthetically or functionally to these compounds. Fatty acids are naturally synthesized via condensation of malonyl coenzyme A units by a fatty acid synthase complex. They usually contain even numbers of carbon atoms in straight chains (commonly C14 to C24), and may be saturated or unsaturated; they can also contain other substituent groups (the AOCS lipid library, http://lipidlibrary.aocs.org/Lipids/whatlip/index.htm). The large variety of functions for lipids (membrane constituents, signaling molecules, energy storage components, etc.) raises the question of how they influence cancer development. To address this question, I would like to first review some specific FAs known to be associated with breast cancer occurrence; then move to the regulatory proteins and enzymes important for cancer formation in *de novo* FAs biogenesis pathway; and, at last, I will discuss current knowledge about the potential link between lipid metabolism and mammary polarity.

Epidemiological data have connected higher breast cancer incidence to higher consumption of ω-6 FAs and lower incidence to higher uptake of ω-3 FAs. These results

unveiled a very interesting pattern between global geographical distribution of higher breast cancer incidence and local diet spectrum regarding the FA types. Namely, foods with higher ω-3 FAs are preferred in the eastern world with lower breast cancer incidence, while high incidence-associated ω-6 FAs enriched diet is prevalent in the western world (Cardenas, JM. Ph.D. Thesis, 2012). The different risk effects on breast cancer incidence from different types of FA have attracted much attention for their potential values in primary prevention. In an effort to understand the preventive effect of FAs, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), extensive studies have been conducted in different cancer models. While the underlying mechanism remains elusive, results showed that ω-3 FAs play inhibitory roles on almost all aspects of the hallmarks of cancer (Stephenson, Al-Taan et al. 2013). Physiologically, ω-3 EPA and ω-6 Arachidonic Acid (AA) are both components of structural membrane phospholipids, and they are also acting as the precursors for the production of eicosanoids mediated by cyclooxygenase (COX) or lipoxygenase (LOX) enzymes. It is believed that the anti-inflammatory molecules generated from EPA are less biologically active than the pro-inflammatory AA-derivatives (Farooqui, Horrocks et al. 2007), which could partially explain the tumor suppressor function of ω-3 FAs, considering that inflammation can promote cancer development.

In addition to the lipids *per se*, some enzymes and regulatory proteins involved in the *de novo* biosynthesis of FAs are important for cancer development as well. As mentioned above, aerobic glycolysis and *de novo* FA biosynthesis are the two featured events of neoplastic reprogramming cross-talking to each other in most cancer cells.

Aerobic glycolysis produces large amounts of pyruvate, which enters mitochondria to turn into citrate, and the latter is eventually transported into the cytosol and used for *de novo* FA biosynthesis. Cytosolic citrate is first converted into acetyl-CoA by ATP citrate lyase (ACLY) followed by carboxylation to malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACACA). The condensation of acetyl-CoA and malonyl-CoA to produce the most common 16-carbon saturated FA palmitate is under the control of fatty acid synthase (FASN). All three enzymes have been reported to be dramatically increased in multiple types of cancer cells and the inhibitory treatments targeting these enzymes could suppress proliferation *in vitro* and limit tumor growth *in vivo* (Hatzivassiliou, Zhao et al. 2005). In contrast to nontransformed murine hematopoietic cells, which are resistant to apoptosis after ACLY is silenced, (Bauer, Hatzivassiliou et al. 2005), breast cancer cells with silenced ACACA undergo apoptosis, but this can be rescued by supplementation of palmitate in the medium (Chajes, Cambot et al. 2006). This observation undermines the potential for ACACA to be an anticancer drug target. Interestingly, immunohistochemistry studies on different stages of breast cancer show that elevated expression of ACACA and FASN already appears in DCIS and LCIS (Milgraum, Witters et al. 1997), the earliest stages that can normally be clinically detected prior to the massive cell proliferation, which suggests an active rather than passive involvement of the activation of *de novo* FAs biosynthesis in early tumorigenesis. In agreement with this postulate, the normal-appearing mammary gland tissues from the same patients showed that a limited number of cells expressing ACACA and FASN are confined to lobules and terminal ducts. Cancer usually initiates in terminal ducts (Milgraum, Witters et al. 1997), where the mammary stem cells are also located (Esslimani-Sahla, Thezenas et al. 2007).

Among the three enzymes, FASN attracted most attention because it catalyzes the last key step of the FAs biosynthesis. In the early days, FASN was used as a predictor for the recurrence of breast cancer, and the expression level of FASN was positively correlated with poor prognosis and mortality (Kuhajda, Piantadosi et al. 1989). Ectopic expression of FASN in nontransformed breast epithelial cell lines, MCF10A and HBL100, significantly promoted cell proliferation via the activation of HER1/HER2 signaling cascade (Vazquez-Martin, Colomer et al. 2008), but there seems to be a positive feedback loop to promote mutual activation between HER1/HER2 and FASN. Indeed, on one hand, it has been shown that FASN is a downstream component of HER1/HER2 (Jin, Yuan et al. 2010; Li, Bu et al. 2012); on the other hand, immunohistological study of breast lesions at different stages revealed that over-expression of FASN is already detected in the nonproliferative benign breast disease lesions where HER2 level is yet to increase (Milgraum, Witters et al. 1997). Given the facts that inhibition of FASN induces cell cycle arrest and over-expression of FASN activates HER2 signaling pathway, FASN behaves more like a "driver" role in cancer initiation. Yet, despite a clear positive correlation, no evidence has been reported to support a causal effect of ACLY, ACACA, and FASN in cancer initiation.

In parallel with the saturated FA (palmitate) pathway, cholesterol biogenesis pathway (mevalonate) is also activated in the reprogrammed lipid metabolism in cancer cells. The two pathways branch after citrate converts into acetyl-CoA. Mevalonate and palmitate pathways are respectively regulated by two transcription factors, sterol regulatory element-binding proteins (SREBP) 1 and 2, from the same gene family.

SREBPs bind to sterol regulatory elements in the promoter regions of many genes coding for the major enzymes participating in lipogenesis to mediate their transcriptions. Currently identified signaling pathways upstream of SREBPs including Pi3K/AKT/mTOR and RAS/Raf/MEK/ERK again suggest a positive correlation of SREBPs expression with cancer development.

Several recent publications may shed light on the connection between the reprogrammed mevalonate pathway in cancer cells and the disruption of cell polarity. The mutation of tumor suppressor gene TP53 is frequently seen in breast cancer. In most cases, *p53* mutation occurs in concert with the elevation of the mevalonate pathway. Using a 3D cell culture system, the Prives group studied mammary gland morphogenesis under the influence of *p53*. They showed that over-expressing mutant p53 in nonmalignant MCF10A cells impaired lumen-like formation, and depletion of endogenously mutated p53 in the metastatic breast cancer cell line MDA-MB-468 could restore some architectural characteristics of *in vivo* mammary acini in 3D cell spheroids, including lumen-like formation as well as the basolateral polarity indicated by cytoskeleton actin and basally localized α6β4 integrins. Interestingly, the mevalonate pathway is the major pathway down-regulated upon the depletion of mutant p53 in reverted MDA-MB-468, and the reverted phenotype can be compromised by supplementation of upstream metabolites in the culture medium (Freed-Pastor, Mizuno et al. 2012). Although it has been shown before that p53 suppresses SREBP as a transcription factor (Yahagi, Shimano et al. 2003), this study is the first one to link the mevalonate pathway to tissue polarity. Unfortunately, the study described here did not

provide evidence regarding the reestablishment of apical polarity in the reverted cells as no information on tight junctions was given.

In many cancerous diseases, sustained activation of the Hippo signaling pathway promotes cell growth and proliferation, while it inhibits differentiation and apoptosis. Upon activation, its downstream effectors YAP/TAZ relocate from the cytoplasm to the cell nucleus where they function as transcriptional factors. Recent screening in breast cancer cell line MDA-MB-231 uncovered that YAP/TAZ nuclear translocation can be abolished by pharmaceutical inhibition of the mevalonate synthesis pathway suggesting a regulatory role of this pathway on the Hippo pathway (Sorrentino, Ruggeri et al. 2014). Surprisingly, numerous apical polarity-related proteins were identified to control the cytoplasmic compartmentalization of YAP/TAZ. The relevant proteins include Crb 1-3, Frmd6, NF2, Kibra, aPKC, PAR3, PAR6, PALS1, Scrib, Dlg, Lgl, AMOT, PEPN14, Ajuba/LIMD1/WTIP, α-catenin, β-catenin, ZO-1, ZO-2, and E-cadherin (Yu and Guan 2013). Given that shutting down the mevalonate pathway can inhibit the Hippo pathway by sequestering YAP/TAZ in the cytoplasm and also restore tissue architecture (at least basolateral polarity (Freed-Pastor, Mizuno et al. 2012), an emerging hypothesis that the mevalonate pathway activates the Hippo pathway by disrupting apical polarity might be formulated.

1.4. The nuclear bodies in cell nuclear organization

In addition to the polarity of the epithelium, another cellular architecture closely reflecting various cellular activities is the cell nucleus. This notion is not new at all since for decades, researchers have characterized several changes within the nuclear

architecture concomitant with transition to malignancy and applied such changes as classic criteria for cancer diagnosis, such as the increased nucleo-cytoplastic ratio and the number of nucleoli. The nuclear architecture of the cell is generally defined as the spatial and temporal arrangement of the nuclear bodies and chromatin within the nucleus. Nuclear bodies are highly dynamic structures raised from the nucleation and interactions among a large group of proteins/RNAs (Dundr and Misteli 2010). Due to the lack of lipid membrane outside of the nuclear bodies, the components, proteins and RNAs, exchange fast between the nuclear body and the surrounding environment. Yet overall, the nucleation, the location, and the composing elements of several types of nuclear bodies appear to remain stable. Thanks to the development of immunolabeling and imaging techniques, a number of new nuclear bodies have been identified like 53BP1 body involved in DNA repair (Panier and Boulton 2014) and polycomb body involved in transcription control (Pirrotta and Li 2012). But even for the nuclear bodies identified many years ago, important information is still elusive, especially regarding structure, biogenesis and biological functions. Because many nuclear bodies are found temporally associated with or close to specific chromosome loci, one of their functions is thought to be protein storage for assigned chromosome activities under particular circumstances (Dundr and Misteli 2010). In Table 1.4.1, I summarized current knowledge about the "classic" nuclear bodies including nucleolus, Cajal body, splicing factor speckles, Promyelocytic leukemia protein (PML) nuclear body, and perinucleolar body. The main reference is the book "the nucleus" published by *cold spring harb perspect biol*: 2010.

Table 1.4.1: Nuclear Bodies

Nuclear bodies	Signature components	Associated chromosomal loci	Major functions	Cancer-related events	reference
Nucleolus	RNAP I, Fibrillarin, UBF,	Nucleolar organizing regions (NORs)	Ribosomal RNA transcription	Increased number of active nucleoli and rRNA transcription in cancer cells	(Pederson 2011)
Cajal body (CB)	Coilin, Fibrillarin, survival motor neuron protein, SnRNAs, SnoRNAs	Preferentially associating with snRNA genes upon their transcriptional activation; transient association with telomeres during S phase; histone gene clusters;	snRNPs modification; telomere synthesis;	Telomerase RNA colocalises with CBs in cancer cells	(Nizami, Deryushev a et al. 2010)
Nuclear speckles/interchr omatin granules	SC35, snRNPs, RNAP II components	No DNA is found within the nuclear speckles, but they can be preferentially associating with highly transcribed chromatin loci.	Presumably coordinating the supply and/or recycling of pre-mRNA processing and transcription factors.	high number and smaller of speckles compared to phenotypically normal cells	(Lelievre, Weaver et al. 1998; Spector and Lamond 2011)
PML nuclear bodies	PML, SUMO, SP100, DAXX	Not associated with chromosomes in general conditions. Under stress or DNA damage, PML NBs can associate with specific loci.	Factory for posttranslational modifications, including acetylation, sumoylation, and phosphorylation of proteins.	PML NBs are disrupted in PML-RARα translation causing acute promyelocytic leukemia	(Lalleman d-Breitenbac h and de The 2010)
Perinucleolar compartments (PNC)	Pol II RNA binding proteins polypyrimidine tract binding protein, CUG-BP, KSRP, Nucleolin; small noncoding RNAs transcribed by Pol III including Alu RNA, hY RNA, MRP RNA, RNase P RNA, 7SL RNA	Localized at the periphery of nucleolus, but it is not clear if associated with the perinucleolar heterochromatin. Further evidence strongly supports PNC associating with specific DNA locus (sequence nonidentified).	Not clear but hypothetically involved in Pol III transcription regulation	Strongly correlated with malignancy in solid tumor	(Pollock and Huang 2010)

For therapeutic purpose, an attractive research field is to understand the biogenesis of nuclear bodies. In contrast to the assembly of cellular polarity that displays a hierarchical order in the recruitment of various protein complexes, current evidence support a stochastic self-organization model for the formation of specific nuclear bodies (Dundr and Misteli 2010). This model highlights the equal importance of the essential components of a nuclear body for its biogenesis. The model was proposed from the study showing that the Cajal body can be generated *de novo* by nucleating a number of its components including proteins and RNAs (Kaiser, Intine et al. 2008). Interestingly, the formation of the PML body was also observed by over-expressing a component protein of the PML nuclear body in the same study, suggesting that this model is not limited to the Cajal body. The randomness of the initiator and the recruitment process is the core concept of the stochastic self-organization model. Currently, the triggers in the first step of nucleation remain unknown for most nuclear bodies.

1.5. Chromatin organization and its biological functions in homeostasis

Our knowledge about chromatin organization in the cell nucleus is greatly improved due to the development of high-throughput molecular biology techniques, especially DNA next generation sequencing, chromatin conformation capture (3C) (Dekker, Rippe et al. 2002), and thereafter 3C derived techniques. An amazing fact about our genome is that the two-meter-long chromatin string has to be packed into a cell nucleus with an average 10-micrometer in diameter. To efficiently respond to various stimuli, the chromatin must be highly organized. Chromatin organization during the interphase of cell cycle is characterized at different levels. The primary structure of

chromatin is the well-known beads-on-a-string composed of histone octamers wrapped by linear DNA. The secondary chromatin organization is the 30 nanometer fiber formed by the interactions between neighbor nucleosomes of the primary structure. One of the supportive pieces of evidence came from X-ray diffraction that unraveled zigzag interaction among four nucleosomes with DNA strands (Schalch, Duda et al. 2005). Beyond the 30-nm fiber are 100-nm fiber structures and even larger fibers that are part of higher chromatin organization. These chromatin fibers are very likely organized into loops anchored to the insoluble nuclear matrix through specific chromatin domains. This proposed loop organization can be visualized protruding out of the nucleus with application of a DNA dye after soluble nuclear proteins are removed (Roti Roti and Wright 1987). DNA loops are essential structures to activate gene transcription by mediating the interaction between nonadjacent enhancer and promoter (Ong and Corces 2011). In addition to these somewhat local interactions, interchromosomal and intrachromosomal long-distance interactions are also executed by the loop structures. For example, genes associated with a particular function are often activated simultaneously, and frequent chromatin interactions among these genes can be detected by 3C although some of these genes are located on different chromosomes, indicating the spatial proximity for these DNA loops during transcription (Schoenfelder, Sexton et al. 2010; Tanizawa, Iwasaki et al. 2010). The mechanism of loop generation is poorly understood, but current evidence suggests a tripartite involvement of chromatin binding proteins, specific chromatin regions and nuclear structural proteins. A group of proteins were identified to engage in chromatin looping including CTCF, the cohesin/codensin complex, mediator, SATB1, etc., among which, CTCF and the cohesin complex are

mostly studied. CTCF binds to the insulator, a DNA domain that is localized either between the promoter and enhancer of a gene to block their communication or at the boundaries of chromatin domains to demarcate the active and the repressive domains (Cuddapah, Jothi et al. 2009; Van Bortle and Corces 2012). In many cases, the cohesin complex and CTCF co-occupy the insulator domains to coordinate transcription regulation (Wendt, Yoshida et al. 2008). The cohesin complex comprises SMC1, SMC3, RAD21, and SA2 proteins that, together, form a ring structure. By tethering two binding sites of cohesin on the same chromosome, the cohesin ring generates a chromatin loop structure. The interchromosomal interaction will be engaged if the binding sites are on two chromosomes. A fraction of cohesin binding on the genome is independent of CTCF. Instead of repressing gene transcription, these cohesin complexes interact with mediator at the promoter regions to initiate transcription (Kagey, Newman et al. 2010). Thus, cohesin seems to be the key factor for chromatin looping *per se* but it is not responsible for determining the activity of the target genes, which is likely controlled by cohesin binding partners as well as the cohesin binding regions. Earlier studies have suggested that the anchorage of the matrix attachment regions (MARs) on the nuclear matrix is the structural basis for chromatin looping (Mirkovitch, Mirault et al. 1984; Heng, Goetze et al. 2004). MARs are AT-rich domains, yet they have no consensus sequence and are dispersed throughout the genome with a localization propensity at gene boundaries (Loc and Stratling 1988; Levy-Wilson and Fortier 1989) or near regulatory elements (Cockerill and Garrard 1986; Gasser and Laemmli 1986). Nuclear matrix is a putative dynamic nuclear skeleton structure formed by insoluble nuclear proteins to support the whole nuclear architecture and thus, nuclear activities. Multiple studies showing the association

of CTCF/cohesin with the nuclear matrix as well as the overlap of their binding regions with MARs (Nabirochkin, Ossokina et al. 1998; Hakimi, Bochar et al. 2002; Dunn, Zhao et al. 2003; Yusufzai and Felsenfeld 2004) have indicated the indispensable role of nuclear matrix proteins in chromatin looping. The binding of MARs onto the nuclear matrix is gene activity and cell type-dependent, which reflects the dynamic nature of the gene loci in 3D context. Consistent with this notion, particular cancer-related genes were shown to reposition in a breast cell line upon acquisition of malignancy (Meaburn, Gudla et al. 2009).

Chromatin self-organizes into globule-like structures within every one-megabase interval in human cells (Dixon, Selvaraj et al. 2012; Nora, Lajoie et al. 2012), termed topologically associating domains (TADs), which was also seen in the fruit fly (Sexton, Yaffe et al. 2012) and budding yeast (Mizuguchi, Fudenberg et al. 2014) at different base length intervals. Very interestingly, CTCF and cohesin were respectively described to bind at the boundaries of TADs in human cells and budding yeast. Although only CTCF was reported to bind about 15% of the boundaries of TADs in human cells so far (Nora, Lajoie et al. 2012), it would not be surprising to see cohesin also bind to these regions at even higher frequency given the results in yeast. These discoveries further reinforce the essential role of CTCF and cohesin in global chromatin organization. Beyond a megabase scale at single chromosome level, it has been shown that multiple gene-rich regions tend to cluster together both with fluorescence *in situ* hybridization (FISH) (Shopland, Lynch et al. 2006) and 4C (Simonis, Klous et al. 2006) techniques. A general agreement is that the gene-rich regions have more chance to localize within the interior of

the nucleus compared to the gene-poor regions that are more often closed to the nuclear periphery (Branco and Pombo 2006), which raised the assumption that the periphery of the nucleus is less permissive for gene transcription. Indeed, this idea was reinforced by the observation that the transcription was decreased when a reporter gene was tethered to the peripheral nuclear lamina. This nuclear structure is composed of Lamin A/C and Lamin B that, together, form a meshwork underneath the nuclear envelope (Dechat, Pfleghaar et al. 2008). About 40% of the genome is associated with the nuclear lamina; and these genomic regions are called lamina-associated chromatin domains (LADs) (Guelen, Pagie et al. 2008). CTCF and cohesin are extensively occupying the chromatin regions adjacent to the LADs, suggesting that a large amount of loops is attached to the lamina.

Genome wide, chromosomes are arranged within chromosome territories. The concept of chromosome territories was proposed by Boveri, T in 1909, who argued that, as in mitosis, chromosome retains individuality during interphase by staying in their own place. The model of chromosome territories was supported by a series studies, and finally beautifully displayed in the human cell nucleus by Bolzer *et al.* using 3D FISH technique combined with multicolor-labeled probes targeting different chromosomes (Bolzer, Kreth et al. 2005). It demonstrated unequivocally that the chromatin is not randomly arranged in the nucleus. It should be noted that although the majority of individual chromosome parts stay in the chromosome territory, it is common to detect intermingling of different chromosomes at the territory boundaries that are thought to be sites filled with most of the types of nuclear bodies mentioned above, notably splicing factor speckles and

Polymerase II transcription factories (Deng, Melnik et al. 2013). Based on transcription activity, nuclear chromatin is characterized into two states, euchromatin (active) and heterochromatin (inactive). The geographic distribution of chromatin shares some similarities among different cell types, for example, heterochromatin is accumulating at the nuclear periphery and the nucleolar periphery upon cell differentiation. Nucleolus-associated chromatin domains (NADs) also have been identified (Nemeth, Conesa et al. 2010); strikingly, they are extensively overlapping with LADs. It is possible that a portion of heterochromatin can randomly associate to the nucleolus or the lamina. If this is the case, the interphase chromatin may display a divergent organization while the cells possess identical functions.

Chromatin organization determines cell function and identity. Maintaining a particular chromatin organization is as important as specific transcription factors in producing induced pluripotent stem cells (Yu, Vodyanik et al. 2007). Even if the over-expressed transcription factors displayed the same binding profile on specific promoter regions, the cell could not be reprogrammed into pluripotent stem cell without chromatin forming the correct loop structure (Wei, Gao et al. 2013; Zhang, Jiao et al. 2013). Altered chromatin organization is one of the remarkable features of cancer cells (Suva, Riggi et al. 2013). Genome-wide sequencing studies based on 3284 tumors have identified a quarter of a million genetic mutations spread on 20,000 protein-coding genes, but only about 125 mutated genes were considered to play the “driver role” in cancer initiation, among which, 20% functions in chromatin regulation (Vogelstein, Papadopoulos et al. 2013). These “cancer-driving” nuclear architectural organizers include enzymes for

epigenetic modifications and chromatin binding proteins, which accordingly determine the degree of local chromatin compaction and 3D chromatin topology, both known to profoundly affect gene transcription activity (Dawson and Kouzarides 2012) as well as genome integrity. To this end, identification and characterization of such architectural proteins will fundamentally improve our knowledge of cancer and thus, its prevention.

1.6. The function of NuMA in chromatin organization control

The nuclear mitotic apparatus protein (NuMA) was first recognized for its well defined role in stabilizing mitotic spindle during cell division (Lydersen and Pettijohn 1980), which makes NuMA an essential protein for cell proliferation and the development of organisms. However, beyond mitosis, NuMA is intensively expressed in the interphase nucleus in multiple cell types (Abad, Lewis et al. 2007), including the neuron cells, years after differentiation and quiescence have occured (Silk, Holland et al. 2009; Radulescu and Cleveland 2010), which raised the interesting postulate that NuMA must perform functions in the nucleus aside from mitosis. NuMA is a large protein of about 240 kilo Dalton with a central coiled-coil domain. The coiled-coil domain provides the structural basis for protein self-polymerization, which was exemplified for many other proteins and protein complexes, like Lamin family members and SMC family members in cohesin/condensin (Herrmann and Aebi 2004; Poon and Mekhail 2011). Indeed, in Hela cells with NuMA transiently over-expressed, hexagon oligomers were formed by the self-organization of NuMA and a large amount of these oligomers arranged into a lattice-like structure filling the nucleus and resistant to detergent extraction (Gueth-Hallonet, Wang et al. 1998; Harborth, Wang et al. 1999). Due to the

insolubility of a fraction of NuMA proteins and the interaction of NuMA with MARs, it has been proposed that NuMA is a nuclear matrix protein (Luderus, den Blaauwen et al. 1994; Nickerson 2001). Several NuMA interacting partners in the nucleus have been reported including GAS41 (Harborth, Weber et al. 2000), NOCA6 (Ko and Chin 2003), and PIM1 (Bhattacharya, Wang et al. 2002), but their role associated with NuMA remains unknown.

Our laboratory has been long focusing on the nuclear function of NuMA after first observing a remarkable redistribution of NuMA in the cell nucleus upon differentiation in non-neoplastic mammary epithelial HMT-3522 S1 cells in 3D cell culture (Lelièvre, Weaver et al. 1998), which led to the assumption that the rearrangement of NuMA is concomitant with nuclear reorganization and tissue architecture. Consistent with this idea, differentially distributed NuMA was observed between non-neoplastic S1 cells and S1-derived invasive T4-2 cancer cells (Knowles, Sudar et al. 2006; Chandramouly, Abad et al. 2007), the S1 cells that were seeded on different extracellular matrix materials, as well as the S1 cells exposed to radiation (Vidi, Chandramouly et al. 2012). In addition, NuMA accumulates at DNA damage spots within 30 minutes (Vidi, Liu et al. 2014). All these subtle redistributions of NuMA corresponding to different environmental stimuli suggested that, at least, a subset of NuMA is dynamic and sensitive to microenvironmental cues.

As discussed earlier, the nuclear architecture comprises nuclear bodies and chromatin organized in a spatial and temporal manner. Interestingly, NuMA colocalization with splicing factors (Lelièvre, Weaver et al. 1998) and association with chromatin (Abad,

Lewis et al. 2007) were both observed in the breast epithelial model. Hence, NuMA possibly plays a global nuclear architectural role by regulating nuclear bodies and chromatin organization together. The involvement of NuMA in higher order chromatin organization, as witnessed by the location of heterochromatin and euchromatin, was established (Abad, Lewis et al. 2007), but the underlying mechanism is still missing. Studies from our group have identified ISWI SNF2h ATPase as one of NuMA's binding partners (Vidi, Liu et al. 2014), pointing out a reasonable direction for further analysis. given the fact that SNF2h participates in several chromatin remodeling complexes (CRCs) (Dirscherl and Krebs 2004) involved in different cellular functions. Following this clue, SNF2h-containing CRCs were assessed for their potential interactions with NuMA, and the B-WICH complex functioning in rDNA transcription was identified (Jayaraman, S. Ph.D. Thesis, 2014). Combined with several other pieces of evidence from our previous work indicating that NuMA might function in the nucleolus (the detailed discussion can be found in Chapter 2), I proposed my hypothesis that NuMA might control the architecture of the cell nucleolus. Consequently, the nucleolar architecture and function is introduced in the next section.

1.7. Cell Nucleolus

"The nucleolus probably should not be considered a relatively simple organelle with a single function, comparable to a machine tool turning out a particular part of an automobile. It is not just 'the organelle where the cell manufactures ribosomes.' It is rather a structure through which materials of several different kinds are flowing, comparable more to a whole production line than to a single machine tool."

The cell nucleolus is the most prominent nuclear body because of its high visibility, even under the light microscope, and its documentation that can be tracked down to the seventeenth century, decades earlier than other nuclear bodies. In nucleolus research history, two breakthrough discoveries probably are the identification of the nucleolar organizing region (NOR) as a fragment of the chromosome where the nucleolus forms, and the demonstration of the nucleolus function in ribosome biogenesis. The generation of nucleoli taking place on NORs was first reported by Heitz, E in 1931, and the link between ribosome biogenesis and nucleolus was established by Donald Brown and John Gurdon in 1964 (Brown and Gurdon 1964). Immediately after the discovery of the nucleolus function in ribosome biogenesis, Conrad Hal Waddington made the comment that I cited at the beginning of this section. I came across these remarks in a memorabilia-like review article (Pederson 2011) and I am surprised too, as much as the author of the article, by the accuracy of Waddington's perspective half a century ago, as the advances in research on the nucleolus are showing every word of the statement to be true.

Electron microscopy analysis of the nucleolus in mammals revealed a tripartite structure including the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). With some disagreement on the functions of these individual regions, a more commonly accepted opinion is that ribosome DNA transcription occurs in DFC as well as at the border of FC and DFC, while GC is the place for ribosomal RNA maturation. Both RNA Polymerase I (Pol I) and rDNA were found in FC, but they are

thought to lack transcription ability. Once the transcription is activated, Pol I-bound rDNA relocates to DFC (Raska, Shaw et al. 2006). Permeability and protein density analysis of the nucleolus in *Xenopus* oocyte showed a higher density of the dense fibrillar component, indicating that more proteins are present in the DFC compared to the granular component (Handwerger, Cordero et al. 2005). Permeability assay showed that GC is a sponge-like structure filled with two types of particles, protein-rich and nucleic acid-rich – likely reflecting its functions in rRNA processing and ribosome assembly.

Outside the nucleolus is a heterochromatin shell. The perinucleolar heterochromatin in human HeLa cervix carcinoma cells (Nemeth, Conesa et al. 2010) and HT1080 fibrosarcoma cells (van Koningsbruggen, Gierlinski et al. 2010) has been isolated and sequenced. The identified chromosomal regions mainly include "satellite repeats, members of the zinc-finger, olfactory receptor defensin, immunoglobulin protein-coding gene families, transcriptionally active 5S rRNA genes and tRNA genes" (Nemeth and Langst 2011) accounting for 4% of the whole genome (unsequenced rDNA-bearing short arms are not included). In addition, a fraction of centromeres (Pluta, Mackay et al. 1995) and telomeres were also reported as the constituents of the perinucleolar heterochromatin, probably due to their physical proximity to NORs on the acrocentric chromosomes. In every sense, the perinucleolus is a subnuclear site characterized as transcription-repressive environment. Interestingly, this "transcription-unfriendly" site is found to transiently associate with many specific nuclear components in unusually high frequency at different cell stages, which implies some potential biological functions still to be elucidated. For example, large heterochromatin bulks

relocating to the perinucleolus is a signature of mammary epithelial cell differentiation (Lelievre 2009). Chromosome $X_{inactive}$ remains silenced through its contact with perinucleolar heterochromatin in S phase (Zhang, Huynh et al. 2007). Some nuclear bodies, like splicing factor speckles (Lelièvre, Weaver et al. 1998), Cajal body, PML, and the perinucleolar compartment, were reported to be close or in touch with the periphery of the nucleolus, yet the functions of these contacts are elusive. With respect to the chromatin relocation to the perinucleolus, several chromatin binding proteins are thought to be important for the procedure, like CENPC1 (Wong, Brettingham-Moore et al. 2007) and CTCF (Yusufzai, Tagami et al. 2004).

Although the nucleolus is considered as one of the nuclear bodies, it possesses a fundamental difference compared to others, which is that the assembly of the nucleolus completely depends on the specific chromosomal domains NORs. NORs are tandem rDNA arrays arranged in head-to-tail coding for ribosomal RNAs. The copy number of rDNA and NORs varies largely among species. The human genome contains about 400 copies of rDNA distributed over five pairs of NORs located between the satellite repetitive elements and the pericentromeric region on the short arms of acrocentric chromosomes 13, 14, 15, 21, and 22. Due to high repetition, DNA sequences of the short arms of these chromosomes are still not deciphered (Shaw and Mckeown 2011). The large copy number of rDNA is believed to meet the requirements of ribosome biogenesis for protein synthesis in proliferating cells. Indeed, ribosomal RNA accounts for 60% and 35% of total RNA synthesis respectively in yeast and dividing mammalian cells (Moss, Langlois et al. 2007). Yet, genetically modified yeast strain with 20-copy rDNAs showed

no difference regarding the rRNA level compared to the strain with 110 copies except for increased sensitivity to DNA damage. Thus, the unusually large copy number of rDNA in the genome, especially the subset kept silent, is hypothesized to protect genome integrity rather than being used for ribosome biogenesis (Ide, Miyazaki et al. 2010). In human cells, each single copy of rDNA encodes a 45S pre-rRNA precursor that contains, in order, 18S, 5.8S, and 28S rRNA coding regions. There are two external transcribed spacers (ETS) at 5' end before 18S and 3' end after 28S in the 45S pre-rRNA. In addition, the 5.8S rRNA coding region is flanked by two internal transcribed spacers (ITS) in the middle of 18S and 28S. Both ETSs and ITSs are cleaved from 45S pre-rRNA during rRNA processing to eventually generate 18S, 5.8S, and 28S rRNAs. Another rRNA 5S is transcribed from another genomic region by RNA polymerease III. Individual rDNAs are separated by the intergenic spacer (IGS). Again, IGS sequences and lengths are highly divergent among different species, from several kb in yeast to 30kb in human. IGS in mouse contains several rDNA regulatory elements including gene promoter, spacer promoter, and gene terminator distributed at both ends of one IGS. The schematic diagram of mouse rDNA and IGS is shown below.

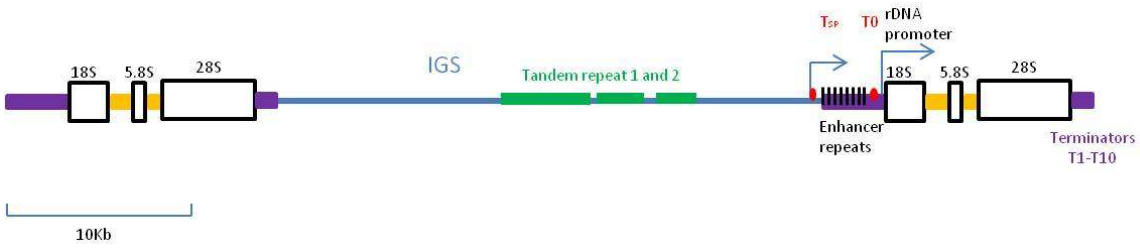


Figure 1.7.1 Diagram of mouse rDNA and IGS. The external transcribed regions are represented by purple frames, the internal transcribed regions are represented by orange

frames, coding regions are represented by white hollow frames, and IGS is the blue line located between two rDNA genes. The locations of some regulatory elements are labeled on the top of the rDNA gene. This figure is modified based on the published scheme (McStay and Grummt 2008).

The ribosomal DNA promoter is located -200bp to +50bp relative to the transcription start site (TSS) at 3' end of IGS, and the terminator region is located at the end of rDNA indicated by the red bars. This region usually contains repetitive terminator elements numbered as T1-T10. T_{sp} is the spacer promoter located at about -2 kb upstream of TSS and its corresponding terminator T0 is immediately upstream of TSS. Tsp drives the transcription of intergenic spacer RNA, which mediates rDNA transcription repression. Tandem repeats 1 and 2 contain large amounts of single repeats and transposable elements, yet their functions are still unclear (Sylvester JE 2004). Furthermore, it should be noted that no alignment study of IGS between mouse and human has been reported so far; therefore, it is not clear whether human IGS contains all the regulatory elements at the corresponding positions.

The mechanism of nucleolus assembly has attracted much interest since the number of the nucleoli and the nucleolar morphology are the most prominent markers of the nuclear architecture correlated with cell proliferation activity. It has been noticed for a long time that the disassembly and reassembly of the nucleolus are controlled by the cell cycle. The Nucleolus starts to dissemble in early prophase, during which, CDK1-cyclin B plays an important role by phosphorylating B23. The phophorylation results in the dissociation of B23 from rRNA. B23, as well as other rRNA processing components,

is released from the nucleolus and distributed on the surface of condensed chromosomes (Okuwaki, Tsujimoto et al. 2002; Negi and Olson 2006). But the upstream binding factor (UBF) and particular Pol I subunits still remains in NORs through the cell cycle. The nucleolus reassembly occurs in telophase, with all the dissociated components re-gathering on the NORs and the reactivation of transcription as well as processing of rRNA. Transcriptional activity of rDNA was once considered as the trigger for nucleolus restoration; however, the study of pseudo-NORs demonstrated that partial formation of the nucleolus can still happen without transcriptional activity (Prieto and McStay 2008). Pseudo-NORs are tandem arrays of a DNA sequence obtained from Xenopus rDNA and incorporated into human chromosomes. They only contain repeated enhancer elements and lack the rRNA coding region, hence it is transcription incapable. By using this model, the authors unarguably showed partial formation of the nucleoli around those pseudo-NORs with components similar to FC. UBF binding to the enhancer elements determines the initiation of nucleolar reestablishment and other procedures are likely following a self-organization mechanism. Interestingly, the restoration of nucleoli in new daughter cells only takes place on the NORs that are actively transcribed in the mother cells (Roussel, Andre et al. 1996). This observation strongly suggested the inheritance of rDNA chromatin organization between cell generations. Epigenetic modifications on DNA and nucleosomes are likely responsible for the inherited rDNA organization in cells from one generation to next.

In early years, scientists had noticed the structural difference between active rDNAs and silent rDNAs on “miller spread” (Christmas tree) in yeast under the electron

microscope, which is that active rDNAs displayed the “Christmas tree” structures with knobs of ribonucleoproteins on the branches while silent rDNAs showed no branches at all (Miller and Beatty 1969). DAPI staining analysis of metaphase chromosomes revealed gaps in active NOR regions, indicating locally decondensed chromatin structure compared to the intense staining on silent NORs that is not distinguishable from heterochromatin. Psoralen intercalates in DNA that is not protected by nucleosomes. Cross-linking assay performed on murine Friend cells showed that psoralen specifically induced DNA cross-linking in active NORs but not in silent NORs, which led the author to conclude that nucleosomes are gone from active rDNAs but still present on IGS and the inactive NORs (Conconi, Widmer et al. 1989). Despite validation of this observation in yeast (Merz, Hondele et al. 2008) and human cells (Zentner, Saiakhova et al. 2011), other studies have found the presence of histones on the active rDNA (Jones, Kawauchi et al. 2007). This discrepancy has not been well explained so far; it is possibly caused by the existence of other types of chromatin-binding histones, in addition to nucleosomes in rDNAs (Prior, Cantor et al. 1983), or by differences in sensitivity and threshold setup among different techniques. Nevertheless, DNA methylation was found to be enriched on the condensed inactive NORs, which indicates an important role of methylation in the maintenance of rDNA silencing (Stancheva, Lucchini et al. 1997). Ribosomal DNA methylation usually occurs on CpGs in the promoter region and it is mediated by DNA methyltransferases DNMT1, DNMT3a, and DNMT3b. Very interestingly, only methylated rDNA chromatin (with the presence of nucleosomes), but not naked rDNA can be repressed *in vitro*, which suggests the importance of chromatin conformation under epigenetically controlled rDNA silencing (Santoro and Grummt 2001). Methylated

CpG at -133 bp impairs UBF binding to the promoter in mouse cells and consequently prevents rDNA transcription. A link between hypomethylation of rDNA promoter and elevated rRNA transcription has been reported in several cancer types (Powell, Mutch et al. 2002; Ghoshal, Majumder et al. 2004), and a causal relationship is thus proposed. However, recent investigations in human prostate cancer tissues (Uemura, Zheng et al. 2012) and breast cancer tissues (Bacalini, Pacilli et al. 2014) either failed to recapitulate a significant change of methylation level in rDNA promoter or revealed hypermethylation of promoter in tumor tissues compared to normal tissues although rRNA level is increased. Even more complex, decreased rRNA level was observed upon the reactivation of former silent rDNA, which was mediated by knocking out methyltransferases or applying a methyltransferase inhibitor, like 5-Azacytidine (5-Aza) (Chen, Comai et al. 1998; Gagnon-Kugler, Langlois et al. 2009). All current evidence concurs to a surprising conclusion that the elevation of rDNA transcription in cancer tissue is not necessarily correlated with rDNA methylation level, or with the number of active NORs. A methylation-independent control of rRNA transcription and a global-monitoring system might exist in parallel to the canonical methylation-dependent mechanism. Depletion of UBF in human NIH3T3 cells did induce silencing of a subset of rDNA and condensation without significant changes in methylation levels after many cell generations (Sanij, Poortinga et al. 2008). It will be important to recapture this phenomenon under physiological conditions.

Another layer of epigenetic control of rDNA activities is posttranslational histone modifications. In fact, rDNA methylation and histone modifications are interdependent.

Study in *Arabidopsis* showed that rDNA promoter demethylation mediated by 5-Aza led to repressive histone marks transiting into active marks. Similarly, blocking the function of histone deacetylase automatically resulted in the decrease of methylation level on rDNA (Earley, Lawrence et al. 2006). The interdependent transition of these epigenetic marks is caused by the interactions and mutual recruitments among distinct epigenetic modification proteins. Novel histone markers specific to rDNA are not reported so far, which suggests a similar epigenetic mechanism for the control of rDNA activities compared to the rest of chromatin.

The recruitment of chromatin remodeling complexes to rDNA determines future epigenetic state and rDNA structure. For example, rDNA silencing and condensation are mainly mediated by nucleolar remodeling complex (NoRC), which contains TIP5 (TTF1-interacting protein 5) and an ATPase protein SNF2h (Strohner, Nemeth et al. 2001). TIP5 binds rDNA promoter and recruits HDAC1 (histone deacetylase 1) (Zhou, Santoro et al. 2002) as well as DNMT1/DNMT3B (DNA methyltransferase) (Zhou and Grummt 2005) to accomplish epigenetic modifications. Depletion of TIP5 not only abolishes rDNA silencing but also impairs the organization of perinucleolar heterochromatin including centric and pericentic repeats (Guetg, Lienemann et al. 2010). SNF2h functions in sliding the nucleosomes on the chromatin to increase the accessibility for other proteins. Environmental energy deprivation induced rDNA silencing is mediated by eNoSC containing nucleomethylin, the NAD^+ -- dependent deacetylase SIRT1, and H3K9 methyltransferase SUV39H1. Coexistence of NoRC and eNoSC complexes that both mediate rDNA silencing suggests distinct rDNA silencing mechanisms in cells

responsive to different stimuli. The activation of rDNA transcription is also mediated by different protein complexes. For instance, the ERCC6 (excision repair cross-complementation group 6) / Pol I / histone methyltransferase G9a complex competitively binds rDNA promoter with NoRC (Yuan, Feng et al. 2007). Another complex B-WICH also interacts with Pol I to mediate rDNA transcription. The B-WICH complex contains NM1 (actin and nuclear myosin 1c) / WSTF / SNF2h and NM1 plays a central role in stabilizing SNF2h and actin on rDNA in addition to recruiting HAT (histone acetyltransferase) (Sarshad, Sadeghifar et al. 2013). Interestingly, SNF2h was found in both rDNA activation complex and silencing complex, which implicates its important but not selective role in altering local rDNA structure.

A large number of active rDNA repeats neighboring the transcription-repressive perinucleolar heterochromatin in the cell nucleolus must require a highly organized chromatin arrangement to avoid interference between euchromatin and heterochromatin. Active rDNAs are located inside the nucleolus while silent rDNA arrays are at the periphery, closed to the perinucleolar heterochromatin (Santoro 2011). This is consistent with previous observation that rDNAs within the nucleolus relocate to the nucleolar periphery upon treatment with actinomycin D, a drug that inhibits Pol I at low concentration. The localization of the proximal regions and the distal regions (relative to centromere) flanking NORs was also studied in the interphase nucleolus recently and the result clearly showed that the flanking regions are located at the periphery of the nucleolus with active decondensed rDNAs stretching into the center (Floutsakou, Agrawal et al. 2013). Several rDNA looping models have been proposed based on the

involvement of different proteins. In the mouse, the terminator sequence motif (T1-T10, see Figure 1.7.1) is recognized by the transcription termination factor TTF-I. In addition to binding to T1-T10 to terminate rDNA transcription, TTF-I also binds to spacer terminator T0 located upstream of the TTS functioning in transcription initiation (Langst, Blank et al. 1997; Langst, Becker et al. 1998). By using 3C technique, Langst *et al.* showed high frequency of interaction between the spacer terminator (T0 region) and the gene terminator (T1-T10 region) indicating that a loop structure is created by tethering these two sites. The authors further showed that TTF-I mediates the loop by binding on these sites (Nemeth, Guibert et al. 2008). Later on, the same group demonstrated that more terminator elements significantly improved transcription efficacy compared to less terminator elements within a single chromatin loop (Diermeier, Nemeth et al. 2013). Yet, neither study addressed the role of IGS in this TTF-I mediated rDNA loops model. In contrast, based on the direct involvement of c-MYC in rDNA transcription (Shiue, Berkson et al. 2009), Shiue *et al.* identified potential matrix attachment regions (MARs) in IGS and proposed a c-MYC mediated r-chromatin looping model in human and rat (Shiue, Nematollahi-Mahani et al. 2014). This model addressed the binding between MYC and IGS upon transcription activation, which allows the transcribed regions to remain in the loops. Because MYC is not characterized as a nuclear matrix component, somehow it has to bridge the association of IGS and the nuclear matrix. The study did not clarify whether MYC attaches to the nucleolar matrix, thus it is not clear how the loops are anchored. In another study of the MARs in IGS, Zillner et al. showed that TIP5 directly binds MARs through its TAM (TIP5/ARBP/MBD) domain as a component of the nuclear matrix (Zillner, Filarsky et al. 2013). This work proposed a model for the

organization of a large number of rDNAs under a transcription permissive environment. Although the cohesin complex is well known for its loop-generating role genome wide and a large body of evidence has confirmed cohesin in rDNA transcription and nucleolar organization control, its potential influence on rDNA looping architecture is not reported.

The coordination of rapid cell division and increased nucleolar activities has been reported in many, if not all, types of cancers. On one hand, the accelerated cell proliferation of cancer requires more ribosome production to support increased demands on protein synthesis. This goal can be achieved by over-expressing a variety of oncogenic proteins. Many oncoproteins simultaneously function in up-regulating rDNA transcription and promoting cell division, for example, c-MYC directly binds rDNA promoter E-box to stimulate rDNA transcription (Arabi, Wu et al. 2005; Grandori, Gomez-Roman et al. 2005), in addition to binding other cell cycle regulatory genes (Bretones, Delgado et al. 2014). Activation of AKT can phosphorylate casein kinase IIα (CK2α) and CK2α further phosphorylates Pol I binding partner TIF-IA to initiate rDNA transcription (Nguyen le and Mitchell 2013). On the other hand, a sufficient number of ribosomes is also thought to be the prerequisite for cell cycle progression from G1 to S phase (Pederson 2011). The underlying mechanism is that the cell mass must reach a certain level prior to the division and this parameter is examined at check point in G1 phase. The cell mass is mainly characterized by the protein amount in a cell, which is further determined by the amount of ribosomes; ribosome biosynthesis is therefore determining cell cycle rate. Indeed, the arrest of cell proliferation induced by impaired ribosome biosynthesis was reported as early as when the nucleolar function was first

described in 1964 (Brown and Gurdon 1964) and thereafter. The nucleolus as the place for rDNA transcription, rRNA processing, ribosome assembly and exporting, is highly sensitive to a variety of external or internal deleterious stimuli that hinder ribosome production, via a cellular reaction termed nucleolar stress. Not surprisingly, any functional disruption of the nucleolus may lead to cell cycle arrest due to ribosome deficiency.

Interestingly, compelling evidence has also unveiled a direct role of the nucleolus in cell cycle control beyond ribosome production. Proteomic analyses performed on purified nucleoli of human HeLa cells have identified over 700 proteins associated with the nucleolus (Andersen, Lyon et al. 2002; Andersen, Lam et al. 2005). This is a large number of proteins identified in a single nuclear body and surprisingly, a large portion of them are irrelevant to ribosome biogenesis, which strongly suggests a multifunctional role of the nucleolus in addition to ribosome biogenesis. Among those 700 proteins, 25 are functionally categorized as cell cycle regulators, yet few studies have been conducted to elucidate their roles in the nucleolus so far. More direct evidence came from the study of P53 activation mediated by nucleolar stress. The expression of P53 remains very low in cells under normal condition; however, the level elevates rapidly upon nucleolar stress. Many ribosome subunit components including RPL5, RPL11, RPL23, PRL26, RPL37, RPS3, RPS7, RPS14, RPS15, RPS20, RPS25, RPS26, RPS27, RPS27L (Golomb, Volarevic et al. 2014), 5S RNA (Sloan, Bohnsack et al. 2013), and nucleolus-localized protein Arf (Weber, Taylor et al. 1999) can be released from the nucleolus upon nucleolar stress and directly bind P53 E3 ubiquitin ligase MDM2 to block P53 degradation. The

increased P53 subsequently leads to cell cycle arrest. Since so many nucleolar components regulate P53 in a similar manner, there is no doubt about the dual-role that the nucleolus plays, ribosome biogenesis and stress sensor, and both roles are tightly linked to the cell cycle.

In this thesis work, I am focusing on two aspects of cellular architecture, polarity and nucleolar organization that are essential for cellular homeostasis to further decipher their role in cancer. My overarching hypothesis is that the function of both of these structures can be further deciphered by studying single proteins that act as nodes for the control of cellular homeostasis. In the next chapter I will present work related to the understanding of the role of the nuclear structural protein NuMA, previously involved in differentiation, response to DNA damage, apoptosis and cell quiescence, in the architectural and functional control of the nucleolus. In another chapter, I will discuss the involvement of CDS1, a protein associated with Pi3K-AKT pathway regulating cell growth and proliferation, in the control of tissue polarity and invasive growth.

CHAPTER 2: NuMA INFLUENCES NUCLEOLAR ARCHITECTURE AND FUNCTION

2.1. Introduction

Nuclear architecture refers to the spatial and temporal organization of the chromatin and the nuclear bodies. NuMA, an organizer of higher order chromatin (Abad, Lewis et al. 2007) was also found to overlap with nuclear bodies like splicing speckles (Lelièvre, Weaver et al. 1998) and Cajal bodies (Gribbon, Dahm et al. 2002), suggesting a potential role for NuMA in the global control of nuclear architecture. The cell nucleolus is the nuclear body for rRNA production, processing, and ribosome assembly, which requires a complex organization. The nucleolus contains rDNA and nucleolar proteins and it is surrounded by a heterochromatin shell (Nemeth, Conesa et al. 2010). Heterochromatin domains such as centromere and pericentromere (Nemeth, Conesa et al. 2010), specific proteins such as CTCF and Tin2 (Yusufzai and Felsenfeld 2004; Kaminker, Plachot et al. 2005), and particular nuclear bodies such as the Cajal body and splicing speckles (Lelievre, Weaver et al. 1998) were observed to be specifically associated with the nucleolus with an unusually high frequency during cell differentiation, suggesting the importance of the nucleolus-related organization in homeostasis and differentiation mediated cell quiescence.

Mounting evidence suggests a link between NuMA and the nucleolus. Formerly, claims were that NuMA was absent from the nucleolus as observed by immunofluorescence microscopy (Gueth-Hallonet, Wang et al. 1998; Zeng 2000), yet a high-sensitivity nucleolar proteomic study revealed an association between NuMA and the nucleolus, which is consistent with our observation using soft X-ray electron microscopy (Lelièvre, unpublished data) as well as electron microscopy analysis of the nucleolus (Jayaraman, S. Ph.D. Thesis, 2014). This discrepancy can be logically explained by the fact that DNA density is extremely low in the nucleolar region, where it usually appears as a black hole when the nucleus is stained with DNA dyes, given the fact that NuMA is a chromatin-associated protein (Abad, Lewis et al. 2007). The B-WICH complex containing SNF2h, WSTF, and NM1 was immunoprecipitated with NuMA (Jayaraman, S. Ph.D. Thesis, 2014), suggesting that NuMA might be involved in ribosomal DNA transcription control, considering the well-known function of the B-WICH complex in rDNA activation (Sarshad, Sadeghifar et al. 2013). The enrichment of NuMA was observed at the perinucleolus to coincide with cell differentiation (Abad, Lewis et al. 2007) and NuMA also accumulated at the periphery of nucleolus in response to RNA Polymerase I inhibition (Abad, P. M.S. Thesis, 2003). These results raised the possibility for NuMA to be involved in rDNA transcription control through the regulation of chromatin organization. Thus, in the major part of my thesis, I have studied the potential role of NuMA in the regulation of nucleolar architecture and rDNA transcription. To elucidate the underlying mechanism of how NuMA might influence chromatin structure, I hypothesized that NuMA and specific chromatin-associated complexes co-organize DNA structure. To this end, the interaction between NuMA and

the cohesin complex and the effect of NuMA on chromatin looping were specifically studied.

Our laboratory previously reported that in multicellular spheroids produced in 3D culture and displaying impaired apical polarity, inducing NuMA redistribution within nuclei pushes quiescent cells into the cell cycle (Chandramouly, Abad et al. 2007). This is an interesting observation since it directly links changes in nuclear architecture to its potential biological consequence - the loss of cell quiescence. Furthermore, the redistribution of epigenetic marks at the perinucleolus was observed when the function of endogenous NuMA was impaired by over-expressing its C-terminal peptide in non-neoplastic breast epithelial S1 cells (Abad, Lewis et al. 2007), which gave rise to the hypothesis that the integrity of the nucleolar architecture maintained by a functional NuMA is necessary for cell quiescence. Thus, among research efforts I also assessed the influence of NuMA on the cell cycle by assessing Ki67 cell cycle marker given its potential involvement in nucleolar functions and the tight correlation between nucleolus and cell cycle.

2.2. Materials and methods

2.2.1. Cell culture

Non-neoplastic S1 HMT-3522 cells (human mammary epithelial cells (HMEC)) (Briand, Petersen et al. 1987) between passages 52 and 60, were plated at 1.75 x 10^6 cells/cm^2 and maintained for 10 days for propagation as monolayers in chemically defined H14 medium (Blaschke et al., 1994; Plachot and Lelièvre, 2004) [DMEM/F12

(Invitrogen Corporation), supplemented with 0.15 IU/ml prolactin (Sigma-Aldrich), 250 ng/ml insulin (Sigma-Aldrich), 2.6 ng/ml sodium selenite (BD Biosciences), $2.67x10^{-5}$ ng/ml β-estradiol (Sigma-Aldrich), 0.5 ng/ml hydrocortisone (Sigma-Aldrich), 10 μg/ml transferrin (Sigma-Aldrich)], including 5 μg/ml epidermal growth factor (EGF; BD Biosciences). Malignant T4-2 HMT-3522 HMECs (Briand et al., 1996) between passages 2 and 20 were plated at 8.75 x 10^5 cells/cm^2 and maintained for 6 days in two-dimensional (2D) culture in H14 medium lacking EGF (H14-EGF). Human mammary epithelial MCF7 cancer cells (ATCC) of metastatic origin were plated at 8.75 x 10^5 cells/cm^2 and maintained for 4-6 days in 2D culture in DMEM medium with 10% fetal bovine serum.

Three-dimensional (3D) cell culture of S1 cells was used in some studies for its merit of mimicking mammary epithelial differentiation; the detailed procedure was followed as described previously (Plachot and Lelievre 2004). Compared to the traditional monolayer (2D) cell culture method, in which cells are proliferating on a plastic surface, cells in 3D cultures are surrounded by extracellular matrix-enriched medium (Plachot and Lelievre 2004). To recapitulate the formation of polarized glandular structures (acini), S1 cells were cultured in the presence of exogenous extracellular matrix (ECM) enriched in basement membrane components (Matrigel™, BD Biosciences). Briefly, S1 cells (35,000 cells/cm^2) were plated on 41 μl/cm^2 matrigel-coated surfaces and cultured in H14 medium containing 5% Matrigel™. Cells were induced to exit the cell cycle upon incubation in H14 medium without EGF for 48 h after day 8 of culture. Acinar morphogenesis, characterized by the formation of a single layer

of epithelial cells surrounding a lumen and delineated by an endogenous basement membrane, was routinely observed by days 9–10. Medium was renewed every 2-3 days. All cells were cultured at 37°C and with 5% CO2.

2.2.2. Actinomycin D treatment

For actinomycin D treatment experiment, S1 HMECs cultured on coverslips on 2D, were propagated initially in H14 medium supplemented with additives for 7 days followed by an additional 3 days in H14 without EGF to induce growth arrest. Cells were then treated with either vehicle Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) or 0.08 μg/ml of actinomycin D (Sigma-Aldrich) for 4 hours at 37° C and processed for immunofluorescence staining as described in section 2.2.3.

2.2.3 Immunoflurescence labeling

Cells cultured on coverslips in 2D were washed in 1X phosphate buffer saline (PBS) followed by the permeabilization treatment in 0.5% Triton X-100 (Sigma-Aldrich) in cytoskeleton (CSK) buffer [100 mM NaCl (Mallinckrodt); 300 mM sucrose (Sigma-Aldrich); 10 mM Pipes (Sigma-Aldrich), pH 6.8; 5 mM MgCl2 (Sigma-Aldrich)] containing protease inhibitor (CSK-PI). After washing twice for five minutes with CSK-PI, cells were fixed for 20 minutes at room temperature with 4% formalin (Sigma-Aldrich). After rinsing thrice for 20 minutes at room temperature with PBS containing 50 mM glycine (PBS-glycine), cells were incubated for 1 hour at room temperature with 10% goat serum (Invitrogen Corporation) in immunofluorescence labeling (IF) buffer [130 mM NaCl; 13.2 mM Na2HPO4; 3.5 mM NaH2PO4; 0.1% (w/v) bovine serum albumin, RIA (Sigma-Aldrich); 0.05% (v/v) Tween 20 (Sigma-Aldrich)]. Cells were

incubated overnight at 4°C with either mouse monoclonal antibody directed against NuMA clone B1C11 (kind gift from Dr. Jeffrey Nickerson, University of Massachusetts), CENPA, rabbit anti-serum against SMC3, RAD21 (kind gift from Dr. Jan-Michael Peters, Research Institute of Molecular Pathology, Vienna, Austria) or rabbit polyclonal antibody against NuMA (Abcam), Pol I (Santa Cruz), SMC1 (Bethyl Labs), Ki67 (Vector Laboratories). Antibodies were diluted in IF buffer containing 10% goat serum to reach the specified concentrations of ½ dilution, 26 µg/ml, 1/50 dilution and 1/50 dilution, respectively. Following incubation with the primary antibody, cells were washed thrice for 10 minutes with IF buffer, and incubated for 50 minutes at room temperature with 12.5 µg/ml fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) and 6.7 µg/ml Alexa-Fluor 568-conjugated goat anti-mouse IgG (InVitrogen Corporation) antibodies diluted in IF buffer containing 10% goat serum. After three 10-minute washes with IF buffer, nuclei were counterstained for DNA with 0.5 µg/ml 4', 6-diamidino-2-phenylindole dihydrochloride:hydrate (DAPI) (Sigma-Aldrich) in PBS for 10 minutes. After removal of excess DAPI, samples were mounted with ProLong® Gold antifade reagent (Invitrogen Corporation).

2.2.4 Co-immunoprecipitation (Co-IP)

S1 HMECs cultured as monolayer (2D) for 12 days in 10 x 150 mm dishes (VWR) were processed for co-IP following the protocol provided by the Universal Magnetic co-IP Kit (Active Motif). The Co-IP products pulled down by the magnetic beads were incubated in the protein loading buffer (10% Glycerol, 1.5% SDS) at 95°C

for 5 minutes to elute proteins from the beads, and subjected to western blot analysis as described in 2.2.5

2.2.5. Western blot analysis

Eluted Co-IP products in protein loading buffer or total protein extracts from cell cultures prepared in Leammli buffer were loaded on SDS-PAGE gels. The percentage of acrylamide was determined by the approximation of protein sample molecular weight. 5% gel was used to visualize proteins larger than 200 kD; 7.5% gel was used for proteins between 40 and 200 kD; and 10% gel was used for proteins smaller than 40 kD. For western blot analysis of proteins in Leammli extracts, 30 µg of sample was loaded per lane and the target protein was detected with primary antibodies and secondary antibodies at the concentrations recommended on the data sheet.

2.2.6 Chromatin immunoprecipitation followed by quantitative PCR (ChIP-PCR)

The protocol for ChIP was adapted from that of Dr. Steve Konieczny's laboratory and modified according to a published article (Lee, Johnstone et al. 2006). Specifically, 4 x 10^7 T4-2 cells cultured in 150 mm dishes were cross-linked with formaldehyde solution (50 mM HEPES-KOH, pH7.5; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 11% formaldehyde) for 10 minutes and quenched with 0.125 M Glycine. After rinsing three times with PBS, cells were harvested by scraping into a 15 ml falcon tube and kept at -80°C until use. Dynabeads Protein G (80 µl) (invitrogen #100.03D) were washed three times in 1 ml blocking buffer (PBS with 0.5% bovine serum albumin (BSA) (Sigma; Cat. No: A7906)) before being split equally and incubated with 2 µg NuMA antibody (Bethyl

Lab, A301-510A) or IgG at 4°C for 6 hours. Antibody-beads (IgG-beads) were then resuspended in 100 µl blocking buffer. Cells were thawed on ice and resuspended in 10 ml lysis buffer (10 mM Tris-HCl ,pH 7.5; 10 mM NaCl; 3 mM MgCl2; 0.5% IGEPAL; 1 mM PMSF (freshly made) by gently pipetting and inverting for 5 minutes, then centrifuged at 700 g for 5 minutes. The cell lysis step was repeated twice and cell pellets were dissolved in 830 µl pre-IP dilution buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 3 mM MgCl2; 1 mM CaCl2; 4% IGEPAL; 0.8% SDS, 1 mM PMSF (freshly prepared); protease inhibitor cocktail, Roche (# 11873580001), freshly prepared). The tube was left on ice for 5 minutes and the solution was diluted with 3x volumes of IP dilution buffer (20 mM Tris-HCl, pH 8; 2 mM EDTA; 1% Triton X-100; 150 mM NaCl; Protease Inhibitor cocktail, freshly prepared). Cells in solution were then subjected to sonication with for 35 bursts, output 10 watt, with 20 seconds per burst and 90 seconds of pause between two bursts in icy water. The sonicated solution was microcentrifuged at 14,000 rpm for 10 minutes to remove the cell debris, and 50 µl of sample was kept aside as input control. The rest of the supernatant was split equally into two parts; each volume was incubated with antibody-beads or IgG-beads by rotating at 4°C. After 2 hours of incubation, the beads were collected and subjected to washes with 700 µl ChIP wash buffer 1 (20 mM Tris-HCl, pH 8; 2 mM EDTA; 1% Triton X-100; 150 mM NaCl; 1 mM PMSF, freshly prepared), buffer 2 (20 mM Tris-HCl, pH 8; 2 mM EDTA; 1% Triton X-100; 0.1% SDS; 500 mM NaCl; 1 mM PMSF, freshly added), and buffer 3 (10 mM Tris-HCl, pH 8; 1 mM EDTA; 0.25M LiCl; 0.5% IGEPAL; 0.5% Deoxycholate). Each washing step was performed by gently inverting the tubes back and forth for 5 minutes at room temperature. The beads were then transferred to a new tube for a final wash with

700 µl of TE buffer. The collected beads were resuspended with 100 µl of elution buffer and incubated at 65°C for 30 minutes. The elution buffer containing cross-linked protein and chromatin products was transferred into a new tube. The beads were washed again with same volume of elution buffer. Reversion of cross-linking was carried out by placing the ChIP products and input sample at 65 °C over-night and all samples were treated with RNase A (0.2 µg/ml) for two hours followed by two hours of treatment with proteinase K (0.2 µg/ml). At last, the chromatin samples were purified with Qiagen PCR Purification Kit and resolved in 50 µl EB.

The ChIP products were analyzed with real-time PCR on ABI 7300 with Sybr green. The PCR program was set up as 55 °C for 2 minutes, 95 °C for 10 minutes, 95 °C for 10 seconds, annealing at 58 °C for 1 minute, extension at 72 °C for 10 seconds, and 40 cycles.

2.3. Results

2.3.1. NuMA influences the nucleolar architecture

Overall, aforementioned evidence has suggested a potential relationship between NuMA and nucleolar function. Nucleolar morphogenesis encompasses three parts, namely active rDNA organization in the middle of nucleolus, the nucleolar-associated proteins, and the perinucleolar heterochromatin. Because NuMA is observed associated with the nucleolus and it is a structural protein, it might be important to maintain nucleolar morphology. To assess nucleolar morphology, a DFC protein Fibrillarin was immunolabeled in NuMA-silenced S1 cells (via small interfering RNA) and in control cells transfected with siNon-Targeting (NT) RNA as shown in Figure 2.3.1.A. Moreover,

a portion of centromere is preferentially associated with the nucleoli in the interphase cell nucleus (Haaf and Schmid 1991) and because the centromere contains highly repetitive DNA sequences, it becomes part of the perinucleolar heterochromatin. The possible change of the perinucleolar heterochromatin organization has been assessed by immuno-labeling the centromeres with an antibody against CENPA, a unique histone H3 variant (Guetg, Lienemann et al. 2010), and we adopted a similar method to evaluate the perinucleolar heterochromatin upon NuMA silencing. I found that while the typical nucleolar protein, fibrillarin is distributed as discrete small dots in NuMA expressing (siNT) control cells, it redistributes into large round foci associated with diffuse staining in NuMA silenced (siNuMA) cells as shown in Figure 2.3.1.A. Because the nucleolar regions are dark with DAPI staining of the nucleus, the approximate number of nucleoli per cells can be determined by the number of these hollow regions combined with the presence of Fibrillarin in these locations in DAPI/Fibrillarin stained cell nuclei. The average number of nucleoli was significantly higher in control cells (2.2 ± 0.1) compared to NuMA silenced cells (1.3 ± 0.1) as shown in figure 2.3.1.B. Overall, the average number of CENPA associated with each nucleolus is significantly different in control cells and in NuMA silenced cells, 5.7 compared to 7.8, which indicates alterations in nucleolar morphology based on the number of nucleoli and the reorganization of centromeric heterochromatin in the perinucleolar region upon NuMA silencing. However, confocal microscopy will be needed to readily determine the exact location of CENPA in relation to the nucleolus.

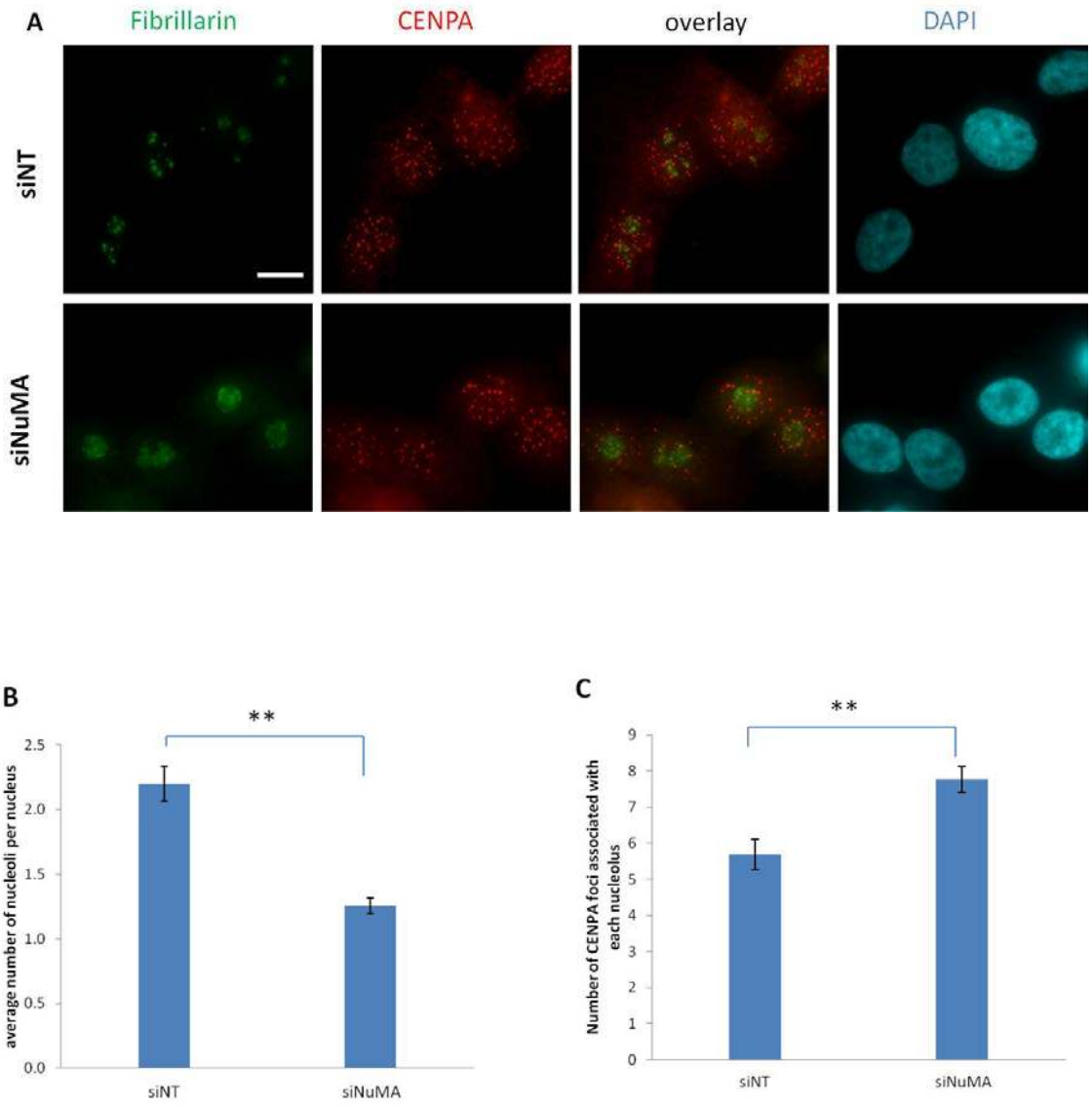


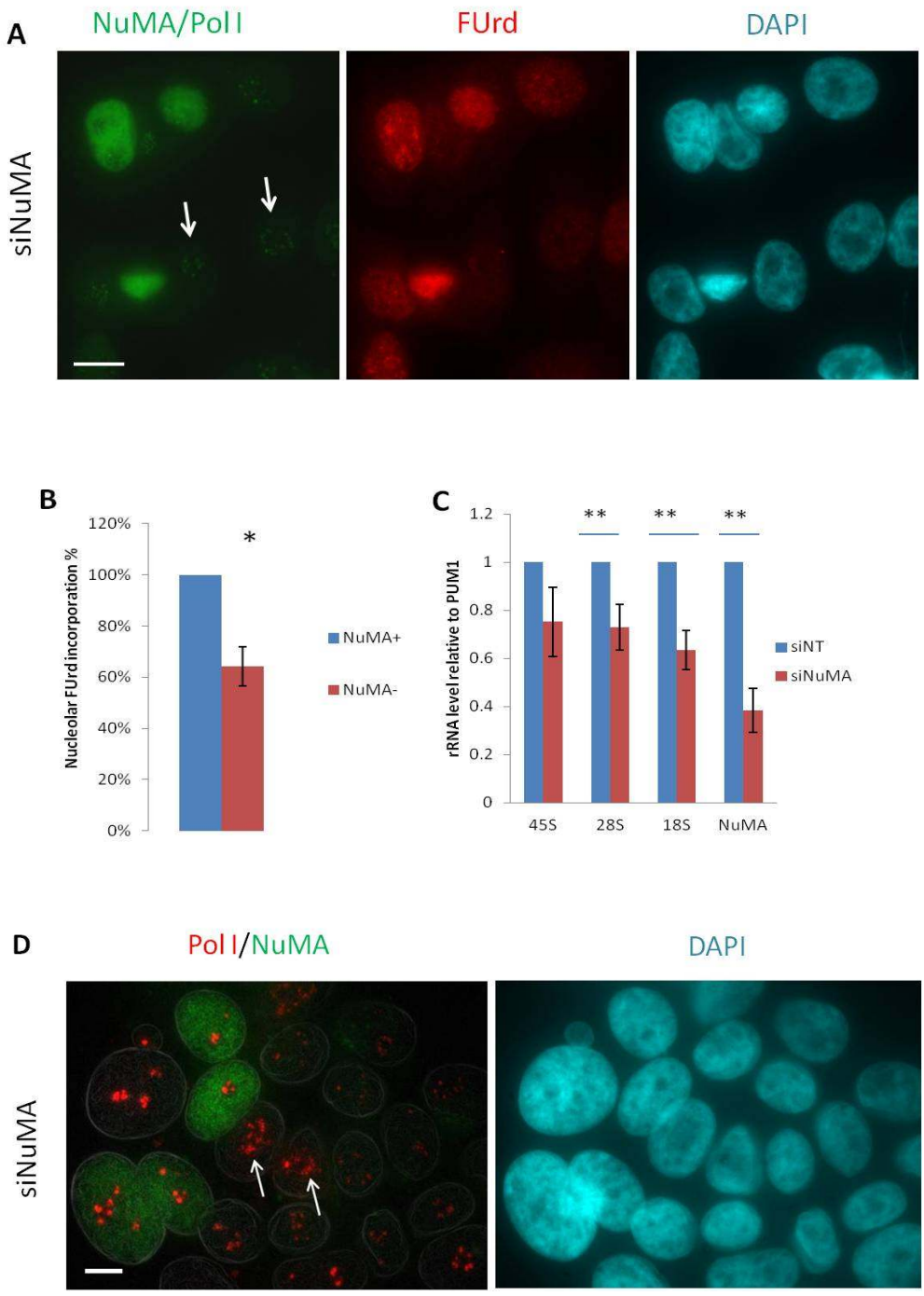
Figure 2.3.1. NuMA silencing influences nucleolar morphology. S1 cells were transfected with siRNA against NuMA or scrambled small RNA as control followed by the immunostaining for NuMA/Fibrillarin/CENPA in siNuMA cells and Fibrillarin/CENPA in siNonTarget transfected cells six days after the transfection (A), size bar, 10 μm. More than 30 cell nuclei were randomly selected for each biological replicate and analyzed for the number of nucleoli per cell nucleus with nucleoli (B) as

well as the number of CENPA foci associated each nucleoli (C) in control and NuMA silenced cells (**$p<0.01$, $n=3$).

2.3.2. NuMA silencing affects nucleolar function with respect to rRNA transcription

The alteration of nucleolar morphology upon NuMA silencing gave rise to the possibility that NuMA might influence nucleolar functions. The nucleolus is responsible for rDNA transcription, rRNA processing, and ribosome assembly and transportation. Our previous work has shown that NuMA is possibly involved in ribosomal biogenesis (Jayaraman, Ph.D. Thesis, 2014). To re-assess NuMA's influence on rDNA transcription, FUrd assay of nascent rRNA level (Sarshad, Sadeghifar et al. 2013) was performed on S1 cells transfected with siRNA against NuMA. To eliminate the variation of FUrd signal intensity due to different experimental treatments, we decided to compare signal intensity in the nucleolar regions between cells with or without NuMA in siNuMA transfected cells instead of between siNuMA and siNT transfected cells. Mean fluorescence intensity in the nucleolar regions in NuMA expressing cells is about two folds higher than that in NuMA silenced cells, which indicates that there is less nascent rRNA in NuMA silenced cells as shown in Figure 2.3.2.A & B. Quantitative PCR assay of 45S pre-rRNA, 18S rRNA, and 28S rRNA was also performed in NuMA silenced S1 cells and control siNT cells. We observed the significant decrease of 28S, 18S, and NuMA RNA level in siNuMA transfected cells, but the reduction of 45S pre-rRNA level was not statistically significant as shown in Figure 2.3.2.C. The function of Pol I is essential for rRNA production, we thereby assessed the distribution pattern as well as the expression level of Pol I in NuMA silenced S1 cells by using immunostaining and western blot assay, respectively. Interestingly, we observed that Pol I changed its distribution pattern, which is more dispersed as indicated by arrows in Figure 2.3.2.D, in NuMA-silenced cells

compared to more congregate staining in NuMA-expressing cells. Western blot analysis of Pol I did not reveal an apparent change of expression in NuMA silenced cells as shown in Figure 2.3.2.E. Given the fact that Pol I binds rDNA on the promoter and coding regions, the dispersed distribution of Pol I likely reflects rDNA rearrangement upon NuMA silencing. Therefore, it is reasonable to speculate that the decreased level of 28S and 18S rRNAs is more likely the consequence of the disruption of rDNA organization induced by NuMA silencing than the result of a change in Pol I expression level. It is difficult to assess the localization of NuMA in the nucleolar region using immunofluorescence. But upon the inhibition of rDNA transcription mediated by actinomycin D, NuMA can be found in distinct perinucleolar regions or caps (Jayaraman, S. Ph.D. Thesis, 2014). To assess the localization of NuMA and Pol I, S1 cells were subjected to actinomycin D treatement and their locations were analyzed by immunofluorescence. As shown in Figure 2.3.2.F, NuMA accumulates in the Pol I-free region at the perinucleolus upon actinomycin D treatment, suggesting that NuMA and Pol I do not interact when rDNA transcription is inhibited. Interestingly, both NuMA and Pol I are both located at the nucleolar periphery, suggesting that they occupy different regions of the perinucleolar chromatin under this particular condition.


A
NuMA/Pol I
FUrd
DAPI
siNuMA
B
Nucleolar FUrd incorporation %
120%
100%
80%
60%
40%
20%
0%
*
NuMA+
NuMA-
C
rRNA level relative to PUM1
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
45S
28S
18S
NuMA
siNT
siNuMA
D
Pol I/NuMA
DAPI
siNuMA

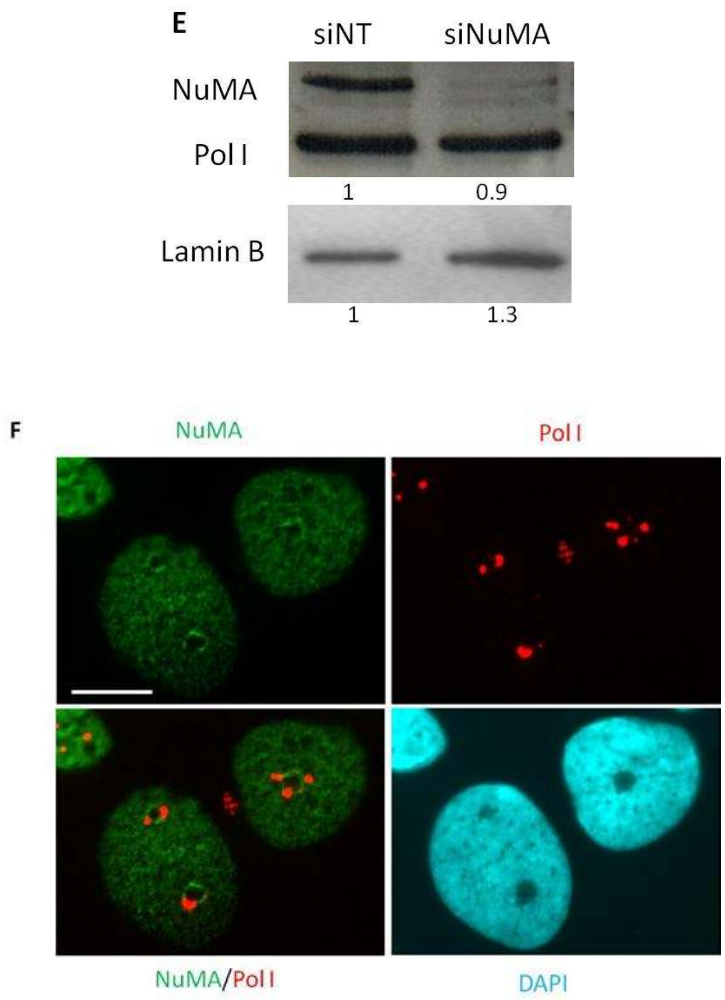


Figure 2.3.2. NuMA silencing impairs rDNA transcription and disrupts Pol I distribution in the nucleolus.

(A) FUrd incorporation assays in S1 cells subjected to NuMA gene knockdown by RNAi. The nucleolar region in NuMA silenced cells are indicated by arrows. Transcription was assessed by short FUrd treatment on cells for 20 minutes to allow its incorporation into nascent nucleolar transcripts.

(B) Quantification of the FUrd foci after immunostaining and immunoflurescense microscopy was performed by measurements on randomly selected nucleolar regions in

the recorded images. The signal was quantified using ImageJ software. The average of the mean grey values in NuMA expressing (NuMA+) cells was determined, and defined as one hundred percent of signal. The average of the mean grey values measured in NuMA silenced cells (NuMA-) was expressed proportionally. Each measurement was performed on more than 30 cells and three replicates were carried out. Error bars represent standard error. * $P<0.05$. Size bar, 10 µm

(C) Real-time PCR assay of 45S, 28S, 18S, and NuMA RNA levels was performed on siNuMA and control siNT S1 cells as described before. The relative RNA level is shown in the bar chart after being normalized to that of the internal reference gene *PUM1*.

(D) NuMA (green) and Pol I (red) dual-immunostaining was performed in S1 cells transfected with siRNA against NuMA in 2D culture. Dispersed staining of Pol I in NuMA silenced cells is indicated by the arrows. Cell nuclear edges are displayed by using function "find edges" in Image J software. Size bar: 10 µm

(E) Western blot analysis of NuMA and Pol I was performed on cells transfected with siNuMA and siNT. Lamin B was used as internal control. Signal intensity relative to siNT is labeled under the bands.

(F) Dual-staining analysis of NuMA and Pol I location in the nucleolus after rDNA transcription was inhibited with actinomycin D. Growth-arrested S1 cells on monolayer culture were treated with actinomycin D at 0.08 µg/ml to specifically inhibit Pol I transcription for 5 hours and subjected to dual-immunostaining for NuMA (green) and Pol I (red). Size bar: 10 µm

2.3.3. NuMA and the cohesin complex interact in interphase

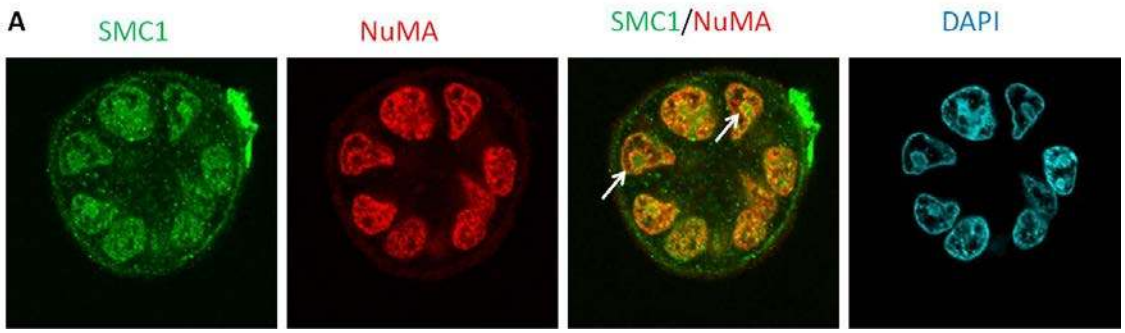
NuMA has been long considered as a component of the nuclear matrix that anchors the genome, but more evidence is still needed to support the original claim of NuMA binding chromatin on the matrix attachment regions (MARs) through its S/TPXX motifs (Luderus, den Blaauwen et al. 1994). Very interestingly, NuMA interaction with the cohesin complex in S phase and M phase of the cell cycle has been reported (Gregson, Schmiesing et al. 2001). Thus, it might be possible that NuMA regulates chromatin organization through its binding with chromatin-associated complexes like cohesin. To further explore this assumption, dual-immunostaining was performed for NuMA and cohesin component SMC1 in non-neoplastic S1 cells induced to differentiate into acini in 3D cell culture. Acini-like multicellular structures generated under 3D cell culture mimic *in vivo* organization of mammary epithelial cells and importantly, the distribution pattern of NuMA *in vivo* is accurately recaptured in acini produced in 3D culture compared to monolayer cell culture on a plastic surface (Lelièvre, Weaver et al. 1998). Confocal imaging of the dual staining (Figure 2.3.3.A) showed that NuMA and cohesin SMC1 are mainly overlapping at the periphery of the nucleolus as well as at the periphery of the nucleus.

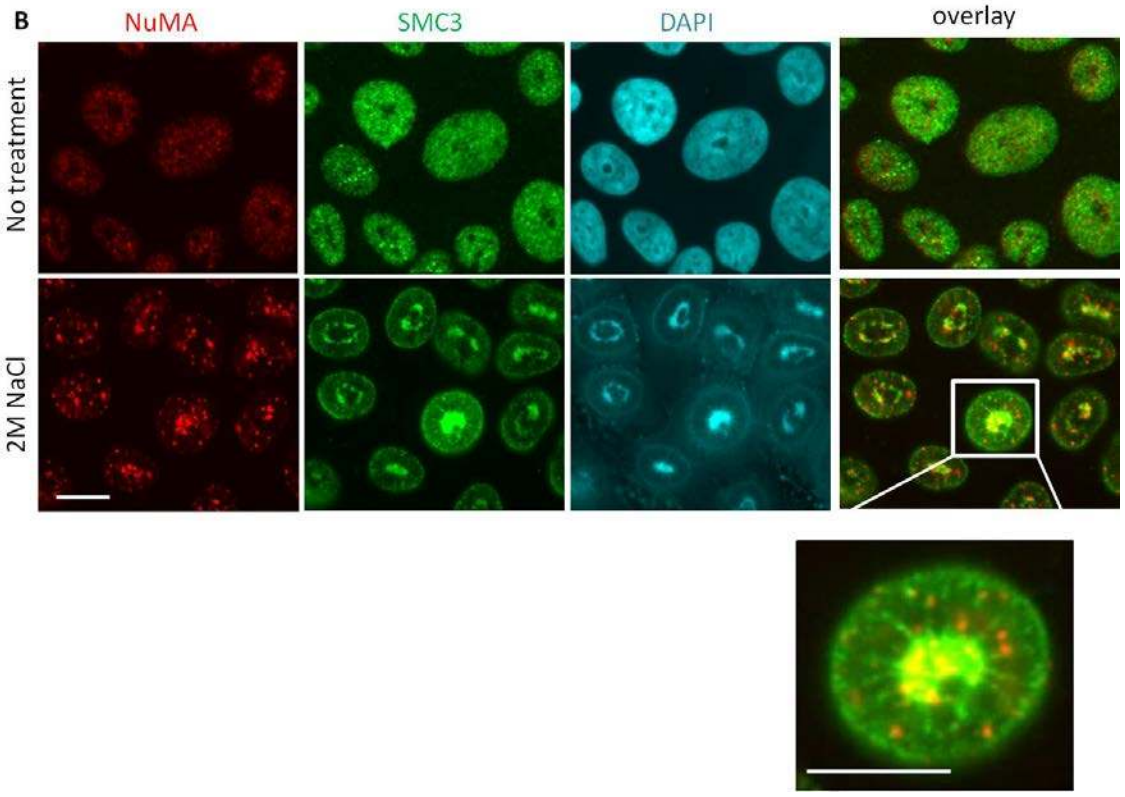
Based on the observation that NuMA and cohesin SMC1 co-localize at the perinucleolus and the former result showing NuMA aggregation at the perinucleolus upon treatment with nucleolar stress inducer, actinomycin D (Patricia A, M.S. Thesis, 2003), I tested whether cohesin could form “nucleolar caps” in response to actinomycin D like NuMA does. Dual staining for NuMA and cohesin SMC3 was performed on

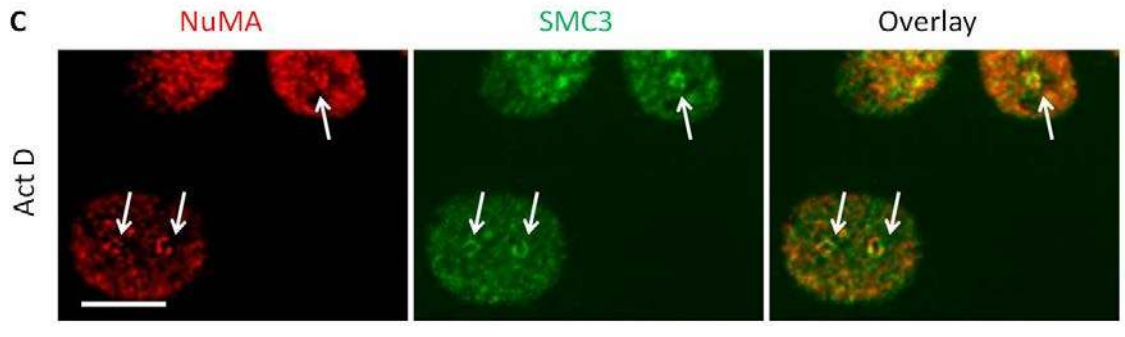
growth-arrested S1 cells in 2D culture following treatment with actinomycin D at 0.08 µg/ml for five hours. Both NuMA and cohesin SMC3 were observed to aggregate at the perinucleolus to form the "nucleolar cap" structure as shown in Figure 2.3.3.B. The scoring results from three replicates (Table 2.3.1) revealed a significantly higher percentage of cells simultaneously displaying both NuMA and SMC3 caps upon the treatment compared to DMSO-treated control, although NuMA caps and SMC3 caps can also be seen to occur independently from each other (i.e., the presence of NuMA caps in one nucleolus does not mean that SMC3 caps are also present in that nucleolus and *vice versa*).

High salt (2M NaCl) removes histones and soluble nuclear proteins to uncover the underlying nuclear matrix. Several lines of evidence indicate that NuMA and cohesin binding partner CTCF both bind to MARs (Luderus, den Blaauwen et al. 1994; Dunn, Zhao et al. 2003; Yusufzai and Felsenfeld 2004). To assess if the insoluble fraction of NuMA and cohesin can overlap, monolayer S1 cells were treated with 2M NaCl followed by dual-staining for NuMA and cohesin SMC3. Figure 2.3.3.B shows that most of the remaining NuMA overlaps with cohesin SMC3 at the edge of the DNA, which suggests that NuMA and cohesin might interact at these locations. Interestingly, both DAPI and SMC3 immunostaining results revealed a radial meshwork structure in the nucleus after NaCl extraction; the NuMA foci located outside of the central dense DNA seem to be present on these strings. Whether this meshwork structure represents the nuclear matrix is unclear.

To investigate whether NuMA could affect cohesin distribution, NuMA-GFP was transiently over-expressed in breast cancer cells MCF7 following transfection with lipofectmine2000. If NuMA indeed interacts with cohesin, I expected to see an influence of NuMA over-expression on cohesin. Immunostaining for SMC3 was performed on the NuMA-GFP transfected cells 24 hours post-transfection. Only GFP was transfected in the control group.


A
SMC1
NuMA
SMC1/NuMA
DAPI


B
NuMA
SMC3
DAPI
overlay
No treatment
2M NaCl


C
NuMA
SMC3
Overlay
Act D

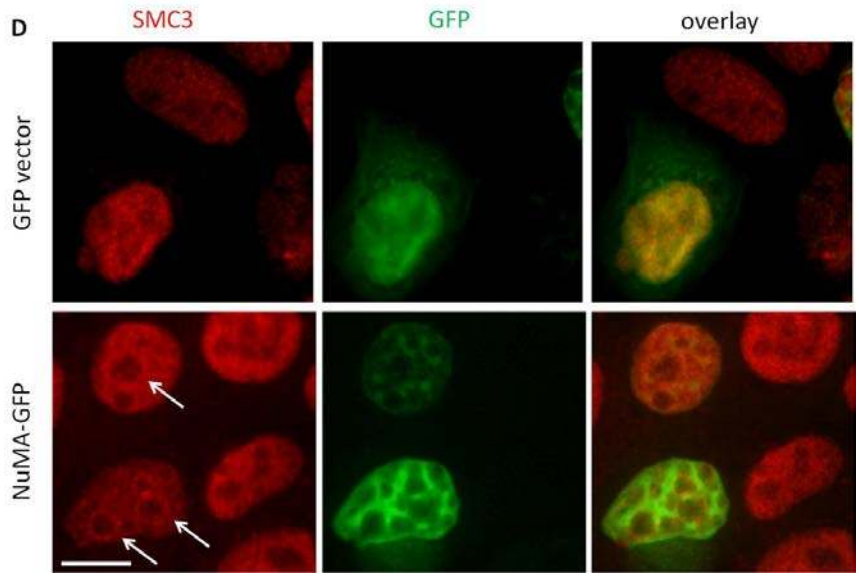


Figure 2.3.3. NuMA and cohesin overlap at the perinucleolus.

(A) Dual-immunostaining for NuMA (red) and cohesin SMC1 (green) in a differentiated glandular structure (acinus) shows that NuMA and SMC1 partially overlap at the perinucleolus as indicated by arrows. S1 cells in 3D culture were subjected to immuostaining on day 12 followed by confocal imaging analysis.

(B) NuMA (red) and cohesin SMC3 (green) relocalize to the perinucleolus upon actinomycin D treatment in non-differentiated (2D culture) growth-arrested cells. S1 cells were cultured as 2D monolayer for 10 days and treated with actinomycin D (0.08 µg/ml) for 5 hrs to halt rDNA transcription. The nucleolar caps are indicated by arrows. Size bar, 10 µm. A minimum of 300 nuclei were analyzed in each treatment and the percentage of cells displaying NuMA nucleolar caps, SMC3 nucleolar caps, and NuMA/SMC3 double nucleolar caps are shown in Table 2.3.3.1.

(C) The distribution of NuMA and SMC3 after 2M NaCl extraction shows partial overlap in the DNA region. Non-neoplastic S1 cells cultured on coverslips were extracted with 2M NaCl and 0.5% Triton for 15 minutes and subjected to immunostaining for NuMA and SMC3 (middle panel) compared to cells without NaCl extraction (upper panel). Size bar, 10 μm. NuMA (red) delineates dense chromatin (blue) in the center as well as at the periphery of the nucleus; while SMC3 (green) entirely overlaps with the DNA in the nucleus. The magnified colocalization of NuMA and SMC3 in one cell from the middle right image (indicated by a white rectangle) is shown in the inset.

(D) Overexpressing NuMA induces aggregation of SMC3 at the perinucleolus in MCF7 cells. MCF7 cells were transfected with NuMA-GFP (lower panel) or GFP vector (upper panel). Immunostaining for SMC3 (red) was performed 24 hours post-transfection. Distinctive rings of SMC3 staining surrounding the nucleoli only occurred in NuMA-GFP over-expressing cells as indicated by arrows in the lower left image but not in GFP vector control cells. Size bar, 10 μm

Table 2.3.3.1. Percentage of cells displaying nucleolar caps from three replicates

	NuMA caps (%)	SMC3 caps (%)	NuMA & SMC3 caps (%)
Rep-1 ActD	27	27	17.5
DMSO	7.8	6	1.8
Rep-2 ActD	18	20	10
DMSO	3.6	1	0.3
Rep-3 ActD	31	25	17.5
DMSO	5.7	4.2	1.5

2.3.4. NuMA and cohesin influence chromatin loops

The partial colocalization of NuMA and cohesin at the periphery of the nucleolus and NuMA's effect on cohesin distribution in the nucleolar region leads to the assumption that NuMA might interact with cohesin. To test this hypothesis, co-immunoprecipitation for NuMA was carried out on growth-arrested S1 cells in 2D culture to assess its interaction with cohesin. Our team previously identified ATPase SNF2h as a binding partner of NuMA, and others also reported the interaction between cohesin and SNF2h (Hakimi, Bochar et al. 2002). Figure 2.3.4.A shows that cohesin components RAD21 and SMC3 were pulled down together with NuMA. SNF2h was co-precipitated as well.

One major function of the cohesin complex is to mediate long distance interaction in chromatin by generating chromatin loops. Silencing cohesin component like RAD21 or SMC3 was reported to increase chromatin loop size that was detected by nuclear halo assay (Guillou, Ibarra et al. 2010). The rationale behind the nuclear halo assay is that after nuclear soluble proteins are removed by 2M NaCl, the underlying nuclear matrix bound by the loosened chromatin is exposed. The unwrapped DNA loops extending out of the permeabilized nuclear envelope can be visualized as a halo around the nuclear core when a fluorescent DNA dye, like propidium iodide, is applied. To test NuMA's potential involvement in chromatin looping, nuclear halo assay was performed on non-neoplastic S1 cells and neoplastic T4-2 cells silenced for NuMA. Both S1 and T4-2 cells showed an increased ratio for the diameter of halo/nucleus in siNuMA cells compared to control siNT, which supports the idea of NuMA's involvement in chromatin looping. The effect

of silencing SNF2h was analyzed in parallel in S1 cells because it interacts with both NuMA and cohesin. No significant difference was observed between siNT-cells and siSNF2h-cells indicating that SNF2h might not be involved in chromatin looping. Yet, the silencing effect of SNF2h in S1 and T4-2 cells needs further validation by both immunofluoresence staining and western blot.

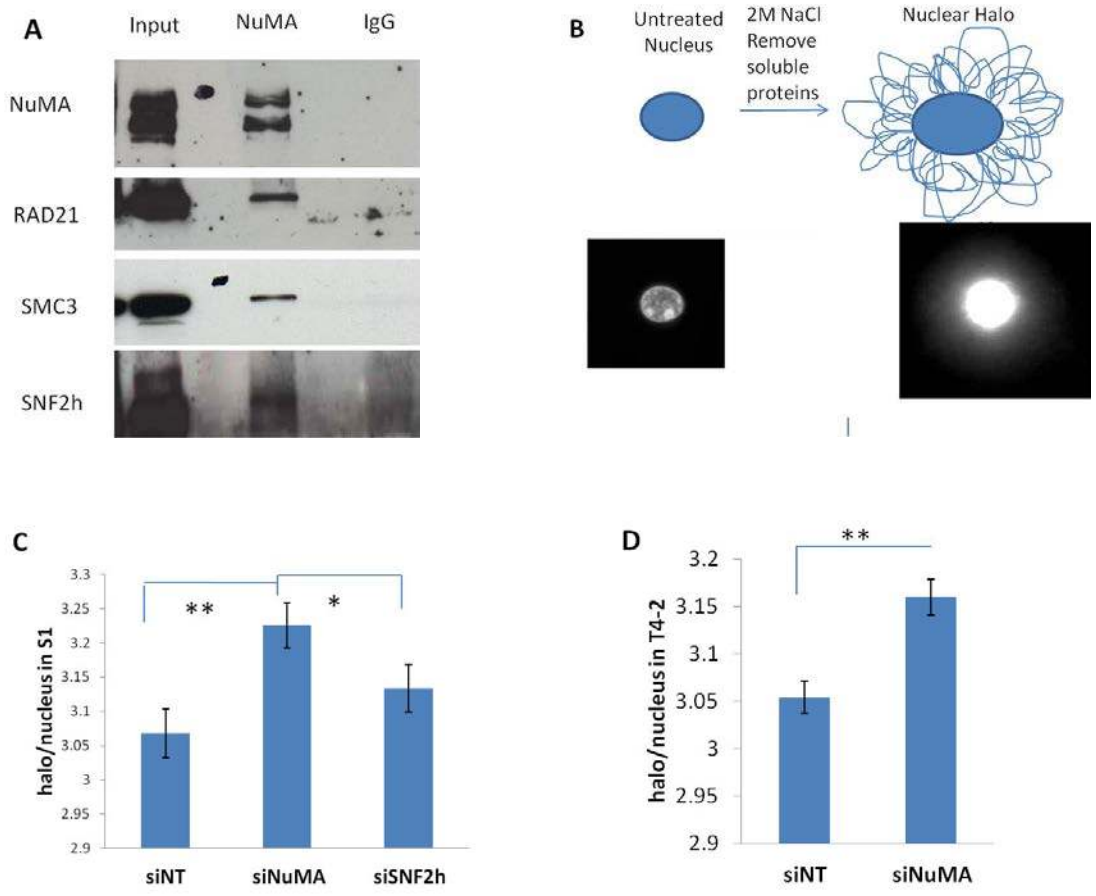


Figure 2.3.4. NuMA interacts with cohesin and influences chromatin looping.

(A) Interaction between NuMA, SNF2h and two components of cohesin (RAD21 and SMC3). Nuclear extracts were prepared from growth-arrested non-neoplastic mammary epithelial S1 cells and incubated with anti-NuMA antibody or non-specific immunoglobulin (IgG) overnight for immunoprecipitation (IP). Protein K coated magnetic beads were used to attract anti-NuMA antibody or IgG. Shown are western blot analyses of NuMA, RAD21, SMC3, and SNF2h on anti-NuMA antibody IP fraction (NuMA IP) and IgG IP fraction.

(B) Comparison of the ratio of halo/nucleus in NuMA silenced S1 and T4-2 cells. S1 (C) and T4-2 cells (D) in 2D culture were treated with trypsin without EDTA on day 6 after siRNA transfection and, following detachment from the culture vessels, they were incubated in CSK buffer containing 2M NaCl, 0.5% Triton x-100, protease inhibitors, and propidium iodide (10 µg/ml) for 3 minutes followed by imaging with a 40x microscope lens. Control groups include siNT (NonTarget), and siSNF2h. The diameter of the halo and the nuclear core were measured from more than 100 cells with Image J under each treatment condition and the ratios of halo/nucleus were compared among siNuMA and control groups (n=3). ** p value< 0.01; * p value<0.05.

2.3.5. NuMA and cohesin are preferentially enriched in specific regions of rDNA

Chromatin immunoprecipitation assay (ChIP) can be used to assess whether the target protein can bind to a specific region of chromatin. Quantitative PCR assay is used to validate the potential enrichment of the target protein on a specific chromatin region. To test whether NuMA and cohesin co-occupy the same region of rDNA, ChIP-quantitative PCR assay was performed to assess the potential enrichment of NuMA and cohesin SMC3 on different regions of rDNA. Primers targeting rDNA promoter region (pro-1), coding regions including 18S (H4), 5.8S (H8), 28S (H13), and nontranscribed IGS regions (H18, H27, H32) were selected for the analysis; their positions are shown in the drawing Figure 2.5.A. Because qPCR is a very sensitive technique, I observed large variations regarding the number of cycles reaching the signal detection threshold when different replicates with ChIP products were subjected to quantification, yet the enrichment trends on the tested rDNA regions are fairly similar. To eliminate this type of variations, two steps of normalization were carried out in my experiment in order to obtain the final relative enrichment fold; i.e. the chromatin amount pulled down by the target protein was first normalized to that of control IgG in each region and the obtained relative fold enrichment at every region was further normalized to the assigned rDNA region H4 to distinguish the potential significance of fold change among these rDNA regions. The enrichment fold of DNA fragment in H4 is picked up as the reference randomly for the second normalization. In fact, since it is the relative difference of different regions that was analyzed in the study, any region could be used as the reference and would not change the trend. The final ChIP-qPCR results showed that both NuMA

(Fig.2.3.5.B) and cohesin SMC3 (Fig. 2.3.5.C) are preferentially enriched in the nontranscribed IGS H27 region compared to coding regions H8 and H13 ($p<0.05$, Table 2.3.5.1 & 2.3.5.2). In fact, the enrichment of NuMA at H27 is more apparent since statistical significance ($p<0.05$) was found in all comparisons between H27 and any other regions (Table 2.3.5.1). The second highest enrichment region for NuMA is at H32 and the T-test showed nearly significance ($p=0.07$) when compared to H8 and H13. SMC3 was also found to be more enriched at H32 based on the average enrichment folds, but it was not statistically significant. Based on the above observations, an overall conclusion is that NuMA and cohesin SMC3 are less enriched at H8 and H13 but co-enriched at H27.

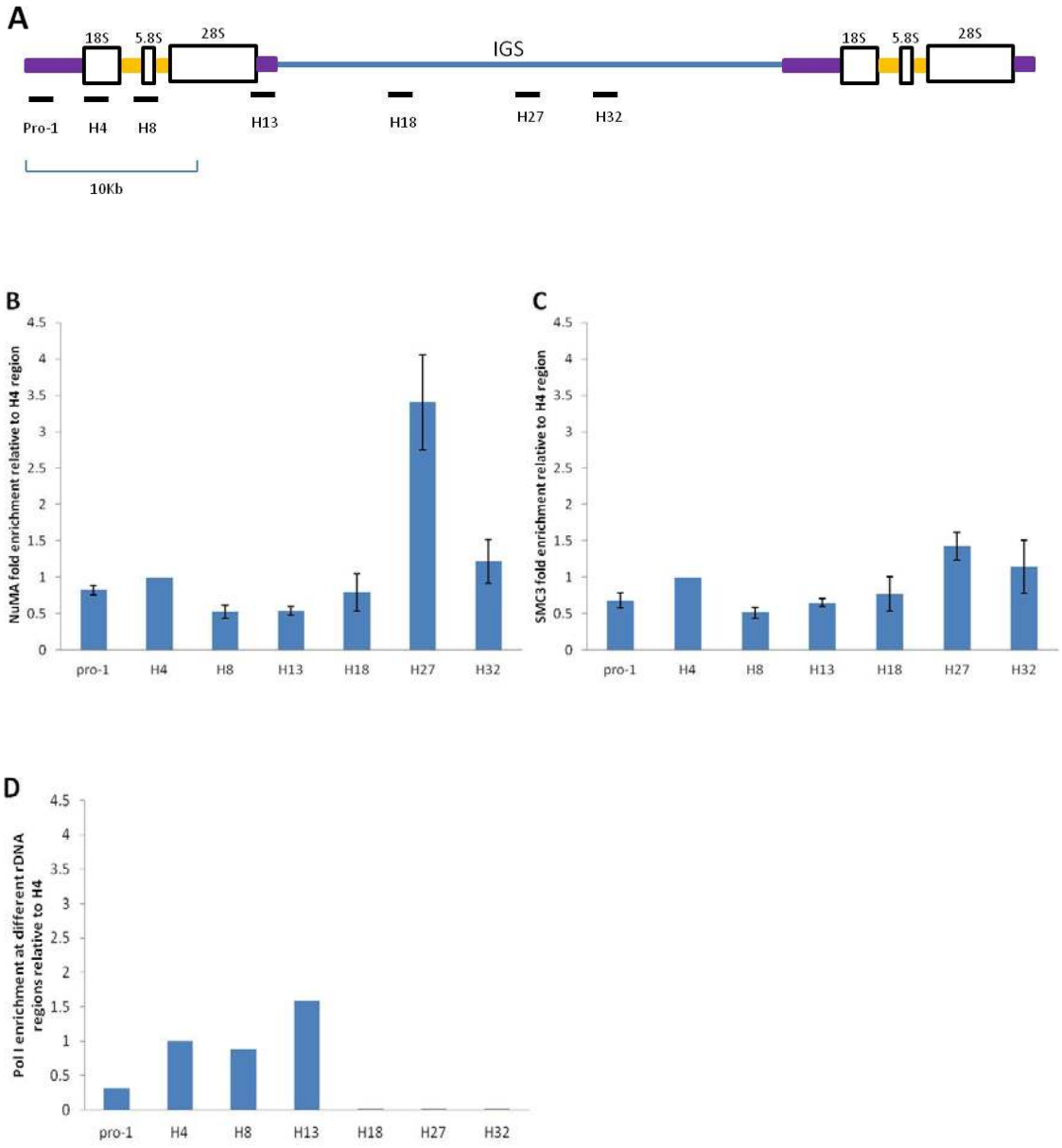


Figure 2.3.5. ChIP-qPCR assay of NuMA, cohesin SMC3, and Pol I on rDNA in T4-2 cells shows that NuMA and cohesin SMC3 are preferentially enriched in specific IGS regions.

(A) Schematic diagram of human rDNA linear structure and the primers used in qPCR assay targeting different regions. Purple bar: external transcribed spacers; yellow bar:

internal transcribed spacers; rectangle: rRNA coding regions; blue line: intergenic spacer; black bar: primers. The ChIP experiments followed by real-time PCR assay of NuMA and SMC3 were performed on monolayer culture of breast cancerT4-2 cells to assess the potential binding to different regions on the rDNA; IgG was used as nonspecific binding control. The enrichment of NuMA and SMC3 at different rDNA regions relative to H4 was obtained from a two-step of calculation. First, the enrichment of NuMA and SMC3 at different rDNA regions relative to IgG was obtained by normalizing the input percent of NuMA and SMC3 to that of IgG. Second, the relative enrichment at different regions from the first step was further normalized to that of region H4. Pol I was used as positive control to validate the ChIP assay on rDNA in two out of four replicate experiments. Similar results of Pol I ChIP assay were obtained and one of them was shown in (D).

Table 2.3.5.1. T-Test p-value of the relative enrichment of NuMA among tested regions on rDNA (p-value has been round off to 2 decimals)

NuMA	pro-1	H4	H8	H13	H18	H27	H32
pro-1	-	0.04	0.04	0.02	0.90	0.01	0.25
H4	-	-	0.00	0.00	0.44	0.01	0.49
H8	-	-	-	0.92	0.36	0.00	0.07
H13	-	-	-	-	0.37	0.00	0.07
H18	-	-	-	-	-	0.01	0.32
H27	-	-	-	-	-	-	0.01
H32	-	-	-	-	-	-	-

Table 2.3.5.2 T-Test p-value (round off to 2 decimals) of the relative enrichment of SMC3 among tested regions on rDNA

SMC3	pro-1	H4	H8	H13	H18	H27	H32
pro-1	-	0.03	0.25	0.82	0.74	0.01	0.27
H4	-	-	0.00	0.00	0.37	0.06	0.71
H8	-	-	-	0.18	0.34	0.00	0.14
H13	-	-	-	-	0.64	0.01	0.23
H18	-	-	-	-	-	0.07	0.42
H27	-	-	-	-	-	-	0.51
H32	-	-	-	-	-	-	-

Tables 2.3.5.1 & 2.3.5.2 show the p values of T-tests between the two groups of enrichment folds from the comparison of any pair of rDNA regions.

2.3.6. NuMA affects Ki67 distribution

After unveiling that NuMA impacts both nucleolar morphology and function, I have explored whether NuMA can influence cell cycle marker Ki67. Ki67 is expressed in all phases of active cells but absent from quiescent cells, which makes it an important marker to indicate cell growth in cancer diagnosis in clinical (Scholzen and Gerdes 2000). Recent studies also showed that Ki67 is involved in rRNA transcription regulation (Bullwinkel, Baron-Luhr et al. 2006; Rahmanzadeh, Huttmann et al. 2007). In addition to the well-known fact of NuMA stabilizing the spindle poles during mitosis, which is essential for the completion of cell division (Silk, Holland et al. 2009), mounting evidence also supports the role of nuclear NuMA in cell cycle progression during the interphase (Chandramouly, Abad et al. 2007; Endo, Moyori et al. 2013; Vidi, Liu et al. 2014). Therefore immunostaining for cell cycle marker Ki67 in NuMA over-expressing MCF7 cells was carried out. NuMA cDNA or GFP-NuMA was transiently expressed in MCF7 cells and empty vector or GFP only was transfected in parallel to be used as control. Ki67 staining displays distinct distribution patterns at different stage of the cell cycle (van Dierendonck, Keijzer et al. 1989), based on which, six patterns (and their roughly corresponding stages) were characterized in this study including no staining (quiescence), speckles (early G1), speckles & foci (G1), two or more foci (late G1, S, and G2), homogenous staining (M), and single spot as shown in the lower panel in Figure 2.3.6.A. Ki67 staining as a single spot within the enlarged nucleolus has been commonly observed in NuMA silenced S1 cells (data not shown) and it is most likely to represent a specific stage of a cell with reduced nucleolar activities before exiting the cell cycle to

enter G0. The percentages of cells in each category based on Ki67 patterns were compared between the treatment groups and control groups as shown in the bar chart in Figure 2.3.6.A. The percentage of control cells with speckles and speckles & foci is significantly higher in control cells than in the treated cells; in contrast, a higher percentage of cells with over-expression of NuMA display a single spot of Ki67 staining. These observations suggest that NuMA affects Ki67 distribution, when NuMA is over-expressed. Although levels of cyclins would have to be measured to correlate changes in Ki67 distribution with an alteration of the cell cycle, the increased presence of cells with a single spot of Ki67 staining might be related to a change in the number of nucleoli that has been correlated with cell proliferation activity (i.e., the more nucleoli the higher the proliferation activity). But as explained above, the exact stage of the cell cycle in which these cells are requires further investigation. We also observed an increased percentage of cells without Ki67 staining in NuMA over-expressing cells, which indicates there is an increased number of quiescent cells upon over-expression of NuMA. These changes are accompanied with a dramatic decrease in the percentage of cells with multiple speckles and small foci of Ki67 staining as well as cells with diffused homogenous staining. Overall, our observations suggest that an excessive amount of NuMA hinders impairs proliferation based on the absence of Ki67 staining on a per cell basis.

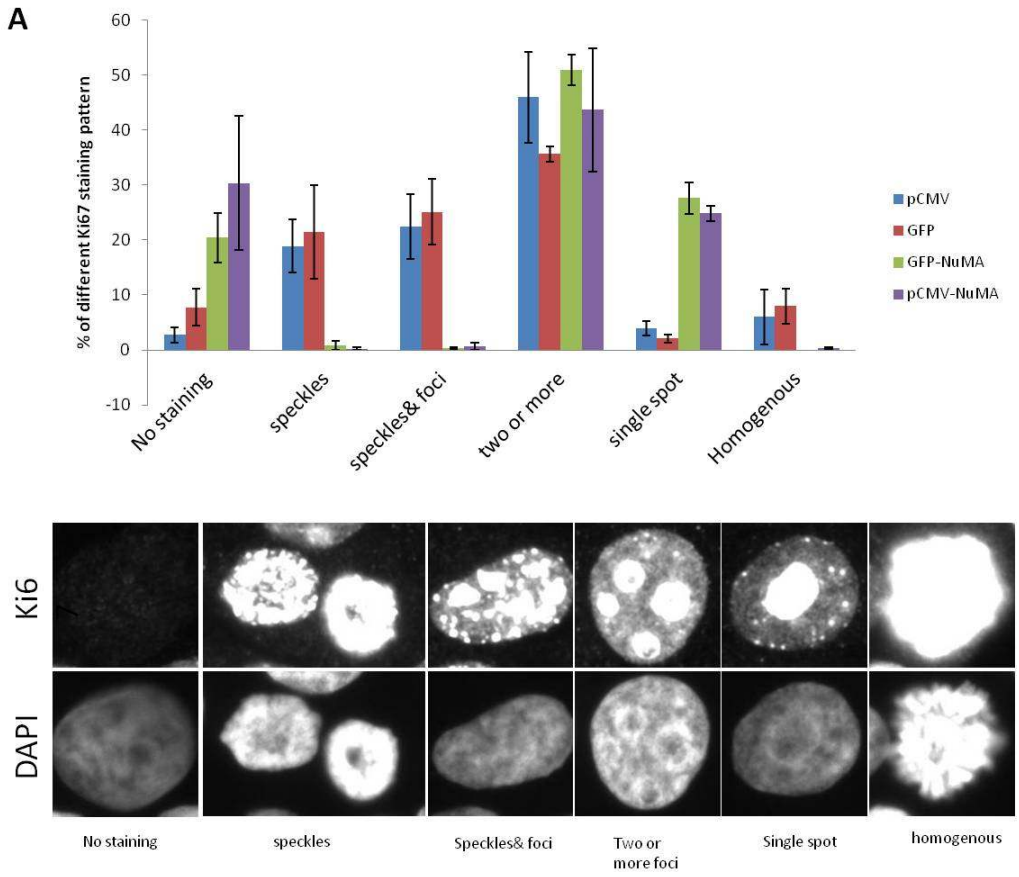


Figure 2.3.6. NuMA influences Ki67 distribution and expression in MCF7 cells

The percentage of cells with different Ki67 patterns upon NuMA over-expression (upper panel) and the characterized Ki67 distribution staining (lower panel) in MCF7 cancer cells. MCF7 cells cultured in 2D were either transfected with GFP-NuMA or with pCMV-NuMA to trigger over-expression of NuMA. Empty vector pCMV and GFP transfected cells were used as controls. Immunostaining for Ki67 was carried out 24 hours after the transfection. Under each treatment, more than 300 cells were scored and the percentages were compared based on the six different distributions of Ki67 as shown in the lower panel. The error bar represents standard error (n=3).

2.4. Discussion

NuMA is a multifunctional protein localized in the cell nucleus beside its presence at the mitotic spindle poles. To this date the possible proposed roles for NuMA in the cell nucleus are an involvement in the building of a nuclear matrix (Berezney and Coffey 1974), apoptosis (Hsu and Yeh 1996), gene transcription control (Harborth, Weber et al. 2000), chromatin organization (Abad, Lewis et al. 2007), and DNA repair (Vidi, Chandramouly et al. 2012). Cell cycle reentry upon alteration of NuMA distribution in reverted malignant mammary epithelial cells, associated with chromatin reorganization, was also reported (Chandramouly, Abad et al. 2007), suggesting a link between NuMA function in chromatin regulation and biological consequences in cell quiescence control. In an effort to elucidate the underlying mechanism of how NuMA controls chromatin organization and thus, possibly exerts its influence on the cell cycle, I conducted the study presented above on the role of NuMA in the regulation of nucleolar architecture and function.

The nucleolar architecture was assessed upon NuMA silencing based on three aspects, such as the number of nucleoli, the distribution of nucleolar proteins Fibrillarin and Pol I, and the number of the centromeres as indicated by CENPA staining associated with the nucleolus. My results showed that while the number of nucleoli is decreased in NuMA silenced cells, the average number of centromeres associated with each nucleolus is increased. The number of nucleoli is positively correlated with the requirement for ribosome biogenesis in these cells, which can be exemplified by the extreme case of cancer cells (Derenzini, Montanaro et al. 2009). Therefore, the decrease of the number of

nucleoli in NuMA silenced S1 cells likely reflected the lesser requirement for ribosomes in these cells probably due to the global reduction in cellular activities. In support of this hypothesis, an overall reduction of rRNAs in NuMA silenced cells was confirmed by qPCR assay of individual rRNA species and the FUrd assay of nascent rRNA. Interestingly, an overall reduction of nascent mRNA was also observed in the FUrd assay (results not shown), suggesting that the influence of NuMA silencing on RNA production is global in the nucleus rather than limited to the nucleolus. The increased average number of CENPA associated with nucleoli in NuMA silenced cells signifies that more centromeres are associated per nucleolus when NuMA is absent, suggesting an impact on heterochromatin organization at the nucleolar periphery. A subset of centromeres are routinely observed to associate or reposition to the nucleoli in the interphase of the cell cycle from *Drosophila* (Padeken, Mendiburo et al. 2013) to human cells (Wiblin, Cui et al. 2005), but the biological function of such phenomenon is not clear so far. Linear proximity between NORs and the centromeres on human acrocentric chromosomes enables these centromeres to associate with the nucleoli, yet it has not been addressed whether centromeres on other chromosomes are equally likely to anchor on the surface of nucleoli. The newly recruited centromeres can be from two sources: either from those acrocentric centromeres that used to be part of other nucleoli, or from any chromosomes recruited by chance. Based on studies from others (Krystosek 1998; Sullivan, Bridger et al. 2001), I favor the first explanation. It would suggest a dynamic microenvironment favoring chromosome repositioning in the nucleus in the absence of NuMA. Nonetheless, this observation may provide a new angle to study how NuMA controls higher-order chromatin organization.

Pol I staining in NuMA silenced cells revealed a less constrained distribution pattern of Pol I foci in the enlarged nucleolar region. Without labeling rDNA, I was not able to conclude whether the reorganization of rDNA occurs upon NuMA silencing. Yet, the unchanged Pol I level assessed by western blotting and the less constrained Pol I foci in NuMA silenced cells leads me to assume that the decrease in rRNA levels that I also measured is more likely caused by the rearrangement of rDNA, although we cannot rule out an impact on complexes involved in transcription and interacting with NuMA like B-WICH (Jayaraman S, Ph.D. 2014). Overall, changes in the nucleolus from these three angles bring enough evidence to conclude that NuMA influences nucleolar architecture.

Both FUrd assay and RT-qPCR were performed to test if the levels of rRNAs are affected in NuMA silenced cells. Results on nascent RNAs with FUrd revealed that rRNA transcription is indeed impaired by NuMA silencing. RT-quantitative PCR results showed that only 28S and 18S but not 45S rRNA levels are significantly decreased in NuMA silenced cells. The 28S rRNA and the 18S rRNA are produced from the 45S pre-rRNA after the cleavage of internal transcribed spacer sequences and external transcribed spacer sequences (Boisvert, van Koningsbruggen et al. 2007). Thus, our results also tend to indicate that the rRNA processing steps are impaired by NuMA silenced cells. However, it should be noted that 45S pre-rRNA was largely decreased in two out of three biological replicates; thus the lack of statistical significance for the average decrease of 45S rRNA level is influenced by the one replicate that displayed no change. The possible reason for the variation among biological replicates is likely the extremely short life-span of 45S pre-rRNA, which can only exist for several minutes (Popov, Smirnov et al. 2013)

before being processed. Further investigation of 45S pre-rRNA level is needed to bring a definitive conclusion.

To understand the mechanism by which NuMA controls nucleolar architecture, especially rDNA organization, a literature search was conducted in order to find out if NuMA has any binding partner functioning in chromatin remodeling. The identified interaction between NuMA and the cohesin complex in S phase and M phase of the cell cycle (Gregson, Schmiesing et al. 2001) has attracted my interest because cohesin is a well-known global player in chromatin organization (Merkenschlager 2010). More importantly, a causal relationship between a dysfunctional cohesin and reduction in the number of ribosomes has been repeatedly observed in a group of diseases termed cohesinopathies (Bose and Gerton 2010). Cohesinopathies are the genetic diseases specifically caused by mutated proteins involved in the cohesin network. Since the cohesion of sister chromatids is not significantly affected in these diseases, it is usually thought that the mutations impair the function of cohesin in chromatin organization and transcription control. In light of the published results showing the interaction between NuMA and cohesin in S and M phases, I also assessed this interaction in the cell nucleus of nonproliferating cells. The overlap of NuMA and cohesin notably occurred at the periphery of the nucleolus and the periphery of nucleus in cells in 3D culture, and in the insoluble nuclear features where the proteins remained closed to the DNA after soluble proteins were removed by high salt. Thus, NuMA and cohesin preferentially overlap at heterochromatin-enriched regions in non-neoplastic differentiated cells. The colocalization of NuMA and cohesin was also assessed in quiescent non-neoplastic S1

cells cultured in 2D (no acinar differentiation), but because both proteins displayed strong fluorescence signal in the nucleus, it was difficult to identify the colocalization pattern (Data not shown). At least a portion of both NuMA and cohesin remains following high salt extraction, possibly as constituents of the nuclear matrix. The merged images of remaining NuMA, cohesin SMC3, and DNA in the nuclei after salt extraction show that cohesin staining is almost completely overlapping with DNA while NuMA is distributed into dispersed foci overlapping with DNA, which suggests NuMA and cohesin might only partially share chromatin functions. This is consistent with the observation from cells in 3D culture that the overlap of NuMA and cohesin occurs in particular locations in the cell nucleus. Hence, we could assume that although these proteins might work in concert under certain circumstances, they also act independently. This hypothesis is supported by results with the formation of nucleolar caps upon halting rDNA transcription with actinomycin D, showing that the two proteins are not always simultaneously involved depending on the cells.

It is known that NuMA and cohesin both bind Rae-1 at the mitotic spindle pole to coordinate the assembly of the mitotic spindle (Wong and Blobel 2008; Kong, Ball et al. 2009) during metaphase, but the functional interpretation for NuMA and cohesin interaction in S phase remains elusive (Gregson, Schmiesing et al. 2001). To rule out other potential chromatin activities mediated by NuMA and cohesin, like DNA replication, and merely focus on the transcription control and subsequently quiescence maintenance, co-IP for NuMA and cohesin was performed exclusively in the nucleus of growth-arrested cells. Co-IP assay results showed that cohesin SMC1 and RAD21 were

pulled down by NuMA together with SNF2h. As mentioned above, SNF2h is an ATPase associated with NuMA (Vidi, Liu et al. 2014), but it is also needed for loading cohesin on the chromosomes by the nucleosome remodeling and deacetylase (NuRD) complex (Hakimi, Bochar et al. 2002). NuRD contains HADC1/HADC2 enzymes that are important to initiate heterochromatinization (Allen, Wade et al. 2013). Thus, it seems logical to observe cohesin appearing in the perinucleolar heterochromatin region given that it works together with NuRD. Unfortunately, the reciprocal IP for cohesin components failed to bring down NuMA (data not shown). A technical explanation would be that the antibody used to IP cohesin disrupted the interaction. Based on the study of NuMA and cohesin in metaphase, their interaction seemed to be mediated by Rae-1 but it was never addressed whether NuMA and cohesin interact directly (Wong and Blobel 2008). If their interaction were indirect, distinct affinities of NuMA and cohesin for a third protein might complicate the coimmunoprecipitation results. Nevertheless, based on current knowledge, several scenarios could be proposed for the interaction between NuMA and cohesin. It is possible that NuMA and cohesin interact through their binding to the same region of chromatin. Published work indicates that NuMA and cohesin bind MARs(Luderus, den Blaauwen et al. 1994). A DNAse treatment prior to co-IP would degrade DNA not embedded in the nuclear matrix and disrupt the association between NuMA and cohesin at these locations, hence the proteins would not co-PI anymore; however, their potential interactions as part of nuclear matrix through MARs could still remain and the two proteins would still co-IP, but possibly to a lesser extent. Another scenario is that NuMA and cohesin interact directly or via a third

protein independently of DNA binding. In this case, the treatment with DNAse would not influence the IP result.

The timing of NuMA and cohesin in their interaction with chromatin also points out to complementary yet independent functions. The binding between cohesin and chromatin at different phases of the cell cycle is relatively well characterized compared to that of NuMA. Cohesin is loaded on chromatin in telophase in metazoans. The binding of cohesin to chromatin in G1 phase is believed to be dynamic rather than "stable" due to the observation that it takes shorter time for the nearly whole population of cohesin to dissociate from chromatin upon photobleaching compared to the time necessary in other phases of the cell cycle (Gerlich, Koch et al. 2006). NuMA enters cell nucleus in telophase as well and at least a fraction of NuMA associates with chromatin. The interaction between NuMA and cohesin in G0/G1 phase was observed in my present study. Work from others also indicates a possibility of NuMA and cohesin co-occupying the same chromosomal regulatory elements to regulate gene transcription (please see Chapter 4 final discussion) (Tarallo, Bamundo et al. 2011) (Tarallo, Bamundo et al. 2011).

Distinct recovery times within the cohesin population upon photobleaching after G1 phase has raised the hypothesis that cohesin is likely present in different pools committed to different functions in S and G2 phases. It is now generally accepted that the dynamic cohesin-chromosome binding is mainly responsible for gene transcription regulation by mediating intrachromosomal interactions. Stable cohesin-chromosome binding is proposed for chromatids cohesion via interchromosomal interactions (Gerlich,

Koch et al. 2006; Jeppsson, Kanno et al. 2014). NuMA and cohesin interaction in S phase has been reported to occur in a small population of both proteins (Gregson, Schmiesing et al. 2001); however, it has not been addressed under which circumstance the interaction takes place. Given the fact that both NuMA and cohesin are also involved in DNA repair, it would not be surprising to see their interaction occur in a specific pool either for transcription regulation or for DNA repair. The release of cohesin from chromosomes and NuMA from the nucleus occurs in prophase. NuMA is likely relocated to the spindle pole area by the end of prophase, while most cohesin detaches from chromosomal arms by the end of metaphase except for centromeric cohesin that is removed at the beginning of anaphase and thus, allows the segregation of sister chromatids (Horsfield, Print et al. 2012). These findings tend to indicate that the interaction between NuMA and cohesin is likely involved in the pool of cohesin that detaches from the chromosomes early on instead of those after the prophase. In addition, a small amount of cohesin accumulates at the spindle poles, hence interacting with NuMA during mitosis; but the biochemical specificities of these cohesin complexes have not been characterized yet, so we cannot rule out the possibility that this fraction of cohesin also represents a different pool in the interphase nucleus.

To my best knowledge, we are the first laboratory to show that both NuMA and cohesin form “nucleolar cap” structures upon Pol I inhibition induced by actinomycin D. The formation of nucleolar caps usually coincides with nucleolar stress, like that induced by the blockage of the ribosome biogenesis network, the inhibition of mRNA transcription and the induction DNA damage (Shav-Tal, Blechman et al. 2005). The

biological functions of these cap structures are not known currently. Interestingly, different proteins were observed to form nucleolar caps depending on distinct stresses, hence indicating a specificity of the stress reaction (Malekkou, Lederer et al. 2010). In this sense, NuMA and cohesin are functionally-related in the stress reaction upon rRNA transcription blockage. Among cells forming caps, over 50% show the simultaneous presence of NuMA and cohesin caps around the nucleoli while other cells only contain nucleolar caps formed by either NuMA or cohesin, indicating that NuMA and cohesin might behave independently from each other. This idea is supported by the fact that the cohesin cap can still form in NuMA silenced cells upon actinomycin D treatment (data not shown). Another interesting observation is that NuMA aggregates at the periphery of the nucleolus without any overlap with caps formed by Pol I (Figure 2.3.2.F) after the treatment with actinomycin D. Under such condition, the formerly active rDNA arrays in the interior of the nucleolus compact and reposition to the nucleolar edge, where they are flanked by the perinuclolar heterochromatin including silent rDNAs, neighboring proximal and distal chromosome regions and other heterochromatin (Guetg, Lienemann et al. 2010; Floutsakou, Agrawal et al. 2013). Thus, it may be concluded that NuMA might exclusively overlap with the perinucleolar heterochromatin after Pol I is inhibited. It should be also pointed out that the reaction of NuMA upon nucleolar stress seems to be cell-type or cell-cycle dependent. NuMA was not reported to form the nucleolar cap structure in some cancer lines used in the study (Shav-Tal, Blechman et al. 2005). Upon comparison between non-neoplastic S1 cells and malignant T4-2 cells, I observed that NuMA caps in T4-2 cells are less obvious than those in S1 cells. This reaction of NuMA seems to be related to cell quiescence status considering that NuMA-formed nucleolar

cap can be clearly observed only in non-neoplastic cells that exited the cell cycle (Abad, P. M.S. Thesis, 2003).

Cohesin functions in chromatin looping, which is illustrated with the nuclear halo assay. Cells with silenced RAD21 or SMC3 produce larger halo size compared to the control cells (Guillou, Ibarra et al. 2010). This may be explained by the loss of integrity of the nuclear matrix structure, so that it cannot provide enough chromatin anchoring points, which leads to the longer chromatin loops reflected by the halo size. Results from halo assays performed on NuMA-silenced S1 cells and T4-2 cells showed a significant increase in halo size compared to control cells. It is likely that the effect is underestimated because of the low transfection efficiency of the HMT-3522 cell series.

The mechanism by which cohesin mutations induce defects in ribosome biogenesis in Cohesinopathies is not clear. Although a claim has been made in a review article that cohesin regulates rDNA loops by binding IGS (Xu, Lu et al. 2014), the related evidence is not seen yet by the time this thesis is written. A recent study of the budding yeast strain with *ECO1* mutation, a homolog of human cohesin acetyltransferase ESCO2 that causes one type of cohesinopathies if mutated, showed that slowed-down sliding of cohesin along the rDNA replication forks decreases rDNA transcription efficiency (Lu, Lee et al. 2014). However, many mysteries still remain. For example, what is the relationship between the cohesin binding sites for rDNA transcription and replication? Moreover, the slowed-down rDNA replication as well as on other genomic regions should directly lead to prolonged S phase, yet this was not reported in the cells from patients so far. ChIP-qPCR was performed in my study to assess the potential binding

regions of NuMA and cohesin on rDNA. A preferential binding at IGS compared to coding regions was revealed for both NuMA and cohesin SMC3 on rDNAs in T4-2 cells and the same pattern was recaptured in MCF7 cells (data not shown). In the statistical analysis, a random region (H4) on rDNA was selected as the reference for the secondary normalization of the relative fold enrichment at different tested rDNA regions. I found that this was a necessary step in the analysis because a similar enrichment trend was seen among the biological replicates of ChIP experiments but the enrichment folds varied largely from one replicate to another. A chromosome region closed to *MYC* gene was selected as the negative binding control for cohesin based on the literature at first, but I soon found out that it is not practical due to the difference in the gene copy number. The normal genome contains only two copies of most genes but there are about 400 copies of rDNA. High background was produced even for the IgG binding at rDNA compared to other regions. To eliminate the variations of antibody (and IgG) binding efficacy at different regions and to eliminate the variations of enrichment folds due to technical issues among the biological replicates, two rounds of normalization were carried out at each individual region in the final statistical analysis.

Human rDNA IGS harbors regulatory elements responsible for rDNA transcription at its 5' and 3' ends adjacent to the transcribed region (seen Figure 1.7.1), but for most parts of the DNA segment between the two terminal regions of IGS potential functions remain largely unknown. In present study, NuMA was found to preferentially bind to region H27 of the IGS, 27kb downstream of the transcription start site. Published sequence analysis revealed that pyrimidine (thymine and cytosine) is almost exclusively

present in the region spanning from 25kb to 27kb (Gonzalez and Sylvester 1995), which might provide a clue of the DNA pattern for NuMA binding. In addition, three tandemly arranged A-containing motif "*ActcA* blocks" are located at 26,866 – 27,125 bp in this pyrimidine-enriched region. A repeat element named "butterfly", due to the pattern shown on the sequencing gel, was also assigned at 27,271 – 27,859 bp. Yet, functional studies related to these motifs are not available, although it was speculated that the IGS fragment containing these putative motifs might contribute to anchoring rDNA at the edge of the nucleolus (Kaplan, Murray et al. 1993; Gonzalez and Sylvester 1995).

My results have shown that NuMA silencing results in rRNA transcription inhibition and NuMA preferentially binds IGS H27. It is reasonable to assume that NuMA silencing will decrease its presence at rDNA IGS regions. This effect would be concomitant with the reduction in rDNA transcription, hence potentially linking NuMA binding to H27 and rDNA transcription. However, the question remains of whether NuMA is present solely in active rDNA or at both inactive and active rDNA. The ChIP assay for Pol I and histone markers specific of active or inactive rDNA in NuMA silenced cells, as well as ChIP (NuMA)-reCHIP (markers of interest) assays in cells expressing NuMA, might help answer this question. Although my current ChIP data supports the preferential binding of NuMA and cohesin to IGS, it does not prove that these proteins are involved in the loop structure in the rDNA. To further test the potential loop organization, 3C assay is required.

At the end of my project, an initial preliminary effort was made to assess the role of NuMA in cell cycle progression. Given that the impairment of rDNA transcription

was observed in NuMA silenced S1 cells, cell cycle arrest should be expected in NuMA-silenced cells. However, it is not that simple. Indeed, others have observed that NuMA silencing does not necessarily lead to cell cycle arrest (Endo, Moyori et al. 2013; Vidi, Liu et al. 2014). This situation is partially caused by the fact that NuMA is a stable protein and it is not easy to completely deplete it from the cells (Silk, Holland et al. 2009), especially in the rapidly growing cancer cells. Flow cytometry quantification of DNA following NuMA silencing with siRNA indicated possibly a cell-type dependent effect on the cell cycle (Vidi, Liu et al. 2014). For example, the percentage of cells in G0/G1 phase was slightly increased in mammary epithelial cell lines, MCF7 and S1. But it decreased slightly in human osteosarcoma U2Os and remained unchanged in leukemia K562 cells. Therefore, in an effort to better understand the relationship between NuMA and proliferation control, cancer cells over-expressing NuMA were analyzed in my experiments. Based on the staining pattern of Ki67, which is also a nucleolar protein during the interphase of the cell cycle, I observed that an increased population of cells accumulates in prolonged G1 and probably also in G0 phase, as shown by the lack of Ki67 expression in these cells. The rRNA level and ribosome level have not been assessed yet in these cells. Future experiments should investigate the expression pattern of different cyclins by dual immunostaining with Ki67 to better correlate the observed changes in Ki67 distribution and an alteration of the cell cycle. Cyclin E reaches its peak as cells enter S phase. Cyclin A and cyclin B elevate to the highest level in G2 and M phase, respectively. Nevertheless, overall results suggest that too much or too little NuMA both impact proliferation. It is possible that, as for NuMA silencing, NuMA over-

expression also disrupts nucleolar structure and function via an imbalance of binding at IGS.

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CHAPTER 3: CDS1 IS FUNCTIONALLY INVOLVED IN CELL POLARITY CONTROL AND CANCER INVASION

3.1 Introduction

Basoapical polarity is a hallmark of functionally differentiated epithelia. The formation of apical polarity acting as a tumor suppressor has been reported previously, and deregulation of some tight junction proteins contributes to cancer invasiveness as well as metastasis (Qiu, Abo et al. 2000; Huang and Muthuswamy 2010). Our laboratory previously demonstrated that the integrity of apical polarity is critical for breast epithelial cells to remain quiescent and proposed that the existence of apical polarity is a "brake" to prevent quiescent cells from reentering the cell cycle (Chandramouly, Abad et al. 2007). To identifiy the gene-expression profile related to apical polarity regulation, whole genome analysis at the mRNA level was performed in glandular structures (acini) produced in 3D culture with and without apical polarity. *CDS1* was one of 30 genes identified to be significantly down-regulated upon apical polarity disruption (Cardenas JM, Ph.D. Thesis, 2012).

CDS1 is critical for phosphatidic acid metabolism and encodes an enzyme that catalyzes the production of CDP-diacylglycerol (CDP-DAG) using cytidine triphosphate (CTP) and PA as substrates. CDP-DAGs react with inositol to generate PIs, which are the

precursors of phosphatidylinositides including phosphatidylinositol phosphate (PIP), phosphatidylinositol biphosphate (PIP_2), and phosphatidylinositol triphosphate (PIP_3). These phophatidylinositides are important molecules involved in multiple signaling pathways conducting various functions (Vivanco and Sawyers 2002; Bharill, Ayyadevara et al. 2013). In mammals, the oncogenic protein AKT is activated after it directly binds to cell membrane-located PIP_3, resulting from the conversion of $PI(4,5)P_2$ by Pi3K (Auger, Serunian et al. 1989). The transition balance between PIP_2 and PIP_3 is controlled by the antagonistic functions of Pi3K and PTEN, both of which are among the most frequently mutated genes in cancer (Watson, Takahashi et al. 2013). Indeed, constitutively elevated PIP_3 is reported in most cancer cells (Vanhaesebroeck, Leevers et al. 2001) , suggesting a critical involvement of the phosphatidylinositide biosynthesis pathway in cancer development and progression. Loss-functional mutated *cds,* the human *CDS1* homologous gene in *Drosophila*, leads to the decrease of PIP_2 level in photoreceptor cells in eyes, but this effect is thought to be specifically caused by the eye-*cds* isoform since it was not observed in other cell types (Wu, Niemeyer et al. 1995). The global impact of CDS to the organism is just getting recognized through a recent study in *Drosophila.* By silencing the CDS gene with RNAi, the Huang group reported an increase of lipid storage indicated by the level of triacylglycerol (TAG) and a decrease of PIs in *Drosophila* salivary gland cells as well as in the larva (Liu, Wang et al. 2014). The production of PIs and TAG in *Drosophila* is carried out by two different pathways using PAs as common precursors and branched thereafter. Loss of CDS function resulted in the blockade of PIs synthesis pathway, hence reinforcing the previous observation in eye photoreceptor cells (Wu, Niemeyer et al. 1995), and therefore diverted most precursor PAs to produce TAG.

Very interestingly, size reduction was observed in the salivary gland cells with silenced CDS while the number of cells was not changed, suggesting that the influence of the decrease in PI level is mainly on cell growth rather than on cell proliferation. Yet, neither cell size nor TAG accumulation was affected by CDS silencing in fat body cells (Liu, Wang et al. 2014), indicating that the lipid metabolism pathways regulating cell growth are cell-type dependent.

In contrast to *Drosophila*, that contains only one CDP-DAG synthase gene, the human genome contains two CDS genes, *CDS1* and *CDS2* sharing 69% identity at the amino acid level (Halford, Dulai et al. 1998). In addition, Tam41 has also been reported recently to perform the same function as CDS in yeast mitochondria (Tamura, Harada et al. 2013), but the role of its homolog in human, Tamm41, is not clear. The investigation of the expression profile of *CDS1* and *CDS2* in mouse embryo and adult tissues shows that *CDS1* only specifically expresses in brain, eye, and smooth muscle in contrast to *CDS2*, which is more universally expressed in multiple tissues (Volta, Bulfone et al. 1999; Inglis-Broadgate, Ocaka et al. 2005). The subcellular localization of CDS1 and CDS2 is similar; both proteins are exclusively expressed in the cytoplasm and concentrated in the endoplasmic reticulum (ER) (Inglis-Broadgate, Ocaka et al. 2005). Recent evidence also suggested that CDS1 and CDS2 might use different PA pools as substrates distinguished by the acyl chain of the lipid (D'Souza, Kim et al. 2014).

Studies performed in vertebrate cells have provided more complicated results regarding the function of CDS. Monkey kidney COS-7 cells transfected with CDS1 cDNA failed to elevate PI levels although the enzyme activity was 7-fold increased

(Lykidis, Jackson et al. 1997). Zebrafish mutant with CDS2 loss-of-function displayed decreased PIP_2 levels that, consequently, caused the failure of vascular morphogenesis (Pan, Pham et al. 2012). The defect in angiogenesis in *cds2* mutants was recaptured by *in vitro* invasion assays in cell culture of human endothelial cells HUVEC with *CDS1* or *CDS2* silenced by RNAi. Moreover, silencing *CDS2* slightly reduced the number of endothelial cells in the zebrafish embryo, yet it was not reported whether cell proliferation was impaired as well *in vitro*.

Collectively, current evidence suggests that loss of CDS function causes PIP_2 reduction, which is likely to hinder cell growth, cell proliferation and even cell invasion depending on the cell type. Therefore, in light of the influence of CDS on major aspects associated with cancer and our recent discovery that *CDS1* might be linked to basoapical polarity, an essential feature of breast homeostasis also involved in proliferation and invasion control, it is necessary to further characterize CDS1's influence in the mammary epithelium in order to better unravel its potential role in breast cancer initiation and progression.

3.2. Materials and methods

3.2.1 Cell culture

3D drip method for cell culture was described as in Chapter 2.2.1.

3D embedded method was applied in all the experiments containing invasive ductal carcinoma MDA-MB-231 cells. Briefly, a thin layer of Matrigel^{TM} was coated on the container surface at 10 µl/cm^2 and the gel was allowed to solidify for 5 minutes at

37ºC. Cells were suspended in Matrigel™ containing 10% H14 medium at the density 830,000/ml for S1 cells (below passage 60) and 415,000/ml for S1 cells at passage 180 (S1p180), S2 cells, T4-2 cells, and MDA-MB-231 cells. The cells in Matrigel™ were incubated at 37ºC for 30 minutes before H14 medium was added to the gel. Regular H14 medium was used to culture S1 cells and H14 without EGF was used to culture the other cells. The culture medium was renewed every 2-3 days.

3.2.2. Drug treatments

MDA-MB-231 cells were incubated with different reagents from day 1 to day 10 of 3D culture. Reagents used included integrin beta 1 antibody AIIB2, 15 µg/ml; Pi3K inhibitor LY294004, 4 µM; mTOR inhibitor rapamycin, 10 nM; DMSO, 0.25% v/v.

3.2.3 Quantitative RT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) was performed by monitoring the increase in SYBR Green fluorescence using a 7300 Real-Time PCR machine (Applied Biosystems, Austin, TX). Primers for PUM1, CDS1 and CDS2 were commercially obtained (SA Bioscience, Frederick, MD). The thermal cycling conditions included one cycle at 50°C, a second denaturing step at 95°C, and 40 cycles with three steps: 95°C for 15 seconds, 60°C for 60 seconds and 72°C for 10 seconds. Gene transcript levels were calculated relative to PUM1 transcripts by the ΔΔCt method (Winer, Jung, Shackel, & Williams, 1999).

3.2.4. Tumor nodule imaging and measurement

Tumor nodules grown within the Matrigel™ were directly imaged with an IX70 microscope (Olympus). Images were taken for nodules located at different planes within the gel layer by progressively adjusting the microscope focus. The edge of each nodule was manually delineated on the pictures using Image J software with "free hand selections" function. Nodule's area and circularity were automatically obtained with the "measurement" function. The number of protrusions from the nodule core was counted manually as well.

3.3. Results

3.2.1. CDS1 and CDS2 mRNA levels are gradually down-regulated in the HMT-3522 cancer progression series.

Our preliminary data have indicated that *CDS1* mRNA is down-regulated in non-neoplastic S1 cells without apical polarity compared to S1 cells with apical polarity. Also, CDS1 protein decreases in S1 cells compared to S1-derived invasive T4-2 cells (Cardenas JM, Ph.D. Thesis, 2012). These findings suggest that the CDP-DAG synthetase 1 is negatively correlated with malignancy. However, since vertebrate animal cells contain two CDP-DAG synthetases, functional compensation might occur when one is absent. To this end, both CDS1 and CDS2 mRNAs were assessed in the HMT-3522 cancer progression series by qRT-PCR. The HMT3522 series (Briand, Petersen et al. 1987; Nielsen, Madsen et al. 1994; Radisky, Muschler et al. 2002) contains non-neoplastic S1 cells at low passage (<60), S1-180 cells (high passage number S1 cell line that mimics an hyperplasia-like structure in 3D culture), ductal carcinoma *in situ* (preinvasive) S2 cells and invasive ductal carcinoma T4-2 cells, which collectively mimic

breast cancer progression. In addition, MDA-MB-231 cells were also assayed as a control due to its extremely low CDS1 level identified previously in the laboratory.

The qRT-PCR results show that both relative CDS1 (Fig. 3.3.1.A) and CDS2 (Fig. 3.3.1.B) mRNA levels are gradually down-regulated in the HMT-3522 cell series when PUM1 is used as the internal reference gene (Lyng, Laenkholm et al. 2008). CDS1 mRNA is significantly reduced in S1-180 cells and further reduced in S2 and T4-2 cells. CDS2 mRNA is significantly reduced in S2 and T4-2 cells but not in S1-180 compared to S1 cells. These qRT-PCR results reinforce our previous hypothesis that the CDP-DAG synthetases are negatively associated with cancer progression in the HMT-3522 model of triple negative breast cancer.

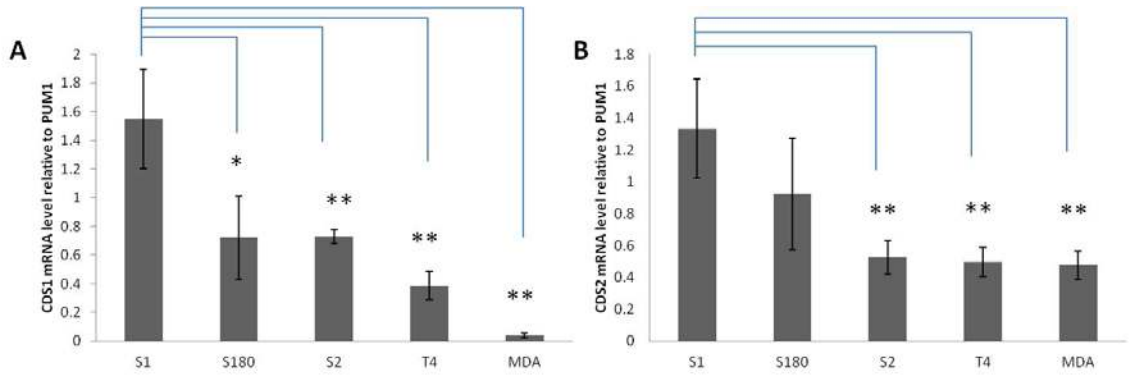

Figure 3.3.1 CDS1 and CDS2 mRNA expressions are down-regulated in cell lines representing different stages of breast cancer progression.

Real-time PCR was performed from tumor nodules formed in 3D culture by the HMT-3522 cancer progression series as well as from MDA-MB-231 cells. S1 represents S1 cells in lower passages (usual passage range: 53 to 60), and S1-180 means S1 cells used in passage numbers between 178 and 187. Cells were cultured embedded in Matrigel^{TM} and collected on day 10, followed by RNA isolation and reverse transcription as described in materials and methods. The mRNA expression was calculated based on the ΔΔCt value, with the results normalized to the average Ct value of the internal control *PUM1* (n=3). T-test analyses were conducted to assess the difference between S1 and any other groups * $p<0.05$; **$p<0.01$.

3.3.2. Re-expressing *CDS1* in MDA-MB-231 cells does not affect cell proliferation, but it partially rescues basal polarity when combined with the inhibition of the Pi3K pathway.

Two of the most important features of cancer cell behavior are uncontrolled proliferation and the acquisition of invasive potential. After confirming that the decrease of CDS1 coincides with cancer progression, I wanted to know whether CDS1 influenced cell proliferation and invasion. Cancer cell invasion is illustrated by cells breaking through the basement membrane. This is accompanied with loss of basal polarity as shown by breaks in the staining or a diffuse cytoplasmic staining of the basal polarity marker beta 4 integrin. I used MDA-MB-231 cells stably transfected with CDS1 cDNA or empty vector available in the laboratory. MDA-MB-231 cell line had been chosen for *CDS1* transfection because it displayed the lowest endogenous CDS1 level among several breast cancer cell lines tested previously (Cardenas JM, Ph.D. Thesis, 2012). Dual-staining for cell cycle marker Ki67 and beta4 integrin was performed in CDS1 re-expressing and control vector tumor nodules produced upon 10 days of 3D cell culture. In addition, the same dual-staining was performed on CDS1 re-expressing and control vector cells that were subjected to several treatments targeting potential signaling pathways that might synergize with CDS1 to tame highly aggressive MDA-MB-213 cells. Indeed it has been shown previously that these cells have to be targeted from different angles to readily revert their aggressive phenotype (Wang, Weaver et al. 1998). As discussed in the introduction, CDS1 pathway mediates the transition from PAs into PIs. First, CDS1 reduction might cause PAs accumulation and PAs are also the signaling

molecules to activate mTOR (Foster 2009), which is critical for cell survival. CDS1 re-expressing and control vector cells were incubated with mTOR inhibitor rapamycin during the cell culture period (10 days). Second, because CDS1 locates upstream of the Pi3K-AKT pathway, CDS1 re-expressing and control cells treated with Pi3K inhibitor LY294002 were also investigated. At last, we incubated CDS1 re-expressing and control cells with integrin beta1 blocking antibody that inhibits MAPK signaling necessary for cell survival (Wang, Weaver et al. 1998).

ANOVA-Tukey analysis of the percentage of cells expressing Ki67 showed no significant differences among any two treatments suggesting that neither re-expressing CDS1 alone nor other combinations of CDS1 and reagents have apparent effect on forcing MDA-MB-231 cells to quit the cell cycle (Fig. 3.3.2.A). Interestingly, when CDS1 re-expression was combined with LY2940004, the inhibitor of Pi3K, basal polarity is partially (schematic is shown in Figure 3.3.2.D) (Vidi, Chandramouly et al. 2012) restored in a significant number of cells compared to the CDS1-MDA-MB-213 cells treated with vehicle DMSO ($p<0.05$) (Fig. 3.3.2.B). Beta4-integrin staining at the basal cell membrane was notably more continuous in LY treated CDS1 re-expressing cell nodules; many of the cell nodules still could not regain full basal polarity defined as a complete circle of basal marker around the nodule. Given that LY alone or CDS1 re-expression alone has no significant effect on the rescue of basal polarity, this result indicates that it is the combination of CDS1 and LY that partially restores basal polarity. Increased *CDS1* mRNA level in CDS1 re-expressing cells compared to control cells was confirmed by RT-PCR as shown in Figure 3.3.2.C.

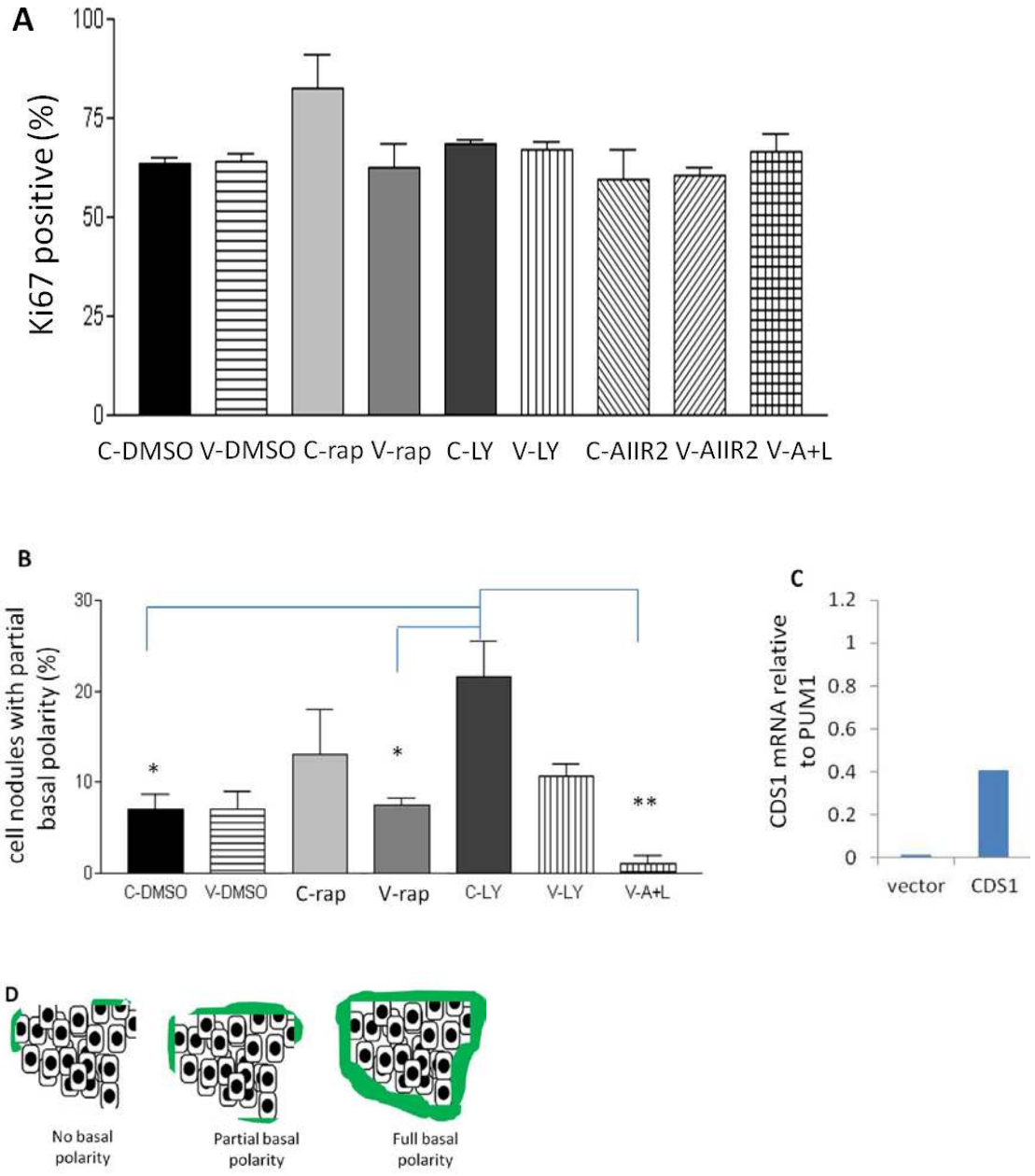

Figure 3.3.2. Assessment of cell cycle activity and basal polarity in MDA-MB-231 cells transfected with CDS1 or vector control. CDS1 re-expressing cells or cells with control vector were plated in Matrigel™ using the embedded method and treated with rapamycin, LY, AIIB2, and the combination of AIIB2 and LY for 10 days, followed by

staining for Ki67 to assess cell cycle status (A) or beta4-integrin to assess basal polarity (B). One-way ANOVA-Tukey was applied to identify the potential significance for a difference between any two treatments. The transcription level of CDS1 in CDS1 re-expressing cells and control vector cells are shown in (C). C-DMSO: CDS1-DMSO; V-DMSO: vector-DMSO; C-rap: CDS1-rapamycin; V-rap: vector-rapamycin; C-LY: CDS1-LY; V-LY: vector-LY; C-AIIB2: CDS1-AIIB2; V-AIIB2: vector-AIIB2; V-A+L: vector-AIIB2+LY (n=3).

3.3.3. CDS1 re-expression combined with LY treatment in MDA-MB-231 cells reduces the size and the circularity of the tumor nodules.

To investigate the role of CDS1 in the invasive phenotype, the percentage of tumor nodules with protrusion were assessed. Also, the size and circularity of tumor nodules formed in 3D embedded culture were measured in MDA-MB-231 cells stably re-expressing CDS1 and compared to cells with vector control. Because all the measurements were carried out on the images, the area size of tumor nodules was represented by the pixels for convenience. An arbitrary length was setup as a standard for determination of invasive protrusion and only those protrusions longer than the standard were counted as true invasive protrusions. The percentage of tumor nodules with invasive protrusion was obtained by comparing the number of tumor nodules containing protrusion(s) to the total number of tumor nodules counted.

The results show that there is no significant difference regarding the percent of nodules with invasive protrusions between LY treated and vehicle DMSO treated transfected cells as shown in Figure 3.3.3.A., suggesting that the treatments do not prevent MDA-MB-231 to grow invasive protrusions. The overall percentage of the cellular nodules containing one protrusion is about 4% in CDS1 cDNA-transfected cells and 6% in vector control cells. But the percentage of nodules containing more than one protrusions is higher in CDS1 re-expressing cells compared to control. Although not statistical significance, this trend is repeatedly observed in three independent replicated experiments and the comparison of the average percentage of three replicates between CDS1 re-expressing cells and control cells is 17% vs. 12% (data not shown). The

percentage in A significant decrease of nodule size is observed on day10 CDS1 re-expressing cells compared to vector control under LY treatment, indicating that higher level of CDS1 expression is negatively correlated with nodule size. The circularity of the nodules is significantly decreased when LY is applied compared to control vehicle. This suggests that a decrease of Pi3K signaling combined with CDS1 expression might impact cell invasion by promoting more cell protrusions at the periphery of the tumor nodules.

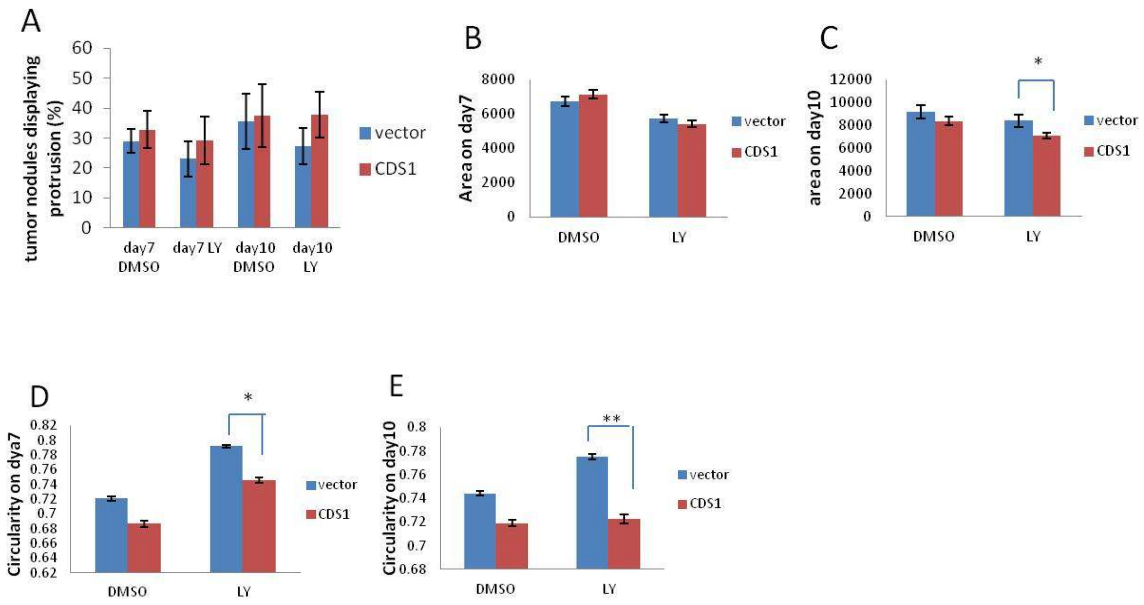

Figure 3.3.3 Assessment of invasion capabilities of the MDA-MB-231 cells over-expressing CDS1. CDS1-MDA-MB-231 and vector control cells were cultured in 3D using the embedded method for 10 days with or without Pi3K inhibitor LY294002. Tumor nodules were imaged on day 7 and day 10, respectively, to measure protrusions (A), size of the nodules on day 7 and day 10 (B and C), and the circularity of the nodules on day 7 and day 10 (D and E). Over 100 cell nodules were measured for each condition (n = 3). The unit of area is represented by pixels. Circularity indicates the roundness of the object. The largest value 1 means perfect circle. * $p<0.05$; ** $p<0.01$

3.4. Discussion

The CDS pathway is so far the only mechanism identified that generates phosphatidylinositols from phosphatidic acid. Because this pathway involves multiple phosphalipid signal molecules, especially phosphatidylinositides, that are pivotal for cell proliferation, growth, survival, cell-cell adhesion, molecules trafficking, and cell skeleton arrangement (Toker and Cantley 1997), deregulation of CDP-DAG synthetase expression is likely to affect cell activities profoundly. At present, CDS2 is known to control angiogenesis during development in zebrafish (Pan, Pham et al. 2012), but the potential similar function of its homologs in higher vertebrates still needs to be confirmed and other functions might also be discovered.

Previous work in our laboratory identified CDS1 to be down-regulated upon the disruption of apical polarity in non-neoplastic S1 cells and its expression further reduced in invasive T4-2 cells (Cardenas JM, Ph.D. Thesis, 2012). To rule out the possibility that CDS2 might compensate for the loss of CDS1 function, I investigated *CDS1* and *CDS2* mRNA levels in HMT-3522 breast cancer progression cell series. My results show that CDS mRNA levels are decreased during cancer progression, which is consistent with the previous finding in our laboratory that CDS1 expression decrease is associated with estrogen receptor negative (ER-) invasive breast cancer in archival clinical samples (Cardenas JM, Ph.D. Thesis, 2012). Especially, to give further details on previously obtained results, CDS1 expression was characterized in normal tissue and breast cancer with a semi-quantitative immunohistochemistry technique by Cardenas (Cardenas JM, Ph.D. Thesis, 2012). The results revealed a decrease of CDS1 in some cancer tissues and

statistical analysis identified a significant association between low CDS1 level and estrogen receptor negative breast cancer. CDS1 mRNA level and protein level in different cancer cell lines were also assessed with qPCR and western blot, respectively. In HMT-3522 series mimicking triple negative breast cancer progression, the decrease of CDS1 mRNA level was concomitant with cancer progression. CDS1 was decreased mostly in a ER negative breast cancer cell line MDA-MB-231, while it was not significantly changed in MCF7 and BT20 cancer cell lines. Western blot analysis of CDS1 revealed an interesting pattern of doublet bands in specific cell lines. Non-neoplastic S1 cells showed a strong expression of lower band while their invasive counterpart T4-2 cells displayed a strong expression of the upper band of the doublet. While MCF7 cell line only showed the lower band of CDS1, the highly invasive cell line MDA-MB-231 only showed higher band. Overall, it seems that the expression of CDS1 in different cell lines is mostly associated with the degree of invasiveness, but more evidence should be obtained to support this hypothesis. These preliminary results logically led us to investigate the role of CDS1 in cell proliferation control and invasion. Over expressing CDS1 in ER(-) invasive breast cancer cell line MDA-MB-231 failed to induce actively dividing cells to exit the cell cycle as indicated by Ki67 immunofluorescence staining, suggesting that CDS1 does not influence proliferation in these breast cancer cells. Ki67 staining in CDS1-re-expressing cells subjected to other potentially phenotype-reverting treatments like the inhibition of mTOR or Pi3K pathways did not reveal a difference either when compared to the corresponding controls. This observation suggests that re-expression of CDS1 is not able to prevent cell from proliferating. In Drosophila *cds* mutant, the cell number in salivary gland is not changed

although a decrease in cell size has occurred (Liu, Wang et al. 2014), which seems in agreement with my result regarding cell cycle activity but it will be important to also assess a possible change in cell size in the MDA-MB-213 cells. However, data from *Drosophila* also indicated that CDS function is cell-type dependent, thus we cannot rule out the possibility that CDS1 affects the cell cycle in other types of human cancer cells. In my present study, Ki67 staining only indicates whether the cells are still in the cell cycle, but without analyzing the distribution pattern for Ki67 or analyze the expression of cyclins, I cannot determine if a specific phase of the cell cycle is extended. I have observed a decrease of total cell number upon the combined treatments with the Pi3K inhibitor and beta1 integrin blocking antibody regardless the re-expression of CDS1 compared to other treatments, but the percentage of cells positive for Ki67 still remains high. This situation could be due to the slow proliferation of these cells without cell cycle exit. Flow cytometry-based cell cycle analysis should provide more information about CDS1's potential role in cell cycle control.

Disruption of the integrity of the basement membrane is the hallmark of cancer progression to the invasive stage. This is usually associated with the redistribution of basal polarity markers like beta4 integrin. It is important to find out if CDS1 re-expression alone or rather combined with other drug treatments that are supposed to tame breast cancer cells can restore polarity. If so, CDS1 might function in preventing invasion. It should be noted that none of the treatments could fully restore the basal polarity in the MDA-MB-213 tumor nodules, but some nodules did display a discontinuous staining of integrin beta4 at the edge, which was defined in a previous

study as partial basal polarity (Vidi, Chandramouly et al. 2012). By re-expressing *CDS1* or inhibiting the Pi3K pathway alone, no significant difference regarding basal polarity was found compared to control cells. But when *CDS1* re-expression was combined with Pi3K pathway inhibition, an improvement of partial basal polarity was observed. This might lead to two possibilities including that both Pi3K activation and lack of CDS1 contribute to cancer invasion via different pathways, or that their effects are additive. We will need to assess the potential changes of PIs level or Pi3K activity in MDA-MB-231 cells after *CDS1* re-expression in order to pin down the signaling pathways influenced by *CDS1* with regards to polarity. The activation of the ERK pathway induces the expression of matrix metalloproteinase-9 (Chung, Lee et al. 2004), which, in turn, degrades the basement membrane components and thus, promotes invasion. In human endothelial HUVEC cells with silenced *CDS1* or *CDS2*, phosphorylated ERK (pERK) is abolished and accordingly, the invasion is alleviated (Pan, Pham et al. 2012). However, in our case, pERK level is decreased in CDS1 re-expressing cells compared to control vector cells (data not shown), indicating that breast epithelial cells might respond differently to changes in CDS1 expression level compared to endothelial cells. The decrease of pERK in CDS1-MDA-MB-231 cells might explain the recovery of basal polarity if MMP activity is changed by such a decrease. Overall, PI and PA levels should be quantified in CDS1 re-expressing cells to narrow down the altered signaling pathways so that we can better understand the contributions from the potential signaling pathways to cancer invasion.

Several invasiveness-related characteristics including protrusion, area, and circularity of the cell nodules were assayed under the combined *CDS1* re-expression and Pi3K inhibition in order to determine the potential preventive effects of the treatment. Our results showed no difference regarding the percentage of nodules with protrusions between treatments and control, suggesting that the observed partial restoration of basal polarity is not able to prevent cells from penetrating into the extracellular matrix. A significant decrease in nodule circularity was found in CDS1 re-expressing cells combined with Pi3K inhibition compared to vector control cells treated with Pi3K inhibition, suggesting that CDS1 promotes the shape of tumor nodules to be more irregular. With the same overall percentages of nodules containing invasive protrusions between treatment and control, a more detailed examination of the number of the protrusions on each nodule revealed that CDS1 re-expressing cells made more nodules with multiple protrusions, while vector control cells made more nodules with a single protrusion. This observation might explain the difference of circularity. In addition, my data showed that CDS1 re-expressing nodules are smaller in size compared to control at day 10 of culture. Several factors could influence nodule size in this study. First, CDS1 expression level was reported to directly determine cell size in *Drosophlia* (Liu, Wang et al. 2014). Second, the existence of basal polarity affects nodule size by protecting the cells from treatment with cytotoxic drugs and promoting cell proliferation, which eventually increases the size of cellular nodules (Weaver, Lelievre et al. 2002). Alternatively, basal polarity generates mechanical force at the periphery of the tumor nodule to maintain multiple cells into a tight spheroid structure. At last, cell death is also a factor contributing to the nodule size. Thus, in future experiments we need to verify that

the smaller size is not due to increased cell death or decreased cell size (Liu, Wang et al. 2014).

Collectively, we found that CDS1 re-expression when the Pi3K pathway is inhibited led to more invasive growth (less circularity) but smaller size for MDA-MB-231 cell nodules. But the mechanisms underlying these phenotypes need further investigation. A logical explanation based on the CDS signaling pathway should be that re-expression of CDS1 increases PIs level, which in turn elevates Pi3K-AKT pathway. It is known that the disruption of apical polarity (Bakin, Tomlinson et al. 2000) as well as the invasive growth (less circularity) are both the consequences of the increased Pi3K activity. Although Pi3K inhibitor was applied to the cells, its effect could be compromised by the re-expression of CDS1 and that could be the reason for the treatments still displaying the difference between CDS1 re-expressing and vector control cells. The correlation of ER- breast cancer at invasive stage and the down-regulation of CDS1 seems to indicate that CDS1 upstream molecules (PAs)-mediated pathways play a more important role at the invasive stage in this subtype breast cancer, possibly involved in the activation of MMPs to further disrupt basal polarity. Thus, the CDS protein could play a dual role in basoapical polarity control. Its expression level might have to increase to disrupt the apical polarity through producing more PIs, but as soon as the apical polarity is impaired, its level quickly decreases to accumulate more PAs to facilitate invasion progression. To this respect, Pi3K-AKT activity and PA mediated pathways corresponding to the change of CDS1 expression should be the targets for the future investigation.

In the present study, I have focused on the role of CDS1 in invasion-related basal polarity. But how CDS1 is correlated with apical polarity still remains unsolved. We have initiated a study to look for genes co-expressing with *CDS1* in Oncomine database (https://www.oncomine.org/). By setting-up the correlation coefficient at greater than 0.7 (a moderate to strong correlation), I cross-checked the selected genes from different datasets. Very interestingly, when the selected genes were arranged by appearing frequency, some of the top genes are tight junction and adherens junction proteins, such as EPCAM, Claudin 4, E-cadherin, and so on (data not shown). Because co-expressing genes are usually considered functionally related, this result supports our hypothesis that CDS1 is linked to apical polarity. Investigating these genes expression profile in CDS1 re-expressing or silenced non-neoplastic cells could bring more information about how CDS1 might regulate apical polarity.

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CHAPTER 4: FINAL DISCUSSION

Cells are the smallest units in the body to conduct basic biological functions such as differentiation, growth, proliferation, aging, and death. All these aspects contain risk to initiate cancer if any function cannot be executed appropriately. Therefore, to understand the cells is a fundamental prerequisite for cancer prevention. Lipids, proteins, and nucleic acids are the essential materials that compose a cell. These cellular entities further assemble into functional complexes at different levels with distinct structures. The arrangement of these functional complexes in a spatial and temporal manner forms the cell organization. Since the cell was first described by Robert Hooke in 1665, every discovery on cell organization has broadened our knowledge of the cell to a new level. This can be best exemplified by the understanding of the double-helix structure of DNA in 1953 that opened the epoch of modern molecular genetics. Indeed, the appropriate cell organization is the structural basis to guide cellular homeostasis. In order to understand the influence of mammary epithelial cell organization on breast cancer initiation and progression, my current research is focused on two proteins, NuMA and CDS1, which are respectively related to cell nucleus organization and basoapical polarity.

Our laboratory previously reported that NuMA organizes higher order chromatin (Abad, Lewis et al. 2007) and affects nucleolar transcription (Jayaraman S, Ph.D. 2014). In the present study, I proposed that NuMA regulates nucleolar organization and function

through its interaction with cohesin. My results show that silencing NuMA can change nucleolar organization and that NuMA and cohesin are preferentially enriched in the rDNA IGS region. Preliminary results further indicate a possible role for NuMA in rDNA looping regulation, thereby, the immediate next step is to test the hypothesis that NuMA is involved in rDNA loops using 3C-qPCR technique (Hagege, Klous et al. 2007). Transcription termination factor TTF-I generates a rDNA loop by tethering its two binding sites flanking the rDNA coding region (Nemeth, Guibert et al. 2008). Yet, NuMA and cohesin were not particularly enriched in TTF-I binding sites (Figure 2.3.5 B&C, region pro-1 and H13) in my study, rather, NuMA/cohesin might control the rDNA loops at IGS regions (Figure 2.3.5 B&C, region H27 and H32). If this is the case, NuMA/cohesin might interact with MYC protein, which was demonstrated to bind rDNA IGS (Shiue, Nematollahi-Mahani et al. 2014). The assessment for the potential interaction between NuMA and MYC (or TTF-I) using co-IP should permit the identification of NuMA's binding partner and thus, provides more information about the rDNA looping structure. It is highly possible that the size of rDNA loops is not uniform given the fact that not all rDNA genes in the nucleolus are active at the same time. Different epigenetic statuses on the rDNA chromatin regions might allow the co-existence of several types of loops mediated by distinct proteins. To this end, NuMA/cohesin, MYC, and TTF-I might co-exist for rDNA looping regulation.

The redistribution of Pol I indicated by staining of Pol I large subunit RPA194 was observed in the nucleolus upon NuMA silencing, but it is not clear if this is due to the direct binding of NuMA and RPA194, or mediated via a third party. Surprisingly, I

also observed a subpopulation of, if not all, RPA194 aggregating at the spindle poles and overlapping with NuMA during mitosis (data not shown). According to the existing evidence, transcriptionally inactive Pol I disassembles during the mitosis: some subunit like RPA43 stay with the NORs during mitosis while other subunits like RPA40, RPA194, and RPA16 are dissociated from the chromosomes (Leung, Gerlich et al. 2004; Chen, Dundr et al. 2005), but the destination of RPA194 was not reported, and other dissociated subunits were not shown to appear at spindle poles based on my literature search. Therefore, this observation might be a novel finding and its biological meaning is not clear. Interestingly, RPA194 behaves similarly as cohesin during the mitosis, which is also aggregating at the spindle poles where it interacts with NuMA through Rae 1 as discussed earlier (Gregson, Schmiesing et al. 2001; Wong and Blobel 2008). Is the presence of RPA194 at spindle poles linked to cohesin or NuMA? Is the presence of RPA194 at spindle poles critical for theformation of the poles and mitotic progression? Is this another mechanism by which the nucleolus controls the cell cycle? All these answers are awaited.

The result of the FUrd assay of nascent RNAs in NuMA silenced cells indicated that the absence of NuMA not only reduces rRNA production but also possibly mRNA production, which suggests a global role of NuMA in RNA transcription. This is consistent with the Lelièvre laboratory earlier results showing that NuMA controls chromatin organization in the whole nucleus. The interaction of NuMA and cohesin possibly exists genome-wide and is not confined in the nucleolar region. Several results from the study of the binding partners for estrogen receptor alpha (ERα) as well as ERα

binding sites on the chromatin might shed light on this aspect. First, NuMA was identified as one of activated ERα binding partners (Cheng, Chang et al. 2010; Tarallo, Bamundo et al. 2011). Second, cohesin and ERα co-occupy at regulatory elements (enhancer and promoter) of hundreds of estrogen responsive genes and cohesin binding to these regions is independent of CTCF (Fullwood, Liu et al. 2009; Schmidt, Schwalie et al. 2010). Collectively, evidence suggests that NuMA and cohesin might coordinate the local chromatin conformation of ERα responsive genes upon estrogen stimulation. This hypothesis can be tested by studying the chromatin loop structure at regulatory elements and gene transcription of one ERα regulated gene, like MYC (McEwan, Eccles et al. 2012). These experiments might also help answer the critical question –of how NuMA influences cell quiescence in mammary epithelial cells.

Finally, with the help of 3C derived high-throughput techniques combined with DNA sequencing and big data analysis, the role of NuMA in higher order chromatin organization will be eventually unraveled.

In the light of the established importance of basoapical polarity on cancer initiation and progression, CDS1 was studied, especially for its role in basal polarity and invasion control. The results suggested that re-expression of CDS1 promotes the integrity of basal polarity but does not prevent the invasive growth in highly invasive MDA-MB-231 cells under our experimental conditions. The signaling pathways influenced by CDS1 re-expression were not identified yet as my goal in this study was to mainly assess the impact of CDS1 on polarity-related phenotypes. In future steps, because many phospholipid molecules and signaling pathways are connected to CDS1's

function, it would be wise to first identify the phospholipid molecules with a significant change in expression level upon CDS1 re-expression in MDA-MB-231 cells.

Based on microarray analysis and CDS1 being related to PI3K pathways, apical polarity establishment or maintenance is another feature that CDS1 might influence and that might link it with cancer initiation. In order to assess CDS1's impact on apical polarity reproduced in a 3D cell culture system, I have tried to constitutively silence CDS1 in HMT-3522 S1 cells with shRNA, yet I could not obtain stably transfected cells due to the difficulty of transfecting the non-neoplastic S1 cells in general. More effort will be conducted in the future to generate S1 cells with CDS1 silenced by using other methods, like viral infection.

In summary, I studied the cell architecture with respect to basoapical polarity and nuclear organization through CDS1 and NuMA, respectively. My work improved our understanding of the cell architecture and cell architecture-controlled activities such as proliferation and invasive growth. Especially for the nucleolus, which is essential for ribosome biogenesis, nuclear stress perception, and cell cycle regulation, I have shown that NuMA influences nucleolar architecture and rDNA transcripition, likely in association with altered proliferation. The elevated activity of the nucleolus is necessary to promote cancer growth and thus, the nucleolus is a promising drug target for cancer intervention (Quin, Devlin et al. 2014). My study of NuMA helps understand more mechanisms by which the nucleolar activities are controlled. My study also suggested that CDS1 protects the integrity of the basal polarity and influences the size and invasiveness of tumor nodules in the cancer cells studied. Given that CDS1 potentially

conducts the regulation of both apical polarity and basal polarity, it plays a unique role in cell architecture control. The biological events corresponding to apical polarity and basal polarity are cell proliferation control and survival. Thus, CDS1 could link both functions on its own, which makes it a potential valuable protein for pharmaceutical purpose.

At the whole cell organization level, the two aspects, basoapical polarity and nuclear organization, are deep down interdependent instead of separated, as earlier studies already established the communication between basal polarity and nuclear organization (Lelievre and Bissell 1998; Lelievre, Weaver et al. 1998; Vidi, Chandramouly et al. 2012), as well as the link between apical polarity and nuclear organization (Abad, Lewis et al. 2007; Chandramouly, Abad et al. 2007). Under these different circumstances NuMA seems to play a central role to sense and react to the signals sent by polarity and to be responsible for the arrangement of nuclear organization accordingly. To this sense, NuMA's behavior fits very well with that of a nuclear stress sensor, which just started to emerge from its functions in regulating the nucleolus. The whole picture of the cell organization reflects the complexity but also the beauty of the cell - everything is connected!

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VITA

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Yunfeng Bai is from Changchun, a big but beautiful city in the northeast part of China. Yunfeng enrolled at Northeast Normal University, which is located in his hometown, and received his B.S. degree in Biotechnology in 1998. He worked in Water Conservancy Committee for Songhua River and Liao River of China for two years after graduation and decided to go back to school to obtain a higher level of education. He received his M.S. degree in Genetics in 2007. Yunfeng entered the Ph.D. program in the Department of Basic Medical Sciences at Purdue University in 2009. He studied the molecular and cellular mechanisms responsible for the alterations of mammary epithelial cell architecture that are important for breast cancer initiation and prevention, in Dr. Sophie Lelièvre's laboratory. He earned his Ph.D. in May of 2015.