EMISSION OF GENOMIC DNA BY EXTRACELLULAR VESICLES

By

Thupten Tsering
Department of Biochemistry, McGill University, Montreal, QC

"A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science"

EMISSION OF GENOMIC DNA BY EXTRACELLULAR VESICLES

Thupten Tsering
Master of Science
Department of Biochemistry, McGill University, Montreal, QC
2018

Abstract

Extracellular vesicles (EVs) are oval membrane-bound structures that play important roles in intercellular communication by transporting their contents between cells over short and long distances. EVs have been shown to carry highly diverse molecular cargo including oncoproteins, transcripts and non-coding RNAs, as well as oncogenic genomic sequences with mutated DNA (gDNA). Indeed, the genesis and roles of EV-associated gDNA in cellular communication and liquid biopsy remain presently unexplained. This thesis characterizes various factors that influence the release of gDNA from cancer cells bearing specific oncogenic mutations. Our results show that H-*RAS*-dependent oncogenic insult results in spontaneous formation of micronuclei enriched in chromosomes 1 and 2 in IEC-18 epithelial cells that undergo malignant transformation (RAS3). However, emission of gDNA through expulsion of micronuclei or related EVs is relatively rare. Instead, our data suggest that the bulk of gDNA emission from viable RAS3 cells occurs through generation of exosome-like EVs likely originating from ample cytoplasmic chromatin associated with cellular transformation. EVs released from RAS3 cells harbour gDNA, both inside and on the external surface and this content is independent of cell cycle and apoptosis. We suggest that autophagy may regulate gDNA entry into the EV biogenesis pathway by degradation of nuclear membrane. Thus, we characterized a process whereby oncogenic H-*RAS* drives gDNA emission through coupling of chromatin deregulation and EV biogenesis. We postulate that this linkage may lead to a better use of EV-associated gDNA as an element of liquid biopsy platform in cancer.

RÉSUMÉ

Les vésicules extracellulaires (VE) sont des structures ovales attachées à la membrane qui jouent un rôle important dans la communication intercellulaire en transportant leur contenu entre cellules sur de courtes et longues distances. Il a été démontré que les VE transportaient des cargaisons moléculaires très diverses, comme des oncoprotéines, des transcrits et des ARN non codants, ainsi que des séquences génomiques oncogènes composées d'ADN muté (ADNg). La genèse et les rôles des ADNg associés à les VE dans la communication cellulaire et la biopsie liquide restent encore actuellement inexpliqués. Cette thèse décrit les différents facteurs influençant la libération de l'ADNg par les cellules cancéreuses porteuses de mutations oncogènes spécifiques. Nos résultats montrent que l'agression oncogène dépendante de H-*RAS* entraîne la formation spontanée de micronoyaux enrichis en chromosomes 1 et 2 dans des cellules épithéliales IEC-18 qui subissent une transformation maligne (RAS3). Cependant, l'émission d'ADNg par expulsion de micronoyaux ou de vésicules extracellulaires apparentées est relativement rare. Nos résultats suggèrent plutôt que la majorité des ADNg relâchés par les cellules RAS3 sont générés par des exosomes ressemblant aux EV provenant probablement de larges portions de chromatine cytoplasmique liée à la transformation cellulaire. Les VE libérées par les cellules RAS3 contiennent de l'ADNg, à la fois à l'intérieur mais aussi sur la surface extérieure des vésicules. Ce contenu est indépendant du cycle cellulaire et de l'apoptose. Nous pensons que l'autophagie pourrait réguler l'entrée de l'ADNg dans la voie de biogenèse des VE par dégradation de la membrane nucléaire. Nous avons donc caractérisé un processus oncogénique au cours duquel l'oncogène H-*RAS* entraine l'émission d'ADNg lié à la dérégulation de la chromatine couplée à la biogenèse d'EV. Nous postulons que ce lien pourrait conduire à une meilleure utilisation des ADNg associés aux VE comme marqueurs associés aux cancers dans les biopsies liquides.

Acknowledgements

First of all, I wish to express my deepest gratitude to my supervisor Dr. Janusz Rak for the opportunity to study and do research in his laboratory. I am indebted to him for his patience, support, knowledge and advice throughout the course of my graduate program. This thesis would not have been possible without his constant encouragement and guidance.

I would like to extend my sincere thanks and appreciation to my committee members Dr. Alain Nepveu and Dr. Loydie Jerome-Majewska for their time, ideas, guidance and feedback during the course of my Master's program.

I have been very lucky to work with wonderful lab members: Brian Meehan, Laura Montermini, Tae Hoon Lee, Shilpa Chennakrishnaiah, Esterina D'Asti, Nadim Tawil, Cristiana Spinelli, Dongsic Choi, Narges Abdian, Saro Aprikian, Catherine Pan and Lata Adnani. I would like to thank them for their support, discussion and feedbacks. I am also grateful to Damien Faury for translating my abstract to French.

I would like to thank the FEMR facility at McGill University, especially Jeannie Mui for her help with the Transmission Electron Microscope. I also owe my thanks to Ming Fu and Shibo Feng from the Molecular Imaging platform at RI MUHC for their excellent technical assistance with the confocal microscope.

Finally, I want to thank my family for their constant love, support and encouragement throughout my academic journey. I also want to take this opportunity to thank my brother Tenzin Gayden for his support and guidance. I would like to dedicate this thesis to my mom Tsering Bhuti for all the sacrifices she has made for me to continue my higher studies.

Contributions

The data presented in the Results section were generated by me unless otherwise indicated. However, several colleagues contributed to this work in various ways, through either helping with assays, by generating preliminary data (some shown) or contributing important ideas.

Shilpa Chennakrishnaiah assisted with the droplet digital PCR assay and also performed the SKY experiment. Saro Aprikian helped with a ddPCR reaction. Laura Montermini assisted with the transfection and filtration experiments. Brian Meehan contributed important ideas and participated in intellectual discussions throughout the project. Jeannie Mui assisted with performing ultra-thin section of cells and extracellular vesicles as well as solving technical issues of immunogold labelling. Shi Bo Feng and Min Fu helped with the set-up of the Zeiss LSM780 laser scanning confocal microscope.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Acidic sphingomyelinase (ASMase)

Acute myeloid leukemia (AML)

Apoptotic bodies (Abs)

ATG (autophagy related genes)

BrdU (5-Bromo-2´-Deoxyuridine)

Cytokinesis-block micronucleus cytome assay (CBMN assay)

Cell-free tumour DNA (CtDNA)

Cerebrospinal fluid (CSF)

Charged multivesicular body proteins (CHMPs)

Chaperone-mediated autophagy (CAM)

Chinese hamster lung CHL

Chromosomal instability (CIN)

Cluster of differentiation 63 (CD63)

Cluster of differentiation 9 (CD9)

Conditioned medium (CM)

Copy number variation (CNV)

Deoxyribonucleic Acid (DNA)

DNA damage response (DDR)

Double minute (DM)

Double-stranded breaks (DSBs)

Droplet digital Polymerase Chain Reaction (ddPCR)

Enzyme-linked immunosorbent assay (ELISA)

Endothelial Growth Factor Receptor (EGFR)

Extracellular vesicles (Evs)

Endosomal Sorting Complex Required for Transportation (ESCRT)

Ethylenediaminetetra acetic acid (EDTA)

Extracellular signal-regulated kinases 1 and 2 (ERK1/2)

Fluorescence-activated cell sorting (FACS)

Fetal Bovine serum (FBS)

Fluorescence In situ Hybridization (FISH)

Genomic Deoxyribonucleic acid (gDNA)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Glioblastoma multiforme (GBM)

Hydroxy urea (HU)

Interleukins (Ils)

Light chain 3 beta (LC 3 B)

Intestinal epithelial cells (IEC)

Intraluminal vesicles (ILVs)

Major histocompatibility complex (MHC)

Mammalian target of rapamycin (mTOR)

Matrix metalloproteinases (MMPs)

Messenger RNA (mRNA)

Mitogen-activated protein kinases (MAPKs)

Micronuclei (MN)

Mitomycin-C (MMC)

Multivesicular bodies (MVBs)

Nanoparticle tracking analysis (NTA)

Non-homologous end joining (NHEJ)

Non-small cell lung carcinoma (NSCLC)

London Right White (LRWhite)

Phosphatase and tensin homolog (PTEN)

Phosphatidylserine (PS)

Radioimmunoprecipitation assay (RIPA)

Ribonucleic acid (RNA)

Reactive oxygen species (ROS),

Small interfering RNA (siRNA)

Spectral karyotyping assay (SKY)

Spindle assembly check point (SAC)

Tetraspanin9 (TSPAN9)

Transmission Electron Microscope (TEM)

Tumour susceptibility gene 101 (TSG101)

CHAPTER 1
General Introduction

Pathways and mediators of intercellular communication

The cell non-cell-autonomous interrelationships between cells govern basic biological processes such as growth, survival differentiation and spatial organization (Wei and Huang, 2013). Mechanisms that control these processes include both intercellular and intracellular pathways that ultimately integrate the behaviours of cellular populations in health as well as disease, including cancer (Bhattacharya et al., 2014). While the emphasis of studies on cancer pathogenesis was traditionally on intracellular signalling pathways, more recently, intercellular communication mechanisms began to receive experimental attention in view of the inability of 'cell-centric' approaches to explain cancer complexity. Among pathways that mediate intercellular crosstalk, the most studied are several modes of mono-molecular messaging involving cell surface receptors and their ligands, such as cytokines and growth factors, as well as adhesion molecules. In addition, more complex structures such as junctions, and membrane bridges including microtubes, cytonemes, tunnelling nanotubes and other membrane formations acting across short or mid-range distances have also recently entered cancer studies (Osswald et al., 2015). Unique among these mechanisms is the exchange of cellular fragments known as extracellular vesicles (Evs) which connect cells across short and long distances as well as systemically (Choi et al., 2017; Kimura et al., 2018). One outstanding question in this regard is how cellular communication pathways can be integrated with the current paradigm of cancer progression under the influence of genetic and epigenetic transformation pathways (Choi et al., 2017).

The link between oncogenic transformation and intercellular communication in cancer

It has been traditionally thought that oncogenic pathways operate within the physical boundaries of cancer (stem) cells affecting largely their intrinsic properties such as growth and survival (Vogelstein and Kinzler, 2004a). This notion is challenged by the mounting evidence that oncogenic transformation may exert multicellular effects, interactive in nature and extrinsic to mutant cancer cells. Such effects include deregulation of angiogenesis (Rak et al., 1995) and formation of tumour stroma (Lisanti et al., 2013) and are attributed to mediators, which remain

poorly defined. In this regard our laboratory has recently shown that cancer cells themselves can emit oncogenic macromolecules in the form of extracellular vesicles (Evs) (Yu et al., 2005; Al-Nedawi et al., 2008; Milsom et al., 2008; Garnier et al., 2012) For example, in the case of epidermal growth factor receptor variant III (EGFRvIII) and mutant H-*RAS* (V12), both oncoproteins and their transcripts exit viable cancer cells in the form of EV-like structures (Al-Nedawi et al., 2008a; Demory Beckler et al., 2013; Lee et al., 2014).

Extracellular vesicles

Evs are oval membrane-bound structures with highly heterogeneous content (Al-Nedawi et al., 2008; Théry et al., 2009; Choi et al., 2017). Evs play important roles in intercellular communication by transporting their contents between cells whereby they alter the properties of both donor and recipient cells (Inamdar et al., 2017). The extracellular shedding of Evs in cancer is regulated and likely dependent on the molecular make-up of cancer cells and cues from the microenvironment (Al-Nedawi et al., 2008). For example, our laboratory discovered that molecular subtypes of human glioblastoma and medulloblastoma differ with respect to the expression patterns of genes known to influence EV biogenesis (vesiculome) (Nakano et al., 2015). These genes are implicated in several pathways of EV formation including membrane blebbing leading to the generation of larger Evs termed "ectosomes" (also known as microvesicles, shed vesicles, or microparticles), or by a more complex, endocytosis-related mechanism resulting in production of smaller Evs known as exosomes (Figure 1) (Théry et al., 2009; Kowal et al., 2016). This initial distinction, while useful, does not reflect the true heterogeneity of Evs which have been recently subdivided into at least 4 categories through the use of proteomic profiling, immunodetection and physical purification methods. According to this protocol certain cancer cells release large Evs, dense small Evs, light small "exosome – like" Evs (many containing CD9 and CD63 tetraspanins), as well as CD81 tetraspanin-enriched exosomes also positive for markers of late endosome and the endosomal sorting complex required for export (ESCRT), such as TSG101 (Kowal et al., 2016). Moreover, the use of asymmetric flow field-flow fractionation (AF4) technology enabled identification of large and small exosomes, as well as non-membranous particles termed exomeres (Zhang et al., 2018).

Indeed, our laboratory predicted that protein profiles of EV preparations from cancer cells may suggest the existence of 10 or more non-overlapping small EV protein profiles (Choi et al.,

2017). In addition, several sets of large Evs have been identified in the secretome of viable cells, including large oncosomes (Los; 1-10 um in diameter), exophers (~4 um), and migrasomes (~1um), while apoptotic bodies (Abs; usually >1um) are Evs related to cellular fragmentation and death (György et al., 2011; Zijlstra and Di Vizio, 2018). It should be noted that the term oncosomes was initially introduced to describe oncogene-containing Evs (Al-Nedawi et al., 2008; Meehan et al., 2016) but it is currently widely misused to describe large Evs emanating from migrating cells (properly called large oncosomes) (Di Vizio et al., 2009). Nonetheless, this partial description adds to the growing understanding of EV diversity and their inferred but poorly understood and complex roles as biological regulators (partially depicted in Figure 1). Each EV type may contain different molecular cargo, including specific proteins, mRNA and microRNA.

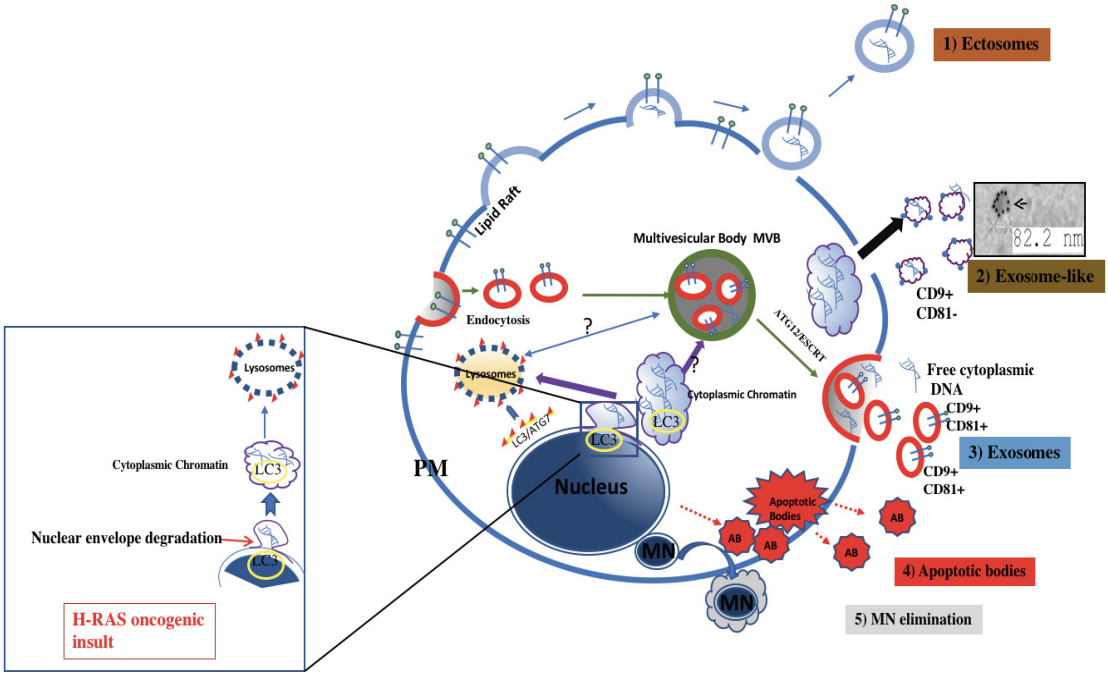


Figure 1.1. The putative role of EV-related cellular export pathways in the emission of extracellular DNA. Several pathways of EV biogenesis have recently been described in the literature including large membrane derived ectosomes and exosomes related to the endocytic pathway. In addition dying cells may give rise to apoptotic bodies or cells may expel micronuclei (MN) (see text) (Zijlstra and Di Vizio, 2018). We postulate that extracellular DNA can be emitted outside a cancer cell by different conventional vesiculation mechanisms (1-4), or as EV-like structures containing micronuclei (MN; 5) Alternatively DNA can exit the cells as free nucleosomes which are thought to contribute to a pool of cell-free cancer DNA (cfDNA) in the

blood of cancer patients. Ectosomes (2) Exosome-like (3) Exosomes (4) Apoptotic bodies (5) micronuclei.

The enigma of extracellular DNA release from cancer cells.

A recent surprising observation is that of the presence of double stranded genomic DNA content in certain EV subsets emitted by viable cancer cells through mechanisms that largely remain obscure. In this regard, our laboratory reported that transformation of non-tumourigenic intestinal epithelial cells (IEC-18) by enforced expression of the mutant V12 H-*RAS* oncogene results in a dramatic tumourigenic conversion of the resulting cells (termed RAS3) (Lee et al., 2014) and profound changes in their vesiculation profile. Notably RAS3 cells manifest increased total EV emission largely in the exosomal size range, inclusion of H-RAS protein, RNA and DNA in the EV cargo and a globally increased emission of genomic double-stranded DNA fragments and histones containing chromatin (Lee et al., 2014, 2016). These properties are of interest for at least two reasons. First, the EV-mediated intercellular transfer of DNA/oncogenic cargo could be biologically consequential due to the implicit self-renewing potential of DNA and the effects of DNA uptake on genetic instability or transformation in recipient cells (currently under study by another student). Secondly, the mechanisms of genomic DNA (gDNA) transit to the EV compartment under the influence of oncogenic transformation remain poorly understood and difficult to conceptualise. In theory, gDNA can exit cancer cells either as cell-free fragments eventually found in circulating plasma (cfDNA), likely related to cellular breakdown, or as one of several classes of Evs, such as ectosomes, exosomes, or exosome-like Evs not related to endosomal transport, or else as programmed cell-death-related apoptotic bodies. In addition, EV-like structures could also mediate the emission of more structured fragments of cellular genome such as micronuclei (MN) (Figure 1.1). In this project, we investigate the contribution of each of these mechanisms to the extracellular DNA pool generated by cancer cells driven by mutant oncogenes.

Oncogenes and EV emission

An oncogene is a dominant mutant of a gene that encodes a protein with a potential to cause cancer (Bafico et al., 2008). Cancer cells are characterized by alterations in oncogenes, tumour suppressor and microRNA genes, either within the genomic sequences or imposed by inheritable epigenetic modification of the cellular chromatin (e.g. DNA and histone methylation) (Fearon

and Vogelstein, 1990; Vogelstein and Kinzler, 2004; Croce, 2008). Tumour suppressor genes are genes whose inactivation leads to cancer progression (Cooper, 2000). While Evs are emitted from normal cells, their production is often elevated in the case of cancer cells, suggesting that changes in key driver genes may affect vesiculation in cancer. Indeed, several studies have shown that mutations in oncogenes and tumour suppressor genes influence vesicular emission in different cancer types (Choi et al., 2017). For example, human colorectal cell lines expressing mutant *K-RAS* gene accompanied by *p53* inactivation exhibit an increased EV-mediated emission of the pro-coagulant receptor called tissue factor (TF) relative to their isogenic counterparts lacking the respective driver mutations (Yu et al., 2005). In addition, defects in exosome biogenesis were reported when p53 target genes such as TSAP6 (tumour suppressor-activated pathway 6) are genetically disrupted (Lespagnol et al., 2008). Similarly, GBM cells carrying oncogenic EGFRvIII showed increased emission of vesicles in terms of both quantity of Evs and their global protein content, as well as repertoire (Al-Nedawi et al., 2008; Garnier et al., 2013). A similar observation was reported in the case of metastatic melanoma harbouring MET proto-oncogene (Peinado et al., 2012). Epigenetic changes in cancer cells driven by oncogenic epidermal growth factor receptor (EGFR) and associated with epithelial to mesenchymal transition (EMT) also changed the output and proteome of cancer-related Evs, including enrichment in procoagulant TF (Garnier et al., 2012, 2013). In the same model system (A431) export of EGFR and gDNA by Evs was altered by pharmacological inhibition of EGFR activity (Montermini et al., 2015). Furthermore, targeting oncogenic fusion product involving promyelocytic leukemia – retinoic acid receptor (PML-RARA) protein in NB_4 cells (acute promyelocytic leukemia cells) using all-trans retinoic acid (ATRA) resulted in changes in the EV emission profile, increases in IL-8 mRNA and protein expression by NB4 cells and their Evs (Fang et al., 2016).

Notably, our laboratory initially reported that cancer cells emit oncogenic proteins themselves as cargo of Evs, which are capable of horizontal transfer or the related signalling and regulatory activity (Al-Nedawi et al., 2008; Lee et al., 2016) This property was described for oncogenic EGFRvIII in glioblastoma (GBM) (Al-Nedawi et al., 2008), amplified EGFR in epidermal cancer cells (Al-Nedawi et al., 2009), and H-*RAS* in the case of transformed intestinal epithelial cells (Lee et al., 2014, 2016). Our group has subsequently confirmed the presence of EGFRvIII

containing Evs in blood of mice harbouring EGFRvIII-driven glioma xenografts and in GBM patients (Montermini et al., 2015). Skog and colleagues (2008) also found oncogenic EGFRvIII transcripts in Evs released by GBM cell lines as well as in biofluids (plasma) of GBM patients (Skog et al., 2008), as did Graner and colleagues (Graner et al., 2009) and similar material was recently recovered from the cerebrospinal fluid of GBM patients (Figueroa et al., 2017).

Recently, our group has demonstrated an increased emission of Evs in RAS-3 cells that are transfected with oncogenic human H-*RAS* gene compared to the non-transformed isogenic parental line IEC-18 (Lee et al JEV 2013 – abstract) (Lee et al., 2014). Notably, these RAS-3 derived Evs harbour double-stranded gDNA containing full-length sequences of the mutant H-*RAS* gene. This finding was later confirmed and duplicated by several studies and multiple (but not all) cancer contexts (Kahlert et al., 2014; Thakur et al., 2014) (Cai et al., 2014; Lazaro-Ibanez et al., 2014; Kanada et al., 2015). Thus, oncogenic pathways must intersect at some level with the cellular machinery responsible for EV biogenesis and this includes abnormal mobilization of chromatin to the process of packaging the EV cargo (Lee et al., 2016; Choi et al., 2017).

RAS signalling

RAS epitomizes processes of cellular transformation and cancer progression (Barbacid, 1990). This potent activity is, at least in part, attributable to the unique role of normal RAS in transmission of intracellular signals and responses to extracellular microenvironment. RAS proteins are members of a large family of small GTPases involved in cell division, migration, proliferation, metastasis, apoptosis and senescence. There are three *RAS* genes in the human genome, namely H-*RAS*, K-*RAS* and N-*RAS* (Downward, 2003), which give rise to four protein products: HRAS, KRAS-A, KRAS-B, NRAS (Barbacid, 1990). Although the biochemical effects of RAS proteins are often regarded as similar, gene targeting studies in mice revealed profound differences, in that only disruption of K-*RAS* expression leads to lethality. RAS proteins interact with a large number of intracellular effectors including RAF, PI3K, RAL-GDS, TIAM1, AF6, PKCz, RIN1 (Rajalingam et al., 2007), of which the best understood are: the effects of mitogen activated protein kinase (MAPK) and phosphoinoside-3-kinase (PI3K) pathways (Nussinov et al., 2014) (Figure 1.2). Since RAS proteins are located on the inner leaflet of the plasma membrane, their signalling is coupled with upstream activation of cell surface

receptors such as EGFR (Zenonos, 2013), and downstream events leading to changes in cellular behaviour, phenotype and gene expression, including genes that mediate intercellular communication, inflammation and angiogenesis (Rajalingam et al., 2007; Rak et al., 2009).

RAS oscillates between guanosine diphosphate (GDP) and guanosine triphosphate (GTP) states, the latter of which signifies activation and binding to aforementioned effectors. Interaction between EGFR and RAS represents a historical and mechanistic paradigm for the role of RAS in cellular regulation and is worth detailing as it includes several proteins with potentially oncogenic activity. Thus, ligand binding to EGFR causes receptor dimerization and autophosphorylation, which creates a number of phosphotyrosine docking sites for adapter proteins containing src homology 2 (SH2) domains. In this regard of crucial importance is recruitment of Shc and Grb2 adaptors, which interacts with Son of Sevenless (SOS) endowed with guanosine exchange factor (GEF) activity for RAS. This activity enables recruitment of GTP to RAS, resulting in its activated state. Such activated (GTP bound) RAS interacts with its aforementioned direct effectors resulting in the onset of several downstream signalling cascades. One such crucial event involves recruitment of kinases of the RAF family to the plasma membrane, which, with the help of scaffolding proteins (KSR1), enables formation of protein complexes and subsequent phosphorylation and activation of MEK, a kinase for mitogen activated kinases (MAPKs) such as ERK1 and 2. These proteins are responsible for transmitting signals for cell proliferation through interaction with transcription factors (Rajalingam et al., 2007; Zenonos, 2013).

Under normal circumstances the weak GTP-ase activity of RAS is accelerated by binding RAS-GAP (GTP-ase activating protein) resulting in hydrolysis of GTP and conversion of RAS to an inactive GDP-bound state (Fernández-Medarde and Santos, 2011). Numerous negative feedback mechanisms regulate the reversal of RAS activation to prevent chronic activation of this potent regulator, or that of its interactors. Thus, MAPKs may phosphorylate and inhibit SOS, while activating Sprouty protein which accelerates recycling and inactivation of EGFR (Lake et al., 2016). However, in cancer cells, mutations in codons (G12, G13 and Q61) of the *RAS* gene lead to changes in protein conformation and its resistance to RAS-GAP activity resulting in RAS

being locked in a persistent GTP-bound and activated state. The profound transforming effect of such events are signified by preponderance of *RAS* mutations in human cancer (Barbacid, 1990).

Mutations in *RAS* oncogenes account for approximately 33% of all human cancers (Woo and Poon, 2004; Karnoub and Weinberg, 2008; Fernández-Medarde and Santos, 2011; Zenonos, 2013; Choi et al., 2017). Alterations in K-*RAS* (21.6%) are more frequent compared to N-*RAS* (8%) and H-*RAS* (3.3%) (Baines et al., 2011). The most common mutational hotspots are at codons G12, G13 and Q61 which occur in evolutionarily highly conserved sequences (Fernández-Medarde and Santos, 2011; Rajasekharan and Raman, 2013; Nabet et al., 2015). As mentioned earlier these gain-of-function mutations impair GTP hydrolysis and constitutively activate Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/mTOR pathways (Figure 1.2), promoting tumour development (Fernández-Medarde and Santos, 2011). It is interesting to note that these *RAS* gene isoforms exhibit some degree of association with specific cancer types. For example, K-*RAS* mutations have been reported in pancreatic (70%–90%), colon (35%–50%) and lung cancers (20%–35%) (Hunter et al., 2015), whereas N-*RAS* mutations predominate in cutaneous melanoma (Johnson and Puzanov, 2015). In contrast, H-*RAS* mutations are frequently found in head and neck squamous cell carcinoma (Hobbs et al., 2016). Defective RAS signalling has also been reported in other human illnesses such as diabetes, inflammatory and immunological disorders (Fernández-Medarde and Santos, 2011). Although drugs that directly target K-*RAS* mutations remain a long-sought aspiration, current therapies are directed against downstream effector kinases such as Raf, MEK and PI3K. Another approach is to inhibit prenyl transferases involved in post-translation modification of RAS proteins and their ability to interact with plasma membrane through the C-terminal CAXX box. In this regard protein farnesyltransferase inhibitors have emerged as plausible anticancer drugs, but they have yet to be approved for clinical use (Alsina et al., 2004; Fernández-Medarde and Santos, 2011; Rajasekharan and Raman, 2013). Thus, while RAS activity is central to malignant transformation, drugability remains a challenge necessitating further study of the biological processes involved.

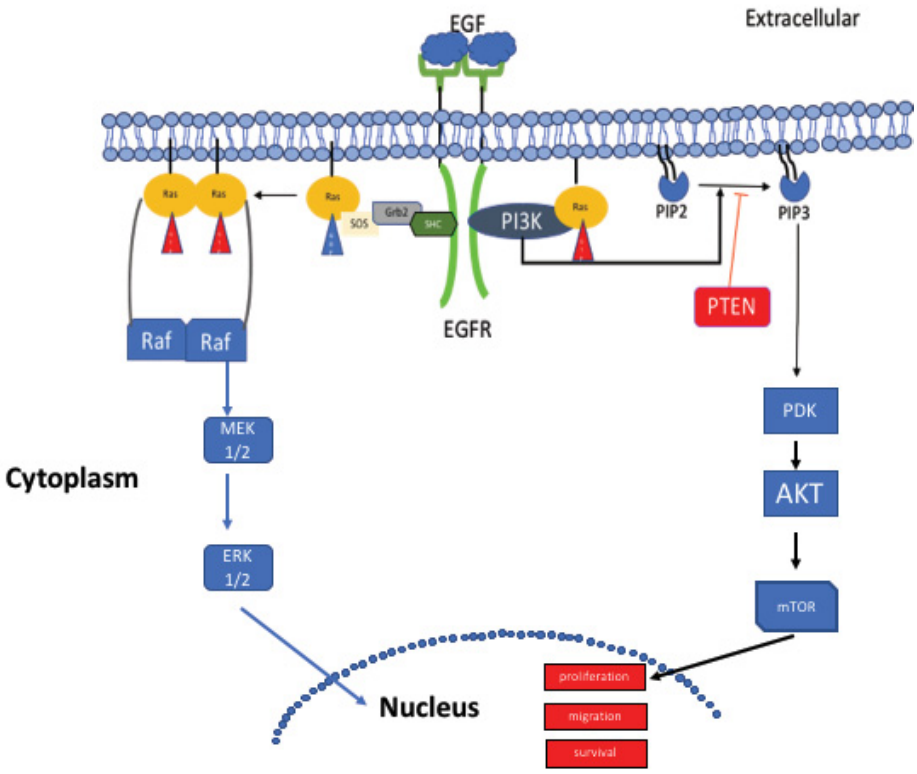


Figure 1.2. RAS downstream signalling pathways. EGF binds to EGFR receptors and activates Raf-MEK-Erk and PI3K-AKT pathways. These pathways play a vital role in cell growth, survival, angiogenesis and regulation of other intercellular interactions. Figure adapted from (Nussinov et al., 2014).

The role of oncogenes in chromatin processing and extracellular emission of DNA by cancer cells

As mentioned earlier certain (but not all) cancer cells spontaneously emit genomic DNA as cargo of their derived Evs (Lee et al., 2016). How genomic DNA could exit the nuclei of cancer cells and enter vesiculation pathways is far from clear. One explanation could be that a low-level apoptotic demise of a small fraction of an otherwise viable cancer cell population may contribute a steady influx of apoptotic bodies to the conditioned media which leads to 'contamination' of exosomal preparations with DNA-containing material. It is also possible that mitotic errors or gene amplification in cancer cells result in formation of large chromosomal or chromatin fragments that may trigger the generation of micronuclei, which are then expelled from cells as EV-like structures by an unknown mechanism. Previous studies have also shown an interplay between autophagy pathways and exosome release (Schmukler et al., 2013; Baixauli et al., 2014;

Papandreou and Tavernarakis, 2017; Zadeh et al., 2017). Autophagy maintains intracellular homeostasis by degrading and recycling cellular constituents. While autophagy occurs constitutively at a basal rate, it can also be induced by stress conditions such as nutrient deprivation and oncogenic activation of the RAS pathway. The autophagy proteins LC3 and ATG8 were found to be present in the nucleus and interact with the nuclear membrane (or envelope) degrading it upon oncogenic *RAS* activation (Dou et al., 2015). This leads to the formation of a cytoplasmic chromatin pool thought to undergo lysosomal degradation. It is known that in the case of overwhelming the lysosomal capacity with protein or other cargo, regulatory mechanisms may redirect vesicular transport to the exosomal release pathway leading to efficient expulsion of this material from cells (Pan and Johnstone, 1983). Whether such a mode of elimination also applies to extranuclear chromatin has not been studied (and is explored in this thesis). We have shown that H-*RAS*-driven cancer cells (RAS3) exhibit multiple nuclear aberrations and higher cytoplasmic chromatin content compared to isogenic control normal (IEC-18) cells. RAS3 cells also express higher levels of LC3 and emit genomic DNA as EV cargo. While the crosstalk between RAS signaling and autophagy is complex, we will discuss the operational link between LC3, RAS and EV-mediated extracellular genomic DNA (gDNA; Figure 1.1).

Putative mechanisms of EV-mediated DNA exit from cancer cells

Extracellular cancer-related mutant DNA found in the biofluids of cancer patients is emerging as a key source of diagnostic information among biomarker platforms often referred to as 'liquid biopsy' (Siravegna et al., 2017). While having access to actionable mutations without invasive sampling of the tumour mass is of great value, the processes that enable the entry of circulating tumour DNA (ctDNA), or cell free DNA (cfDNA) into extracellular fluid spaces is often taken for granted and poorly understood (Lee et al., 2016). In theory, there may be several sources such as material including cellular breakdown and necrosis, apoptotic body release from dying cancer cells and an active release of chromatin or nucleosomes by viable cancer cells through poorly understood mechanisms, including formation of DNA-containing Evs (Lee et al., 2016). The notion of EV-mediated extracellular DNA exit is predicated on the observation in cellular cancer models that this material is found largely or exclusively in a fraction of conditioned media that is sedimentable by ultracentrifugation at approximately 100,000 x g force (Lee et al., 2014). Since

the respective pellets contain different membrane-bound, EV-like structures, including those resembling ectosomes or exosomes, it was suggested that there is a link between manners of nuclear DNA processing and one or more pathways of cellular vesiculation. These may include surface budding of viable cells to form DNA-containing ectosomes or smaller exosome-like structures. It is also conceivable that the entry of DNA into the endosomal trafficking pathway that generates *bona fide* exosomes may occur in some instances (Figure 1.1). It is of note that such processes are largely restricted to cancer cells harbouring oncogenic mutations, such as those affecting *RAS*, which are among the best studied (Lee et al., 2014, 2016). Indeed, *RAS* affects all aspects of chromatin homeostasis that may potentially play a role in gDNA exit including DNA synthesis, cell cycle progression and checkpoints, cell survival machinery, genetic stability and mutation rates, autophagy and maintenance of the cellular envelope (Dou et al., 2015).

The impact of oncogenic mutations on deregulation of cell cycle

At any given point of time, approximately 100 million cells undergo cell division in our body (Alberts et al., 2014) and it is estimated that 1 out of 100 cells undergo abnormal cell division. In normal cell division, chromosomes segregate in a controlled manner and two daughter cells are formed. However, in cancer cells several mechanisms that control the integrity of cell division are compromised, ranging from tolerance to accumulation of point mutations and checkpoint defects (e.g. due to loss of TP53, INK4A and other suppressors) to the accuracy of chromosomal separation being compromised, defects leading to microsatellite (MIN) or chromosomal instability (CIN), respectively. In addition, activation of dominant acting oncogenes, such as *RAS*, propels the cells through deregulated cell cycle and affords them altered survival properties, increasing the level of genetic instability. For example, studies show that mutant *RAS* can lead to generation of drug resistant cellular mutants within one population doubling (Denko et al., 1994). Finally, loss or deregulation of genes that control the function of DNA repair pathways, such as mismatch repair genes (MMR) may either initiate or exacerbate these processes (Vogelstein and Kinzler, 2004b). Taken together these processes are generally regarded as a source of genomic diversity within cancer cell populations and the mechanism of their progressive evolution toward increasingly malignant and intractable state (Nowell, 1976; Gerlinger et al., 2012).

Genetic instability may generate extrachromosomal and extranuclear chromatin over the course of the cell cycle. Thus, whole chromosomes or chromosome fragments may lag behind during cell division resulting in chromosomal imbalances, losses and translocations. This loss of normal chromosomal repertoire (aneuploidy) is observed in many types of cancer cells, especially those with the CIN-type genetic instability (Woo and Poon, 2004). According to Geigl et al. (Geigl et al., 2008), CIN refers to the rate at which a whole or part of a chromosome is either gained or lost, which is not tantamount to aneuploidy, but could be causative of it (Geigl et al., 2008). Several factors have been suggested to underlie the CIN in cancer including 1) mitotic defects, 2) cell cycle checkpoint defects and 3) oncogene induced mitotic stress (Maleki and Röcken, 2017).

Mitotic defects leading to CIN can be attributed to four main defects in mitosis, namely a) error in spindle assembly checkpoint (SAC) (Kops et al., 2004), b) defects in chromosome attachment on microtubules (defects in kinetochore-microtubule attachments) (Thompson and Compton, 2008), c) two or more centrosomes leading to multipolar spindle formation (Bakhoum and Compton, 2009) and d) defects in centromere geometry and structure (Sakuno et al., 2009). Tumour suppressors and oncogenes also affect the mitotic process resulting in chromosomal aberration. Tumour suppressor *TP53* maintains genomic stability and it is altered in more than half of human cancers underscoring its role as the 'guardian of the genome' (Vogelstein et al., 2000). In the event of endogenous and exogenous insults, TP53 responds to DNA damage or cell cycle checkpoint failure by either promoting cell cycle arrest for repair or initiate apoptosis to eliminate the damaged cells. Inactivation or loss of TP53 have been shown to cause centrosome amplification leading to abnormal chromosome numbers (Tarapore and Fukasawa, 2002). Similarly, a recent study (Perera and Venkitaraman, 2016) showed oncogenes such as K-*RAS* and *MYC* induce errors in chromosomal alignment and segregation during mitosis. In keeping with this observation pancreatic ductal adenocarcinoma (PDAC), which is characterized by more than 90% frequency of K-*RAS* mutations, displays chromosomal abnormalities (Wido et al., 1990; Caldas and Kern, 1995). Further, overexpression of oncogenic H-*RAS* G12V reportedly caused multiple mitotic errors including centrosome duplication, micronuclei formation and weakening of the SAC in specific cell types (Knauf et al., 2006; Perera and Venkitaraman, 2016). While the role of RAS in tumorigenesis through MAPK signalling is well studied and its

role in causing genetic instability amply documented, very little is known about how exactly RAS is involved in inducing genomic aberrations. Yang et al. (Yang et al., 2013) showed that RAS-driven ovarian epithelial cells can exhibit amplification of *AURKA* (Aurora kinase A) gene and concomitant decrease in *BRCA2* expression, resulting in genomic instability through abnormal cytokinesis. Aurora kinase A plays an important role in different mitotic events, such as centrosome duplication, chromosomal alignment, and SAC among others, and amplification of this gene has been reported in multiple cancers (Sen et al., 2002; Li et al., 2003). Another study (Abulaiti et al., 2006) reported that overexpression of oncogenic H-*RAS* (V12 and L61) in rat thyroid cells causes their escape from cell cycle checkpoints due to the impaired DNA damage response, leading to micronuclei formation and chromosomal instability.

Likewise, *MYC* is deregulated (mostly amplified or overexpressed) and acts as a potent oncogene in many cancers (Kumari et al., 2017). Abnormal expression of *MYC* affects several downstream genes involved in the cell cycle and DNA synthesis (e.g. cyclin D1), thus promoting genomic instability. In keeping with this notion, overexpression of *MYC* is linked to a variety of chromosomal aberrations, including production of extrachromosomal elements, centromere and telomere fusions and aneuploidy (Mai et al., 1996; Kuttler and Mai, 2007). Deregulated *MYC* can also induce double-stranded DNA breaks (DSBs) through various mechanisms including accumulation of reactive oxygen species (ROS), an increase in replication stress and reduction in DSB repair potential (Kumari et al., 2017).

Micronuclei as a plausible source of extranuclear chromatin

Micronuclei (MN) are small, extranuclear chromatin bodies enclosed by a nuclear membrane (Luzhna et al., 2013; Hintzsche et al., 2017). The size of MN ranges from 1μm-3μm (Yasui et al., 2010; Shimizu, 2011; Ji et al., 2014; Hintzsche et al., 2017), occupying approximately one-third of the main nucleus (Mure et al., 1996) and their number may vary from one to several in a cell. MNs are usually round to oval in shape (Mamat et al., 2008) and contain fragmented and compacted chromosomes, therefore affording a stronger DAPI signal compared to the one emanating from the main nucleus (Bhattacharya et al., 2015). MNs were discovered more than a century ago by William Howell and Justin Jolly (Sears and Udden, 2012) and they were known as Howell-Jolly bodies. Later in 1961, Howell-Jolly bodies were observed in bone marrow in

association with folic acid and Vitamin B (12) deficiency state (Dawson and Bury, 1961). The significance of MNs grew because they became widely used as signs of genotoxicity. For example, cells treated with genotoxic agents such as cytochalasin-B (inhibitor of spindle assembly) (Fenech and Morley, 1985) and Mitomycin C (Mure et al., 1996) increase MN formation. An *in vitro* MN test named CBMN assay (Cytokinesis-block micronucleus cytome assay), where cytochalasin-B (Cyt B) is used to prevent cytokinesis, was developed to detect for the presence of MNs in cells (Fenech, 2007) and visualise chromosomal abnormalities.

MNs can also form as a result of spontaneous mitotic defects. During abnormal cell division, fragmented chromosomes or whole chromosomes may acquire their own nuclear membrane and form MNs. Furthermore, overexpression of oncogenes, such as n-Myc (Sugihara et al., 2004), v-ras (Saavedra et al., 1999) and H-*RAS* (Woo and Poon, 2004), may cause chromosomal instability which leads to formation of MNs containing amplified or altered genomic sequences.

The fate of MNs depends on their nature including the presence of nuclear envelope, its integrity (Geraud et al., 1989), the state of nuclear pore complexes (Sukegawa and Blobel, 1993) and the content of whole chromosomes (Labidi et al., 1987). MNs with an intact nuclear envelope can undergo DNA replication (Obe et al., 1975) and are capable of DNA damage repair (DDR) having contained the respective enzymatic activities (Medvedeva et al., 2007), while MNs without a nuclear envelope are usually degraded (Hintzsche et al., 2017). In addition, MN containing whole chromosomes may have transcriptional activity, but they fail to produce mRNA in the case of acentric chromosomes (Luzhna et al., 2013).

Errors during mitosis leave the cells susceptible to continuous accumulation of genetic mutations and other structural defects, including the occurrence of chromosomal shattering known as chromothripsis (Becker, 2015). It has been reported that 2-3% of all cancers undergo massive rearrangement of a single chromosome (Stephens et al., 2011). In contrast to sequential rearrangements and translocations, chromothripsis involves fragmentation of a chromosome into many parts, which become simultaneously randomly reintegrated into a rearranged new structure, mainly due to non-homologous end joining (NHEJ) mechanisms (Stephens et al., 2011). Chromothripsis may be a relatively common phenomenon in certain cancer cells, where

rearrangement of an entire chromosome results in variation in DNA copy number (Bignell et al., 2010; Zhang et al., 2015). The mechanism involved in chromothripsis remains unknown, but several scenarios are being investigated. Stephens et al., (Stephens et al., 2011) put forward two such scenarios. In the first instance, the shattering of chromosomes may be due to ionizing radiation and double-strand breaks. These breaks trigger a DNA repair mechanism (NHEJ) but lead to multiple errors resulting in fragmentation followed by re-alignment. Second scenario is dependent on telomere shortening and erosion resulting in end-to-end fusions between chromosomes. Such events would cause formation of anaphase bridges and ultimately chromosome breakage that could lead to chromothripsis (Tusell et al., 2010). However, other mechanisms are also being considered. For example, using live cell imaging and single cell sequencing, Zhang et al., reported chromothripsis in MNs of osteosarcoma cells (U2OS) that are treated with nocodazole (Zhang et al., 2015).

FISH (Fluorescent *in situ* hybridization) and SKY (Spectral Karyotyping) are important tools for detecting the content of MN within various cells. For example, FISH was used to detect amplified *MYC* sequence in the micronuclei of the human colorectal cancer cell line COLO 320DM (Shimizu, 2011), while the SKY assay has been employed to identify the contribution of specific chromosomes in MN derived from lymphocytes treated with Cyt-B (Leach and Jackson-Cook, 2001). Taken together, aneuploidy, CIN and chromothripsis are examples of processes that may lead to large-scale mitotic errors and generate deposits of extranuclear chromatin such as MNs. These mitotic errors may cause perpetual defects in DNA replication and their impact could last throughout the lifespan of the tumour cell (Lengauer et al., 1997), thereby generating a pool of displaced gDNA that cancer cells must be equipped to process.

Mitotic and epigenetic determinants of micronuclei formation

The human body contains more than 10 trillion cells (Chan and Hickson, 2011). To maintain normal cellular function, strict choreography of cell division is of utmost importance. As mentioned earlier, aberrations in mitotic division can result in the presence of MNs in dividing cells. MNs are usually formed during anaphase (Figure 1.3), when a whole chromosome or fragments of chromosomes that lag behind become separated from the nuclear genome (Luzhna et al., 2013). Cells that undergo missegregation of chromosomes during anaphase often acquire

aneuploidy known to be both a consequence and the cause of malignant transformation and cancer progression, especially due to loss or gain of function of certain crucial cellular genes (Potapova and Gorbsky, 2017).

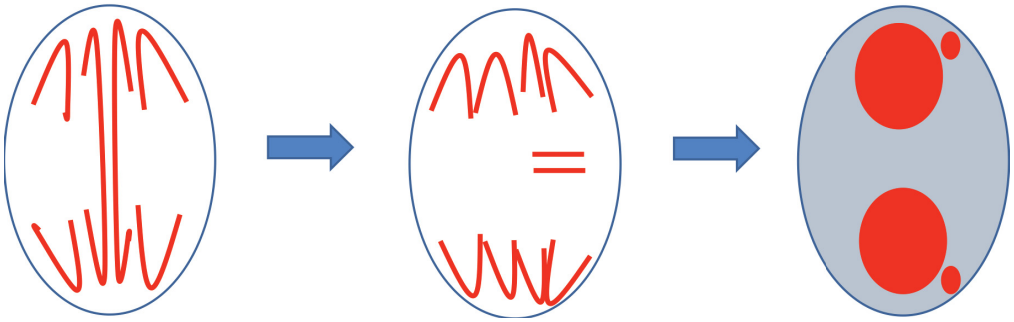


Figure 1.3. Micronuclei formation during mitosis (anaphase). Schematic diagram describing the irregular chromosome separation and micronuclei formation.

Another possibility of forming MNs is during interphase stage. It is then that a MN could be formed by a nuclear blebbing mechanism. This poorly understood phenomenon may occur in the presence of double minute chromosomes (DM), such as is the case in certain colorectal cancer cell lines containing *c-Myc* amplicons (COLO 320DM) (Orr and Compton, 2013). DMs are fragments of extrachromosomal chromatin consisting of the amplified genomic sequence (e.g. c-Myc). These extrachromosomal bodies are left behind during mitosis (anaphase stage) since they do not contain centromeres and they may aggregate and become emitted into the cytoplasm as overt MNs during the subsequent interphase (Shimizu, 2011). For example, MNs in COLO 320DM cells are formed from double minute/extra chromatin bodies without centromeres and such micronuclei carry an amplified, expressed and oncogenic *c-Myc* (Shimizu, 2011).

During mitosis, microtubules of the mitotic spindle are attached to sister chromatids via kinetochores. This allows sister chromatids to separate and move to the opposite poles of the dividing cell. Therefore defects in microtubules or kinetochore attachment result in states where chromatids cannot properly separate and this may lead to elongation of the affected chromosome to form what is known as a chromosomal bridge (Ganem and Pellman, 2012; Pampalona et al., 2016). This chromosome bridge may break and its ends may fuse with other chromosomes to

form break-fusion bridges (Terradas et al., 2010). Chromosome fragmentation in this process may lead to the formation of MNs (Kisurina-Evgenieva et al., 2016). Interestingly, in the case of a lagging chromosome, the opposite spindle poles apply equal amount of force to pull the chromosome, which is attached to microtubules via a single kinetochore, thus stopping the motion to prevent formation of a lagging chromosome. Failure of this mechanism and formation of lagging chromosomes leads to their encapsulation during telophase, a process that forms MN with their own nuclear membrane (Ganem and Pellman, 2012).

The epigenome plays a vital role in cancer progression and may contribute to MN formation. Thus, hypomethylation of centromeres and the pericentromeric DNA region may lead to incorrect kinetochore orientation and attachment to mitotic spindle fibers. This may precipitate missegration of the affected chromosome resulting in MN formation (Ehrlich, 2002; Luzhna et al., 2013; Kisurina-Evgenieva et al., 2016).

MN can also be formed due to genotoxic and cytotoxic stress. For example, agents used to treat cancer are often described as clastogens and anuegens due to their impact on the genomic structure (Kalsbeek and Golsteyn, 2017). Clastogens are defined as genotoxic agents that disturb DNA repair and replication, causing chromosome breakage. Such agents include cisplatin, which may increase MN concentration in glioblastoma (GBM) cells (Lewis and Golsteyn, 2016). In addition, radiation is also known to cause DSB and increase the level of MNs in human fibroblasts (Terradas et al., 2009). In the case of Hydroxyurea (HU) treatment, experiments with COLO 320DM cells revealed enhanced formation of MNs enriched for *c-Myc* sequences (Shimizu, 2011). In addition, Mitomycin-C (MMC) at higher concentrations leads to higher numbers of MNs formed in the Chinese hamster lung (CHL) fibroblast cell line (Hashimoto et al., 2010). Alternatively, aneugens cause disorders in chromosome numbers by interfering with the cell division machinery (Hashimoto et al., 2010). For example, taxol perturb the stable chromosome separation by affecting mitotic spindle formation resulting in an increase in MN content (e.g. 20% increase in MNs within human breast carcinoma cells (MCF-7) (Kisurina-Evgen'eva et al., 2006). It was reported that aneugens can promote centromere-positive MN formation whereas clastogens induce centromere-negative MNs (Hashimoto et al., 2010).

Determinants of MN maintenance and elimination

It has been previously reported that DMs containing amplified *c-Myc* sequences can be eliminated from COLO 320DM cells and COLO 320HSR cells after HU treatment (Shimizu et al., 2000). This treatment increases the elimination of *c-Myc* oncogenic content from COLO 320DM cells and reduces their tumourigenicity (Von Hoff et al., 1992). Shimizu et al. reported that when COLO 320DM cells containing MNs are treated with HU, the MNs are eliminated while surrounded by a cytoplasmic membrane (Shimizu et al., 2000). However, the mechanism of DM and MN elimination is not known at the moment and the generality of MN emission to the extracellular space has not been extensively investigated. Shimizu discussed a possibility that the expelled MNs (exo-micronuclei) containing amplified *c-Myc* could be transferred between cells (horizontal gene transfer) with the possibility of secondary transformation (Shimizu, 2011). In 2006, Fernandes also reported that the *Allium cepa* species generates MNs and emits them outside of the cells after treatment with trifluralin herbicide. Our interest was to determine the contribution of MNs to extracellular DNA emission from RAS-transformed cells where we observed their formation. We also focused our attention on cancerous cells that are known to emit gDNA spontaneously and the role of MN export during this process (without treatment of clastogenic and aneugenic agents) to understand the biological consequences of these events, such as the impact on neighbouring cells (Lee et al., 2016) as well as diagnostic implications for liquid biopsy (Bardelli and Pantel, 2017).

The plausible role of autophagy as a putative mechanism of extracellular emission of gDNA

As shown in the course of this thesis project, MN formation is a minor albeit fascinating element in the extracellular release of gDNA by cancer cells harboring mutant oncogenes. While reflecting on this puzzling finding, we noted three relevant circumstances: (i) Earlier studies from our laboratory documented a major role for small exosome-like Evs as carriers of extracellular gDNA (Lee et al., 2014); (ii) Formation of exosome-like Evs has recently been linked to pathways of autophagy (Murrow et al., 2015) and (iii) The emission of gDNA from cells could be expected to generate a transient pool of cytoplasmic chromatin, a process which has recently been implicated in RAS-transformed cells (Dou et al., 2015). While the latter observation was mainly linked to lysosomal degradation of extranuclear chromatin, there are multiple examples of pathways re-routing such traffic toward exosomal release (Pan and

Johnstone, 1983). Thus, autophagy could represent a link between the generation of gDNA and its transit to the extracellular space as cancer Evs (Lee et al., 2016).

While we consider EV-related emission of gDNA as a different mechanism than the aforementioned formation and expulsion of MN, both may be linked to autophagy. Indeed, autophagy maintains cellular homeostasis by degrading cellular contents (Sagona et al., 2014) and some related proteins such as LC3 and LAMP1 are adjacent to MNs which may be degraded by autophagosomes (Rello-Varona et al., 2012). In addition, CHMP4B (ESCRT-III proteins) has been implicated in both lysosome and autophagosome-dependent degradation of MNs (Sagona et al., 2014). Since MNs could be harmful to the host cell due to potential perturbations in subsequent mitoses, the existence of different MN elimination pathways is not surprising. As mentioned earlier, these pathways may include emission from host cells, degradation by autophagy or degradation by Dnase (Kisurina-Evgenieva et al., 2016) but their role is not clear. Nonetheless, autophagy represents a largely understudied mechanism of extracellular gDNA release (whether EV- or MN-mediated) and deserves some commentary.

Determinants and mechanisms of autophagy

In 2016, the Nobel Prize in Medicine and Physiology was awarded to Yoshinoru Ohsumi for his work on autophagy and in recognition of the central importance of this mechanism in the maintenance of cellular homeostasis and contribution to disease (The Nobel Assembly, 2016). Indeed, autophagy is a complex process of degradation of cellular components and foreign pathogens to maintain cellular homeostasis and energy balance (Reggiori et al., 2012a). Several cellular structures and organelles play a role in formation of autophagosomes and their fusion with lysosomes. Christian de Duve discovered lysosomes for the first time in 1949 and he too was awarded the Nobel Prize in Physiology or Medicine in 1974 (Castro-Obregon, 2010). Since then, there have been tremendous developments in studies on functions and pathways leading to lysosomes. Fundamentally, there are three pathways to degrade cytoplasmic components by delivering them into lysosomes: microautophagy, chaperone-mediated autophagy (CAM) and macroautophagy (Singh and Cuervo, 2011; Antonucci et al., 2015). Firstly, the microautophagy pathway has been described in the yeast and involves internalization of cytoplasmic cargo through direct invaginations of the lysosomal membrane which resembles Multivesicular Bodies

(MVB) formation by the late endosome (Marzella et al., 1981; Mizushima et al., 2008). Microautophagy is also known to depend on the endosomal sorting complex required for transport (ESCRT I and III) proteins (Sahu et al., 2011). However, the molecular mechanism involving microautophagy and its role in delivery of cytoplasmic cargo to lysosomes in mammalian cells is still unclear. Secondly, CAM, as the name indicates, relies on a cytosolic chaperone, such as HSC70, for identification of a penta-peptide sequence (KFERQ) in the cytosol and delivers it, one at time, across the lysosomal membrane resulting in the degradation of the respective protein in the lysosome (Sahu et al., 2011; Reggiori et al., 2012b). Lastly, macroautophagy (here after referred to as autophagy) involves a distinct family of ATG (autophagy-related genes) proteins and LC3 (microtubule-associated protein 1A/1B-light chain 3) protein as well as other players to either selectively or randomly tag the cargo protein. This triggers formation of vesicular structures referred to as autophagosomes, which later fuse with lysosomes or MVB (Kadandale and Kiger, 2010; Farre and Subramani, 2017).

Autophagy consists of four steps: induction, autophagosome formation, degradation and recycling of cellular contents. Autophagosome formation can be divided into initiation, nucleation and expansion (Lamb et al., 2013). In general, macroautophagy is sub-classified into induced-autophagy (e.g. by amino acid starvation) and endo-autophagy (recycling of cytoplasmic proteins). However, this is oversimplified and cannot be applied to more complicated roles of the biological machinery involved (Mizushima, 2007). Autophagy is an integral pathway of the lysosomal degradation process and it is known to protect the cell from functional and energetic collapse during starvation. Depending on context, this basic role may promote cell survival or lead to apoptotic death, preserve cellular function and health or lead to disease (Farre and Subramani, 2017). For example, autophagy is responsible for degrading aggregated proteins which cause neurodegenerative diseases such as dementia (tau proteins) and Parkinson's disease (α-synuclein). Likewise, autophagy promotes disposal of infectious agents such as Mycobacterium tuberculosis (Rubinsztein et al., 2015; Bento et al., 2016), but it can also enable cancer cells to survive in the face of chemotherapy (Yang et al., 2011; White, 2015).

The emerging link between autophagy and cytoplasmic chromatin in cancer

How does gDNA exit outside the cytoplasm from the nucleus and ultimately into the culture medium or biofluids? It is implicit that to transit from the nucleus to the cell exterior gDNA has to pass through various barriers such as nuclear membrane, cytoplasm and finally through the plasma membrane, and both the occurrence and mechanisms of such passage remain poorly understood. In this regard cancer supplies multiple interesting clues. For example, oncogenic insults such as activation of RAS may lead to nuclear membrane disruption as seen in mouse embryonic fibroblast (IMR90) and HEK293T cell lines (Dou et al., 2015). Furthermore, activation of oncogenes leads to an increase in cytoplasmic chromatin content resulting in high amounts of ROS and ultimately causes DNA damage. Recent literature suggests that the cytoplasmic content of gDNA and DNA-binding proteins is regulated by autophagy, whose effectors are also targets of malignant transformation (Mrakovcic and Fröhlich, 2018).

Autophagy plays complex roles in human cancer. Depending on tumour type, cancer progression stage and cellular context, autophagy can either promote or inhibit tumourigenesis (Schmukler et al., 2014). For example, autophagy promotes tumour growth in K-*RAS*-driven (G12D) non-small cell lung carcinoma (NSCLC), as well as in breast cancer by suppressing p53 activation stimulated by DNA damage (Guo et al., 2013). On the other hand, spontaneous regression of neuroblastoma occurs through apoptosis via RAS-induced autophagy and cell death (Kitanaka et al., 2002). RAS is also known to induce cytotoxic autophagy in glioma cancer cell line (U251) and gastric cancer cell line (MKN1) (Chi et al., 1999). Autophagy plays a vital role in thyroid cancer (Netea-Maier et al., 2015) in the context of activation of the RAS signalling pathway as shown in Figure 1.4. The latter describes how PI3K inhibits autophagy via mTOR, whereas, p53 and PTEN activate autophagy by inhibiting mTOR and PI3K-AKT pathways. RAS signalling cross-talk with autophagy is highly complex. Depending on the cell type, RAS activates autophagy through MAPK pathway or it inhibits autophagy via PI3K (Schmukler et al., 2014; Netea-Maier et al., 2015). *RAS* oncogenes increase MN formation (during anaphase) and promote accumulation of cytoplasmic chromatin (nuclear membrane blebbing) as per our observation and those described by Dou et al. (Dou et al., 2015) in relation to oncogene-induced senescence. The cytosolic chromatin fragments activate inflammation (cGAS – STING pathway) (Di Micco, 2017; Harding et al., 2017; MacKenzie et al., 2017) and RAS activates autophagy

which degrades the cytoplasmic content (Adams et al., 2013) which may interfere with the inflammatory effect. Dou et al. (Dou et al., 2015) also reported that autophagy proteins (LC3/ATG8) mediate degradation of the nuclear membrane upon oncogenic insult, such as enforced expression of mutant *RAS* (Dou et al., 2015). This is proposed to involve LC3 and ATG8 proteins found in the nucleus where they bind to nuclear lamin b1 and interact with lamin associated domains (LAD) on chromatin (Steensel and Belmont, 2017). Thus, it is possible that oncogenic transformation may lead to the disruption of the nuclear envelope through mobilization of autophagy proteins while some of these proteins may also manage the ingress of chromatin into the cytosol and its degradation in the lysosome. We posit that some of these processes may also 'spill over' into the exosome biogenesis and release pathways.

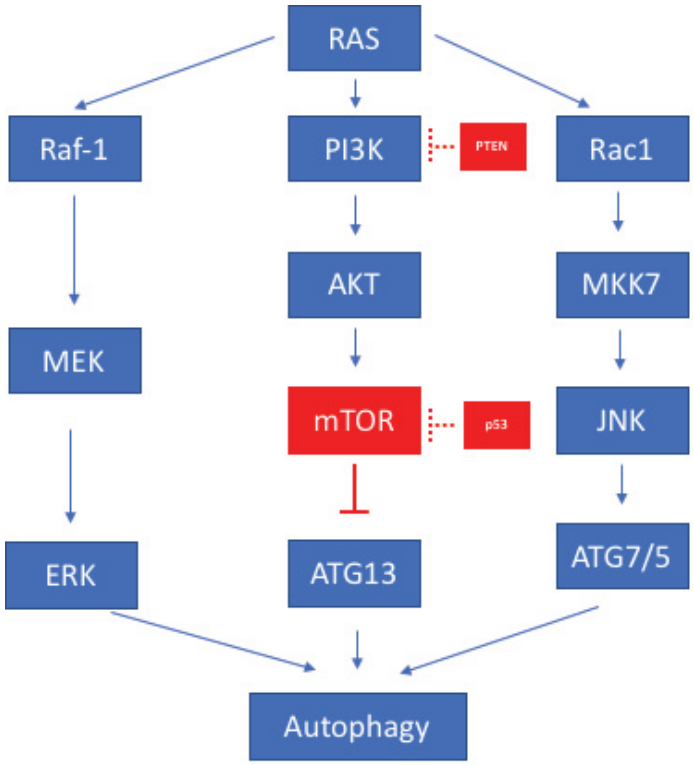


Figure 1.4. Cross-talk between RAS and autophagy signalling. Figure is adapted from Schmukler (Schmukler et al., 2014) and Netea-Maier (Netea-Maier et al., 2015).

Intersecting pathways of autophagy and exosome biogenesis

Recent studies linked autophagy to formation of extracellular vesicles originating from the endosome (exosomes) (Murrow et al., 2015). Still more evidence is emerging regarding the interplay of the autophagy and exosome biogenesis pathways (Gruenberg and Stenmark, 2004;

Ojha et al., 2017). As mentioned earlier, autophagy is initiated by maturation of pre-autophagosomal structures (PASs) to autophagosomes. Lysosomes fuse with autophagosomes and internal materials are degraded (Mizushima, 2007). Recently, it has been shown that autophagy proteins are involved in phagocyte-mediated endocytosis and exocytosis (Münz, 2017). Whereas, exosome biogenesis may be linked to Micropinocytosis and endocytosis of the extracellular material (Lim and Gleeson, 2011).

Endocytosis is a part of the normal plasma membrane recycling process, and can result, for example, from the activation of growth factor receptors (Tomas et al., 2014; Johannes et al., 2015). The formation of clathrin or caveolin regulated plasma membrane invaginations (pits) may engulf membrane proteins and their complexes, including capture of monoubiquitinated proteins by the ESCRT system) and lead to formation of endosome which then evolves to form secondary intraluminal vesicles to generate MVB (Figure 1.5) (Colombo et al., 2014). Larger amounts of extracellular material and fluid may be engulfed by macropinocytosis and phagocytosis (Mulcahy et al., 2014). MVBs can either fuse with lysosomes for degradation or fuse with the plasma membrane for exocytosis (emission of exosomes) (Gruenberg and Stenmark, 2004).

Interference with autophagy may directly or indirectly influence exosome release. For example, disrupting the fusion of autophagosomes with lysosomes may lead to binding of autophagosomes to MVB and increased exosome emission. In the case of autophagy induction, such as under starvation conditions, recycling of cellular content may lead to decreased exosome emission (Hessvik and Llorente, 2017; Ojha et al., 2017). Moreover, autophagy protein ATG7 deletion can increase exosome emission of GAPDH (Sahu et al., 2011). In line with this observation, ATG5, ATG7, ATG16L1 have been known to regulate secretion of granule contents of Paneth cells (Papandreou and Tavernarakis, 2017). Phosphoinositide kinase PIKfyve is involved in fusion of lysosome with multivesicular bodies and autophagosomes. Inhibition of PIKfyve results in reduced autophagy and an increase in exosome emission (Hessvik et al., 2016).

There are several pathways involved in the emission of exosomes carrying diverse molecular cargo (proteins, lipids and nucleic acids) (Colombo et al., 2014). These pathways are highly

regulated but still poorly understood (Baixauli et al., 2014). For example, autophagy proteins ATG12-ATG3 interact with Alix protein, which is involved in late endosome formation. Therefore, deletion of ATG12-ATG3 proteins impairs the basal autophagy flux, leading to accumulation of MVBs and impaired exosome release (Murrow et al., 2015).

Furthermore, ISGylation decreases MVB formation and promotes aggregation of cytoplasmic contents. The cytoplasmic aggregations are further degraded by the autophagosome-lysosome pathway and hence, exosome emission may also be limited by autophagy processes (Villarroya-Beltri et al., 2016). In yeast, autophagy genes such as ATG5, ATG7, ATG8 and ATG12 are involved in emission of Acb1 protein. In addition, Acb1 emission requires Vps23 which is a component of the ESCRT I complex, and t-SNARE Sso1 involved in vesicular plasma membrane docking pathway (Duran et al., 2010).

The aforementioned ESCRT protein complex is required for MVB formation (Schmidt and Teis, 2012) and it is also responsible for completion for autophagy (Rusten and Stenmark, 2009). Mutation in ESCRT subunits inhibits autophagy pathways, resulting in accumulation of aberrant proteins (Filimonenko et al., 2007). Other pathways may also mediate the cross-talk between autophagy and EV biogenesis. One example is in the age-related macular degeneration (AMD) loss of central vision usually seen in senior patients (Wang et al., 2009) caused by accumulation of proteinaceous material (drusen) in the retina. It has been shown that an increase in autophagy and exosome emission occurs in both elderly human patients and in mouse models of AMD, including the presence of autophagy proteins (ATG12-ATG5) and exosomal markers (CD63 and CD81) in drusen (Wang et al., 2009).

In general, the balance between intracellular and extracellular content is a subject of several regulatory processes involving both soluble secretome and vesicular transport, processes of regulation, degradation, and export. While these events have been subjects of extensive studies, cellular pathology, such as cancer, poses new challenges among which the release of material normally protected from outside influences such as gDNA represents a fascinating and important puzzle, which motivated some of the tenets of this thesis project.

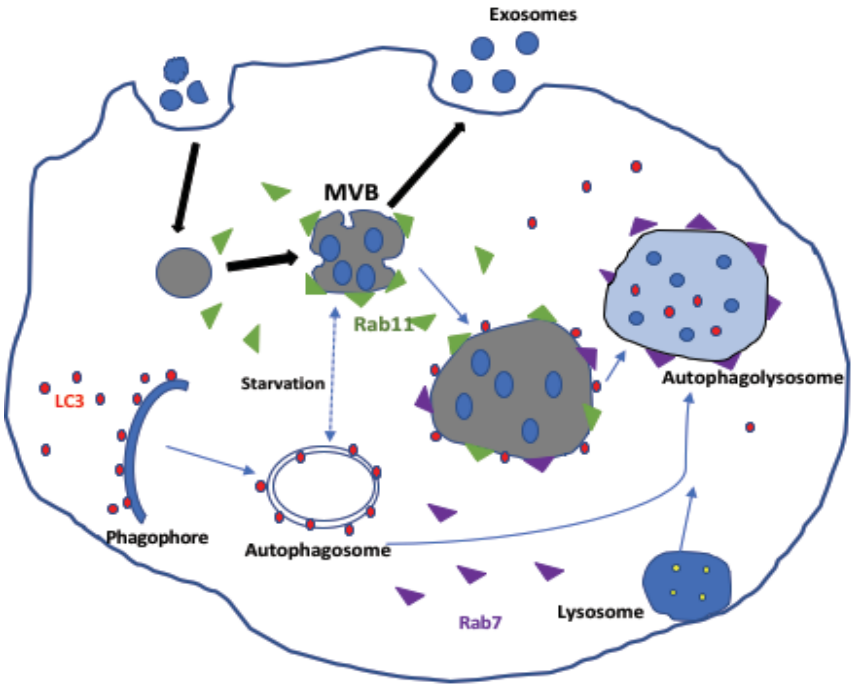


Figure 1.5. Schematic diagram depicting interrelation between exosome biogenesis and autophagy process. Adapted from Fader et al. 2007.

CHAPTER 2

Rationale, Hypotheses and Research Plan

Rationale

My Masters project concentrates on characterization of the basic determinants that affect the emission of gDNA from cancer cells harbouring specific oncogenic mutations. Indeed, the literature contains multiple examples of oncogenic DNA emission from cancer cells (Balaj et al., 2011). Indeed, circulating cell free tumour DNA (ctDNA) has emerged as a powerful platform of liquid biopsy with which to molecularly define, prognosticate and design treatments for cancers on the basis of real-time remote monitoring of genomic events through the analysis of cancer fingerprints in blood and other biofluids (Wan et al., 2017; Kaiser, 2018; Kalinich and Haber, 2018). Circulating cancer DNA also has poorly-understood functional implications as a mediator of biological events. For instance, García-Olmo et al. (García-Olmo et al., 2010) demonstrated the presence of functional K-*RAS* mutant DNA in the plasma of colorectal cancer patients and this material caused oncogenic transformation of cultured fibroblasts. Similar suggestions were also made by other authors with regards to horizontal DNA transfer between cancer and normal cells in different experimental settings (Holmgren et al., 1999, 2002, Cai et al., 2013, 2014). On the other hand, studies from other groups suggest that such processes may have transient transforming effects on stromal cells (Lee et al., 2014, 2016) and trigger thrombo-inflammatory responses in circulating phagocytes in mice (Chennakrishnaiah et al JTH published), rather than secondary cancers. In all these settings the emerging realisation is that extracellular gDNA is not merely a by-product of cell death. Rather, it can be released from live cancer cells in a form and quantity that is likely a subject of cellular regulation, which may in itself be informative.

The form of extracellular DNA that is being released from cells under these various circumstances is not always clear, but at least in some cases it appears to be material with EV properties. Thus, Balaj et al. (Balaj et al., 2011) observed the presence of single-stranded DNA containing the c-Myc oncogene in tumour microvesicles. Our laboratory has documented the presence of chromatin in exosome-like Evs derived from RAS3 cells and in other models (Lee et al., 2014, 2016) while similar findings were also reported in cells of leukemic, melanoma, pancreatic and other origins (Cai et al., 2014; Kahlert et al., 2014; Lazaro-Ibanez et al., 2014;

Thakur et al., 2014). In cases where this material has been sequenced, a representation of the whole cellular genome seems to predominate suggesting lack of sequence specificity (Kahlert et al., 2014; Lee et al., 2014), even though 'spikes' in the DNA profile may suggest regional enrichment in some cases, and the contribution of RNA/DNA complexes (Sansone et al., 2017). Other studies also highlighted the EV content of oncogenic, mitochondrial or plasmid DNA, sometimes distributed unevenly between EV subtypes (Kahlert et al., 2014; Lazaro-Ibanez et al., 2014; Sanz-garcia et al., 2014; Thakur et al., 2014; Kanada et al., 2015). Even though the respective emission processes have not been established or described in detail, multiple lines of evidence from our and other laboratories point to the possibility that an important, or main, mechanism of gDNA exit from cancer cells involves cellular vesiculation pathways.

We reasoned that multiple pathways could exist which enable the exit of gDNA from cancer cells through the EV compartment. For example, in addition to small exosome-size Evs produced by the aforementioned RAS3 cells, we observed that these cells (unlike their non-transformed isogenic IEC-18 counterparts) harbour major perturbations in the structure of their nuclear envelope (shown by TEM and confocal microscopy) and also produce MNs as defined by SKY, FISH, lamin b1 immunofluorescence and BrdU-labelling experiments. As mentioned earlier MNs are small extra-nuclear bodies, ranging in size from 1-3 μm and thought to originate from the acentric chromatid/chromosome fragments or whole chromosomes that lag during anaphase (Fenech and Natarajan, 2011). This is of interest as MNs often contain oncogenic amplicons, such as *MYC* (e.g. in COLO 320DM cells) and were found to be released from cancer cells through an unknown mechanism (Shimizu et al., 2000). Interestingly, amplicons of oncogenic sequences encompassing EGFR and mutant EGFRvIII in glioblastoma cells have also been reported to undergo a genomic loss in cell culture, either spontaneously or in the presence of EGFR inhibitors (erlotinib) (Bigner et al., 1990; Nathanson et al., 2014). On the other hand, the gDNA content associated with RAS3-derived Evs was earlier characterized as relatively sequence-unspecific and representative of almost the entire genome of donor cells (Lee et al., 2014). These reports and observations suggest that gDNA emission may occur in either sequence-selective form (as MN content) or sequence-non-selective form (as small Evs containing gDNA of the entire genome), possibly through several different mechanisms.

We also posit that the emission of extracellular DNA implicitly requires a level of superfluous DNA replication, as otherwise the cancer cell genome would undergo progressive quantitative disintegration. This raises the question as to whether interference with DNA synthesis could prevent/alter extracellular DNA release. Alternatively, it is possible that as a cancer cell population retains its viability (as is the case for RAS3 cells and other DNA emitting cell lines), a small subset of cells regularly undergoes apoptosis. This would be expected to generate a steady influx of apoptotic bodies into the EV population produced by cancer cells and result in 'contamination' of other EV fractions, including exosomes, with DNA-containing material. While apoptotic bodies are believed to be large, we did observe a caspase-dependent formation of exosome-sized DNA-containing vesicles in cancer cells subjected to treatment with targeted anticancer agents (Montermini et al., 2015). Hence, if such a process occurred spontaneously in RAS-transformed cells, it stands to reason that blocking apoptosis by the use of caspase inhibitors might obliterate the release of extracellular gDNA.

Finally, as mentioned earlier, exosome biogenesis has recently been linked with elements of the cellular circuitry that regulates autophagy, notably through interactions of Atg12 protein and Syntenin/Alix (Murrow et al., 2015). At the same time, other ATG proteins have been implicated in generation of cytoplasmic chromatin in cells acutely expressing mutant *RAS* oncogene. It is proposed that LC3/Atg8 dependent degradation of the nuclear envelope with the involvement of Atg7 leads to a release of genomic content to the cytoplasm where it serves as an inducer of stress responses before being degraded by the lysosome (Dou et al., 2015). We surmise that alternative processing of this material may include vesiculation pathways regulated by ATG proteins and leading to extracellular expulsion of cytoplasmic chromatin.

My project was designed to operationally characterise some of these patterns, and their relative contribution to extracellular DNA pool, using a panel of cancer cell lines with known oncogenic transformation mechanisms and partially catalogued for extracellular DNA emission profiles.

Hypotheses

Based on the argument presented in the previous section the formal hypotheses of this thesis project are: (i) Oncogenic transformation triggers genomic DNA emission from cancer cells through at least two different types of membrane structures (extracellular vesicles and/or micronuclei). (ii) Mechanistically, extracellular DNA formation is linked to aberrations in DNA synthesis and cellular mitogenesis (superfluous intracellular DNA) resulting in formation of micronuclei or other reservoirs of cytoplasmatic chromatin, which undergo active expulsion from cells through a vesiculation pathway. (iii) Alternatively, extracellular DNA may be a function of low-level apoptosis that leads to co-purification of exosome-like extracellular vesicles with DNA-containing apoptotic bodies. (iv) Processes of autophagy involved in exosome biogenesis and turnover of cytoplasmic chromatin may contribute to the emission of extracellular DNA.

Research Plan

Building on the results of my predecessors in the laboratory, the emerging literature and my own preliminary data I formulated three tasks (aims and thesis chapters) driving my experimental explorations. These were designed to

(i) Assess the contribution of MNs to extracellular DNA emission from RAS3 cells.

(ii) *Assess the contribution of Evs to extracellular DNA emission from RAS3 cells.*

(iii) Assess the nature of extracellular DNA and its dependence on DNA synthesis, apoptosis and autophagy processes.

CHAPTER 3
Materials and Methods

Cell lines and culture conditions

We used three different cell lines, namely IEC-18, RAS3 and COLO 320DM. IEC-18 is a non-tumourigenic and immortalized cell line originating from rat intestinal epithelial cells as previously described by Quaroni and Isselbacher (Cultures and Isselbacher, 1981). The RAS3 cell line is tumourigenic and derived from IEC-18 cells transfected with an oncogenic H-*RAS* gene carrying a G12V mutation (Rak et al., 1995). COLO 320DM is a human colorectal cancer cell line that contains double minute (DM) chromosomes. COLO 320DM was purchased from ATCC. IEC-18 and RAS3 cells were maintained as monolayers in AMEM medium with 5% FBS, 1% pen/strep, 20mM glucose, 4 mM L-glutamine and 10μgml^{-1} insulin. COLO 320DM cells were cultured in complete media (RPMI 1640 with 10% FBS) as recommended by the ATCC product sheet. For EV isolation experiments, EV-depleted FBS was used (110,000g spun overnight, followed by 0.22 μm filtration).

EV isolation

Evs were isolated using ultracentrifugation (Lee et al., 2014, 2016; Montermini et al., 2015) or size-exclusion chromatography techniques (Lobb et al., 2015; Gámez-Valero et al., 2016). In the ultracentrifugation method, conditioned media was collected and spun at 400g for 10 minutes. It was then filtered with a 0.22 μm filter and centrifuged at 110,000g with a Type 70 Ti rotor for 1 hour at 4°C (k-factor: 111.4) (Cvjetkovic et al., 2014; Musante et al., 2014). The EV pellet was washed with PBS. In size-exclusion chromatography (qEV column from Izon Science), conditioned media was first concentrated using 100kDa Amicon ultra-15 centrifugal filter unit (Millipore # UFC910024). The concentrate was poured on top of the qEV column and 16 fractions were collected from the column. Fractions 7, 8, 9, 10 were EV fractions which was confirmed using whole-mount negative staining on Transmission Electron Microscope (TEM) (Figure 3.1).

DNA Extraction

Cells or extracellular vesicles were treated with DNA lysis buffer (see below) and DNA was precipitated using isopropanol. The concentration and purity of extracted DNA was determined spectroscopically at 260 nm against distilled water as a blank, using the 260/280 nm and 260/230 nm ratio respectively. The measurements were performed using both nanodrop spectrophotometer and fluorometric Qubit system (Thermo Fisher Scientific, Germany).

DNA Lysis Buffer (Laird et al., 1991; Long, 2002)

100mM Tris HCl pH 8.5	5ml
0.5M EDTA	0.5ml
10% SDS	1ml
5M NaCl	2ml
20mg/ml Proteinase K	0.25ml

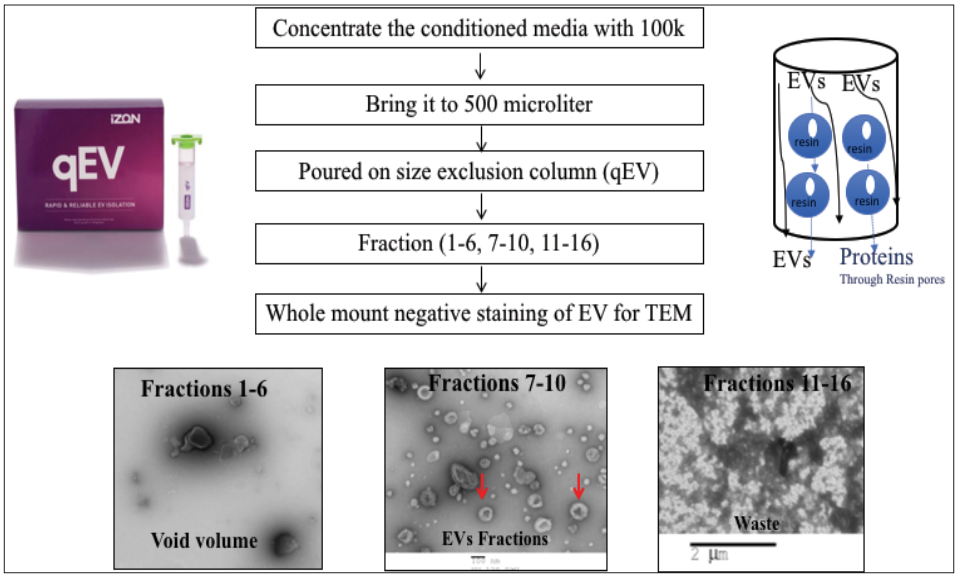


Figure 3.1. Evidence of EV emissions from RAS3 cells based on size exclusion chromatography purification and TEM imaging.

PCR

PCR was performed using 2µl of genomic DNA preparation containing 5-10ng DNA, as template. The reaction mixture also contained MyTaq^{TM} HS Red Mix, DNAse free water, and 10µM of each primer in a total reaction volume of 20µl. A total of 35 PCR cycles were carried

out with the following conditions; 95°C for 10 minutes, 94°C for 30 seconds, annealing temperature 64°C for 30 seconds, 72°C for 1 minute, repeat the cycle 34 times and 72°C for 5 minutes. Annealing temperature varies with Tm of primers (Table 3.1) and was optimized using gradient PCR. PCR products were loaded into 2% agarose gel stained with ethidium bromide and ran at 100V in TBE buffer. Separated DNA samples were visualized with gel documentation system (Bio-Rad).

Gene	Primer sequences (5'-3')	Size (bp)	Annealing (°C)
H-*RAS*(Human)	Forward: GCAGGAGACCCTGTAGGAGGACCC Reverse: TGGCACCTGGACGGCGGCGCCAG	188	64
MYC (Ji et al., 2014)	Forward: GATTCTCTGCTCTCCTCGAC Reverse: GCCCGTTAAATAAGCTGC	178	58
GAPDH	Forward: AGGGCCCTGACAACTCTTTT Reverse: AGGGGTCTACATGGCAACTG	231	60
Beta-actin (Human)	Forward: GGCATCCTCACCCTGAAGTA Reverse: CCACTCACCTGGGTCATCTT	215	58
Beta-actin (Rat)	Forward: ACCCCAGCCATGTACGTAG Reverse**:** ATGAAGTGTGACGTTGACATC	128	62

Table 3.1. Primer sequences, amplicon sizes and annealing temperatures for PCR reactions.

Droplet digital PCR (ddPCR)

Droplet digital PCR (ddPCR) assay was performed according to the manufacturer's protocol. Briefly, each reaction consisted of ~10ng of the template DNA, 1x ddPCR Eva Green Supermix, 1µM forward and reverse primers and Dnase free water to bring the reaction volume to 20µl. For each reaction, 60µl of Droplet Generation Oil (Bio-Rad) was applied, loaded onto cartridge and droplets were generated using the QX100 Droplet Generator (Bio-Rad). The droplets were transferred to a 96-well plate, sealed and PCR was performed with the following conditions: 1x 95 °C for 5 min, 45x (95 °C for 30 sec, 62 °C or 64 °C for 60 sec and 72 °C for 30 sec), and 1x 90 °C (5 min). After the PCR reaction was completed, the plate was transferred and read in

QX100 Droplet Reader (Bio-Rad) and data were analyzed with QuantaSoft droplet reader software (Bio-Rad).

Transfection

The pEGFP-C1 plasmid with LC3 insert was gifted from Dr. Kabeya. Transfection was performed using Lipofectamine ® 2000 reagents. Mock transfection was carried out using an empty vector. A total of 5,000 cells were plated in each well in a 6-well plate and grown in a 37 °C incubator overnight. The next day, the cells were transfected with a mixture of 2µl of plasmid DNA containing LC3 gene and 4 µl of Lipofectamine 2000 reagent (1:2). After 48 hours, cells were fed with selective media containing G418 (750µg) (Wisent Inc, Cat # 450-130-QL) for 7 days or sorted for GFP using the BD FACSAria Fusion (488nm) instrument. Transfection was further confirmed with western blot against LC3 antibodies (Table 3.2).

Western blot

Cells were trypsinized, centrifuged at 400g for 5 minutes and the pellet was washed with PBS. The cells were lysed in RIPA buffer (BioRad, 2016) (50mM Tris-HCl (pH = 8), 1% NP40, 150mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1mM sodium orthovanadate and 1mM NaF) containing protease inhibitor (1 tablet for 7 ml, Roche Cat # 11836153001). Lysates were quantified using Micro BCA Protein Assay Kit (Thermo Fisher Scientific Cat # 23235). Proteins were separated by SDS-PAGE (12%), electrotransferred to polyvinylidene difluoride membranes overnight at 4°C (30V) and blocked for 1 hour at room temperature with 5% non-fat dry milk in TBST (pH = 7.5). Membranes were washed and incubated overnight at 4°C with primary antibodies anti-LC3B (1:1000) and anti-Lamin b1 (1:500). Anti-β-actin (1:5000) was used as a loading control (Table 3.2). Membranes were washed thrice with TBST for 5 minutes each and then probed with HRP-conjugated anti-rabbit IgG secondary antibody (1:1000) for 1 hour at room temperature (Table 3.2). Images were revealed using Amersham ECL prime detection (Amersham Biosciences: Mississauga, ON) and the ChemiDoc MP imaging system (BioRad: Mississauga, ON).

Primary Antibodies	Dilutions	Company and Cat #
Alpha tubulin mouse monoclonal	IF (1:1000)	Abcam, ab7291
BrdU mouse monoclonal	IF (1:1000), TEM (1:1)	Abcam, ab8152
BrdU Rabbit polyclonal	IF (1:1000)	Abcam, ab152095
Histone H3 Mouse monoclonal	TEM (1:1)	Abcam, ab24834
Lamin B1 Mouse monoclonal	IF (1:1000), WB (1:1000)	Abcam, ab8982
LCB rabbit polyclonal	WB (1:1000)	Abcam, ab48394
Anti-beta actin Mouse monoclonal	WB (1:10000)	Sigma, A45441
Tetraspanin 9 rabbit polyclonal	WB (1:1000)	Abcam, ab113775

Secondary antibodies	Dilutions	Company and Cat #
Chicken anti-mouse Alexa Fluor 594	IF (1:1000)	Thermo life technologies, Ref # A21201
Chicken anti-mouse Alexa Fluor 594	IF (1:1000)	Thermo life technologies, Ref # A21201
Donkey Anti-rabbit Alexa Fluor 594	IF (1:1000)	Thermo life technologies, Ref # A21207
Donkey anti-rabbit Alexa Fluor 488	IF (1:1000)	Thermo life technologies, Ref # A21206
Goat anti-rabbit IgG H&L (10nm Gold)	TEM (1:20)	Abcam, ab272234
Goat anti -Mouse IgG H&L (20nm Gold)	TEM (1:20)	Abcam, ab27242
Goat anti mouse Alexa Fluor 488	IF (1:1000)	Thermo life technologies, Ref # A11001
Anti-rabbit IgG HRP linked antibody	WB (1:1000)	Cell signalling Ref # 09/2016
Goat anti-mouse IgG H+L HRP conjugate	WB (1:5000)	BioRad, 170-6516

Table 3.2. List of antibodies used.

Live Cell Imaging

JuLI Stage live cell imaging system (NanoEnTek) was used to track micronuclei in real time. Cells were cultured in a 6-well plate (Thermofisher) overnight and stained with Hoechst 33342 dye (0.5μg/ml) for 15 minutes. Cells were washed with PBS buffer and fresh media was added. Images were taken every hour for a 24-hour period. For Confocal live cell imaging, cells were grown in a 35mm dish (MatTek Part # P35G-0.170-14-C) and incubated with Hoechst 33342 dye (0.5μg/ml) for 15 minutes at 37°C. Cells were washed, and fresh media was added. Time-lapse images were taken using a Zeiss LSM780 confocal microscope at every two hours for a period of 12 hours.

Confocal Immunofluorescence Imaging

Adherent cells were grown in a 35mm dish (MatTek) overnight. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.01% NP40. Subsequently, fixed cells were washed with PBS and incubated in blocking buffer for an hour (1% BSA in PBS). Primary antibody was diluted (1:1000) in blocking buffer and incubated overnight at 4°C. The cells were washed with PBS for 10 minutes and incubated in secondary antibody (1:1000) for 1 hour at room temperature. The secondary antibody was removed by washing with PBS for 10 minutes and nucleus was stained with DAPI. Cells were imaged with LSM780 confocal microscope with a 60x oil immersion objective lens. The settings were: zoom=0.6, pixel dwell time=0.7μsec, average=8, master gain=812, digital gain=1.0, digital offset=0, pinhole=128.1, laser line 488 nm=15, laser line 405 nm=0.7 and laser line 594 nm=12.

Fluorescence In Situ Hybridization (FISH)

The FISH protocol was followed according to the Metasystem guidelines. The probe for the MYC gene was purchased from Metasystem (Cat # D-6008-100-OG). Interphase FISH for MYC gene amplification was performed on COLO320DM cells. A FISH assay for rat chromosomes 1 (probe XRP1 green D-1501-FI) and 2 (XRP2 orange D-1502-050-OR) was carried out at the metaphase stage, where cells were pre-treated with 10 ml of hypotonic solution (KCl 75mM) (Deng et al., 2003). Briefly, cells were fixed with methanol and acetic acid (3:1). Fixed cells were spotted on glass slides and spread uniformly, then the slides were dried overnight. 10μl of XL MYC amp probe was added on each slide and covered with a coverslip. Rubber cement was applied on the edges to avoid drying. Using the ThermoBrite system from Abbott Molecular, the sample and probe were denatured at 75°C for 2 minutes and the temperature was then lowered to 37°C to allow the probe to hybridize for 48 hours. The slide cover was removed and washed with 0.4x SSC buffer (pH 7.0) to remove excess probes or unspecific staining at 75°C for 2 minutes. The slides were washed again with 2x SSC and 0.05% Tween-20 (pH 7.0) at room temperature for 30 seconds. The slides were rinsed in double distilled water to avoid crystal formation, counter stained with anti-fade DAPI (D-0902-500-DA) and covered with cover slip. Images were viewed under confocal microscope (Zeiss LSM780 laser scanning confocal microscope) with objective set at 63x and fluorescence at absorption 552nm and emission 576nm.

Immunogold Labelling of Cells and Evs

Cells were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde. The fixed cells were processed for LRWhite embedding. LRWhite embedded blocks were cut into ultrathin sections (100nm) using diamond knives. The sections were stained with primary antibody (H3 total Histones, abcam ab24834) and dsDNA antibody (Santa Cruz Biotechnology sc58749) (BrdU antibody abcam ab8152), followed by gold-conjugated secondary antibody (10nm and 20nm), which would bind to their respective primary antibodies (Table 3.2).

The isolated Evs (110,000g for 1 hr ultracentrifugation) were washed with the wash buffer (0.1M sodium cacodylate buffer) (pH7.4) and fixed with 2.5% glutaraldehyde. A volume of 10µl of Evs in fixative solution was placed on the negatively-charged copper grids. Primary antibody (anti-Tetraspanin 9/TSPAN9) was incubated with the slides overnight at 4 degree and gold-conjugated secondary antibody (10nm gold) was incubated at room temperature for 30 minutes.
Images were taken using the Tecnai 12 BioTwin 120kV Transmission Electron Microscope. Jennie Mui at the Facility for Electron Microscope Research (FEMR) unit of McGill University provided expert assistance in preparing LRWhite embedded sections.

Immunogold Double Labeling of LRWhite Ultrathin Section of Evs

Evs were washed with wash buffer and fixed with 4% paraformaldehyde with 0.5% glutaraldehyde solution. Fixed Evs were further centrifuged at 110,000 x g for 1 hour and the pellets were resuspended in 2% low melting point agarose gel. (Note: 2% agarose is melted in distilled water at 70 degrees Celsius). Agarose containing Evs underwent a series of dehydration steps i.e 1x 30% EtOH, 1x 50% EtOH, 1x 70% EtOH, 1x 80% EtOH, 1x 90% EtOH, 1x 95% EtOH, and 1x 100% EtOH. Each step was carried out for 8 minutes and within 4 hours at room temperature. Following this, the specimens were embedded in LRWhite resin (Figure 3.2), cut into ultrathin sections and labelled with antibodies against TSPAN9 and against dsDNA marker (Table3.2).

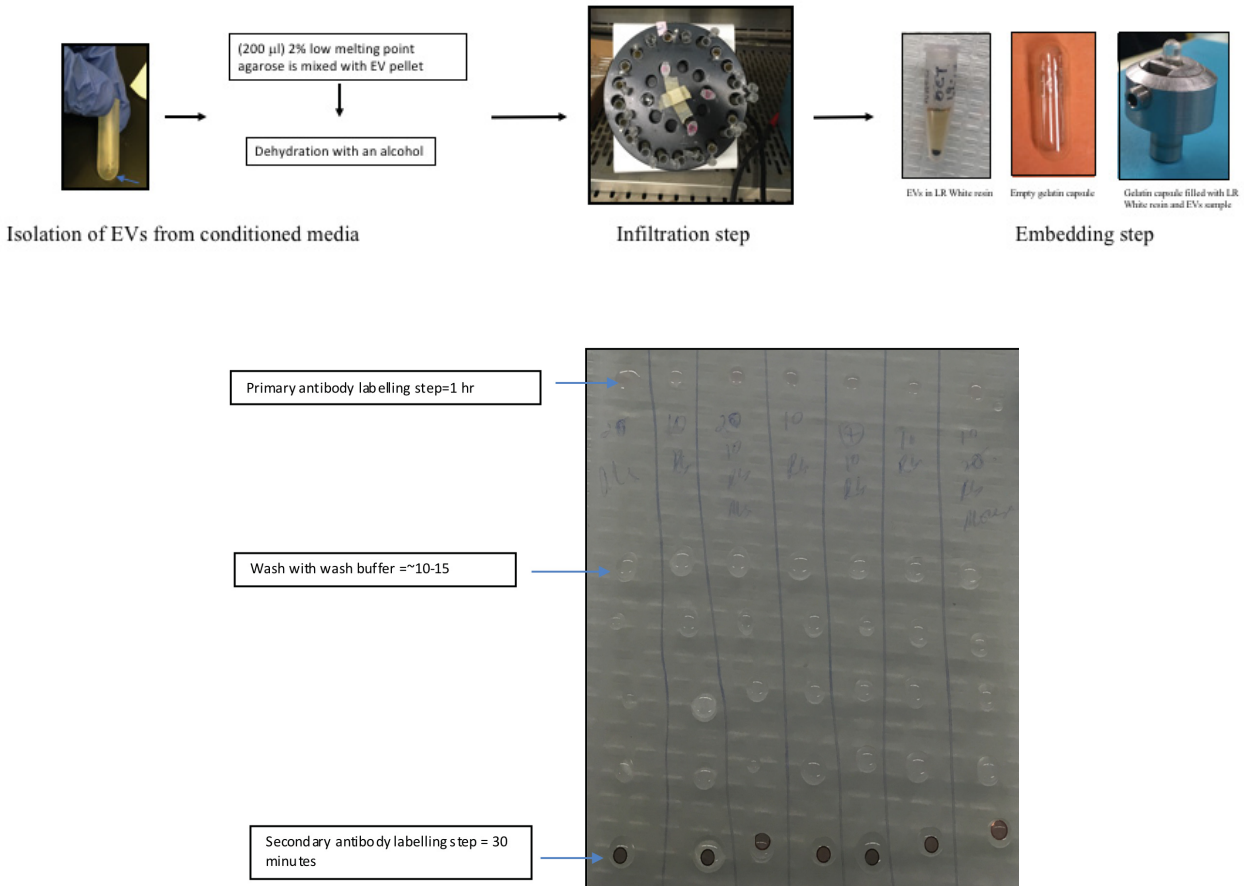


Figure 3.2. LRWhite-TEM steps of preparing cells and extracellular vesicles for double labelling using immunogold method.

Inducing Cell Cycle Arrest Using Mitomycin-C

To find the optimal concentration of Mitomycin-C (MMC) to inhibit the cell cycle at G1 phase, we treated RAS3 and IEC-18 cells with different concentrations (10µg/ml, 25µg/ml, 50µg/ml, 100µg/ml) of MMC (Sigma # M4287) for 2 hours. Cells treated with 10 µg/ml of MMC for 2 hours showed the least toxicity and coupled with arrest in G1 phase (MTS assay and flow cytometry data). Treated cells were washed, and fresh EV-depleted media was added and incubated for 12 hours. Cell cycle analysis was confirmed by staining with propidium iodide (PI) and flow cytometry (BD FACSCalibur). For the latter, cells were harvested, washed with PBS, fixed in 4% paraformaldehyde for 30 minutes and permeabilized with 0.01% NP40 (mild detergent) for 30 minutes. The cells were again washed two times with PBS and 1µg/ml of PI (Sigma # P4864-10ml) was added to the cell suspension. FL2 flow cytometry histograms were

generated to document a prominent G1/G0 peak in MMC-treated cells in a relative absence of S-phase.

Inhibition of the Apoptotic Pathway using ZVAD Peptide

Z-VAD-FMK (Promega Corporation) is an irreversible pan-caspase inhibitor. RAS3 and IEC-18 were grown in fresh EV-depleted media containing ZVAD (20μM) for 24 hours and Evs were isolated from the conditioned media as described earlier (Montermini et al., 2015).

Interference with Autophagy using Chloroquine Treatment

RAS3 and IEC-18 cells were treated with different concentrations (25μM, 50μM, 100μM) of chloroquine (Cell Signaling Technology # 14774) to inhibit the autophagy process at different time points (4, 6, 8, 10, 14, 16 hours). At 50μM concentration for 16 hours we found an optimal effect without causing overt toxicity to cells. Evs were collected from conditioned media of both chloroquine-treated and untreated cells and then DNA was isolated, quantified and assayed.

MTS – Cell Growth and Viability Assay

To measure cell proliferation and viability, MTS assay (Promega, Madison, WI) was performed on RAS3 cells treated as indicated. For this purpose, 5,000 cells per well were seeded in a 96-well microtiter flat bottom plate. Agents such as MMC (10μg/ml) or as otherwise indicated were mixed with the culture media and incubated for different durations (2 hours, 4 hours, 6 hours, and 8 hours), followed by a wash with PBS. Fresh medium was added and incubated overnight. MTS reagent was added and incubated at 37°C for 2 hours as per the manufacturer's instructions. Absorbance was read at 570nm using Biotek spectrophotometer (EPOCH).

ELISA for Histones-DNA Complexes

ELISA protocol was followed according to the manufacturer's guidelines (Roche Cat # 11774425001). While this is an assay designed to detect apoptotic bodies, the same principle can be used to detect chromatin-associated histone-DNA complexes in EV preparations (Lee et al., 2014). Briefly, Evs from RAS3 and IEC-18 were lysed using the provided lysis buffer (40 μl) and incubated for 30 minutes at room temperature. Lysates were centrifuged at 200g for 10 minutes. 20 μl of the supernatant and culture media were transferred along with positive and

negative controls provided in the kit into the microplate coated with anti-histone monoclonal antibody. To each well containing 20µl of samples, 80µl of the immunoreagent was added. The microplate was covered with adhesive cover foil provided with the kit and incubated at room temperature on gentle shaker at 300 rpm for 30 minutes. The solution was removed thoroughly by tapping/blotting on clean tissue papers. Each well was rinsed with 250µl of incubation buffer (3 times) and 100µl of ABST solution was added. The plate was incubated again on the shaker at 300rpm for 30 minutes. Finally, 100µl of ABST stop solution was pipetted to each well. Microplate was then read at 405 nm absorbance.

Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA, #NS500 NanoSight) utilizes light scattering and Brownian motion to determine size distribution and concentration of nanosized particles (Malloy, 2011). Evs were isolated by ultracentrifugation and EV pellets were resuspended in PBS (500µl). For each sample 10µl of Evs was taken and further diluted in PBS at a 1:500 ratio. Diluted samples were loaded onto the NTA chamber and three recordings of 30 seconds were taken under NTA processing settings of the software (NTA version 3.1) to analyze the concentration and size distribution of the particles.

Data Analysis

All results were repeated at least 3 times with similar results, unless otherwise indicated (provided as N). The numerical values were presented as mean +/- SD and the statistical analysis was performed using Student's t-test to compare between IEC-18 and RAS3 cells for all the experimental outcomes, unless otherwise specified. *P* value of 0.05 or less was considered significant.

CHAPTER 4

Micronuclei formation in oncogene-driven cancer cells and their contribution to extracellular DNA

Introduction – Micronuclei as a source of superfluous chromatin

We chose to first explore the link between sequence-specific and non-specific EV-mediated gDNA emission in RAS3 cells, in which an oncogenic mutation triggers formation of MNs with preferential accumulation of chromosomes 1 and 2 (our prior unpublished observation). Notably the same cells emit Evs containing gDNA sequences representative of the whole genome. While this shows that micronuclei and EV formations may represent two different pathways, in our preliminary experiments we suspected that some RAS3 cells containing MN may have a higher likelihood of undergoing apoptosis-like cell death (as suggested by live confocal microscopy). This suggests that the genesis of the DNA content in small Evs may have a cell death component, but that the majority of MN-containing cells (40-50% of the population) actually do survive. Therefore, the MN contribution to extracellular gDNA would likely represent a non-death process. Indeed, exit of MNs from cancer cells has been documented by Shimizu et al (Shimizu et al., 2000). We chose to examine this in RAS-transformed cells.

Results – Oncogenic Dependent Aberrations in Cellular Mitogenesis and Micronuclei Formation

Emission of MNs from RAS3 cells

To assess whether MN-related DNA sequences exit cancer cells as described by Shimizu et al. (Shimizu et al., 2000), I used the JuLI Stage live cell imaging system and recorded time-lapsed images of RAS3 cells stained with Hoechst 33342 to visualise MNs at every hour for a period of 24 hours. This robust imaging platform does not use UV laser, but is sensitive enough to detect MNs. We tracked approximately 35 micronuclei and only one micronucleus was emitted out from the parental RAS3 cell during cell division (Pointed arrow in Figure 4.1). Live imaging revealed the lagging chromosome and MN formation during mitosis (anaphase stage) (Figure 4.1, **hour 4**). We further tracked the lagging chromosome and observed that MNs may occasionally exit the cell and interact with cellular processes of neighbouring cells (Figure 4.1, **hours 5, 6 and 7**). In addition, MNs were inserted into the intact cytoplasm as suggested by

Shimizu et al. (Shimizu et al., 2000). This was further confirmed with confocal live cell imaging microscope and TEM (Supplementary Figure 1).

In brief, our experiment showed for the first time a spontaneous elimination of MN from the cell using real-time JuLI Stage live cell imaging system. Shimizu's group had also shown the extrusion of MNs from COLO 320DM cells, however this was performed on fixed cells, which does not provide a full real-time picture of the dynamics of the process involved. In addition, they used Hydroxy Urea (HU) to induce micronuclei formation, which is not compatible with a spontaneous elimination of MNs under the influence of the oncogenic pathway (Shimizu et al., 2000). In the future, additional real-time events involving either spontaneous or induced elimination of MNs could help understand the number and the state of cells which actively eliminate these structures. Furthermore, tracking the eliminated MNs will provide insight into the fate of expelled MN in the extracellular space and possibly *in vivo*. While these are attractive possibilities our observations indicate that the expulsion of MNs from RAS3 cells is a relatively rare event and may play a minor role in formation of the extracellular gDNA pool. This is consistent with a lack of overt enrichment in chromosome 1 and 2 sequences in the extracellular DNA in this model (MNs are enriched in these sequences), suggesting another pathway of chromatin exit.

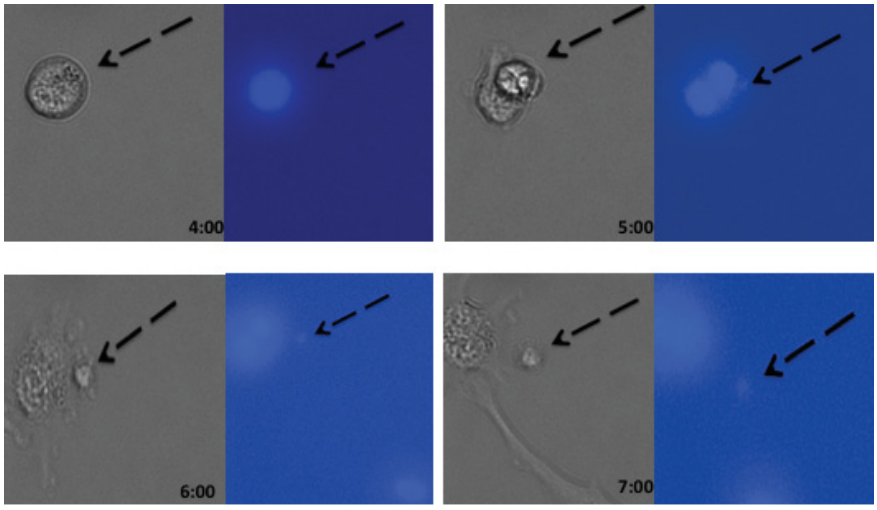


Figure 4.1. Enlarged images from Figure 4.2 (hours 4:00, 5:00, 7:00).

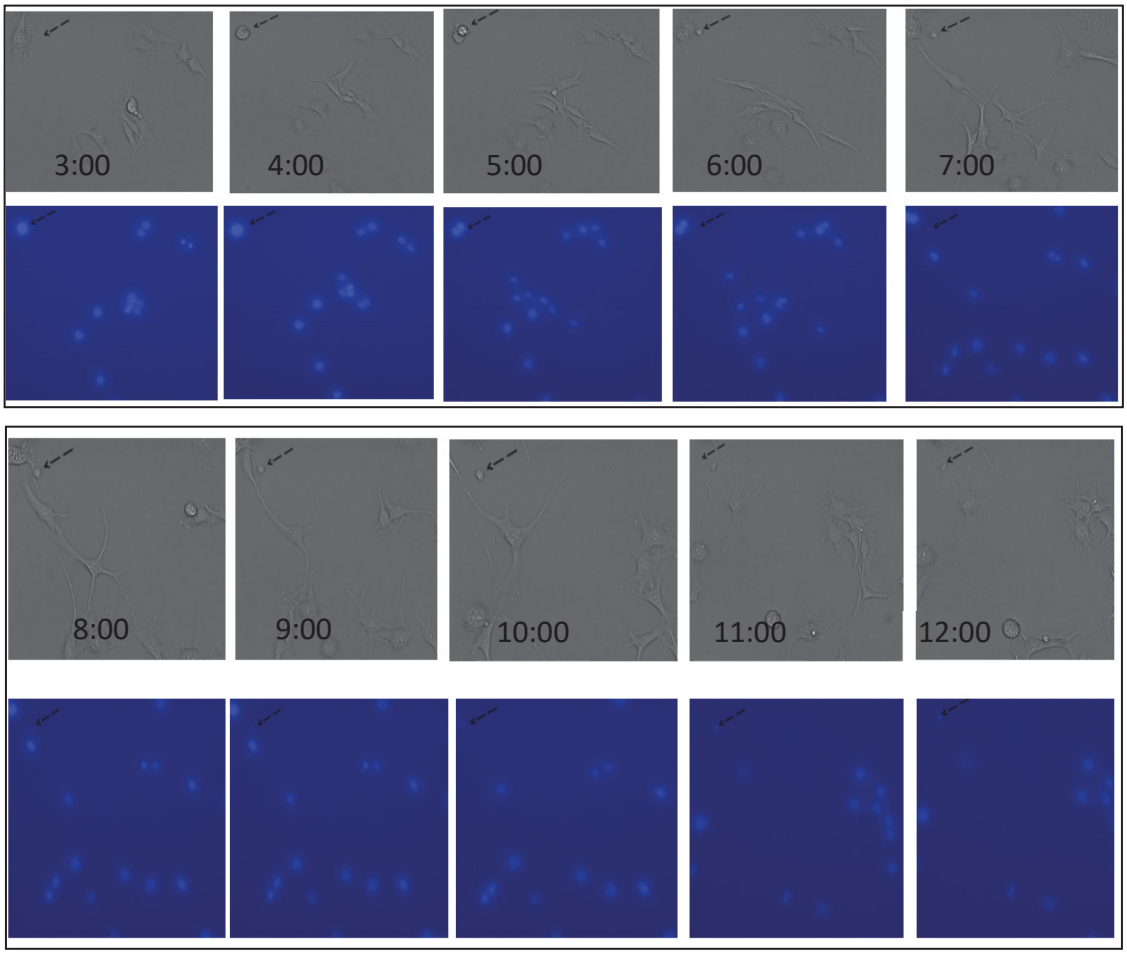


Figure 4.2. Spontaneous micronucleus elimination from RAS3 cells. JuLI Stage live cell imaging system showing that RAS3 micronuclei undergo an occasional expulsion from cancer cells. Images were captured every hour but only hours 3 to 12 are shown. Arrow shows the micronucleus that was tracked. The upper images were taken in phase contrast mode and lower images were in fluorescent mode – cells stained with Hoechst33342 DNA binding dye.

Frequent Defects of Mitotic Spindle in Cancer Cells Driven by Oncogenic RAS

The infrequent expulsion of MNs from RAS3 cells is somewhat paradoxical in light of impressive aberration in the mitogenic apparatus of these rapidly dividing cells, possibly a contributing factor to MN biogenesis. Indeed, using confocal fluorescent microscopy I observed numerous RAS-related anomalies in mitosis of RAS3 cells, that may likely contribute to the formation of extranuclear and possibly extracellular DNA. Overexpression of oncogenic *RAS* has been reported to destabilize chromosomes and cause MN formation also in human colon carcinoma cell lines, (de Vries et al., 1993), rat mammary carcinoma cells (Ichikawa et al., 1991), and thyroid PCLL3 cells (Saavedra et al., 2000). In my own studies, tripolar spindle

formation was observed in a large fraction of RAS3 cells (~50%) (Figures 4.3 and 4.4), suggesting that approximately half of RAS3 cells face difficulties in separating chromosomes in a controlled manner during every cell cycle they enter. However, we did not observe tripolar spindle formation in their parental IEC-18 cells (Figures 4.3 and 4.4) which also grow rapidly in culture, indicating that mitotic defect is driven by the H-*RAS* oncogene expressed in RAS3 cells. Interestingly, it has already been shown that multipolar spindle formation can lead to micronucleation in various systems (Schultz and Önfelt, 1994; Utani et al., 2010) and this parallel was also true for RAS3 cells, albeit without a formal proof of causality. Nonetheless, microscopic evidence in our hands does not support frequent export of MNs from RAS3 cells and this process is not a major contribution to extracellular gDNA.

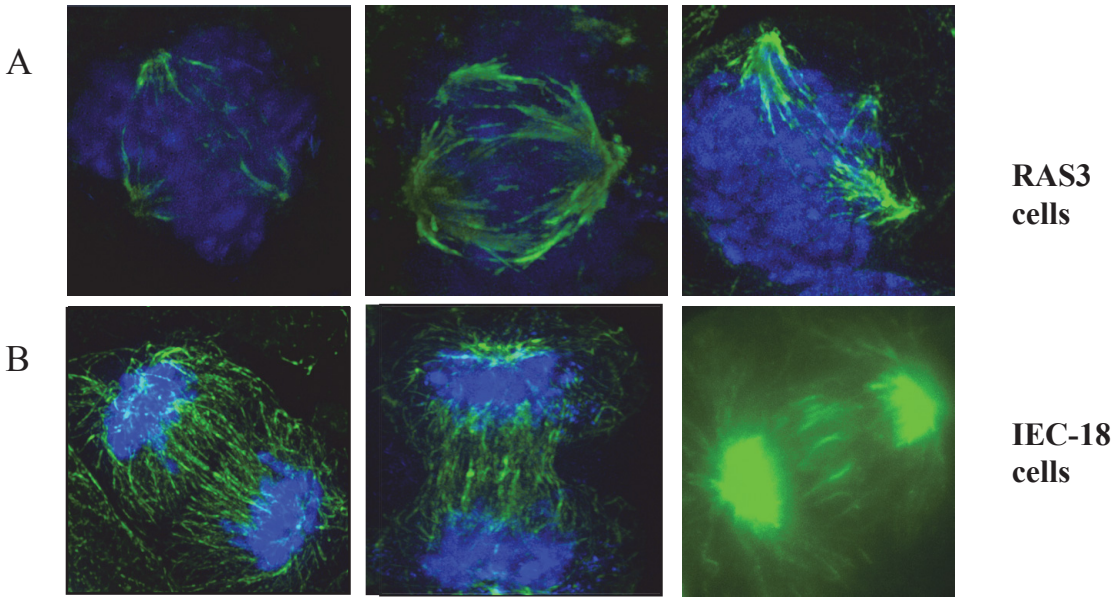


Figure 4.3. *RAS* oncogene causes mitotic aberrations. (A) RAS3 cell undergoing mitotic errors (tripolar spindle formation), while IEC-18 cells are unaffected (B).

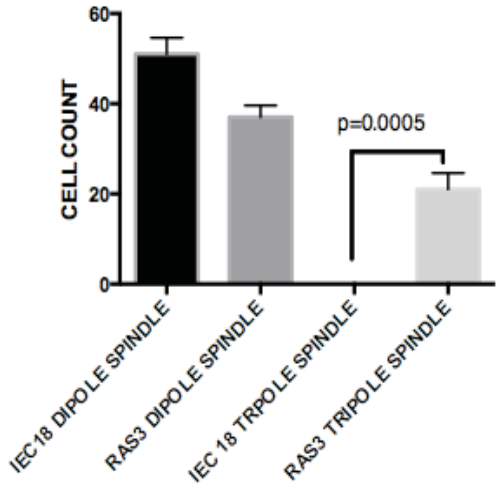


Figure 4.4. RAS3 cells undergo tripolar spindle formation.

Fractionation of Particulate Carriers of Extracellular DNA using Progressive Filtration Method

Real-time live cell imaging is an impressive but mostly a qualitative tool to visualize MN formation and their exit from cells. However, it can track only a few cells at a time and it is also time-consuming and expensive. Another limitation of imaging is that the cells move out of focus and their movement causes difficulties in tracking the cells of interest. Therefore, to assess whether MN-related sequences exit cancer cells as described by Shimizu et al. (Shimizu et al., 2000), we invested in fractionation protocols that would enable purification of MNs and other gDNA-containing fractions of cellular conditioned media to estimate where this material actually resides.

To develop MN purification protocols, I chose to use as a positive control (calibrator) the COLO 320DM cell line containing MN known to carry (and be highly enriched in) the MYC sequence (confirmed by FISH assay: Figure 4.5). Thus, the relative MYC signal as detected by PCR could be used in this case as a marker of MN-derived extracellular gDNA (and their purity) versus other putative DNA carriers such as Evs and soluble supernatant. We reasoned that this approach could be used to assess the efficiency and purity of our MN isolation protocol and, once optimized, applied to other systems. Notably, if EV preparations from such conditioned media indicated a preponderance of MYC sequences over control genes (beta actin) this may mean that

this source of gDNA is related to MNs, e.g. as a consequence of their fragmentation and degradation before or after their emission from cells.

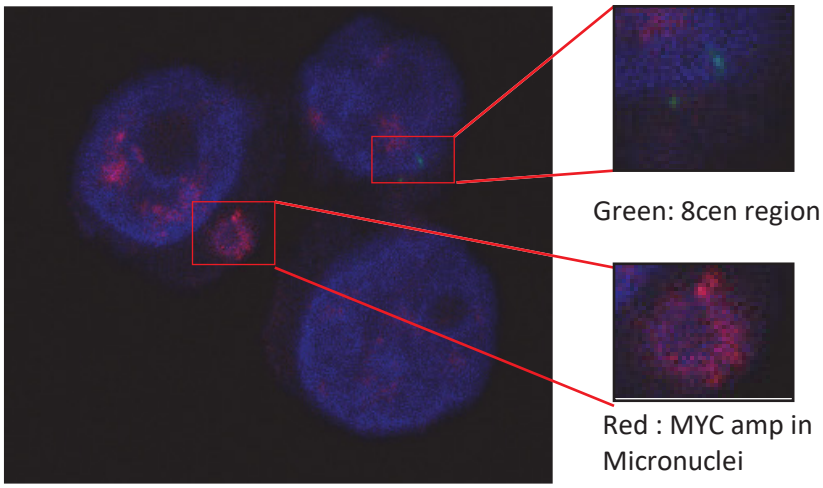


Figure 4.5. FISH assay of COLO 320DM cells containing micronuclei with highly amplified *MYC* gene. FISH signals of *MYC* and centromere (cen) are displayed in red and green, respectively, with nuclei counterstained with DAPI.

As shown in Figure 4.6 we initially isolated MN from COLO 320DM conditioned media using three different filter pore sizes in sequential order: 3 μm, 1 μm and 0.22 μm to yield different fractions consisting of large apoptotic bodies, micronuclei, ectosomes and Evs plus soluble DNA, respectively (Figure 4.6). Following this filtration sequence EV-related and soluble extracellular DNA could be directly purified from the 0.22 μm filtrate by ultracentrifugation. Specifically, for the 1 μm filter size, where MN were expected to be retained (based on size), we performed FISH assay targeting the *MYC* gene. Indeed, we detected high level of *MYC* amplification in this material (Figure 4.7 a and b). In addition, DNA was extracted from the unfiltered material retained on each filter and quantified with Qubit. Notably, certain amounts of DNA were obtained from these fractions at each filter pore size, as well as from EV pellets collected in the flow through (FT) following the ultracentrifugation (Figure 4.6). This was further confirmed using the Bioanalyzer 2100 (Agilent) (Supplementary Figure 4) and PCR for *MYC* gene (Figure 4.7 c)

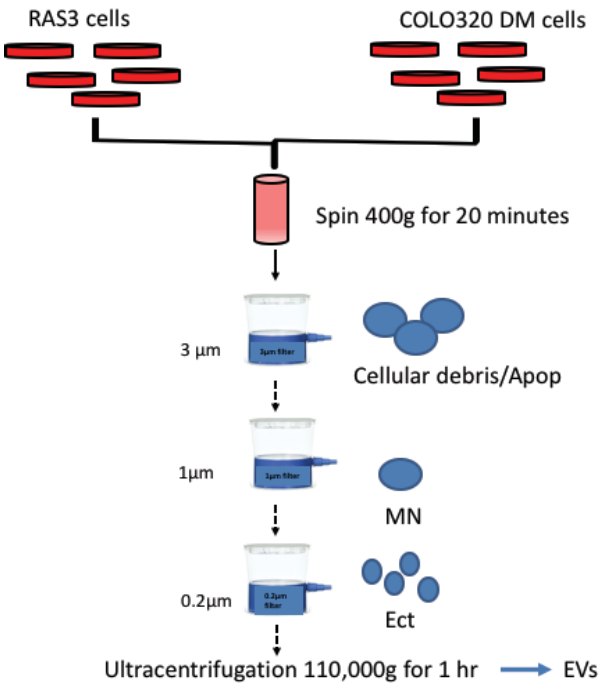


Figure 4.6. Filtration protocol for purifying micronuclei from conditioned media.
Filtration of conditioned media with 3µm, 1µm and 0.2µm filters. CM: Conditioned Media, Apop: Apoptotic bodies, MN: Micronuclei, Ect: Ectosomes and Evs: Extracellular vesicles.

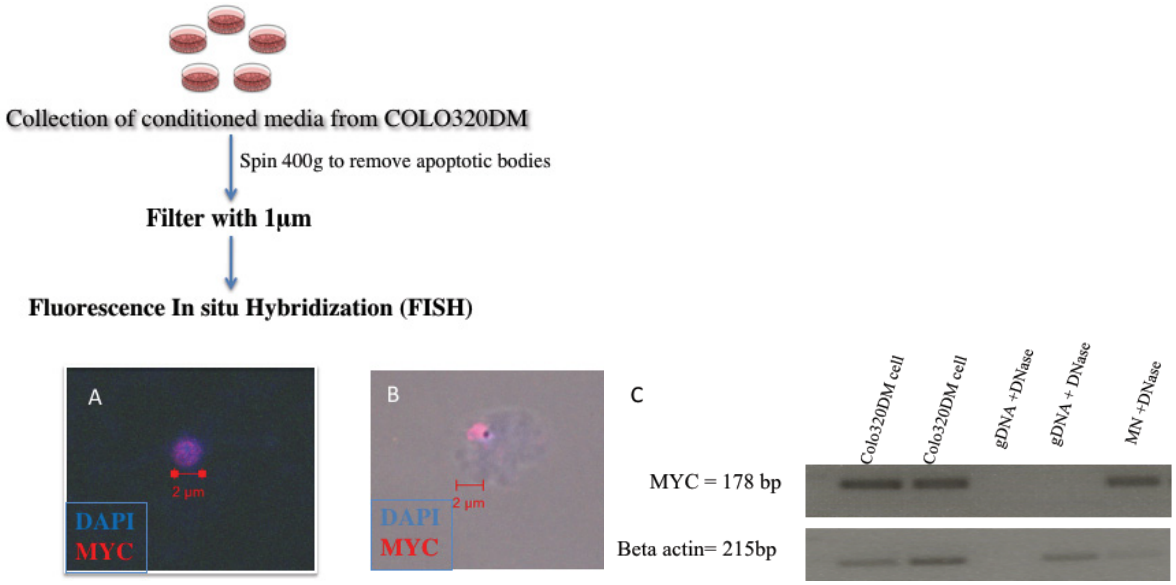


Figure 4.7. Assessment of micronuclei purified from 1µm filter by FISH and PCR assays.
Filtration protocol to purify micronuclei (MN) from conditioned media of COLO 320DM cells (top panel). (A-B) MN with *MYC* amplification (FISH assay) isolated from conditioned media using filtration protocol. C) PCR amplification of *MYC* gene from genomic DNA extracted from COLO 320DM cell and extracellular MN (exo-MN). DNAse was used to eliminate surface DNA external to MNs.

Enrichment of chromosome 1 and 2 in micronuclei of RAS-expressing cancer cells.

Since MNs are enriched in specific DNA sequences, namely amplified *MYC*, in COLO 320DM cells, we asked whether cells harbouring multiple copies of oncogenic H-*RAS* produce MNs with a random or non-random DNA composition. This is relevant as we observed higher incidence of MN formation in RAS3 cells (20%) compared to their parental non-transformed IEC-18 counterparts (5%) as measured during the anaphase stage of the cell cycle (Figures 4.8 and 4.9).

Several studies have examined the contents of MNs in various cancer cells (Leach and Jackson-Cook, 2001; Norppa and Falck, 2003; Okamoto et al., 2012; Ji et al., 2014). The composition of MN is influenced by the types of insults cells sustain due to physical (radiation), chemical (clastogenic and aneugenic) and biological (age and gender) factors (Lindberg et al., 2008). Therefore, characterization of MN content is important to understand the cause of their formation. For example, as mentioned earlier, clastogenic exposure would result in MNs with acentric chromosomes and thus can be detected using the FISH assay targeting telomeric region. Further, in older females, a higher frequency of X chromosome centromeres in MNs was detected using centromeric FISH in cultured lymphocytes (Richard et al., 1994). In addition, Y chromosome positive centromeres were seen in MNs of lymphocytes from older males (Guttenbach et al., 1994). Interestingly, according to some studies more than half of 119 total MNs detected in lymphocytes of healthy women contained chromosome 2. However, the micronucleation of chromosome 2 is not clearly defined (Peace et al., 1999; Norppa and Falck, 2003).

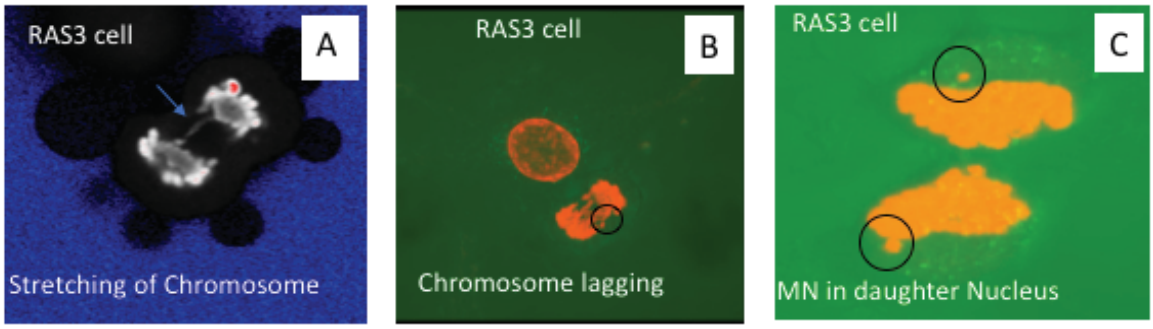


Figure 4.8. Micronuclei formation in RAS3 cell. (A) Abnormal anaphase stage (chromosome stretching) (B) Late anaphase stage (lagging chromosome) (C) Telophase stage (daughter cells with MN). Cell nuclei and MN were stained with DAPI.

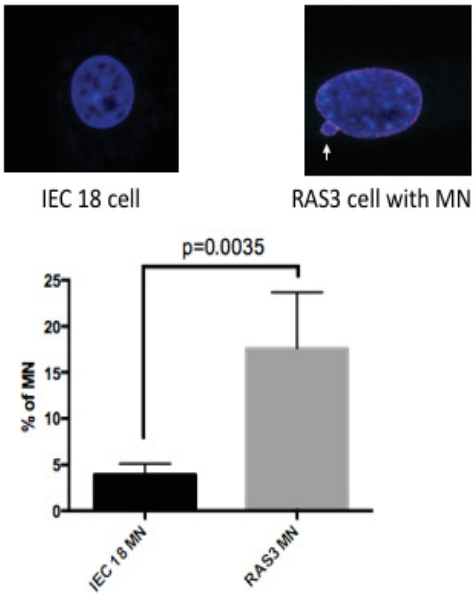


Figure 4.9. Comparison of micronuclei content of RAS3 cells and their non-transformed IEC-18 counterparts.

Notably, preliminary spectral karyotyping (SKY) experiments conducted in the laboratory of Dr. Sabine Mai at the University of Manitoba by a member of our laboratory detected a non-random distribution of chromosomal sequences in MNs of RAS3 cells. These results suggested the specific accumulation of chromosomes 1 and 2 (~40%) in these MNs (Chennakrishnaiah et al, unpublished data). SKY is a multicolour fluorescence chromosome painting technique, in which all 23 pairs of chromosomes are stained simultaneously with multiple probes resulting in unique colour combinations (Bayani and Squire, 2001; Imataka and Arisaka, 2012). SKY has been extensively used in cancer cytogenetics and offers detailed information about abnormal chromosomes (Bayani and Squire, 2001; Imataka and Arisaka, 2012). To further confirm the SKY results, we performed the FISH technique to label chromosomes 1 and 2 in RAS3 cells and their MNs. In keeping with previous observations, we observed high numbers of MNs containing chromosomes 1 and 2 (Figure 4.10). While it is not clear why chromosomes 1 and 2 are selectively enriched, it is possible that exogenously introduced H-*RAS* oncogene may be involved in driving this preferential packaging by being integrated into these two largest chromosomes. In contrast, we did not observe any specific chromosomes in rare MNs of indolent IEC-18 cells. In summary, we confirmed our previous findings using the FISH assay to show that chromosomes 1 and 2 preferentially enter the MNs of RAS3 cells.

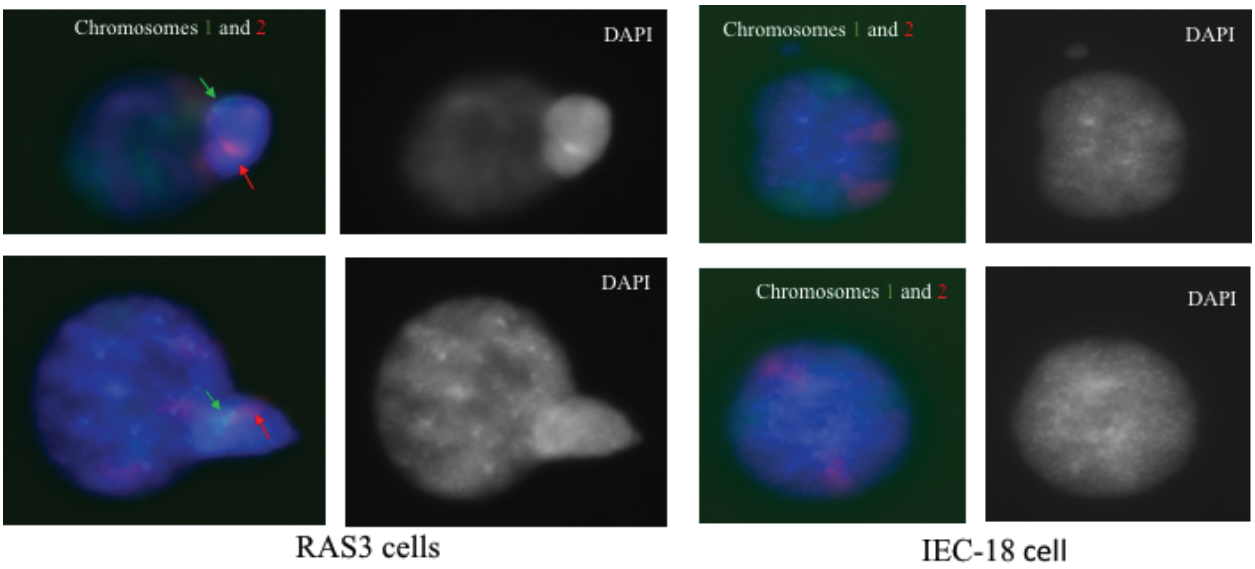


Figure 4.10. FISH assay confirms the enrichment of chromosomes 1 and 2 in micronuclei of RAS3 cells but no specific chromosomes are observed in IEC-18 micronuclei.

Translocations observed in chromosomes 1 and 2 of H-RAS transformed cells as a plausible contributing factor to non-random micronuclei composition.

We attempted to investigate the plausible reasons why chromosomes 1 and 2 are enriched in MNs of RAS3 cells. We reasoned that aneuploidy, an imbalance in chromosome numbers which is a frequent occurrence in cancer, is caused by an increased rate of chromosomal instability (van Jaarsveld and Kops, 2016). The RAS signalling pathway is known to be involved in promoting cell proliferation and genomic instability (Kamata and Pritchard, 2011; Maleki and Röcken, 2017) (Denko et al., 1994). Indeed, *RAS* oncogenes induce chromosome missegregation and additional defects in *TP53* and other genes which may result in the survival and expansion of aneuploid cancer cells (Kamata and Pritchard, 2011). Mutations in *RAS* genes (H-*RAS*, K-*RAS*, N-*RAS*) in human cancer can lead to chromosome rearrangement such as deletions, duplications and translocations (Thompson and Compton, 2011a; Orr and Compton, 2013). For example, expression of mutant N-*RAS* in certain cell types may track with translocations between chromosomes 6 and 5, and gains of chromosomes 10 and 17 (Potapova and Gorbsky, 2017). The *RAS* mutation also leads to improper chromosome separation during mitosis. A defect in mitosis is due to several reasons such as kinetochore malfunctions, merotelic kinetochore attachments, faulty sister chromatid separation, and centrosome amplification (Maleki and Röcken, 2017).

Thus, we surmised that oncogenic H-*RAS* expressed in RAS3 cells may lead to chromosomal instability and/or mitotic errors resulting in chromosome 1 and 2 aberrations and their subsequent packaging in MNs. We have already documented tripolar mitoses occurring in these cells with extraordinary frequency (Figure 4.3). To address this hypothesis further, we performed FISH on metaphase spreads of RAS3 cells using probes designed to label chromosomes 1 and 2. Surprisingly, we observed translocations of chromosomes 1 and 2 (Figures 4.11 and 4.12) in 100% of RAS3 cells, while only 40% of IEC-18 cells showed translocations of these two chromosomes (Table 4.1). Chromosomal translocations are associated with aneuploidy and cancer (Kamata and Pritchard, 2011). Moreover, we also observed aneuploidy in RAS3 cells (data not shown). Altogether, our data suggest that cellular chromosomal translocation driven by H-*RAS* oncogene may contribute to the enrichment of chromosomes 1 and 2 in micronuclei of RAS3 cells.

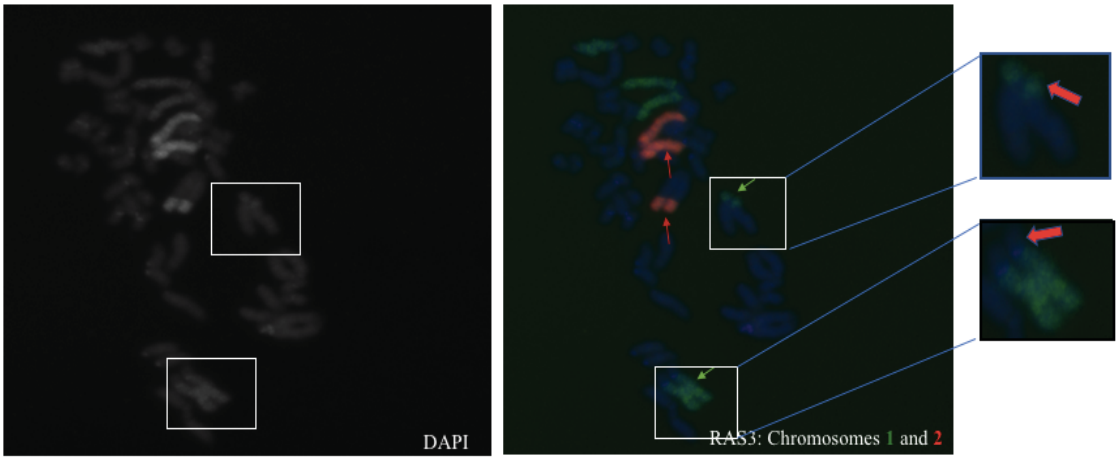


Figure 4.11. Metaphase spread of RAS3 visualizes the translocation of chromosomes 1 and 2. FISH signals of Chromosomes 1 and 2 are displayed in green and red, respectively.

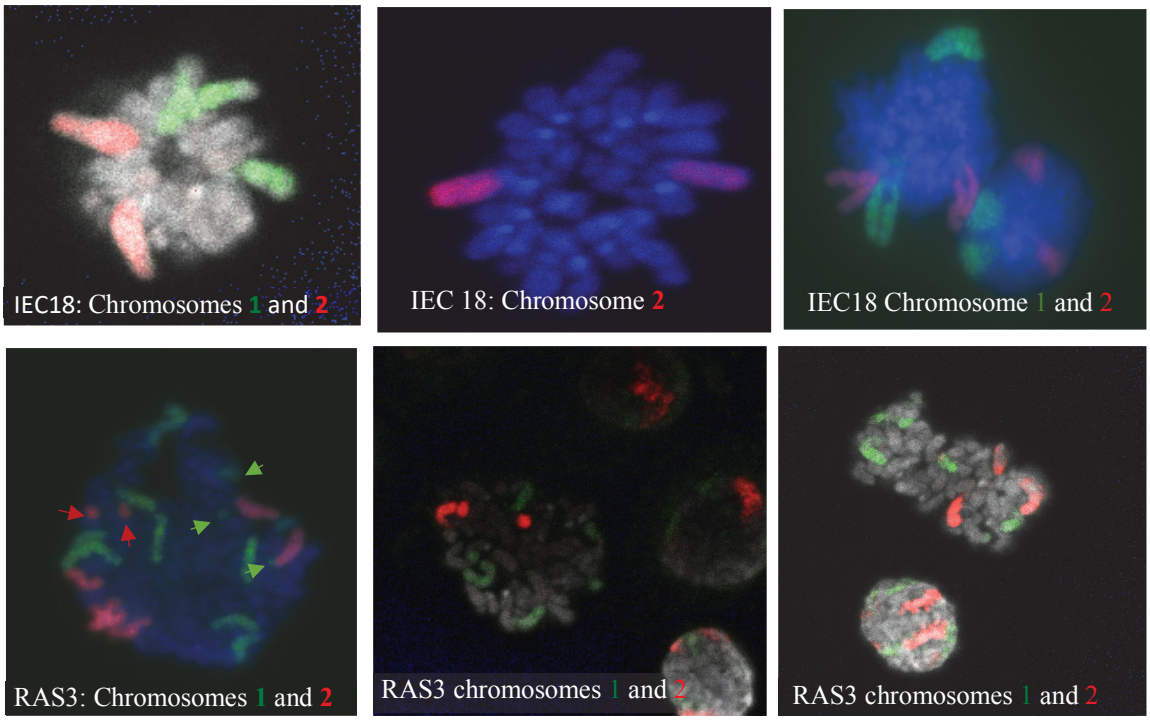


Figure 4.12. Translocation of chromosomes 1 and 2 (arrows) is specific to RAS3 cells and absent in the majority of their non-transformed IEC-18 counterparts.

Cell lines	Translocation of chromosomes 1 and 2	Normal chromosomes 1 and 2	Total cells
RAS3	33	-	33
IEC-18	10	25	35

Table 4.1. FISH assay on RAS3 and IEC-18 metaphase spreads shows translocation of chromosomes 1 and 2.

Active DNA synthesis in micronuclei of RAS3 cells

The fate of MNs depends on their content and nature (Utani et al., 2010; Hintzsche et al., 2017). DNA in MNs undergoes replication when enclosed with a nuclear membrane. However, micronuclei without a nuclear membrane are inactive and undergo degradation (Thompson and Compton, 2011b; Okamoto et al., 2012). We queried whether or not MNs in our RAS3 model cells undergo DNA synthesis. This was accomplished using BrdU (5-Bromo-2´-Deoxyuridine) labelling visualized with the fluorescent confocal microscope and electron microscopy (TEM). BrdU is incorporated into DNA of actively dividing cells and can be detected with an anti-BrdU

antibody. To take advantage of this property RAS3 cells were grown in media containing BrdU and the cells were fixed with 4% paraformaldehyde and labeled with the antibodies anti-BrdU (to detect newly synthesized DNA), and anti-lamin b1 (to detect nuclear envelope). The samples were also counterstained with DAPI to visualise the nuclei. In the confocal microscope, we observed incorporation of BrdU into both nuclei and MNs of RAS3 cells as well as some staining for nuclear envelope marker, lamin b1, suggesting an active DNA synthesis in MNs and their possibly intact nuclear membrane (Figure 4.13).

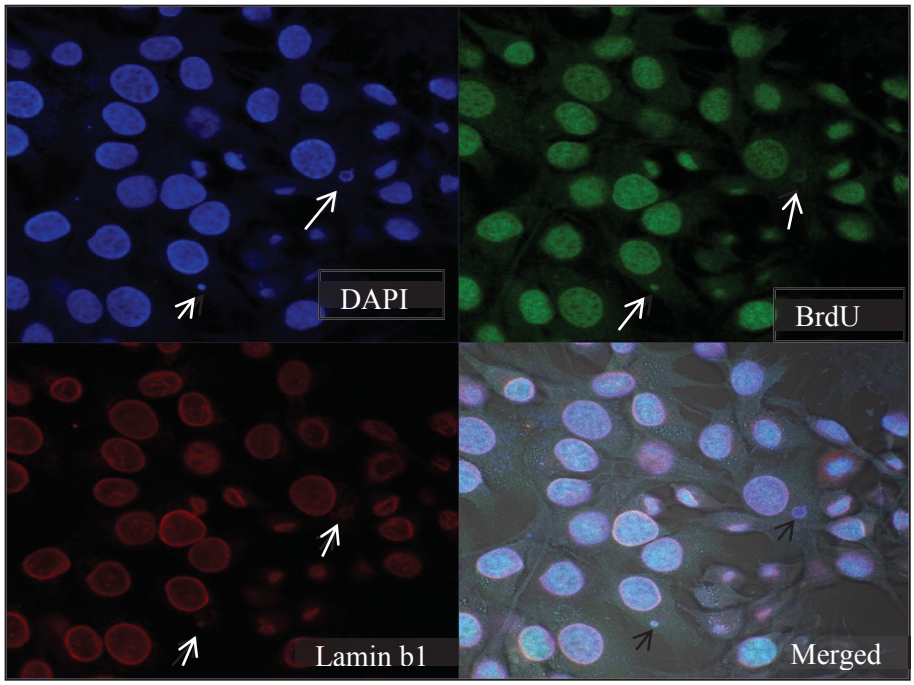
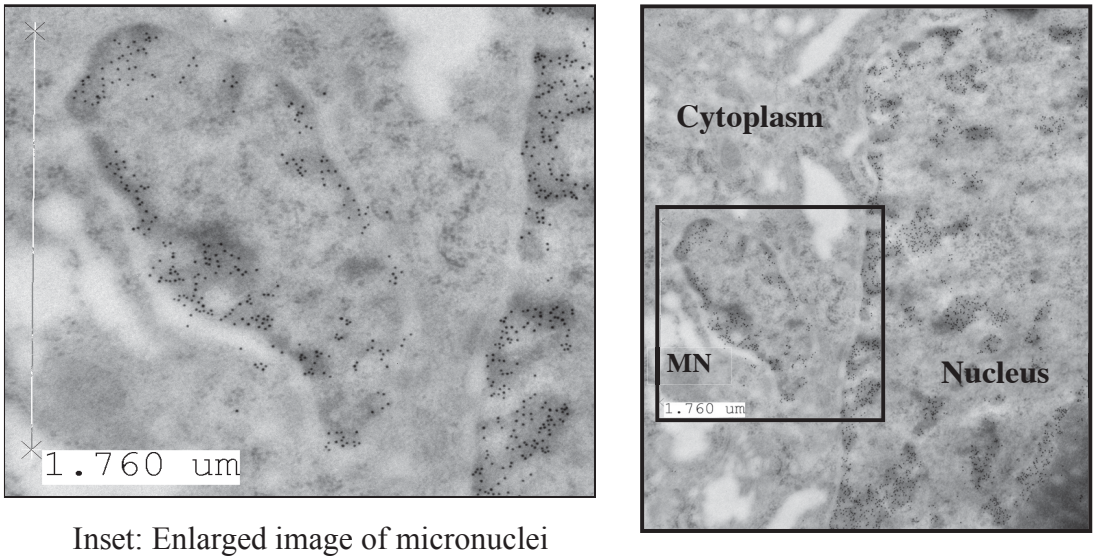


Figure 4.13. Confocal images of RAS3 micronuclei to visualize BrdU and lamin b1 staining.

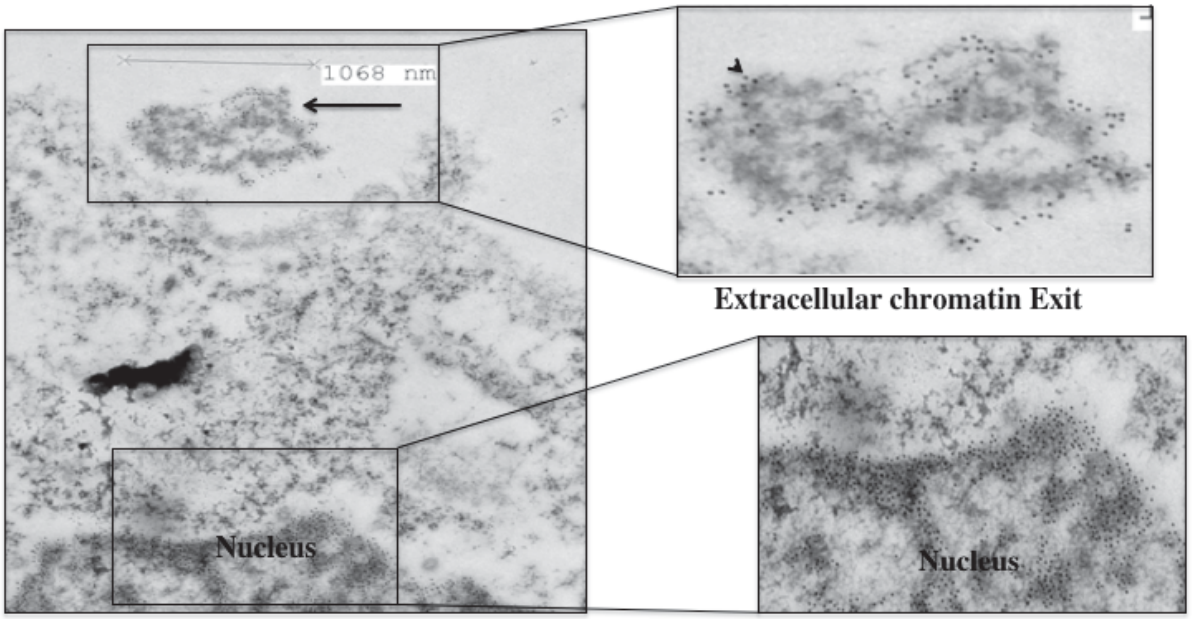
TEM imaging of cells stained with immunogold-labelled antibodies confirmed BrdU positivity of MNs associated with RAS3 cells (Figure 4.14). Briefly, the cells were processed for LRWhite sections and labelled with anti-BrdU primary antibody and secondary antibody conjugated with 10nm gold particles. The latter are detected as dark spots in both the nucleus and MNs of RAS3 cells, suggesting incorporation of BrdU during DNA synthesis (Figure 4.14). To our knowledge this technique was performed for the first time for MN detection. This immunogold technique can be used in the future to track MNs in RAS3 cells. Using this approach, we also observed extracellular MN-like structures (exo-micronuclei) adjacent to COLO 320DM cells stained with immunogold-labelled antibody against another chromatin-related antigen, histone H3 (Figure

4.15). In addition, we observed that all RAS3 cells contain cytoplasmic chromatin when labeled with dsDNA marker and histone antibodies (Figure 4.19, Figure 4.20, Figure 4.21), whereas only a few IEC-18 cells contain cytoplasmic chromatin (Figure 4.16, Figure 4.17, Figure 4.18). Furthermore, JuLI Stage live cell imaging validated the persistence of MN for at least one cell cycle (data not shown). Overall, our data suggest that MNs, which maintain intact nuclear membrane undergo active DNA synthesis or form from newly synthesised DNA following S phase of the cell cycle.



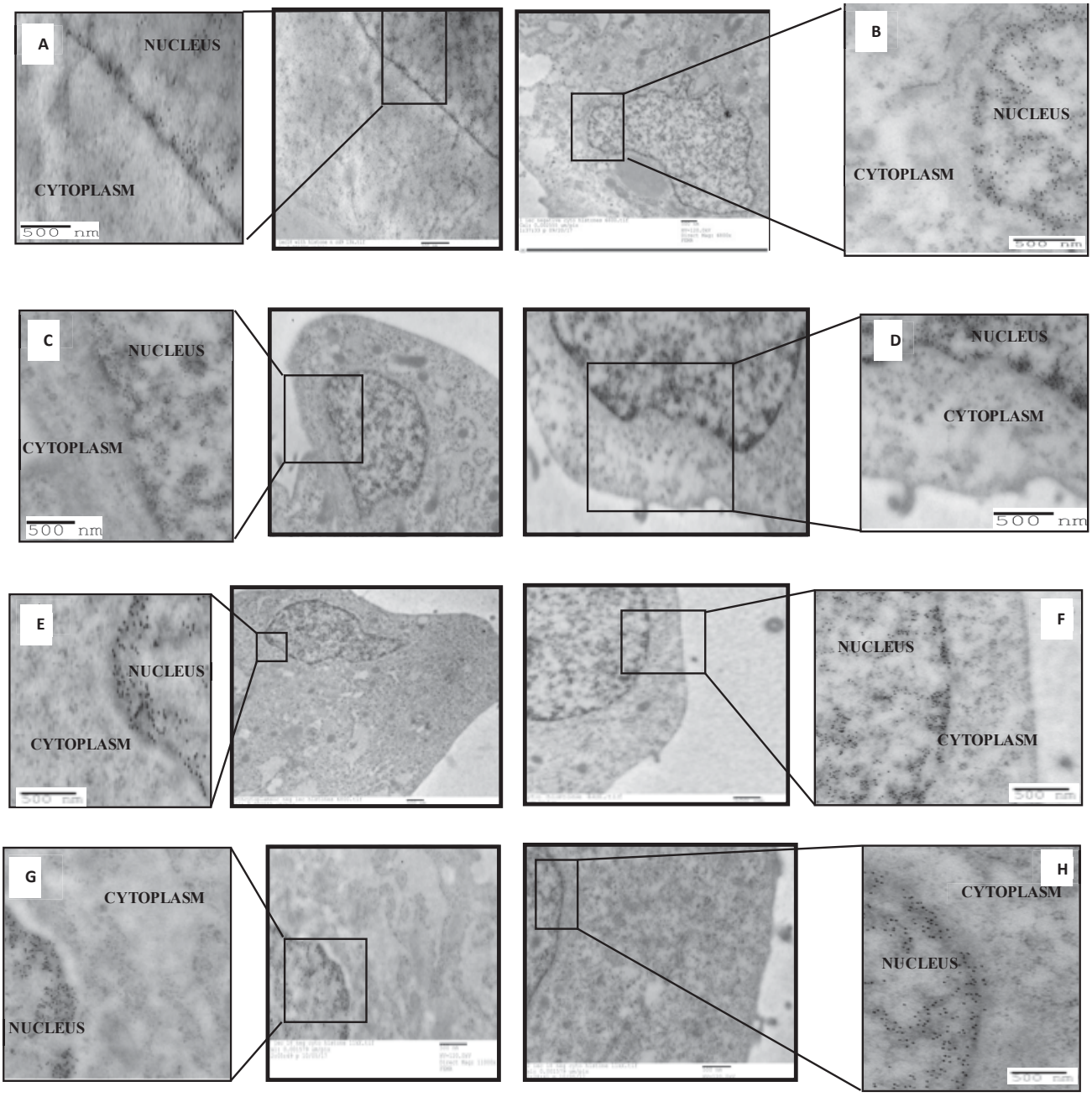
Inset: Enlarged image of micronuclei

Figure 4.14. TEM images of RAS3 cells. Nucleus and micronuclei labeled with BrdU and secondary antibody conjugated with gold particles (10nm). Magnified 6800X. Gold particles are detected as individual dark spots and can be seen in both nucleus and micronuclei. Scale bar indicates 1.760μm.



TEM images labelled with primary antibody (H3 total Histones) conjugated with secondary antibody (10nm gold)

Figure 4.15. TEM image of COLO 320DM cells immunogold stained for extranuclear chromatin. Scale bar indicates 1068nm**.**


A
NUCLEUS
CYTOPLASM
500 nm
B
NUCLEUS
CYTOPLASM
500 nm
C
NUCLEUS
CYTOPLASM
500 nm
D
NUCLEUS
CYTOPLASM
500 nm
E
NUCLEUS
CYTOPLASM
500 nm
F
NUCLEUS
CYTOPLASM
500 nm
G
CYTOPLASM
NUCLEUS
H
CYTOPLASM
NUCLEUS
500 nm

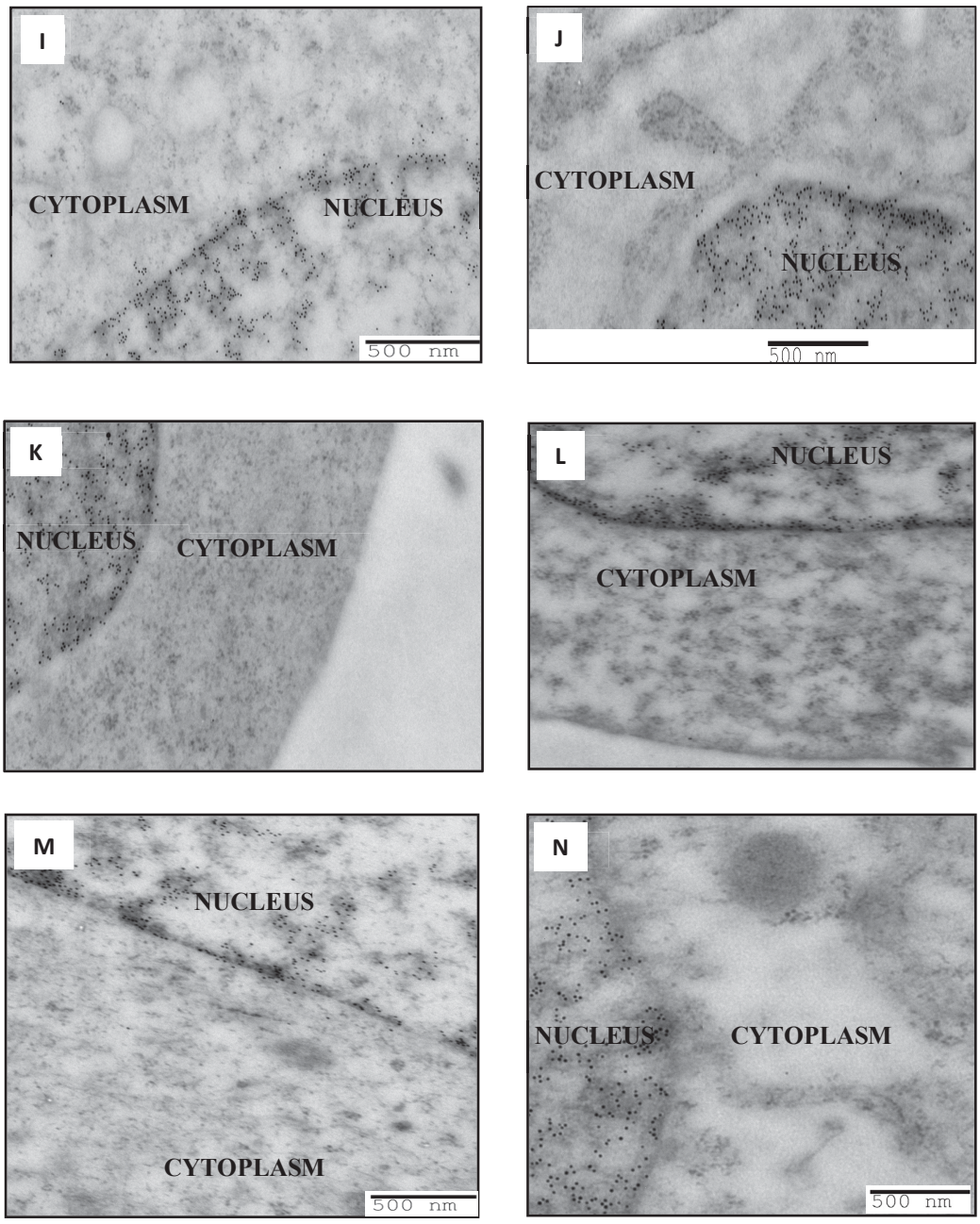


Figure 4.16. TEM images (A-N) of IEC-18 cells without cytoplasmic histones labelled with antibody against H3 total histones. (secondary antibody conjugated with gold 10nm). Scale bars indicate 500nm. Figure inset (enlarged images) show the cytoplasm without immunogold stain.

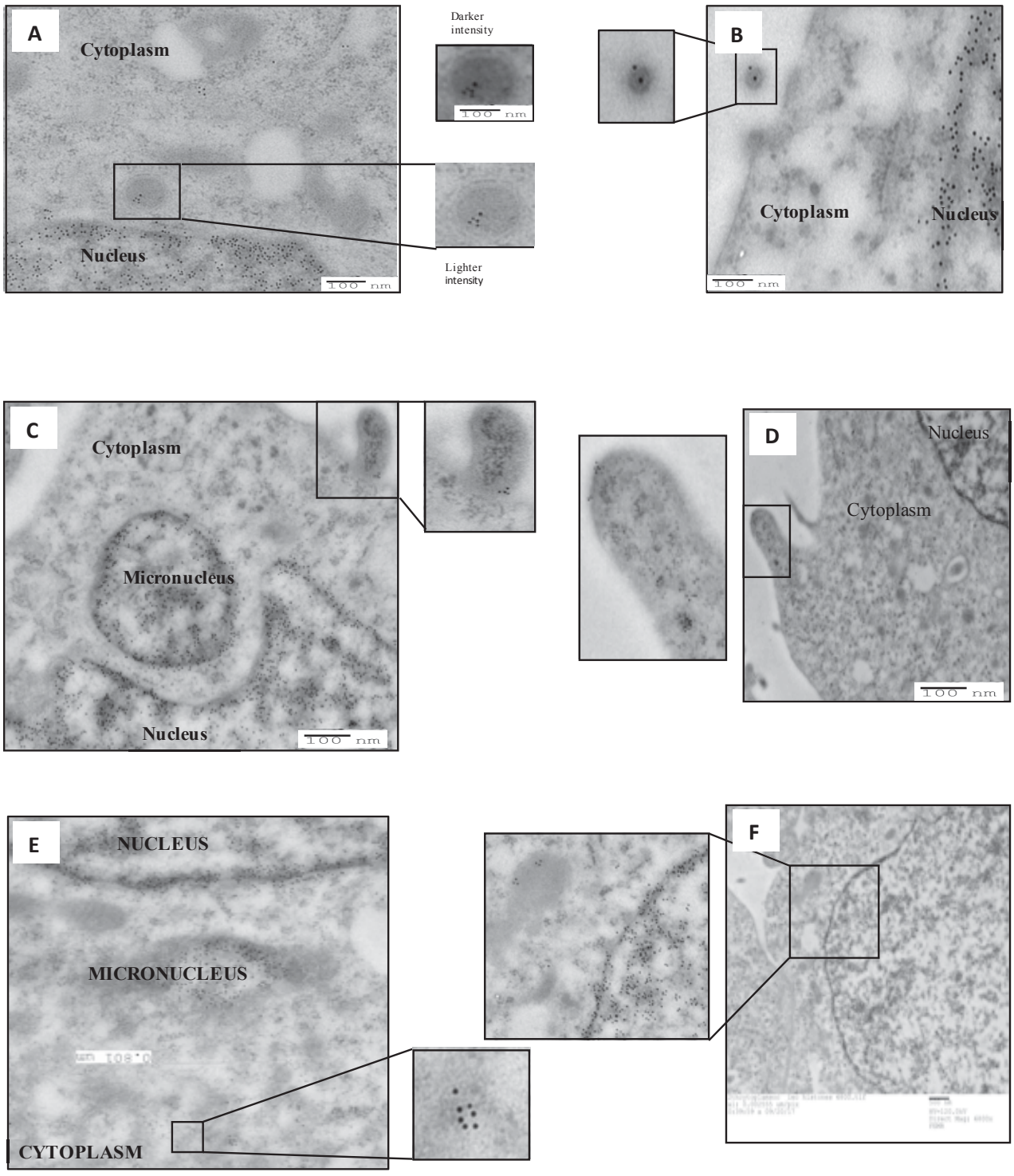


Figure 4.17. TEM images (A-F) of IEC-18 cells with cytoplasmic histones labelled with anti-Histone H3 total histone antibody. (secondary antibody was conjugated with gold particles - 10nm). Figure inset (zoom images) shows the presence of cytoplasmic histones (chromatin) inside IEC-18 cells.

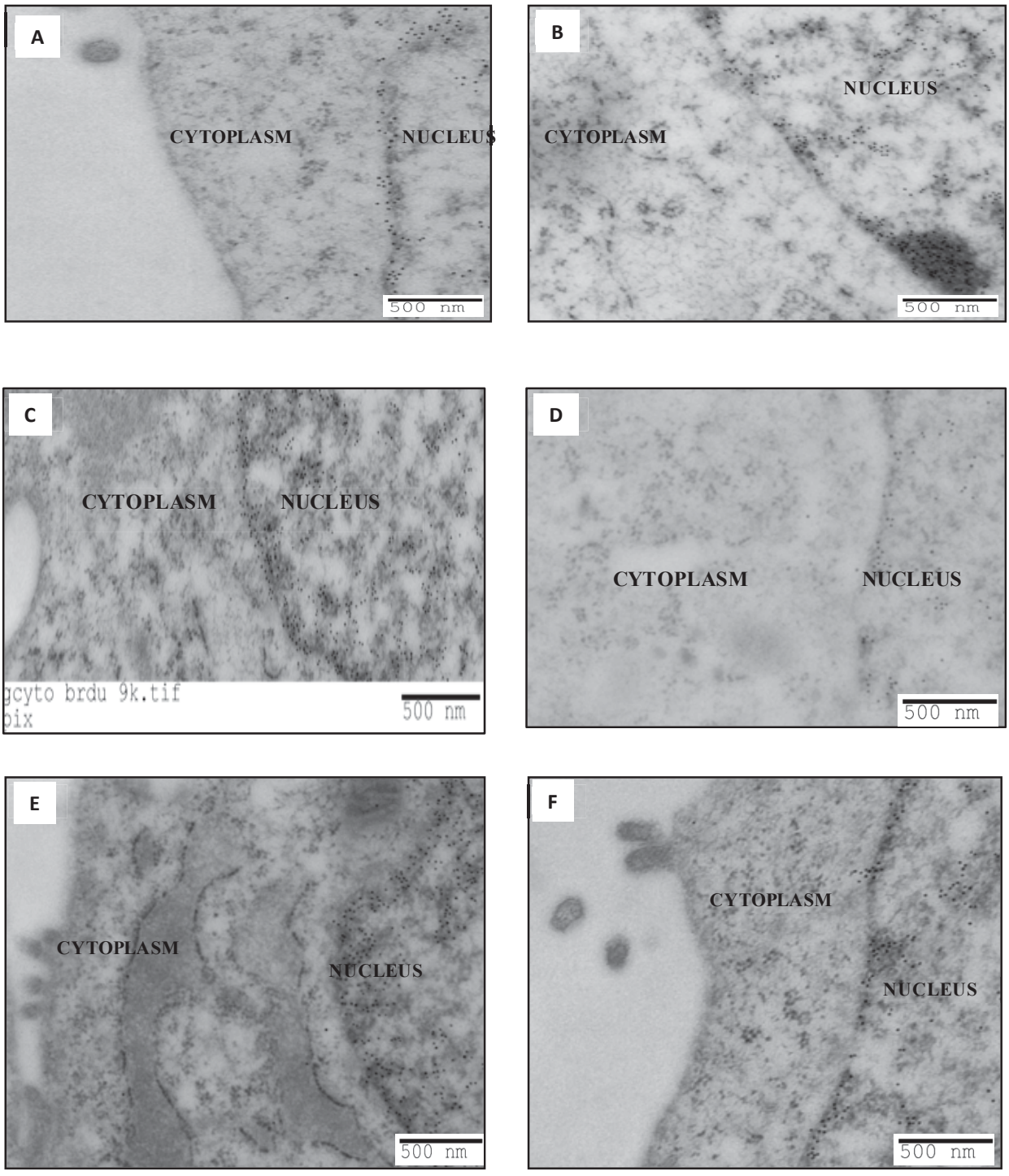


Figure 4.18. TEM images (A-F) of IEC-18 cells with no cytoplasmic staining with anti-BrdU antibody. (secondary antibody with gold 10nm). Scale bars indicate 500nm.

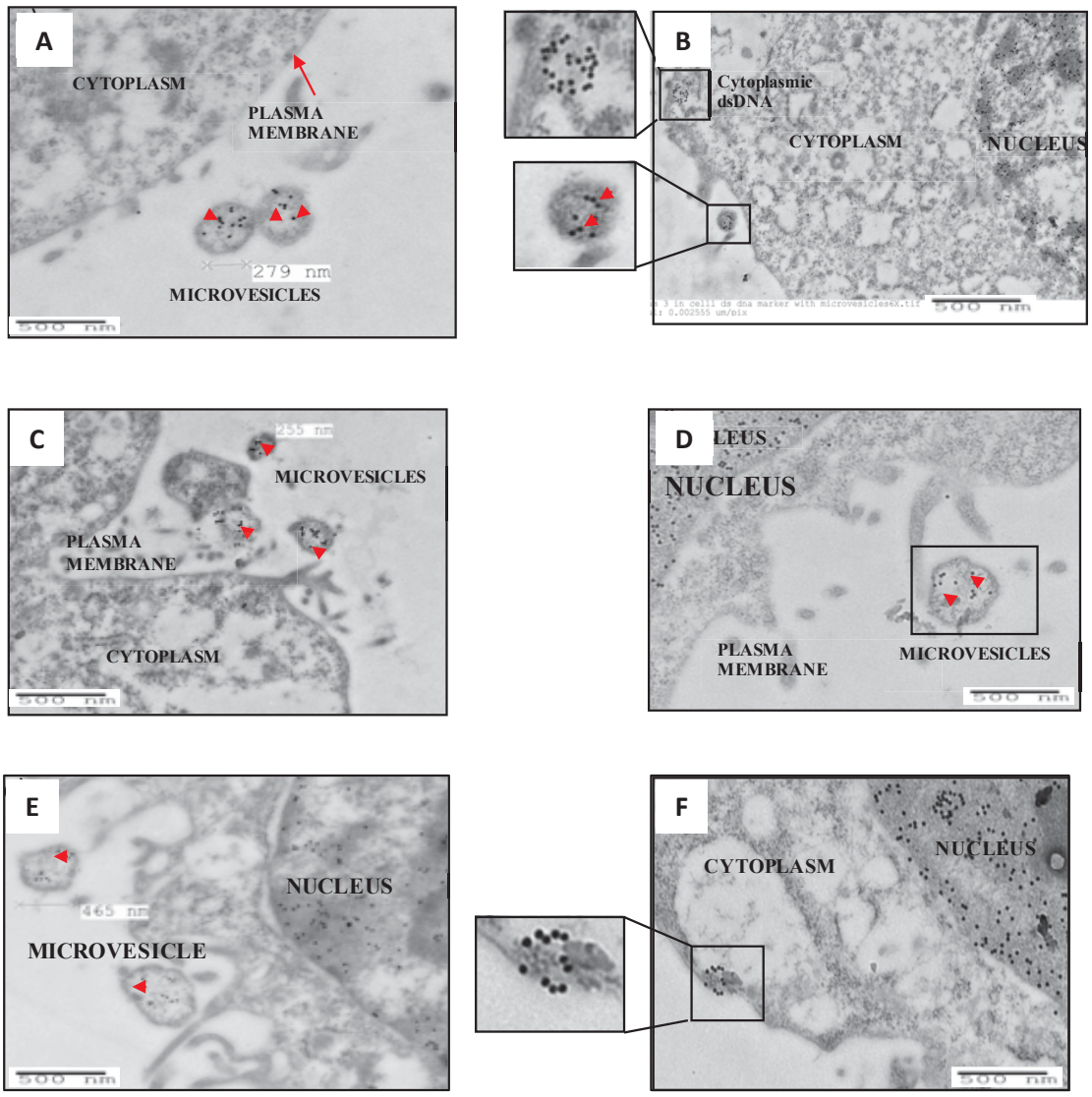
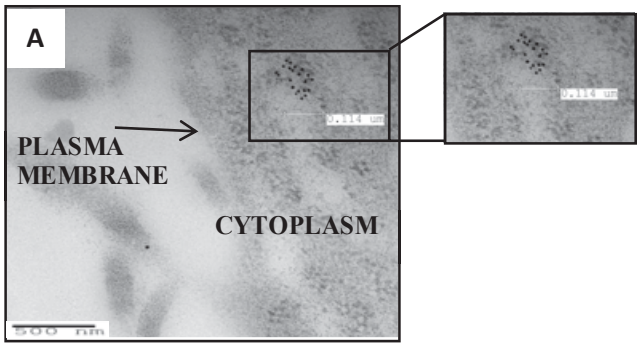
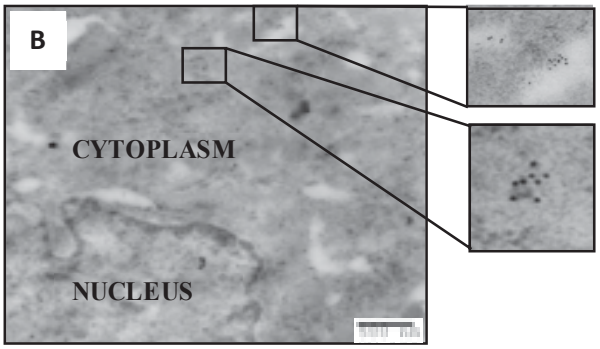
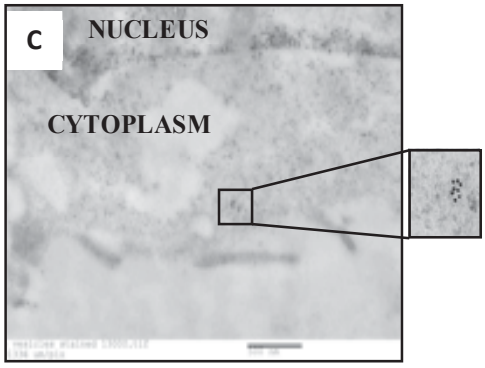
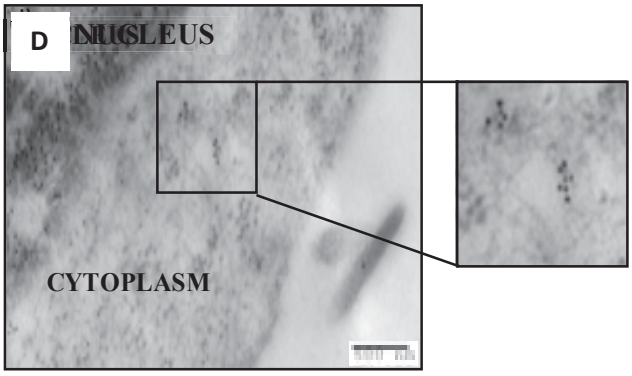


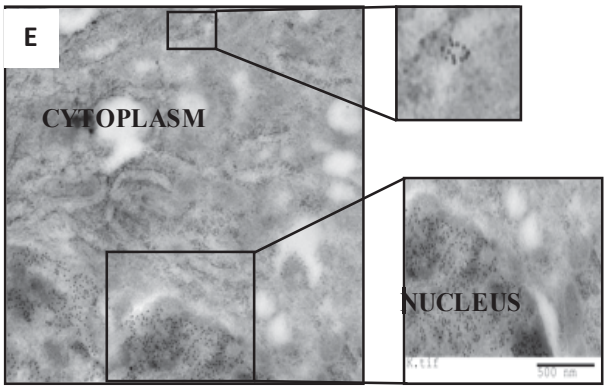
Figure 4.19. TEM images (A-F) of RAS3 cells with microvesicle-like structures stained for ds-DNA marker. (secondary antibody with gold 20nm). Scale bar indicates 500nm.

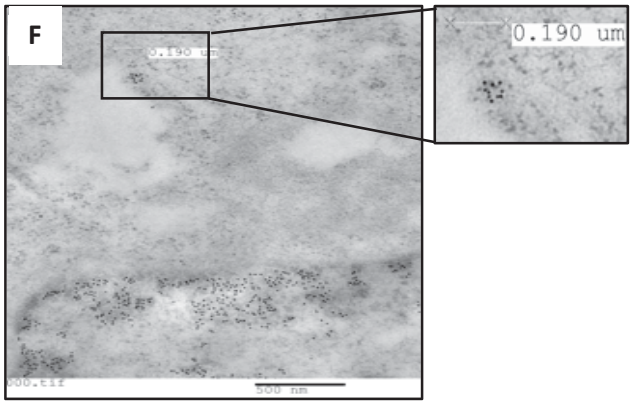

A
PLASMA
MEMBRANE
CYTOPLASM


B
CYTOPLASM
NUCLEUS


C
NUCLEUS
CYTOPLASM


D
CYTOPLASM


E
CYTOPLASM
NUCLEUS


F
0.190 um

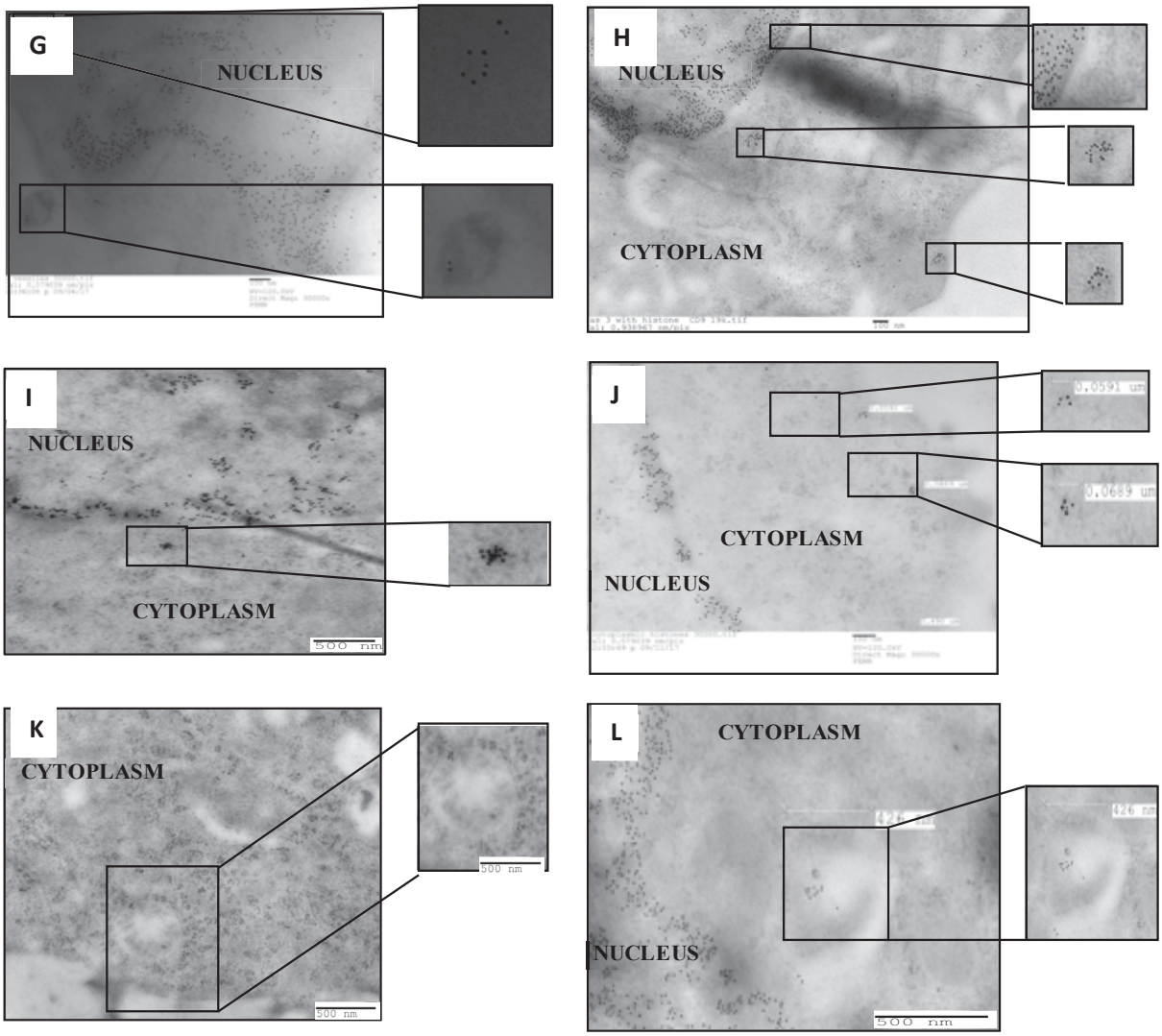


Figure 4.20. TEM images (A-L) of RAS3 cells with cytoplasmic histones visualized using anti-Histone H3 total Histone antibody. (secondary antibody conjugated with gold particles - 10nm). Figures inset (zoom images) shows the presence of cytoplasmic histones stained with primary antibody (anti-histones) and secondary antibody (immunogold- 10nm). Scale bar indicates 500nm.

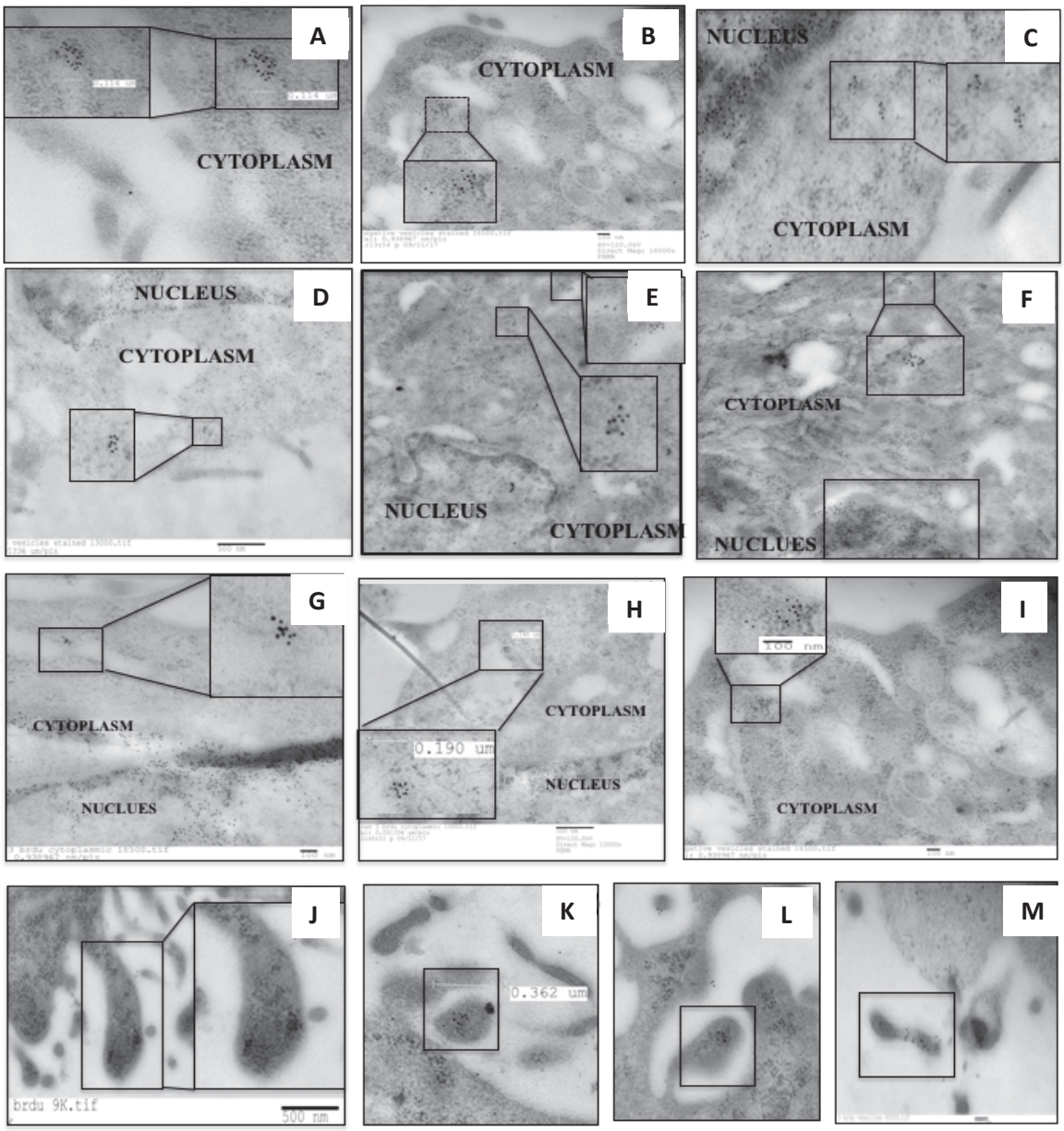


Figure 4.21. TEM images (A-M) of RAS3 cells with cytoplasmic BrdU signal.
Cytoplasmic DNA stained with 10nm gold particle-conjugated anti BrdU antibody. Figure inset (enlarged images) shows the presence of BrdU (newly synthesized DNA) in the cytoplasm. Scale bar indicates 500nm.

Micronuclei account for a minor part of the extracellular DNA released from RAS-driven cells.

Finally, we employed the aforementioned filtration protocol to quantitatively assess the contribution of MNs and other particles to the content of extracellular gDNA. Thus, conditioned media of RAS3 cells was collected and fractionated as described earlier and the flow through of the 0.22 μm filter was further separated by ultracentrifugation into EVs (pellet) and soluble DNA (supernatant). Surprisingly, we did not detect significant DNA content in unfiltered materials retained by all three filter pore sizes (3 μm, 1 μm and 0.22 μm), which should have trapped even the smallest MNs and large EVs, but we found DNA in the small EV pellet after 0.22 μm filtration. Traces of this material containing mutant H-*RAS* sequences were also found in the soluble supernatant, probably as a result of release from small EVs or their incomplete sedimentation. These results are consistent with earlier reports from our laboratory where differential centrifugation of RAS3 conditioned media resulted in the strongest chromatin signal in a fraction of small EVs (P4) (Lee et al., 2014). We further confirmed these results using ddPCR with the H-*RAS* gene (Figure 4.22) and the Agilent 2100 Bioanalyzer (Supplementary Figure 2), which is a more sensitive method of detecting dsDNA than fluorometric quantification such as Qubit. Altogether, our results show that there was virtually no global MN contribution from RAS3 cells to extracellular gDNA.

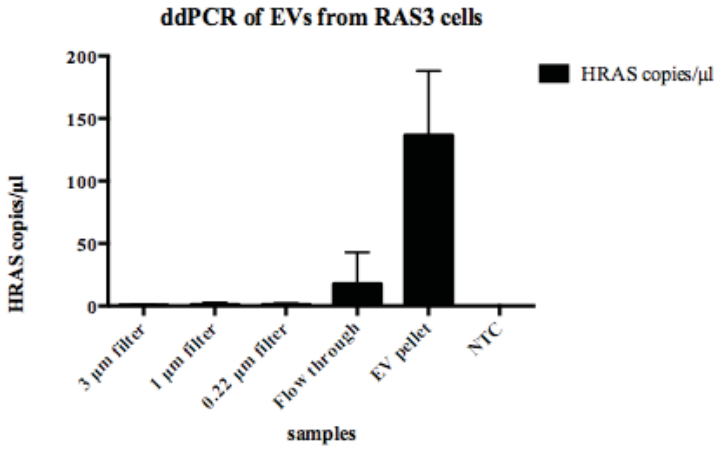


Figure 4.22. Droplet digital PCR (ddPCR) documenting preponderance of extracellular DNA in the EV pellet. EV fraction of serial filtration experiment exhibits the highest copy numbers of the H-*RAS* gene. N=2.

Discussion – Oncogenic RAS Directs Extracellular DNA Emission to Small Vesicles and not to Micronuclei

Thus, our analysis of MN formation by *RAS*-driven cancer cells suggests that this process, while prominent, does not contribute in a measurable way to the ample release of gDNA from these cells to their surroundings. Indeed, MN formation may signify or parallel other alterations in the chromatin processing processes that occur in *RAS*-driven cells. While the presence of MNs is a good predictor of the ability of our cellular models to emit extracellular gDNA it is not tantamount to it and instead this material exits cancer cells as small particles passible through a 0.22μm filter and sedimentable under ultracentrifugation conditions normally used to isolate exosomes.

As will be discussed in-depth later, these results rule out MNs as a major factor in the pool of extracellular gDNA. Such conclusion is in line with the discrepancy between the obvious enrichment of MNs generated by RAS3 cells for chromosomes 1 and 2, and the lack of this enrichment in the whole genome sequence of the corresponding extracellular gDNA (Lee et al., 2014). Our results also mirror and extend earlier studies in our laboratory where differential centrifugation protocols were used to isolate RAS3 ectosomes (P2-3 fractions) and exosome-like EVs (P4 fraction). The analysis of H-*RAS* content, total gDNA content and the levels of chromatin in these fractions pointed to exosome-like vesicles as carriers of extracellular chromatin (Lee et al., 2014).

While informative, these results require positive confirmation that exosomes or exosome-like EVs indeed carry gDNA and further exploration of their properties. Moreover, the mechanisms of gDNA incorporation to EVs and their extracellular exit are of utmost interest as predictors of how this material may contribute to circulating cell-free DNA in cancer patients (Bardelli and Pantel, 2017). Finally, the influence of oncogenic transformation mediated by mutant *RAS, M*YC and other genes is of considerable importance as determinants of vesiculation processes, rates of gDNA emission from affected cancer cells, as well as biological activity of EV-related gDNA in cancer (Lee et al., 2016). Some of these questions will be explored in the remaining chapters of this thesis.

CHAPTER 5

Extracellular vesicles as mediators of cellular DNA emission by cancer cells driven by oncogenic *RAS*

Introduction - EVs as putative mediators of extracellular gDNA emission in cancer

EVs emitted from cells were once considered as cellular debris and artefacts (Lamichhane et al., 2015; Hromada et al., 2017) but the discovery of their multiple regulatory functions irreversibly changed this perception (Théry et al., 2009). In particular, finding oncogenic material in the EV cargo, including active oncoproteins (Al-Nedawi et al., 2008), transcripts, microRNA (Skog et al., 2008), and DNA (Holmgren et al., 1999, 2002; Bergsmedh et al., 2001; Kahlert et al., 2014; Lee et al., 2014), opened up a new area of research and became the focal point of interest amongst cancer research communities (Gámez-Valero et al., 2016; Hromada et al., 2017).

The seminal development in the search for EV function occurred in 1983 when Pan and Johnston from the Department of Biochemistry, McGill University showed for the first time that vesicles carrying transferrin receptors are emitted out as a necessary step during sheep reticulocyte maturation (Pan and Johnstone, 1983). This development also led to the establishment of the term "exosomes" for EVs studied in this context. In 1996, Raposo reported that the B-lymphocytes secrete MHC II positive exosomes as demonstrated through the use of cryo-immunogold electron microscopy (Raposo, 1996). EV research picked up an even higher pace when Ratajczak (Ratajczak et al., 2006) and Valadi (Valadi et al., 2007) published their seminal findings on exosomes containing mRNA and microRNA, with functional capacity to transfer these macromolecules to recipient cells and alter their function. EVs involved in these studies were heterogeneous and referred to as either microvesicles or exosomes. Since then extensive studies have been carried out on EVs and their ability to carry RNA (Crescitelli et al., 2013; Hill et al., 2013; Quesenberry et al., 2015; Berardocco et al., 2017; Kim et al., 2017), however there are still much fewer studies on other EV-associated nucleic acids, especially DNA (Lee et al., 2014; Thakur et al., 2014). Indeed, DNA in exosome preparations was once thought to be a sign of their impurity and contamination with cellular debris.

Studies conducted during the past 7 – 10 years conclusively established the ability of certain viable and intact cells to emit either mitochondrial or genomic DNA as cargo of EVs that in many instances resemble exosomes. It is still unclear how gDNA is entrapped in these EVs and released outside of the cells. Nevertheless, our laboratory and others have shown that EVs carrying DNA cover the whole genome sequence of the host cell line (Kahlert et al., 2014; Lee et al., 2014). In addition, we suggested that disruption of acidic sphingomyelinase (membrane budding regulator) and the p53/Rb (cell cycle regulator) pathways did not inhibit the emission of EVs carrying oncogenic DNA (Lee et al., 2014). Numerous reports have shown that EVs have been found in body fluids such as saliva (Palanisamy et al., 2010; Gallo et al., 2012; Ogawa et al., 2013), blood (Harding et al., 1984; Pan et al., 1985; Witwer et al., 2013), urine (Pisitkun et al., 2004; Dear et al., 2013), cerebrospinal fluid (CSF) (Marzesco, 2005; Street et al., 2012), breast milk (Lässer et al., 2011; Zhou et al., 2011), and amniotic fluid (Jean-Pierre et al., 2006; Keller et al., 2007; Asea et al., 2008). This is relevant because EVs themselves as well as their DNA content may constitute a unique biomarker and molecular diagnosis platform in cancer. In addition, it has been reported that EVs carry full mitochondrial genome and restore the impaired metabolism(Sansone et al., 2017).

EVs are highly heterogeneous (Crescitelli et al., 2013; Zijlstra and Di Vizio, 2018). Depending on their contents, EVs may perform numerous biological functions in both health and disease (Colombo et al., 2014). These functions rely on the unique ability of EVs to act as multiplex hubs of biological regulators, as carriers of cell-associated and otherwise non-secretable bioactive macromolecules, and/or as shuttles of molecules from the point of synthesis to the point of activity, often located away from the cell of origin. In doing so, EVs protect their cargo (e.g. RNA) from degradation, dilution and consumption, and may deliver their cargo to molecularly-defined addresses, such as specific target cells or extracellular matrix (ECM) sites (Hoshino et al., 2015).

Fundamentally, EVs perform three major cellular functions: (i) cell-autonomous removal of superfluous molecules from the cellular interior (‘dumping’); (ii) non-cell-autonomous transfer of molecules from donor to recipient cells, as a form of intercellular networking (communication); (iii) modification of acellular aspects of the microenvironment, for example by

delivery of proteolytic and other activities to ECM sites or by forming migratory tracks in tissues for collective cell movement (delivery) (Choi et al., 2017). The effects of these processes in complex physiological and pathological settings is increasingly well-documented. For example, EVs from breast milk may boost infant immune system (Zhou et al., 2011) and EVs in blood or platelet-derived EVs are involved in hemostasis and coagulation (Owens and Mackman, 2011). As mentioned earlier, reticulocyte maturation is dependent on EV-mediated dumping of transferrin receptors (Harding et al., 1983; Pan and Johnstone, 1983; Johnstone et al., 1987), and EVs in CSF protect cells against amyloid beta accumulation (An et al., 2013). EVs are also central to several key processes in cancer such as angiogenesis, metastasis and disease progression (Al-Nedawi et al., 2008; Peinado et al., 2012; Hoshino et al., 2015; Choi et al., 2017). The accessibility of EVs in body fluids may serve as important tools for biomarker development and their entry into fluid spaces may present considerable therapeutic opportunities (Pisitkun et al., 2004; Alvarez-Erviti et al., 2011; Sun et al., 2017; Zhang et al., 2017; Halvaei et al., 2018).

It is increasingly clear that EVs are highly heterogeneous and are generated through a number of biogenetic processes, each regulated in a distinct manner and resulting in different types and molecular repertoires of the resulting EVs. However, the nomenclature to describe this diversity and classify EVs has not been well-standardized (Lotvall et al., 2014). The commonly used terminology ‘EVs’ is largely based on generic features of known vesiculation pathways, physical properties and some molecular markers (Colombo et al., 2014). This convention usually distinguishes apoptotic bodies (ABs), large oncosomes (LOs), microvesicles (MVs)/ectosomes, exosomes, and exosome-like structures (Akers et al., 2013; Kowal et al., 2016; Zijlstra and Di Vizio, 2018). There are stark size differences between these EV classes in that ABs are usually larger than 1000 nm, MVs range between 150 and 1000nm, and exosomes are 30 – 150 nm in size. This is not an absolute criterion as small ABs could be generated by cancer cells treated with targeted agents (Montermini et al., 2015), while exosomes may occur as larger and smaller species, complemented by other particles such as exomers and probably many others (Zhang et al., 2018). Another distinction is that ABs generally come from cells that succumbed to apoptotic demise while all other EVs (especially exosomes and ectosomes) are actively released from life cells (Lee et al., 2011).

The contribution of different EV categories to the extracellular pool of gDNA is a matter of considerable debate (Kanada et al., 2015) and uncertainty as to the contact points between gDNA processing and EV biogenesis pathways. The most obvious connection can be drawn in the case of ABs which, by definition, harbour fragmented chromatin of their preceding cell. In addition, ABs carry cellular organelles with cytoplasm and nuclear membrane.

There could be different entry points of gDNA into different vesiculation pathways. Large oncosomes (1000-10,000 nm) exit cells as a result of abscission of plasma membrane blebs from which they originate (Di Vizio et al., 2009). Membrane blebbing process is also at the heart of MV formation where vesicles may directly bud from the plasma membrane as a result of different cellular signals leading to calcium fluxes and formation of phospholipid asymmetry sites owing to the action of acidic sphingomyelinmases, scramblase and other enzymes (Lee et al., 2011; Akers et al., 2013). The budding of MVs could be induced by stimulation of cellular signalling and the completion of this process results in the exposure of phosphatidylserine (PS) prior to and after release of these EVs via a myosin-dependent process (Abels and Breakefield, 2017) (Figure 5.1). Due to this cell-external mechanism of MV formation they are also often referred to as ectosomes. MV properties include a size range between 150nm to 2,000nm, molecular composition resembling the cell of origin, sedimentation at centrifugal forces between 10,000 and 20,000 x g and lack of exosome-specific markers (Kowal et al., 2016). MVs are highly heterogeneous and they carry lipids, proteins and nucleic acids (Lamichhane et al., 2015; Abels and Breakefield, 2016). In cells harbouring exogenous DNA, MVs may represent a preferential type of EVs whereby this material could be released (Kanada et al., 2015) but spontaneous packaging of DNA into MVs has also been described (Lazaro-Ibanez et al., 2014).

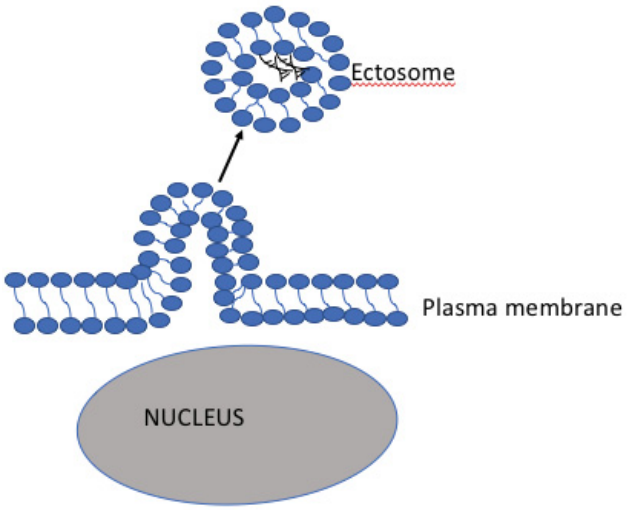


Figure 5.1. Ectosome emission from cells.
A diagram showing biogenesis of plasma membrane-derived vesicles (ectosome/microvesicle-like structures).

Exosome biogenesis is relatively well-studied among other EVs. Almost all cells release exosomes (Robbins and Morelli, 2014; Kadota et al., 2017). However, their numbers depend on cell type and stimulation. Exosomes are oval, membrane-enclosed and usually within the size range of 30-100 nm. In spite of this uniform appearance, exosomes are molecularly and functionally heterogeneous (Abels and Breakefield, 2016; Freitas et al., 2018). Their biogenesis is linked to the endocytic pathway, where ligation of cell surface receptors and their internalization (Tomas et al., 2014) leads to invagination of the plasma membrane and entry of the resulting vesicle into the early endosome, whose maturation to MVB may lead to secondary invagination to form intraluminal vesicles (ILV) from which exosomes ultimately originate (Wollert and Hurley, 2010; Akers et al., 2013; Witwer et al., 2013). The endosomal pathway is divided into three domains: early, late and recycle endosomes (Akers et al., 2013). Late endosomes are often tantamount to MVB, which serve to direct the ILV content to either the plasma membrane (exocytosis) or lysosomes (degradation). The fate of ILV bound to be released as exosomes depends on tetraspanins, such as CD63 (Pols and Klumperman, 2009) and CD9 (Akers et al., 2013), the overexpression of which stimulates cellular EV output. These two tetraspanins are also used as EV markers and have been employed in several exosome purification protocols. It should be noted however that they are not specific to bona fide MVB-derived exosomes and can be found on other types of EVs (Kowal et al., 2016).

Biogenesis of exosomes is well-controlled by both the state of the cell and the state of constituent macromolecules. For example, post-translational modification of macromolecules regulates their entry into exosomes, including: mono-ubiquitination, NEDD-ylation (Putz et al., 2012), phosphorylation (Montermini et al., 2015) and ISGylation of ESCRT-I (TSG101) (Villarroya-Beltri et al., 2016). Another mechanism of exosome emission is through ESCRT proteins such as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. ESCRT proteins are involved in exosome biogenesis by their recruitment to the site of intraluminal vesicles (Wollert and Hurley, 2010; Akers et al., 2013). ESCRT protein employment depends on phosphatidylinositol trisphosphate (PIP3) and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) expression. Indeed, phosphatidylinositol phosphate (PIP3) is enriched in early endosomes (Akers et al., 2013). ESCRT-0 identifies the presence of ubiquitinated proteins on the early endosome membrane (Abels and Breakefield, 2017) while ESCRT-I and II initiate the membrane budding and ESCRT-III finishes the budding process. Furthermore, ESCRT-III is recruited by Alix protein which is further bound to TSG101 (Bebelman et al., 2018). Although, there has been great progress in unravelling the mechanisms of EV biogenesis and cargo assembly, the exosome pathway is still under investigation and many questions remain. For example, inhibition of ESCRT proteins does not stop MVB formation. This shows the existence of another route of exosome biogenesis which is independent of ESCRT complex (Stuffers et al., 2009; Abels and Breakefield, 2017). How these processes intersect with mechanisms that control extranuclear transport, packaging and extracellular expulsion of gDNA is presently unknown and of great interest.

Results - Direct evidence for the presence of cellular DNA in cancer-derived EVs

In the previous chapter we have documented that it is not MNs but the EV fraction of the RAS3 cell conditioned media that contains the vast majority of the extracellular gDNA. This has been inferred from the PCR analysis and does not address questions surrounding the nature/properties of EVs carrying gDNA and whether the signal is localized in the EV lumen (an indication of active packaging) or on the surface (a possibly passive mode of gDNA transportation). While prior data from our laboratory suggest that external digestion of RAS3 EVs with exonuclease I did not remove their gDNA content, this content could have been protected by protein complexes.

To address some of these questions, we chose to employ electron microscopy to directly visualise gDNA and exosome marker proteins associated with RAS3 EVs. The pathways of EV emission are still under investigation and the molecular profiles of EV subtypes remain under discussion (Lotvall et al., 2014). Nonetheless, tetraspanins are a family of membrane proteins involved in a wide range of biological activities including cell signalling, adhesion, migration and fusion (Hemler, 2005; Levy and Shoham, 2005) as well as biogenesis of EVs (Zöller, 2009). In fact, tetraspanins such as CD9, CD63 and CD81 are used as classical markers of EVs to understand their emission (e.g. endocytosis mechanisms) and function (Colombo et al., 2014). While CD9 is not considered specific for exosome biogenesis (Kowal et al., 2016), a previous study (Chairoungdua et al., 2010) showed that CD9 knockout disrupts the exosome emission in bone marrow dendritic cells while overexpression promotes vesiculation. CD9 is ubiquitously expressed in a number of systems (Montermini et al., 2015; Kowal et al., 2016), including those where EV emission is upregulated by oncogenic transformation (Garnier et al., 2013). Therefore, we hypothesized that CD9 will likely be expressed at a high level in EVs from RAS3 cells. To this effect, we employed our TEM protocol with immunogold labelling against TSPAN9 (CD9) in EVs collected from both RAS3 and IEC18 cell lines. We observed that EVs derived from RAS3 cells were enriched for TSPAN9, whereas those collected from IEC-18 cells were fewer and scarcely labelled with the TSPAN9 antibody (Figure 5.2). This finding suggests that EVs emitted from RAS3 cells follow a different (RAS-dependent) biogenetic pathway as compared to IEC-18 EVs. This result was further confirmed via western blot and mass spectrometry by Dr. Choi, a postdoctoral fellow in our laboratory, a notion relevant to the possibility of the selective EV-mediated gDNA exit from cancer cells (Choi – unpublished).

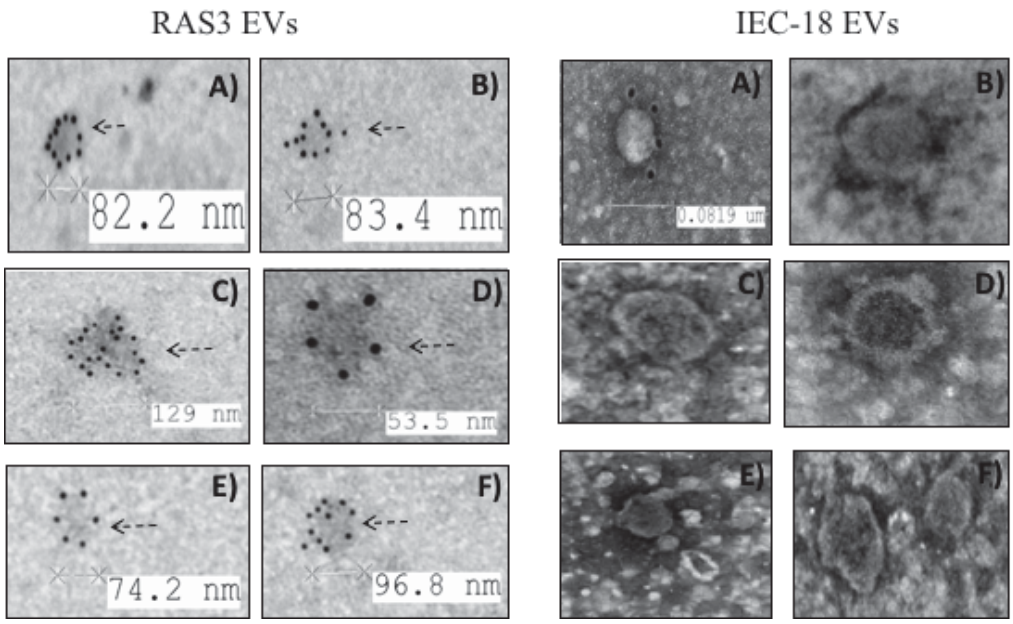


Figure 5.2. Expression of EV marker tetraspanin 9 (CD9) on single EVs.
(A-F) TEM of exosomes derived from RAS3 conditioned media was performed upon staining with anti-tetraspanin 9/CD9 antibody. Images document exosome-like EVs (size < 100nm) (Left panels). Exosome-like EVs derived from IEC-18 conditioned media and stained with anti-tetraspanin 9 (Right panels) exhibit different morphology staining and properties (contrast).

Our laboratory had previously reported the presence of dsDNA in EVs (Lee et al., 2014). To directly confirm the presence of dsDNA either within or on the surface of these structures, ultra-thin sections of EV pellets derived from RAS3 cells were labelled with primary anti-DNA antibody (dsDNA marker) and anti-TSPAN9 antibody followed by the respective gold-conjugated secondary antibodies with different sizes of gold particles (Figure 5.3). Only a few EVs were positively labeled with the dsDNA marker and TSPAN9 confirming our earlier observation (Lee et al., 2014) (Supplementary Figure 3) and a more recent finding that only a subset of RAS3 EVs carry gDNA, as revealed by Nano-flow cytometry (Choi et al – unpublished). Indeed, a low number of EVs staining positive for the dsDNA marker may be due to the heterogeneity of EV populations and selective encapsulation of dsDNA in some but not all EV subtypes (Thakur et al., 2014). This plausible specificity is currently under further study in our group.

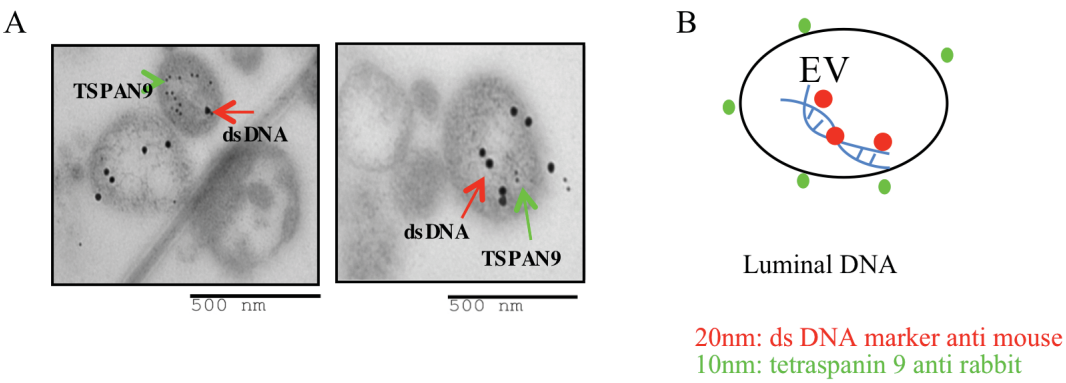


Figure 5.3. Double labelling of ultrathin sections of RAS3 EVs with immunogold.
(A) Ultrathin section of EV pellet labelled with TSPAN9 and dsDNA marker. (B) Schematic diagram of an EV containing DNA and surface labeled with dsDNA marker and tetraspanin 9, respectively.

In addition, I was able to show visually that dsDNA-positive EVs are emitted outside the cells. Studies conducted by Shelke et al. (Shelke et al., 2016), and other groups (Guescini et al., 2010; Thakur et al., 2014; Fischer et al., 2016) have suggested that DNA is on the surface of EVs while earlier studies from our group implied exclusively luminal location within EVs (Lee et al., 2014). Nonetheless, no visual evidence was reported to either effect. Our observations involving ultrathin sections suggest that at least a portion of gDNA is contained in the EV lumen (Fig. 5.3). However, when we used the whole mount preparation of EVs coupled with immunogold staining we observed that both the TSPAN9 and dsDNA markers were often co-localized and appeared on the surface of EVs (Figure 5.4). These observations require additional and more extensive confirmation to understand the prevailing pattern. Yet, even these limited findings raise several important questions regarding the gDNA elimination via the EV pathway. First, is the encapsulation of dsDNA/gDNA random or does it indicate that different biogenesis pathways are responsible for the formation of gDNA-containing and gDNA-free exosome-like EVs? Secondly, how does dsDNA interact with the surface of EVs in the cell microenvironment and what mechanism serves to emit such material outside the cells? Finally, these preliminary results would require extensive and independent morphological and biochemical confirmation. Once again, our earlier study suggests that treatment of intact RAS3-derived EVs with DNase does not remove the gDNA content from this material suggesting luminal or at least enzymatically inaccessible packaging of extracellular chromatin. My experiments expand our ability to formulate and interrogate these important questions at the single EV level.

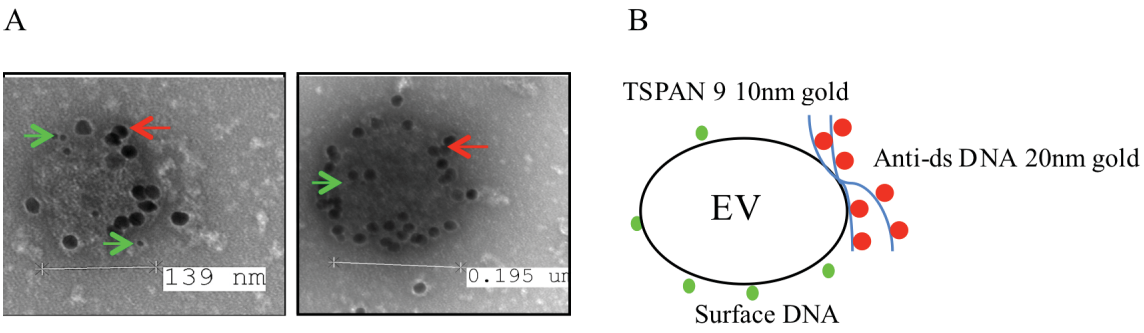


Figure 5.4. Double labelling of whole mount EVs.
(A) Whole mount of EV pellet labeled with TSPAN9 (green arrow) and dsDNA marker (red arrow). (B) Diagram of an EV containing DNA and surface TSPAN9/CD9 as labeled with dsDNA marker and TSPAN9 antibodies, respectively.

Discussion - Differential mechanisms of genomic DNA emission from cancerous cells, the roles of extracellular vesicles and micronuclei

Using JuLI Stage live cell imaging and TEM, we directly observed two different mechanisms of gDNA emission from RAS3 cells via MN (Figure 4.1) and through exosome-like EVs (Figure 3.1; 5.3; 5.4). We have also estimated the contribution of these various pathways to the overall level of extracellular gDNA, which points to EVs as a predominant mechanism (Fig. 4.25). These results represent an important validation and extension of our prior studies, and lead to similar conclusions.

Thus, we have previously shown that genomic DNA emission via the EV pathway covers the whole genome of RAS3 cells (Figure 5.5) (Lee et al., 2014) whereas MN from RAS3 cells encapsulates gDNA enriched for sequences of chromosomes 1 and 2 (Chennakrishnaiah, unpublished data). Interestingly, there were no apparent enrichment of specific sequences from chromosomes 1 and 2 in the EV genome of RAS3 cells (Lee et al., 2014). This is likely due to the fact that MNs are minor contributors to the pool of extracellular gDNA. However, additional circumstances may also need to be considered. For example, in our sequencing experiments we strove to eliminate any possibility of cell debris entering our extracellular gDNA preparation. To do so, we chose to use a 0.2 μm filter to prepare our EV isolates after initial differential centrifugation runs. In this setting, EV sizes larger than 0.2 μm (ABs, ectosomes/MVs, large

oncosomes/LOs, MNs etc.) are excluded from the sample possibly leading to the loss of chromosome 1 and 2 signal associated with MNs. However, when we used sequential filtration steps using 3μm, 1μm and 0.22μm to determine if larger EVs, including exo-MNs, were represented in the extracellular gDNA pool, we were unable to detect measurable amounts of such material in any other fractions than the 0.22μm filtrate (mainly EVs). Indeed, we did not detect measurable amounts of dsDNA (Qubit assay) on any of the filters (3μm, 1μm and 0.22μm). Furthermore, we used the Bioanalyzer to observe DNA in the EV pellet, but no DNA fragments were found on any of the filters (3μm, 1μm and 0.2μm), which would be expected if larger structures were trapped. This confirms that neither large ABs nor MNs significantly contribute to gDNA emission under control of oncogenic *RAS*. Instead, we found DNA in EV pellets after ultracentrifugation and Bioanalyzer QC analysis confirmed the presence of larger fragments of gDNA in the EV pellets (Supplementary Figure 2). In addition, as shown in chapter 4, we detected highest H-*RAS* signal in the EV fraction using the ddPCR (Figure 4.22) assay.

As a proof of principle, we also tested the efficacy of our filtration protocol in isolates of conditioned media of COLO 320DM cells. In this case we expected that MNs of these cells could be isolated on larger filters according to previous reports by Shimizu et al. (Shimizu et al., 2000). These investigators have already reported that COLO 320DM cells reduce their intrinsic tumourigenic capacity by emitting DMs containing highly amplified *MYC* genes in the form of extracellular MNs. Indeed, we did observe MNs on the 1 μm filter and we quantified DNA extracted from these samples (Qubit) to determine whether they correspond to MNs enriched for MYC using FISH (Figure 4.7 a and b) and ddPCR (Figure 5.6) techniques. These results were congruent with MYC being present in large extracellular structures, likely MNs, released by COLO 320DM cells. This shows that our filtration protocol was effective, and the results obtained in the case of RAS3 cells were not biased or artefactual. Moreover, similar filtration protocol to purify MNs has been used by other researchers (Mortimer and Hawthorne, 1975; Labidi et al., 2018). In summary, the results confirm that only EVs contribute to genomic DNA emission by RAS3 cells and not MNs. These observations raise important questions as to the mechanisms and processes that lead to formation of gDNA-containing EVs under the control of mutant H-*RAS*.

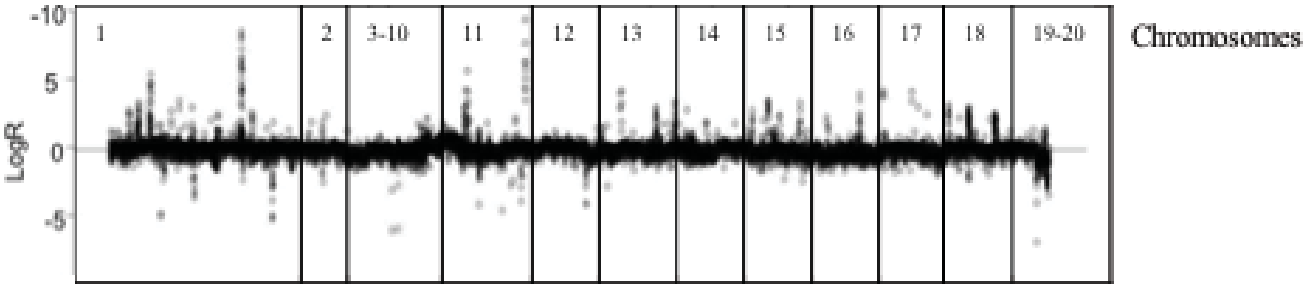


Figure 5.5. EVs derived from RAS3 contain whole genomic sequence.
Copy Number Variation (CNV) of EVs and whole genome of RAS3 cells. Notice that there is no enrichment of any chromosomes. Figure was adapted from Lee et al. (Lee et al., 2014).

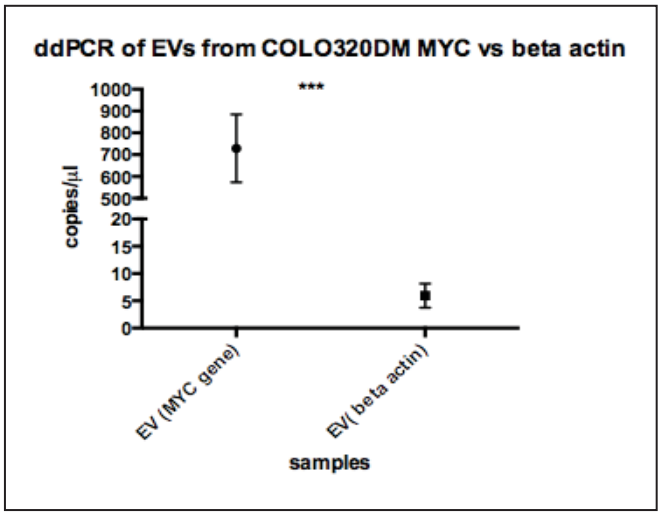


Figure 5.6. Droplet digital PCR analysis of gDNA of EVs isolated from COLO 320DM cells.
Droplet digital PCR results showing higher expression of *MYC* gene in EVs derived from COLO320DM cells compared to beta actin gene. (n=3 and *** P <0.0004)

CHAPTER 6

Putative mechanisms of extracellular vesicle mediated emission of genomic DNA: Aberrant DNA synthesis, apoptosis and autophagy

Introduction – Candidate Mechanisms of Genomic DNA Emission through the Vesiculation Pathway

Operationally, the EV-mediated emission of gDNA can result from abnormal DNA synthesis, formation of apoptotic bodies due to the death of a subset of cells or through recently described events involved in cytoplasmic chromatin processing by autophagy-related pathways regulated by *RAS* (Dou et al., 2015; Takahashi et al., 2017). The latter is intriguing as autophagy has already been linked to vesiculation pathways through interactions of Atg12 with EV proteins such as Alix and syntenin (Murrow et al., 2015).

The role of DNA synthesis and its link to cell cycle progression is an implicit factor to consider in the context of exosome-like EV-associated gDNA (exoDNA) emission. First, cancer cells, and RAS3 are no exception, are programmed to divide at high rate while they also produce exoDNA. If DNA synthesis was decoupled from, or lower than, the output of exoDNA from cells the integrity of the cellular genome would be expected to be progressively compromised leading to losses in genetic material and potentially cellular demise. This is not the case for RAS3 cells, which maintain their DNA content at a steady level, comparable, if not higher, than that of their non-transformed IEC-18 counterparts, which do not emit exoDNA. Second, we have already shown that at least one form of aberrant DNA, such as MNs, irrespectively of their contribution to exoDNA, participates in DNA synthesis. Thus, multiple anomalies in RAS3 chromatin maintenance, such as abnormal mitosis, chromosomal translocations, MNs and others occur in the presence of, with possible dependence on, a sustained and high rate of DNA synthesis. This leads to the question whether interference with this process in a form of cell cycle block, either natural or pharmacologically induced, would affect formation and release of exoDNA?

While RAS3 cells exhibit extreme resistance to cell death-inducing stimuli, whether biological (Rak et al., 1995) or mechanical (Lee et al., 2015), *RAS* and other oncogenes may activate both survival and death programs (Serrano et al., 1997; Dou et al., 2015). Therefore, it is possible that

while the RAS3 population exhibits close to 100% viability in cell culture, a marginal but sustained stochastic cellular demise may generate a population of ABs that may become admixed to our EV preparations. Although, ABs are usually regarded as larger structures between 1 and 2μm in diameter (György et al., 2011), we and others have documented the existence of exosome-sized apoptotic 'nano-bodies' induced in cancer cells by some targeted agents (Montermini et al., 2015). If any of these scenarios were applicable to exoDNA we would expect that inhibitors of apoptotic or other death pathways could reduce or eliminate gDNA from the RAS3 EV cargo.

As mentioned earlier the very existence of exoDNA suggest a process whereby this nuclear material would transit the cellular cytoplasm en route to extracellular space. This would require a breakdown of the nuclear containment and formation of the cytoplasmic pool of chromatin. Indeed, recent aforementioned studies suggest that *RAS* transformation upregulates elements of the autophagy pathway such as LC3 protein (Dou et al., 2015). In this setting, nuclear LC3 causes defects and degradation of the nuclear envelope, which leads to the entry of chromatin into the cellular cytoplasm and the onset of oncogene-induced senescence (Dou et al., 2015). This process is controlled by lysosomal degradation of cytoplasmic chromatin, but in primary cells expressing RAS this breakdown is incomplete and does not suffice to avert cell death. Nonetheless, the pathway delineated by these studies links autophagy, oncogene expression and cytoplasmic chromatin without asking whether this chromatin may also be a subject of EV-mediated export, an aspect central to this thesis project. If this LC3-Atg7-driven mechanism applies to RAS3 cells, a blockade of autophagy would be expected to deplete or reduce the level of exoDNA in their EV population. Conversely, upregulation of this pathway (e.g. by overexpression of LC3) in IEC-18 cells may provoke them to release exoDNA-containing EVs. These questions will be explored in the current chapter.

Results – Testing the role of DNA Synthesis, Apoptosis and Autophagy in the EV-mediated Release of DNA

Independence of EV-mediated Emission of gDNA from DNA Synthesis and Apoptosis

We surmised that one or more of the aforementioned processes (DNA synthesis, apoptosis or autophagy) are required for the biogenesis of exosome-like EVs containing exoDNA. It follows

that the respective pharmacological inhibitors (Mitomycin-C, ZVAD or Chloroquine) could reduce/eliminate gDNA content in the EV fraction of RAS3 cells.

To test this assumption RAS3 cells were first treated with Mitomycin-C (MMC), arrested in G1 phase (Figure 6.1) and analysed for H-*RAS* gene sequence emission (PCR) in their EVs. Using NTA/Nanosight measurements, we observed a higher concentration of EVs in conditioned media of MMC-treated RAS3 cells compared to untreated ones. PCR results showed the presence of H-*RAS* gene in both of these EV subsets (Figure 6.2). While this may suggest that gDNA emission by RAS3 cells is independent of DNA synthesis, we noticed that effective doses of MMC trigger cancer cell death as a function of both time and duration of the G1 arrest, suggesting a possible contribution of ABs to the EV preparation under these conditions. Moreover, this analysis suggested that protracted inhibition of DNA synthesis does not eliminate gDNA from EV preparations and thereby it is unlikely that increased DNA synthesis per se is the main reason for gDNA emission via EVs.

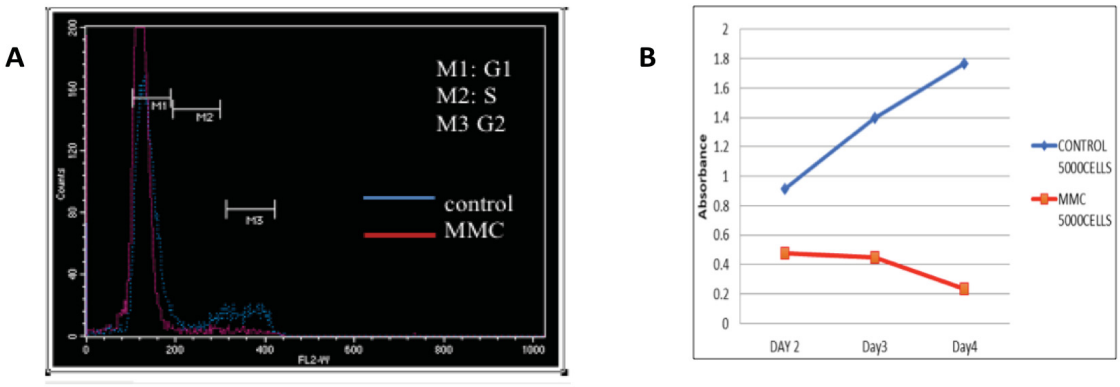


Figure 6.1. FACS data and MTS analysis of Mitomycin-C induced growth arrest in RAS3 cells. (A) Cell cycle analysis using flow cytometry and Propidium Iodide. RAS3 cell cycle arrest at G1 stage after MMC treatment. (B) MTS assay of control and treated RAS3 with MMC.

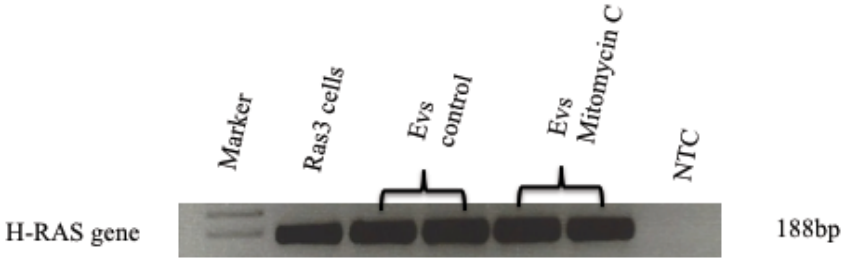


Figure 6.2. Emission of gDNA through EVs is independent of DNA synthesis and cell division. The genomic DNA of RAS3 cells, their derived EV controls and EVs from RAS3 cells treated with MMC were amplified using primers specific for H-*RAS* and PCR products (188bp) were separated on 2% agarose gel electrophoresis. NTC= Negative control.

We then reasoned that RAS3 cells could undergo spontaneous apoptosis at a low rate or that MMC treatment could induce an apoptotic process resulting in the contamination of EV preparations with ABs. It should be re-emphasized that RAS3 cells are highly viable during the whole cycle of EV preparation, but we cannot exclude that a small fraction of these cells undergoes death processes. While apoptotic bodies are considered to be larger than the majority of gDNA-containing EVs we observed that formation of exosome-size (< 220 nm) apoptotic bodies is readily demonstrable using signal transduction inhibitors in cancer cell cultures (Montermini et al., 2015). In view of these observations we explored the effects of a caspase inhibitor (ZVAD) on gDNA emission of MMC-treated and untreated RAS3 cells. Interestingly, ZVAD pre-treatment of EV donor cells did not eliminate gDNA from EV fractions of intact RAS3 cells and these cells were >90% viable (as judged by trypan blue exclusion). While MMC treatment reduced the viability of RAS3 cells to below 90%, ZVAD pre-treatment reduced somewhat but did not abolish gDNA content in EV isolates of these cells. These observations suggest that RAS3 cells are resistant to protracted, MMC-mediated growth arrest and, importantly, they emit gDNA-containing EVs in a manner independent of apoptotic cell death (possibly actively), at least insofar as this death process is amenable to inhibition by anti-caspase peptides (Figure 6.3).

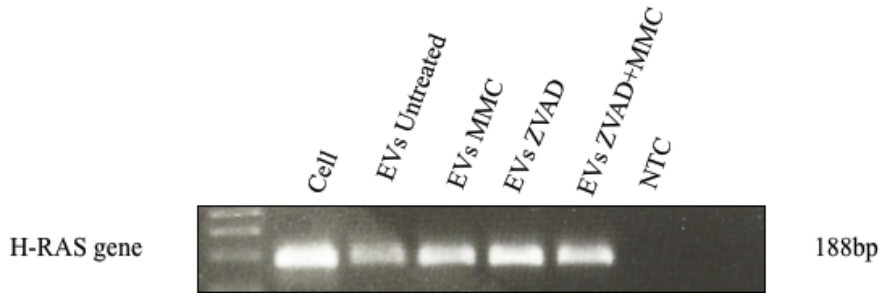


Figure 6.3. Emission of EVs containing genomic DNA is independent of apoptotic cell death. The genomic DNA isolates from RAS3 cells, their EVs from untreated cells and EVs of treated with ZVAD were amplified using H-*RAS* primer (to generate 188bp PCR product) and separated on 2% agarose gel electrophoresis. NTC= Negative control.

A Possible Role of Autophagy in Vesicular Emission of gDNA from RAS-transformed Cells

To explore the role of autophagy in formation of exoDNA by RAS3 cells, we initially used chloroquine. This agent is known as an anti-malaria drug (Slater, 1993), but also exerts a number of effects on eukaryotic cells. One of these activities is the ability of chloroquine to inhibit the autophagy process by accumulating in acidic lysosomes and increasing their pH (Loughran et al., 2013). While this effect may lead to apoptosis or necrosis, these are not normally observed in short-term experiments (Xiaoyan et al., 2012). Likewise, we did not observe any cell death in RAS3 cells treated with chloroquine, as judged by trypan blue assay and confocal microscopy results. In addition, chloroquine is known to disrupt the endocytic pathway, however, chloroquine with KCl (depolarization) treatment increases exosome emission (Yuyama et al., 2008).

As expected, following addition of chloroquine to cultures of RAS3 and IEC-18 cells, confocal microscopy revealed a build-up of large vacuoles in the cellular cytoplasm (Figure 6.4). In addition, nanoparticle tracking analysis (NTA) showed that chloroquine treatment increased RAS3 EV emission, compared to untreated RAS3 cells. Interestingly, H-*RAS* gene quantification using ddPCR suggested that exosome-like EVs emitted from chloroquine-treated RAS3 cells carried significantly less gDNA (H-*RAS* gene) in comparison to untreated controls (Figure 6.5A). We have also observed that overexpression of LC3 in IEC-18 cells led to an increase in their production of gDNA-containing EVs (data not shown).

Vesicular transport in the context of autophagy is complex and multicompartmental. In the process MVBs may fuse with autophagosomes and form amphisomes, which then may fuse with lysosome for degradation (Morvan et al., 2009; Papandreou and Tavernarakis, 2017). This may result in the reduction of exosome emission from cells under starvation conditions (Hessvik et al., 2016; Ojha et al., 2017). Where in this cascade is the entry point for gDNA is presently uncertain, but in light of our data, it is possible that autophagy may contribute to extracellular emission of gDNA (Boya et al., 2013).

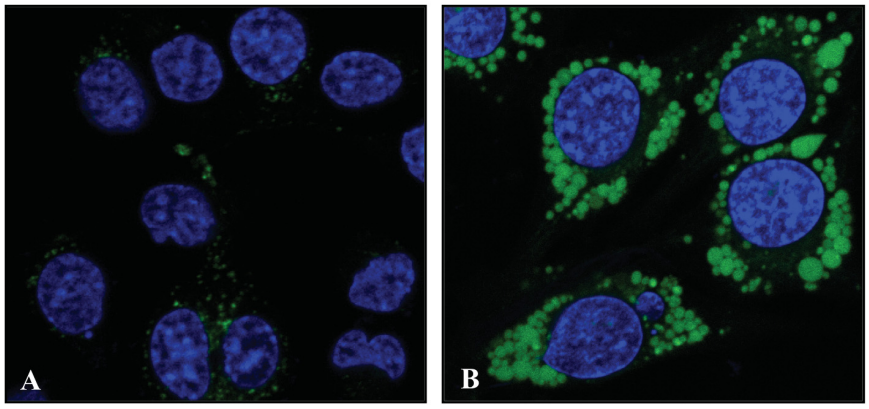


Figure 6.4. Chloroquine treatment leads to build-up of large vacuoles in the cytoplasm of RAS3 cells. Confocal microscope images of RAS3 cells: control (A) and cells treated with 50μM chloroquine (B). Chloroquine induces build-up of large vacuoles corresponding to lysosomes (labeled with LysoTracker Green) in the cytoplasm of cancer cells.

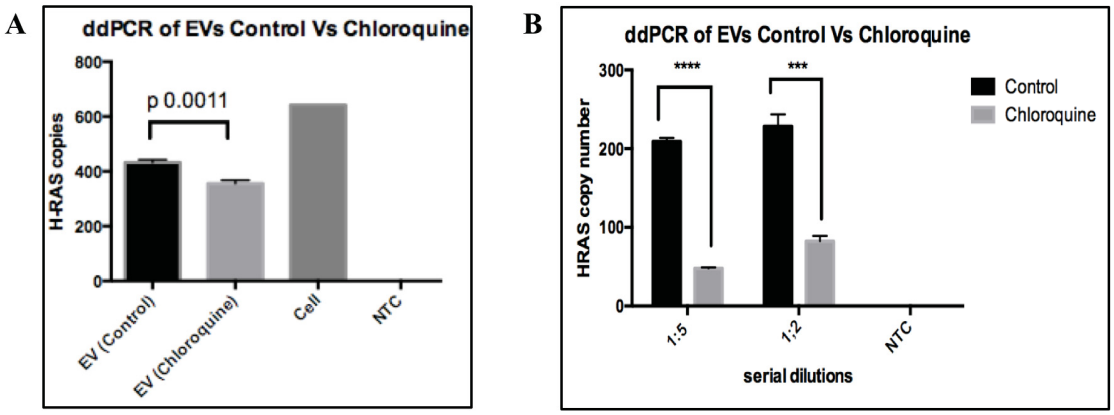


Figure 6.5. Analysis of EV-associated gDNA by ddPCR reveals the impact of chloroquine on DNA emission by RAS3 cells. (A) EVs derived from RAS3 cells treated with chloroquine contain fewer copies of the H-*RAS* gene compared to untreated control. (B) Serial dilutions (1:2, 1:5) of EVs from untreated and treated RAS3 cells reveals the effect of chloroquine on the H-

RAS ddPCR signal. Subsequent reduction of H-*RAS* copy number was achieved with serial dilution of the template DNA. A) N=4 and B) N=3.

As mentioned earlier this notion is consistent with the emerging role of autophagy in vesiculation processes. For example, several reports have shown a crosstalk between autophagy proteins (Atg5, 7, 8, 12) (Schmukler et al., 2013) and exosome emission pathways (Baixauli et al., 2014; Papandreou and Tavernarakis, 2017; Zadeh et al., 2017). However, it is not understood how autophagy proteins (e.g. LC3) may influence EVs and their gDNA content, and the role of oncogenic *RAS* in these interrelationships has not been studied.

RAS-transformation Leads to Defects in Nuclear Envelope and Formation of Cytoplasmic Chromatin.

Even if interactions with autophagy proteins are capable of triggering the EV-mediated extracellular removal of the cytoplasmic DNA, the reasons why this material is released from the nucleus in the first place remain puzzling. Interestingly, we noticed that RAS3 cells have a higher expression of LC3 protein compared to their non-transformed IEC-18 counterparts (Figure 6.11). Notably, LC3 and ATG8 protein in the nucleus are involved in nuclear envelope degradation (Dou et al., 2015).

This observation is consistent with our data suggesting that RAS3 cells exhibit aberrant nuclear structure, which is not the case for their parental IEC-18 cell line. Thus, we observed nuclear membrane ruffles, folds and irregularities of the nuclear envelope of RAS3 cells using dSTORM and TEM imaging systems (Figures 6.6 and 6.7). Also, nuclear membrane degradation is consistent with the presence of cytoplasmic chromatin (CCM) in RAS3, but not (or minimally) in IEC-18 cells, as can be seen by labelling cells with gold particle-conjugated antibodies against histones, dsDNA and BrdU followed by TEM (Figures 6.8 and 6.9) (Figure 4.16 - Figure 4.21).

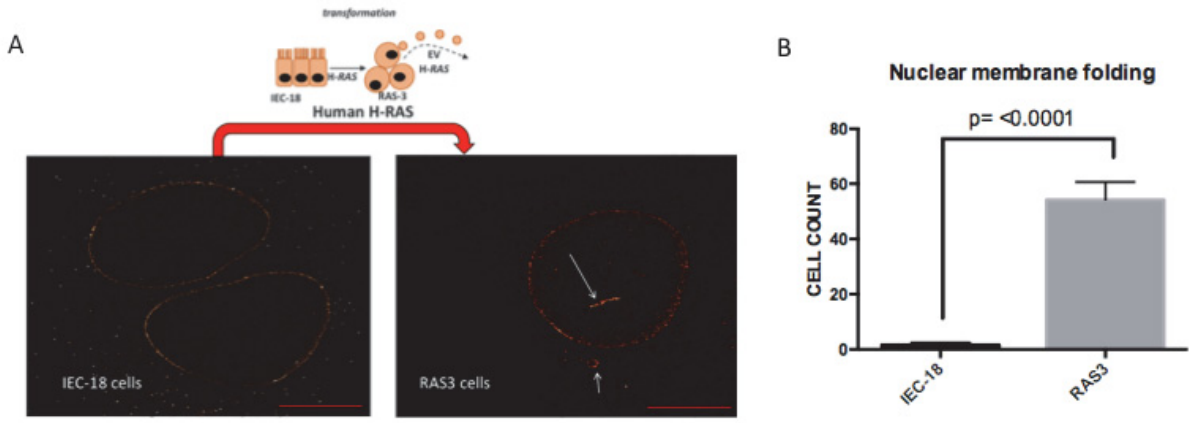
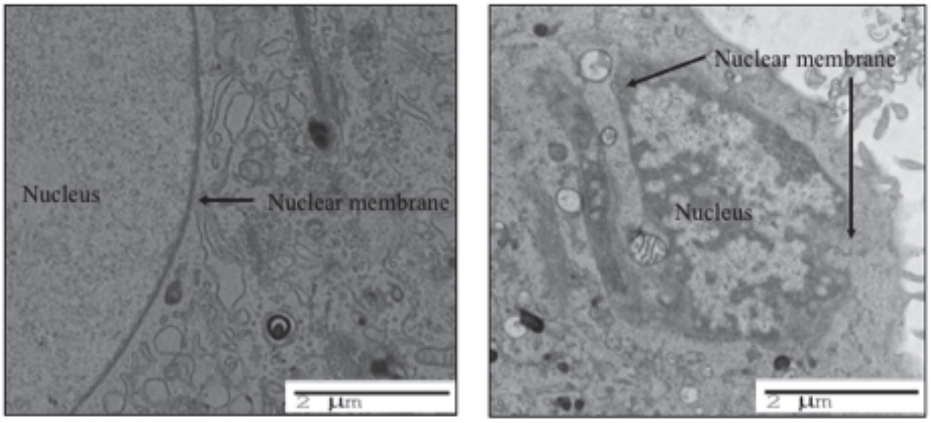


Figure 6.6. Perturbation of nuclear membrane morphology due to H-*RAS* transformation. (A) dSTORM super resolution imaging of nuclear membrane (lamin b1). IEC-18 cells (left panel) RAS3 cells (right panel). (B) Approximately half of RAS3 cells (~50/100) show malformed nuclear membrane. Scale bar indicates 5μm.



TEM of IEC-18 stable nuclear membrane 1900X (scale bar=2μm)

TEM of RAS3 nuclear membrane disruption 2900X (scale bar=2μm)

Figure 6.7 TEM reveals differential features of nuclear envelope in IEC-18 and RAS3 cells. IEC-18 nuclear membrane (left panel) appears to be stable and intact whereas RAS3 nuclear membrane (right panel) shows disruption, confirming the super resolution dSTORM image (see Figure 6.6).

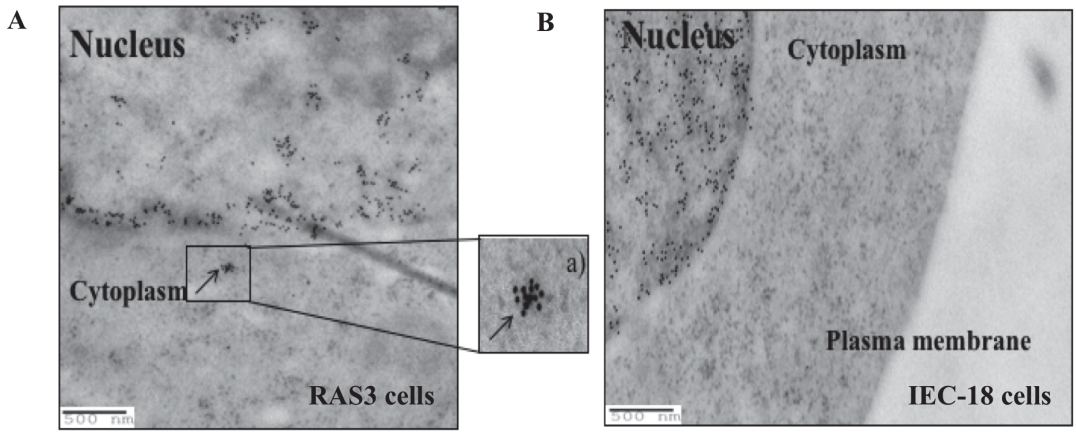


Figure 6.8. Evidence of cytoplasmic chromatin content in *RAS* transformed cells.
TEM of RAS3 (A) and IEC-18 cells (B) immunostained with anti-histone antibody. RAS3 cytoplasmic histones are stained with 10nm gold particles (a) Figure inset (zoom image) shows the presence of cytoplasmic chromatin while IEC-18 cells are without cytoplasmic histones. Scale bar 500nm.

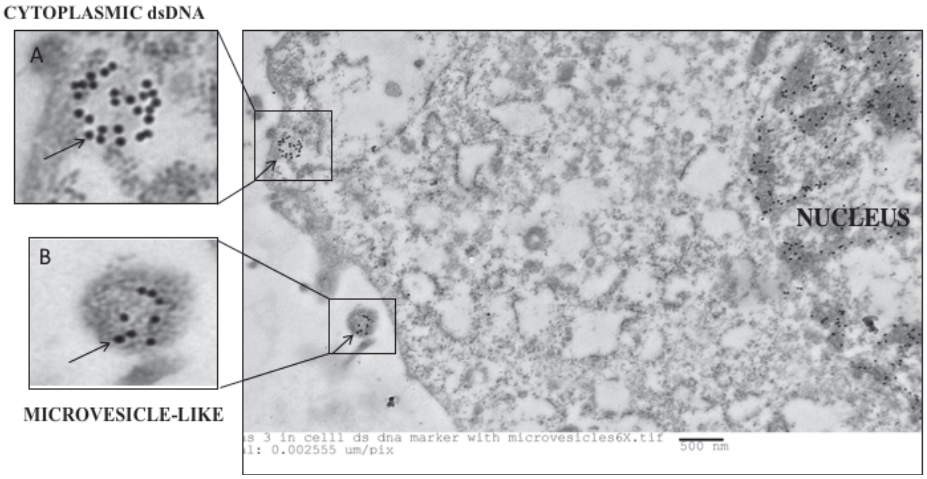


Figure 6.9. TEM of RAS3 cells stained with anti-dsDNA antibody reveals cytoplasmic chromatin and dsDNA. (A) Cytoplasmic dsDNA immunolabeled with 20nm gold particles. (B) Microvesicle-like structure stained positively for dsDNA. Magnification 6000X. Scale bar 500nm.

LC3 B protein is highly expressed in RAS3 cells

We were able to corroborate the previously published data suggesting that mutant *RAS* may upregulate LC3 B protein levels in cells undergoing malignant transformation. This elevated expression of LC3 B protein in RAS3 cells versus their non-transformed IEC-18 counterparts

(Figure 6.10) prompted us to test the impact of this increase on the emission of exoDNA. To accomplish this, GFP-tagged *LC3B* sequence was overexpressed in RAS3 and IEC-18 cells (Figure 6.11) and the corresponding knockdowns were also initially included in the research plan. As discussed previously, only RAS3 cells display aberrant nuclear structures, suggesting a possible role of LC3 B in autophagy-mediated nuclear membrane degradation (Figures 6.6 and 6.7). If the aforementioned autophagic damage to the nuclear envelope of *RAS*-driven cells is critical to EV-mediated emission of gDNA, this process is expected to be decreased upon diminution of the LC3 protein in RAS3 cells. This study will aid in understanding the crosstalk between LC3 and gDNA elimination via EVs. Alternatively, overexpression of LC3 B protein in IEC-18 cells would be expected to result in nuclear membrane degradation, increased cytoplasmic content of chromatin and higher rate of vesicular emission of exoDNA. Indeed, IEC-18 cells engineered to overexpress LC3 B proteins show an increase in EV emission and preliminary data suggest an increase in EV-associated gDNA (data not shown). Taken together, our data with perturbation of the autophagy pathway using chloroquine and manipulation of LC3 suggest a scenario where the damage to nuclear envelope in *RAS*-transformed cells may lead to accumulation of cytoplasmic chromatin that may overwhelm the lysosomal degradation pathway and redirect this material to exosome emission, as has been reported for other molecular cargo (Hessvik and Llorente, 2017).

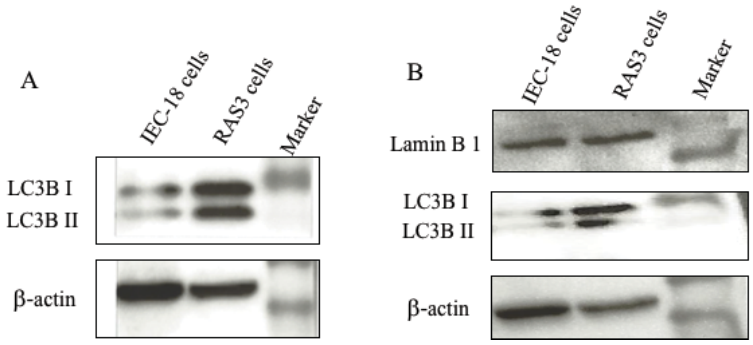


Figure 6.10. Upregulation of LC3 B by oncogenic H-*RAS* expressed in RAS3 cells.
(A) Western blot analysis for LC3B and beta-actin protein lysate from RAS3 and IEC-18 cells.
(B) Western blot documenting no difference in Lamin b1 expression in parallel to changes in LC3 B proteins levels between IEC-18 and RAS3 cells.

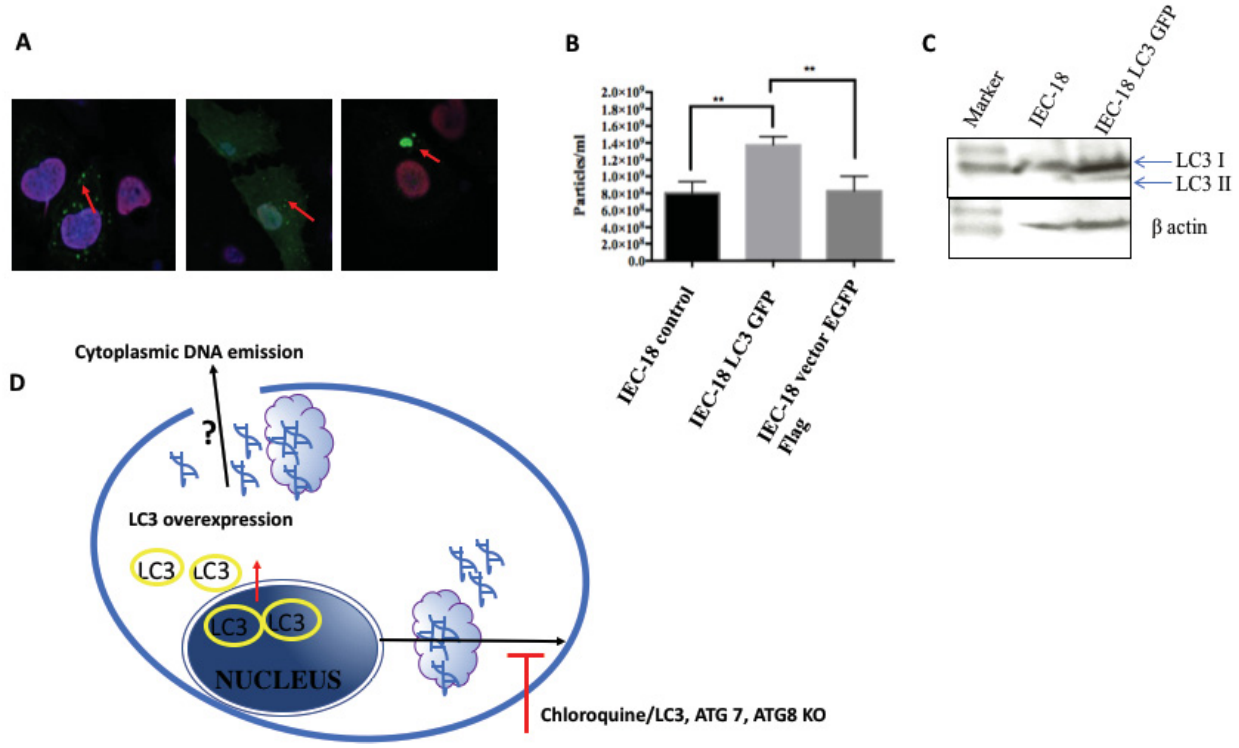


Figure 6.11. Enforced overexpression of LC3 B in IEC-18 - preliminary data and working hypothesis. LC3 expression was conducted according to previously published protocols with the same reagents (Kabeya, 2000). (A) IEC-18 cells transfected with LC3 GFP – fluorescent puncta visible under confocal microscopy. (B) Higher amounts of EV emission in LC3-overexpressing IEC-18 cells. (C) Western blot results confirming the overexpression of LC3 B proteins in IEC-18 cells. (D) Schematic diagram depicting the overexpression of LC3 proteins and its predicted results on EV emission.

The Presence of Nucleosomes in the EV Cargo

We have previously shown that cytoplasmic histones and dsDNA are present in RAS3 and (to a lesser extent) in IEC-18 cells (Figure 6.8 and 6.9). To determine whether DNA and histones occur in the form of nucleosomes in EVs as previously reported (Simpson et al., 2008; Lee et al., 2014), we performed DNA-Histone complex detection using ELISA assay. Our results revealed that EVs from RAS3 cells, indeed, contain dramatically higher amounts of such complexes (nucleosomes) compared to IEC-18 EVs (Figure 6.12).

This finding is not consistent with Balaj et al. (Balaj et al., 2011), who showed that EVs derived from tumour cells harbour single stranded DNA. These contrasting results could be attributed to different model systems used in these respective studies. A recent study also reported that EVs derived from mouse melanoma cells (B16-F1) are enriched in histones, heat shock proteins and the tetraspanin CD81, as detected by mass spectrometry (Muhsin-Sharafaldine et al., 2016). These histones include H2A, H2B, H3.1 and H4, which may likely serve as chaperones for nucleic acid content in EVs (Muhsin-Sharafaldine et al., 2016). In addition, changes at methylation levels in histone H3 (H3K4 and H3K9) regulate the release of exosomes in rat and human adipocytes (Müller et al., 2012). Furthermore, H1.0 linker histone variant and its corresponding mRNA have also been reported in EVs released from oligodendroglioma cells (Schiera et al., 2013). More recently, H1.0-binding RNA binding proteins (RBPs) such as MYEF2 have been shown in EVs emitted from A375 melanoma cells (Schiera et al., 2016). Thus, epigenetics appears to play a role in EV emission and warrants further investigation to understand the underlying mechanism.

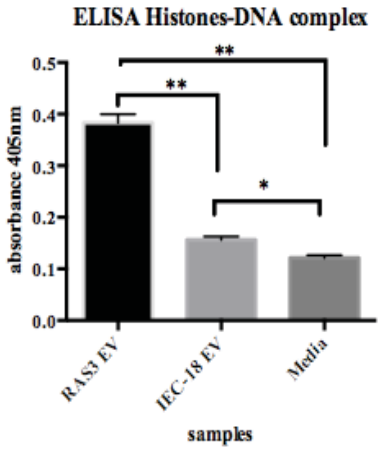


Figure 6.12. ELISA analysis of DNA-Histone complexes in EVs released by RAS3 and IEC-18 cells. Unpaired two tailed t-test: *p* value =0.0032 (RAS3 EV and IEC-18 EV) *p* value=0.0023 (RAS3 EV and conditioned media), *p* value= 0.0255 (IEC-18 and conditioned media).

CHAPTER 7

General Discussion, Conclusions and Future Directions

In this Masters project, I investigated the underpinnings of the vesicular exoDNA release under the influence of mutant H-*RAS*. The most important conclusion of this work is that oncogenic transformation is associated with potentially interlinked processes of nuclear envelope disruption, accumulation of cytoplasmic chromatin, and emission of EVs containing genomic DNA. These processes may be linked to *RAS*-dependent alteration of the autophagy pathway and EV biogenesis, still to be fully explored at the molecular level.

My initial assumption had been that both MNs and EVs may play significant (perhaps different) roles in the process of gDNA emission from cancer cells. The involvement of MNs was inferred from the high frequency with which these structures appear in *RAS*-transformed cells, their linkages to deregulation of chromatin, and from prior literature (Saavedra et al., 1999; Shimizu et al., 2000; Abulaiti et al., 2006; Kamata and Pritchard, 2011), which suggested a possibility of MN release from certain cancer cell lines. Therefore, I proceeded to characterize the properties, occurrence and DNA content of RAS3 MNs. In the process I developed and adapted several imaging techniques such as TEM, immunogold labelling, FISH, confocal microscopy, high resolution microscopy and live cell imaging using JuLI Stage technology. These techniques were essential to be able to directly observe MNs rather than base our conclusions on biochemical surrogates. I have also developed protocols for ultracentrifugation and sequential filtration to purify EVs with different properties and to assay them for general DNA content and specific sequences. The overall conclusion from this work as summarized in Chapter 3 is that MNs play a negligible role in exoDNA emission even though they are closely associated with the process of *RAS* transformation and aberrations in mitogenesis.

These results prompted us to focus on EVs as the main pathway of exoDNA emission from *RAS*-transformed cells. In this regard my study thus far reports a small but essential step towards a better understanding of the key determinants of exoDNA emission from cells harbouring mutant oncogenes. For example, my work suggests that DNA synthesis *per se* is not the key trigger of exoDNA formation and neither is stochastic cell death within the RAS3 cell population. Rather, I

have obtained preliminary evidence for the *RAS*-dependent activation of nuclear envelope damage, extranuclear chromatin formation and the emission of exoDNA, as a function of autophagy-related processes.

This is important as exoDNA (gDNA) is likely a significant part of liquid biopsy biomarker approaches involving the detection of mutant cfDNA in the blood of cancer patients for the purpose of molecular diagnosis, real time monitoring of cancer progression, cell population dynamics under therapy and ultimately a greater personalization of patient care (Giulietti and Occhipinti, 2015; Chronopoulos et al., 2017; Zhang et al., 2017; Halvaei et al., 2018).

It should also be considered that EV-related gDNA may possess unique biological activities worthy of exploration from the therapeutic perspective (Lee et al., 2016). As exoDNA/gDNA is likely associated with a specific subset of EVs which are likely a 'signature' of certain cancer-related processes, such as the action of potent oncogenes including *RAS*, so too their biological effects could be unique and different than those of other populations of cancer EVs (Choi et al., 2017). These questions are being explored in our laboratory.

In the future, we need to explore more cell lines such as RAS4, RAS7 and SRC-3, all related to IEC-18, as well as realistic human models harbouring *RAS* and other oncogenes, including cancer stem cells. In addition, inducible clones such as Clone25 which can induce *RAS* gene by dexamethasone (Rak et al., 1995) can be used to validate and compare with RAS3 cell EVs, in terms of properties and biogenesis. In such a system the acute (primary) effects of *RAS* could be isolated from those that are secondary to long-term *RAS*-transformation, as is the case for RAS3 cells.

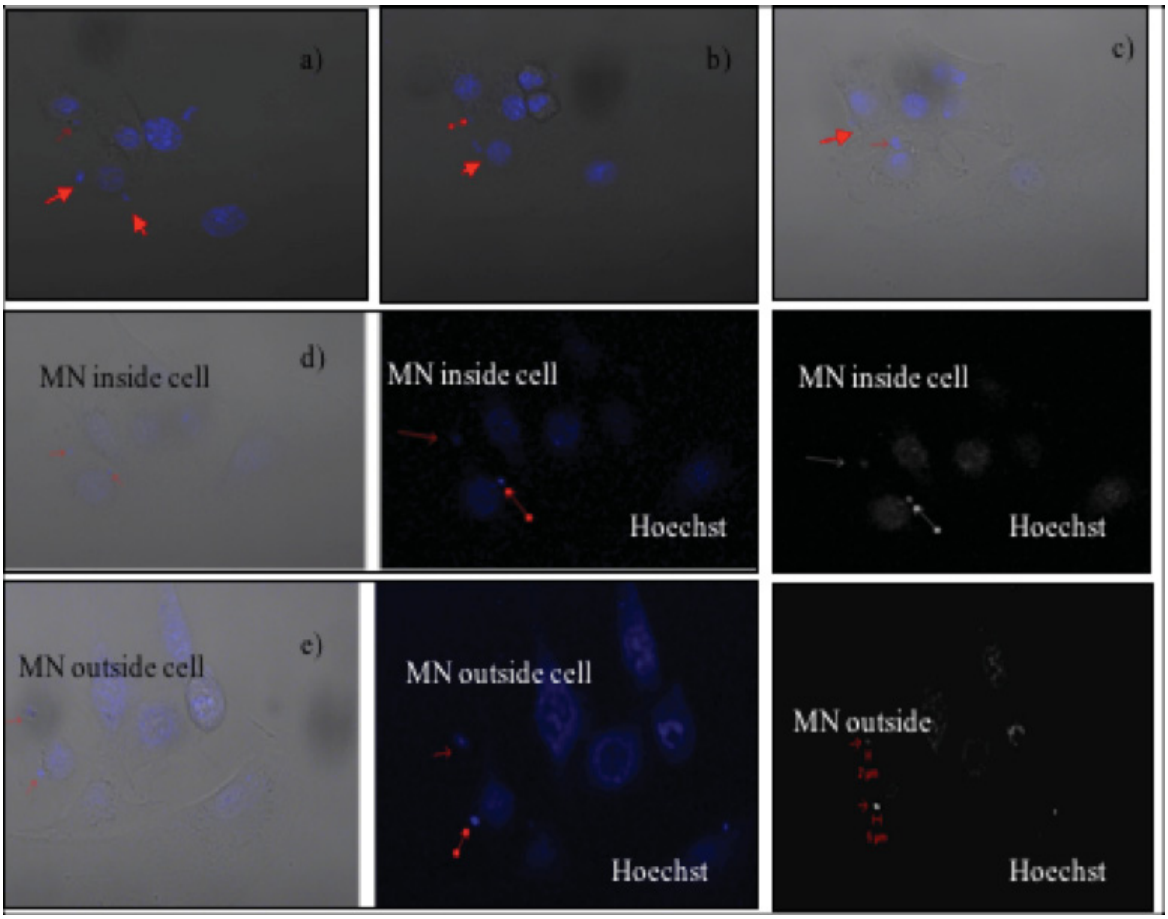
Our future explorations should also include evidence for the role of autophagy proteins in regulating the levels of ctDNA in cancer patients and the influence of therapies on them, both cancer-directed and supportive treatments. The role of such circumstances on the diagnostic performance of ctDNA assays is presently unknown. Moreover, these tests would likely benefit from enrichment for specific EV subsets carrying exoDNA, and those remain to be identified and purified (again this work is ongoing in our laboratory).

Finally, exoDNA should be examined *in vivo* as biological regulators affecting tumour microenvironment, both locally and systemically in animal models and in cancer patients. Of note is the fact that DNA may trigger coagulation system activation, while histones possess proinflammatory properties, all of which may propel development of paraneoplastic syndromes (thrombosis) (Geddings et al., 2014) and propagation of the metastatic disease (Hoshino et al., 2015).

Indeed, studies on extracellular emission of the cellular genome remain a fascinating area of investigation, as this process defies the notion of intracellular confinement of the genetic material. Therefore, the related explorations in cancer are rich in conceptual novelty and translational opportunities.

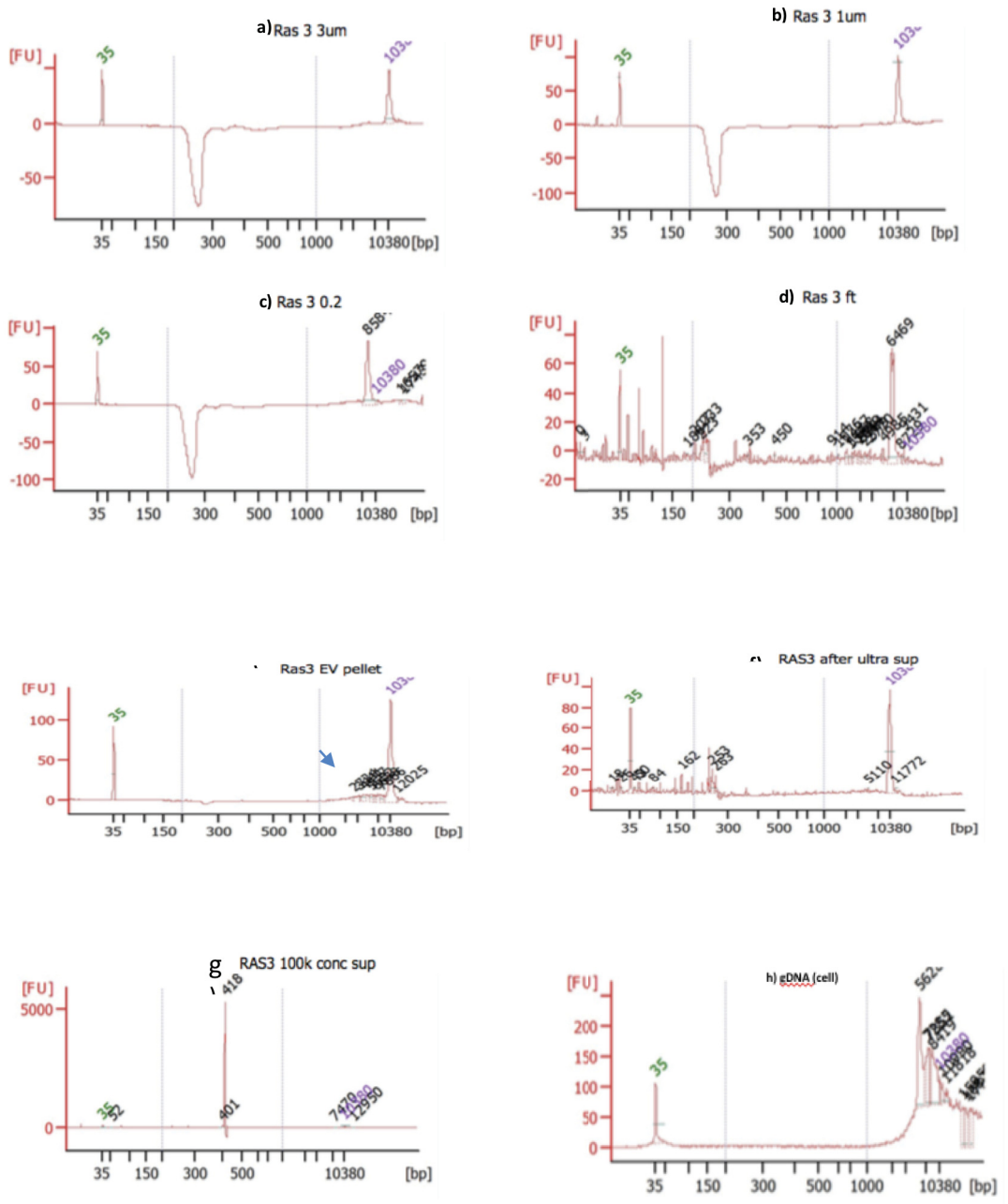
Supplementary Data

Confocal live cell imaging confirms the emission of MN from RAS3 cells



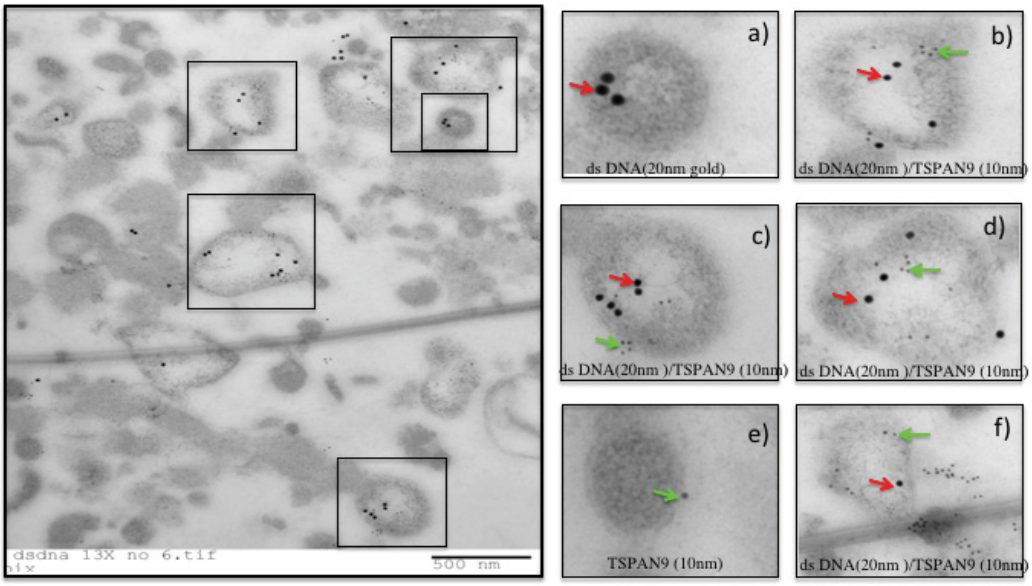
Supplementary Figure 1. Time lapse images of RAS3 cells stained with Hoechst 33342. a-c) arrows indicate the presence of micronuclei in RAS3 cells. d) Two micronuclei are inside the cell pointed with arrows e) one of the micronuclei is emitted out of the cell.

Bioanalyzer result of extracellular vesicles from RAS3 cells



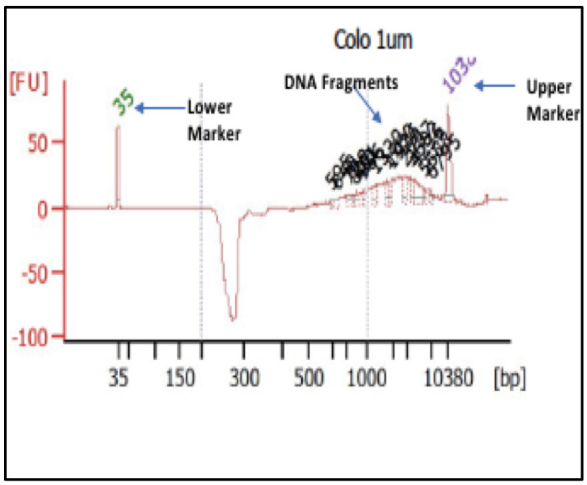
Supplementary Figure 2. Bioanalyzer profiles of EV DNA. Absence of DNA fragments on a) 3µm filter, b) 1µm filter, c) 0.2µm filter, d) Flow through of 0.2µm filtrate. Notably, larger DNA fragments are seen in e) EV pellet after 110,000g ultracentrifugation (Arrow pointed) f) supernatant after 110,000g ultracentrifugation (smaller DNA fragments) g) 100kDa concentrated supernatant. h) IEC-18 cells.

EV heterogeneity as revealed by TEM



Supplementary Figure 3. TEM images of heterogeneous population of RAS3 EVs. Very few EVs are labelled with dsDNA marker and TSPAN9 antibodies. Magnification 13,000X and Scale bar 500nm.

Analysis of genomic DNA on 1μm filter by Bioanalyzer system

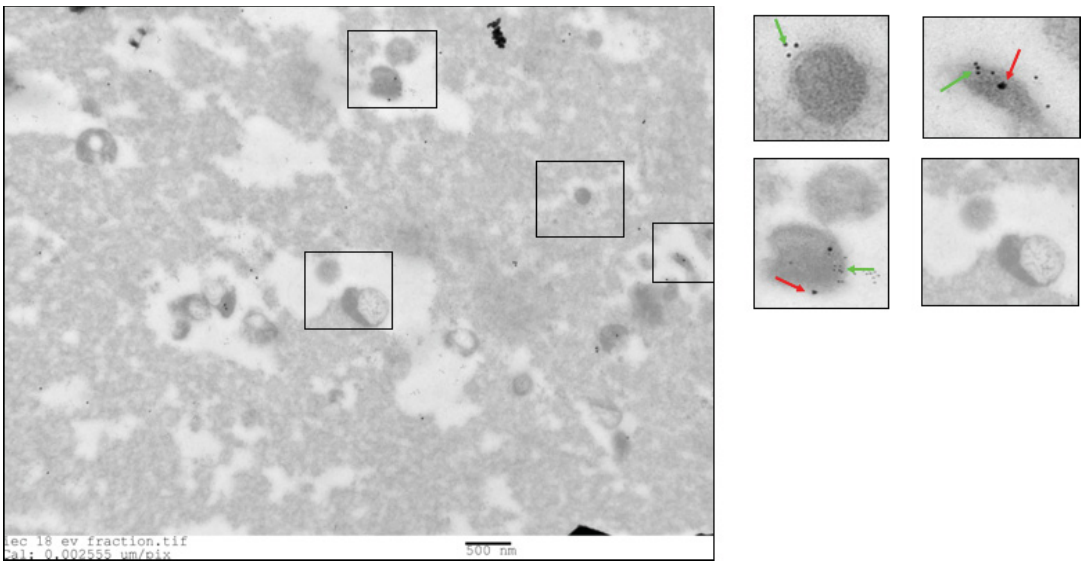


Peak table for sample 6 : Colo 1um

Peak	Size [bp]	Conc. [pg/μl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	595	6.36	16.2	
3	640	7.02	16.6	
4	728	11.56	24.1	
5	820	10.60	19.6	
6	861	10.31	18.1	
7	966	11.42	17.9	
8	1,320	18.01	20.7	
9	1,760	14.65	12.6	
10	1,926	16.63	13.1	
11	2,797	27.47	14.9	
12	3,682	14.34	5.9	
13	4,236	41.36	14.8	
14	5,688	11.13	3.0	
15	6,795	6.63	1.5	
16	10,380	75.00	10.9	Upper Marker

Supplementary Figure 4. Bioanalyzer data analysis of genomic DNA captured on 1μm filter as putative micronuclei. DNA fragments present on 1μm filter from COLO320DM conditioned media between two markers (lower marker=35 bp, upper marker=10380 bp). Supplementary Table 1: different size fragments of DNA on 1μm filter (green arrow head is lower marker =35bp and purple arrow head is upper marker = 10,380 bp).

Minimal labeling of TSPAN9 and dsDNA marker antibodies on IEC-18 EVs



Supplementary Figure 5. TEM images of ultrathin sections of IEC-18 EVs. The sections are stained with TSPAN9 and dsDNA marker antibodies. However, very few EVs are labeled with these antibodies suggesting multiplicity of EV subpopulations and the impact of mutant H-*RAS* on the EV phenotype. Green arrow pointed to TSPAN9 10nm gold and red arrow pointed to dsDNA marker 20nm gold.

References:

Abels ER, Breakefield XO. 2016. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. Cell Mol Neurobiol 36:301–312.

Abels ER, Breakefield XO. 2017. Introduction to Extracellular Vesicles: Biogenesis , RNA Cargo Selection, Content, Release and Uptake. Cell Mol Neurobiol 36:301–312.

Abulaiti A, Fikaris AJ, Tsygankova OM, Meinkoth JL. 2006. Ras induces chromosome instability and abrogation of the DNA damage response. Cancer Res 66:10505–10512.

Adams PD, Ivanov A, Pawlikowski J, Manoharan I, Tuyn J Van, Nelson DM, Singh Rai T, Shah PP, Hewitt G, Korolchuk VI, Passos JF, Wu H, Berger SL. 2013. Lysosome-mediated processing of chromatin in senescence. J Cell Biol 202:129–143.

Akers, C. Jhonny , Gonda, David, Kum, Ryan, Carter, Bob Chen C. 2013. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies Johnny. Neuro Oncol.

Akers JC, Gonda D, Kim R, Carter BS, Chen CC. 2013. Biogenesis of extracellular vesicles (EV): Exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol 113:1–11.

Al-Nedawi K, Meehan B, Kerbel RS, Allison AC, Rak J. 2009. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. Proc Natl Acad Sci [Internet] 106:3794–3799. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.0804543106

Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J. 2008a. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol 10:619–624.

Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A, Rak J. 2008b. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Lett Nat Cell Biol 10:619–624.

Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, Walter P. 2014. Molecular Biology of the Cell 6e.

Alsina M, Fonseca R, Wilson EF, Belle AN, Gerbino E, Price-Troska T, Overton RM, Ahmann G, Bruzek LM, Adjei AA, Kaufmann SH, Wright JJ, Sullivan D, Djulbegovic B, Cantor AB, Greipp PR, Dalton WS, Sebti SM. 2004. Farnesyltransferase inhibitor tipifarnib is well tolerated, induces stabilization of disease, and inhibits farnesylation and oncogenic/tumor survival pathways in patients with advanced multiple myeloma. Blood 103:3271–3277.

Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJA. 2011. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 29:341–345.

An K, Klyubin I, Kim Y, Jung JH, Mably AJ, O'Dowd ST, Lynch T, Kanmert D, Lemere CA, Finan GM, Park JW, Kim TW, Walsh DM, Rowan MJ, Kim JH. 2013. Exosomes neutralize synaptic-plasticity-disrupting activity of Aβ assemblies in vivo. Mol Brain [Internet] 6:1. Available from: Molecular Brain

Antonucci L, Fagman JB, Kim JY, Todoric J, Gukovsky I, Mackey M, Ellisman MH, Karin M. 2015. Basal autophagy maintains pancreatic acinar cell homeostasis and protein synthesis and prevents ER stress. Proc Natl Acad Sci [Internet] 112:E6166–E6174. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1519384112

Asea A, Jean-Pierre C, Kaur P, Rao P, Linhares IM, Skupski D, Witkin SS. 2008. Heat shock protein-containing exosomes in mid-trimester amniotic fluids. J Reprod Immunol 79:12–17.

B L, M G, Verdun-Hernanadez D, M B. 2018. Procedure for isolating micronuclei from rat

kangaroo cultured cells containing individualized chromosomes . Eur J Cell Biol 38:165–170.
Bafico A, Grumolato L, Aaronson SA. 2008. Oncogenes and Signal Transduction. In: The Molecular Basis of Cancer. . p 17–30.
Baines AT, Xu D, Der CJ. 2011. Inhibition of Ras for cancer treatment: the search continues. Future Med Chem [Internet] 3:1787–1808. Available from: http://www.future-science.com/doi/10.4155/fmc.11.121
Baixauli F, López-Otín C, Mittelbrunn M. 2014. Exosomes and autophagy: Coordinated mechanisms for the maintenance of cellular fitness. Front Immunol 5:1–6.
Bakhoum SF, Compton DA. 2009. Cancer: CINful Centrosomes. Curr Biol [Internet] 19:R642–R645. Available from: http://dx.doi.org/10.1016/j.cub.2009.06.039
Balaj L, Lessard R, Dai L, Cho Y, Pomeroy SL, Breakefield XO, Skog J. 2011. tumour microvessicles contains retrotransposone elements and amplified oncogene sequences. Nat Commun 2:180–189.
Barbacid M. 1990. Ras Oncogenes: Their Role in Neoplasia. Eur J Clin Invest 20:225–235.
Bardelli A, Pantel K. 2017. Liquid Biopsies, What We Do Not Know (Yet). Cancer Cell 31:172–179.
Bayani J, Squire J. 2001. Advances in the detection of chromosomal aberrations using spectral karyotyping. Clin Genet 59:65–73.
Bebelman MP, Smit MJ, Pegtel DM, Baglio SR. 2018. Biogenesis and function of extracellular vesicles in cancer. Pharmacol Ther [Internet]:#pagerange#. Available from: http://linkinghub.elsevier.com/retrieve/pii/S016372581830038X
Becker PS. 2015. Shattered Chromosomes : A Cataclysmic Origin of Cancer. Hematol:2–5.
Bento CF, Renna M, Ghislat G, Puri C, Ashkenazi A, Vicinanza M, Menzies FM, Rubinsztein DC. 2016. Mammalian Autophagy: How Does It Work? Annu Rev Biochem [Internet] 85:685–713. Available from: http://www.annualreviews.org/doi/10.1146/annurev-biochem-060815-014556
Berardocco M, Radeghieri A, Busatto S, Gallorini M, Raggi C, Gissi C, D'Agnano I, Bergese P, Felsani A, Berardi AC. 2017. RNA-seq reveals distinctive RNA profiles of small extracellular vesicles from different human liver cancer cell lines. Oncotarget [Internet] 8. Available from: http://www.oncotarget.com/fulltext/20503
Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz AL, Holmgren L. 2001. Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc Natl Acad Sci U S A [Internet] 98:6407–11. Available from: http://www.pnas.org/content/98/11/6407.long
Bhattacharya A, Prakash YS, Eissa NT. 2014. Secretory function of autophagy in innate immune cells. Cell Microbiol 16:1637–1645.
Bhattacharya H, Bhattacharya D, Ghosal SR, Roy S, Sugunan AP. 2015. Status of hepatitis B infection - A decade after hepatitis B vaccination of susceptible Nicobarese, an indigenous tribe of Andaman & Nicobar (A&N) islands with high hepatitis B endemicity. Indian J Med Res 141:653–661.
Bignell GR, Greenman CD, Davies H, Butler AP, Edkins S, Andrews JM, Buck G, Chen L, Beare D, Latimer C, Widaa S, Hinton J, Fahey C, Fu B, Swamy S, Dalgliesh GL, Teh BT, Deloukas P, Yang F, Campbell PJ, Futreal PA, Stratton MR. 2010. Signatures of mutation and selection in the cancer genome. Nature [Internet] 463:893–898. Available from: http://dx.doi.org/10.1038/nature08768
Bigner SH, Humphrey PA, Wong AJ, Vogelstein B, Mark J, Friedman HS, Bigner DD. 1990.

Characterization of the epidermal growth factor receptor in human glioma cell lines and xenografts. Cancer Res [Internet] 50:8017–8022. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2253244

BioRad. 2016. General Protocol for Western Blotting. Boletín 6376 [Internet] 20:7–8. Available from: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf

Boya P, Reggiori F, Codogno P. 2013. Emerging regulation and functions of autophagy. Nat Cell Biol 15:713–720.

Cai J, Han Y, Ren H, Chen C, He D, Zhou L, Eisner GM, Asico LD, Jose PA, Zeng C. 2013. Extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells is a novel mechanism for genetic influence between cells. J Mol Cell Biol 5:227–238.

Cai J, Wu G, Tan X, Han Y, Chen C, Li C, Wang N, Zou X, Chen X, Zhou F, He D, Zhou L, Jose PA, Zeng C. 2014. Transferred BCR/ABL DNA from K562 extracellular vesicles causes chronic myeloid leukemia in immunodeficient mice. PLoS One 9:1–11.

Caldas C, Kern SE. 1995. K-ras mutation and pancreatic adenocarcinoma. Int J Pancreatol 18:1–6.

Castro-Obregon S. 2010. The Discovery of Lysosomes and Autophagy. Nat Educ 9:49.

Chairoungdua A, Smith DL, Pochard P, Hull M, Caplan MJ. 2010. Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling. J Cell Biol 190:1079–1091.

Chan KL, Hickson ID. 2011. New insights into the formation and resolution of ultra-fine anaphase bridges. Semin Cell Dev Biol [Internet] 22:906–912. Available from: http://dx.doi.org/10.1016/j.semcdb.2011.07.001

Chi S, Kitanaka C, Noguchi K, Mochizuki T, Nagashima Y, Shirouzu M, Fujita H, Yoshida M, Chen W, Asai A, Himeno M, Yokoyama S, Kuchino Y. 1999. Oncogenic Ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. Oncogene 18:2281–2290.

Choi D, Lee TH, Spinelli C, Chennakrishnaiah S, D'Asti E, Rak J. 2017. Extracellular vesicle communication pathways as regulatory targets of oncogenic transformation. Semin Cell Dev Biol [Internet] 67:11–22. Available from: http://dx.doi.org/10.1016/j.semcdb.2017.01.003

Chronopoulos A, Lieberthal TJ, del Río Hernández AE. 2017. Exosomes as a platform for "liquid biopsy" in pancreatic cancer. Converg Sci Phys Oncol [Internet] 3:13005. Available from: http://stacks.iop.org/2057-1739/3/i=1/a=013005?key=crossref.abaf7dead9f7ca3bdde2abfdcd34eb49

Colombo M, Raposo G, Théry C. 2014. Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. Annu Rev Cell Dev Biol [Internet] 30:255–289. Available from: http://www.annualreviews.org/doi/10.1146/annurev-cellbio-101512-122326

Cooper G, Sunderland M. 2000. The Cell: A Molecular Approach 2nd Edition. Sinauer Assoc:1–820.

Crescitelli R, Lässer C, Szabó TG, Kittel A, Eldh M, Dianzani I, Buzás EI, Lötvall J. 2013. Distinct RNA profiles in subpopulations of extracellular vesicles: Apoptotic bodies, microvesicles and exosomes. J Extracell Vesicles 2:1–10.

Croce CM. 2008. Oncogenes and cancer. N Engl J Med 358.

Cultures EC, Isselbacher KJ. 1981. Cytotoxic Effects and Metabolism of Benzo [a] pyrene and 7 , 12-Dimethylbenz [a] anthracene in Duodenal and Ileal. J Natl Cancer Inst 67:1353–

1362.
Cvjetkovic A, Lötvall J, Lässer C. 2014. The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. J Extracell Vesicles 3:1–11.
DAWSON DW, BURY HP. 1961. The significance of Howell-Jolly bodies and giant metamyelocytes in marrow smears. J Clin Pathol [Internet] 14:374–80. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=480237&tool=pmcentrez&rendertype=abstract
Dear JW, Street JM, Bailey MA. 2013. Urinary exosomes: A reservoir for biomarker discovery and potential mediators of intrarenal signalling. Proteomics 13:1572–1580.
Demory Beckler M, Higginbotham JN, Franklin JL, Ham A-J, Halvey PJ, Imasuen IE, Whitwell C, Li M, Liebler DC, Coffey RJ. 2013. Proteomic Analysis of Exosomes from Mutant KRAS Colon Cancer Cells Identifies Intercellular Transfer of Mutant KRAS. Mol Cell Proteomics [Internet] 12:343–355. Available from: http://www.mcponline.org/lookup/doi/10.1074/mcp.M112.022806
Deng W, Tsao SW, Lucas JN, Leung CS, Cheung ALM. 2003. A new method for improving metaphase chromosome spreading. Cytometry [Internet] 51A:46–51. Available from: http://doi.wiley.com/10.1002/cyto.a.10004
Denko NC, Giacciat AJ, Stringer JR, Stambrooku PJ. 1994. The human Ha-ras oncogene induces genomic instability in murine fibroblasts within one cell cycle (NI 3T3 ce/Indud lb -/chromoomes). Genetics 91:5124–5128.
Dou Z, Xu C, Donahue G, Shimi T, Pan JA, Zhu J, Ivanov A, Capell BC, Drake AM, Shah PP, Catanzaro JM, Ricketts MD, Lamark T, Adam SA, Marmorstein R, Zong WX, Johansen T, Goldman RD, Adams PD, Berger SL. 2015. Autophagy mediates degradation of nuclear lamina. Nature [Internet] 527:105–109. Available from: http://dx.doi.org/10.1038/nature15548
Downward J. 2003. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 3:11–22.
Duran JM, Anjard C, Stefan C, Loomis WF, Malhotra V. 2010. Unconventional secretion of Acb1 is mediated by autophagosomes. J Cell Biol 188:527–536.
Ehrlich M. 2002. DNA methylation in cancer: Too much, but also too little. Oncogene 21:5400–5413.
Fang Y, Garnier D, Lee TH, D'Asti E, Montermini L, Meehan B, Rak J. 2016. PML–RARa modulates the vascular signature of extracellular vesicles released by acute promyelocytic leukemia cells. Angiogenesis 19:25–38.
Farre J-C, Subramani S. 2017. Mechanistic insights into selective autophagy pathways: Lesson from yeast. Nat Rev Mol Cell Biol 17:537–552.
Fearon ER, Vogelstein B. 1990. A genetic model for colorectal tumorigenesis. Cell 61:759–767.
Fenech M. 2007. Cytokinesis-block micronucleus cytome assay in lymphocytes. Nat Protoc 2:1084–1104.
Fenech M, Morley AA. 1985. Measurement of micronuclei in lymphocytes. Mutat Res Mutagen Relat Subj 147:29–36.
Fenech M, Natarajan AT. 2011. Molecular mechanisms of micronucleus , nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26:125–132.
Fernández-Medarde A, Santos E. 2011. Ras in cancer and developmental diseases. Genes and Cancer 2:344–358.
Figueroa JM, Skog J, Akers J, Li H, Komotar R, Jensen R, Ringel F, Yang I, Kalkanis S,

Thompson R, Loguidice L, Berghoff E, Parsa A, Liau L, Curry W, Cahill D, Bettegowda C, Lang FF, Chiocca EA, Henson J, Kim R, Breakefield X, Chen C, Messer K, Hochberg F, Carter BS. 2017. Detection of wild-Type EGFR amplification and EGFRvIII mutation in CSF-derived extracellular vesicles of glioblastoma patients. Neuro Oncol 19:1494–1502.

Filimonenko M, Stuffers S, Raiborg C, Yamamoto A, Malerød L, Fisher EMC, Isaacs A, Brech A, Stenmark H, Simonsen A. 2007. Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. J Cell Biol 179:485–500.

Fischer S, Cornils K, Speiseder T, Badbaran A, Reimer R, Indenbirken D, Grundhoff A, Brunswig-Spickenheier B, Alawi M, Lange C. 2016. Indication of horizontal DNA gene transfer by extracellular vesicles. PLoS One 11.

Gallo A, Tandon M, Alevizos I, Illei GG. 2012. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7:1–5.

Gámez-Valero A, Monguió-Tortajada M, Carreras-Planella L, Franquesa M, Beyer K, Borràs FE. 2016. Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents. Nat / Sci Reports [Internet] 6:1–9. Available from: http://dx.doi.org/10.1038/srep33641

Ganem NJ, Pellman D. 2012. Linking abnormal mitosis to the acquisition of DNA damage. J Cell Biol 199:871–881.

García-Olmo DC, Domínguez C, García-Arranz M, Anker P, Stroun M, García-Verdugo JM, García-Olmo D. 2010. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. Cancer Res 70:560–567.

Garnier D, Magnus N, Lee TH, Bentley V, Meehan B, Milsom C, Montermini L, Kislinger T, Rak J. 2012. Cancer Cells Induced to Express Mesenchymal Phenotype Release Exosome-like Extracellular Vesicles Carrying Tissue. 287:43565–43572.

Garnier D, Magnus N, Meehan B, Kislinger T, Rak J. 2013. Qualitative changes in the proteome of extracellular vesicles accompanying cancer cell transition to mesenchymal state. Exp Cell Res 319:2747–2757.

Geddings JE, Aleman MM, Wolberg AS, von Brupsilonhl ML, Massberg S, Mackman N. 2014. Strengths and Weaknesses of a New Mouse Model of Thrombosis Induced by Inferior Vena Cava Stenosis. J Thromb Haemost.

Geigl JB, Obenauf AC, Schwarzbraun T, Speicher MR. 2008. Defining "chromosomal instability." Trends Genet 24:64–69.

Geraud G, Laquerriere F, Masson C, Labidi B. 1989. Three-Dimensional Organization of Micronuclei Colchicine in PtK , Cells Induced by. Methods 181:27–39.

Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C. 2012. Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. N Engl J Med [Internet] 366:883–892. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa1113205

Giulietti M, Occhipinti G. 2015. Employment of Exosomes for Liquid Biopsies. Transl Biomed [Internet] 6:17–19. Available from: http://www.transbiomedicine.com/translational-biomedicine/employment-of-exosomes-for-liquid-biopsies.php?aid=8010

Graner MW, Alzate O, Dechkovskaia AM, Keene JD, Sampson JH, Mitchell DA, Bigner DD. 2009. Proteomic and immunologic analyses of brain tumor exosomes. FASEB J [Internet] 23:1541–1557. Available from: http://www.fasebj.org/cgi/doi/10.1096/fj.08-122184
Gruenberg J, Stenmark H. 2004. The biogenesis of multivesicular endosomes. Nat Rev Mol Cell Biol 5:317–323.
Guescini M, Genedani S, Stocchi V, Agnati LF. 2010. Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. J Neural Transm 117:1–4.
Guo JY, Xia B, White E. 2013. Autophagy-Mediated tumor Promotion. Cell [Internet] 155:1216–1219. Available from: http://dx.doi.org/10.1016/j.cell.2013.11.019
Guttenbach M, Schakowski R, Schmid M. 1994. Aneuploidy and ageing: sex chromosome exclusion into micronuclei. Hum Genet 94:295–298.
György B, Szabó TG, Pásztói M, Pál Z, Misják P, Aradi B, László V, Pállinger É, Pap E, Kittel Á, Nagy G, Falus A, Buzás EI. 2011. Membrane vesicles, current state-of-the-art: Emerging role of extracellular vesicles. Cell Mol Life Sci 68:2667–2688.
Halvaei S, Daryani S, Eslami-S Z, Samadi T, Jafarbeik-Iravani N, Bakhshayesh TO, Majidzadeh-A K, Esmaeili R. 2018. Exosomes in Cancer Liquid Biopsy: A Focus on Breast Cancer. Mol Ther - Nucleic Acids [Internet] 10:131–141. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2162253117302986
Harding C, Heuser J, Stahl P. 1983. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol 97:329–339.
Harding C, Heuser J, Stahl P. 1984. Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. Eur J Cell Biol [Internet] 35:256–63. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6151502
Harding SM, Benci JL, Irianto J, Discher DE, Minn AJ, Greenberg RA. 2017. Mitotic progression following DNA damage enables pattern recognition within micronuclei. Nature [Internet] 548:466–470. Available from: http://dx.doi.org/10.1038/nature23470
Hashimoto K, Nakajima Y, Matsumura S, Chatani F. 2010. An in vitro micronucleus assay with size-classified micronucleus counting to discriminate aneugens from clastogens. Toxicol Vitr 24:208–216.
Hemler ME. 2005. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 6:801–811.
Hessvik NP, Llorente A. 2017. Current knowledge on exosome biogenesis and release. Cell Mol Life Sci 75:193–208.
Hessvik NP, Øverbye A, Brech A, Torgersen ML, Jakobsen IS, Sandvig K, Llorente A. 2016. PIKfyve inhibition increases exosome release and induces secretory autophagy. Cell Mol Life Sci 73:4717–4737.
Hill AF, Pegtel DM, Lambertz U, Leonardi T, O'Driscoll L, Pluchino S, Ter-Ovanesyan D, Nolte-'t Hoen ENM. 2013. ISEV position paper: extracellular vesicle RNA analysis and bioinformatics. J Extracell Vesicles [Internet] 2:22859. Available from: https://www.tandfonline.com/doi/full/10.3402/jev.v2i0.22859
Hintzsche H, Hemmann U, Poth A, Utesch D, Lott J, Stopper H. 2017. Fate of micronuclei and micronucleated cells. Mutat Res - Rev Mutat Res [Internet] 771:85–98. Available from: http://dx.doi.org/10.1016/j.mrrev.2017.02.002
Hobbs GA, Der CJ, Rossman KL. 2016. RAS isoforms and mutations in cancer at a glance. J Cell Sci [Internet] 129:1287–1292. Available from:

http://jcs.biologists.org/lookup/doi/10.1242/jcs.182873
Von Hoff DD, McGill JR, Forseth BJ, Davidson KK, Bradley TP, Van Devanter DR, Wahl GM. 1992. Elimination of extrachromosomally amplified MYC genes from human tumor cells reduces their tumorigenicity. Proc Natl Acad Sci U S A [Internet] 89:8165–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=49877&tool=pmcentrez&rendertype=abstract
Holmgren L, Szeles a, Rajnavölgyi E, Folkman J, Klein G, Ernberg I, Falk KI. 1999. Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood 93:3956–3963.
Holmgren L, Szeles A, Rajnavölgyi E, Folkman J, Klein G, Ernberg I, Falk KI. 2002. Horizontal transfer of DNA by the uptake of apoptotic bodies. Vox Sang 93:305–306.
Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmer L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Jørgen Labori K, Kure EH, Grandgenett PM, Hollingsworth MA, De Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D. 2015. Tumour exosome integrins determine organotropic metastasis. Nature 527:329–335.
Hromada C, Mühleder S, Grillari J, Redl H, Holnthoner W. 2017. Endothelial extracellular vesicles-promises and challenges. Front Physiol 8.
Hunter JC, Manandhar A, Carrasco M a, Gurbani D, Gondi S, Westover KD. 2015. Biochemical and Structural Analysis of Common Cancer-Associated KRAS Mutations. Mol Cancer Res [Internet]:1325–1336. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26037647
Ichikawa T, Schalken JA, Ichikawa Y, Steinberg GD, Isaacs JT. 1991. H-ras expression, genetic instability, and acquisition of metastatic ability by rat prostatic cancer cells following v-H-ras oncogene transfection. Prostate 18:163–172.
Imataka G, Arisaka O. 2012. Chromosome Analysis Using Spectral Karyotyping (SKY). Cell Biochem Biophys 62:13–17.
Inamdar S, Nitiyanandan R, Rege K. 2017. Emerging applications of exosomes in cancer therapeutics and diagnostics. Bioeng Transl Med [Internet] 2:70–80. Available from: http://doi.wiley.com/10.1002/btm2.10059
van Jaarsveld RH, Kops GJPL. 2016. Difference Makers: Chromosomal Instability versus Aneuploidy in Cancer. Trends in Cancer 2:561–571.
Jean-Pierre C, Perni SC, Bongiovanni AM, Kalish RB, Karasahan E, Ravich M, Ratushny V, Skupski DW, Witkin SS. 2006. Extracellular 70-kd heat shock protein in mid-trimester amniotic fluid and its effect on cytokine production by ex vivo-cultured amniotic fluid cells. Am J Obstet Gynecol 194:694–698.
Ji W, Bian Z, Yu Y, Yuan C, Liu Y, Yu L, Li C, Zhu J, Jia X, Guan R. 2014. Expulsion of micronuclei containing amplified genes contributes to a decrease in double minute chromosomes from malignant tumor cells. 1288:1279–1288.
Johannes L, Parton RG, Bassereau P, Mayor S. 2015. Building endocytic pits without clathrin. Nat Rev Mol Cell Biol 16:311–321.
Johnson DB, Puzanov I. 2015. Treatment of NRAS-Mutant Melanoma. Curr Treat Options Oncol 16.

Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. 1987. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem 262:9412–9420.
Kabeya Y. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J [Internet] 19:5720–5728. Available from: http://emboj.embopress.org/cgi/doi/10.1093/emboj/19.21.5720
Kadandale P, Kiger AA. 2010. Role of selective autophagy in cellular remodeling: "Self-eating" into shape. Autophagy 6:1194–1195.
Kadota T, Fujita Y, Yoshioka Y, Araya J, Kuwano K, Ochiya T. 2017. Emerging role of extracellular vesicles as a senescence-associated secretory phenotype: Insights into the pathophysiology of lung diseases. Mol Aspects Med [Internet] 60:92–103. Available from: https://doi.org/10.1016/j.mam.2017.11.005
Kahlert C, Melo SA, Protopopov A, Tang J, Seth S, Koch M, Zhang J, Weitz J, Chin L, Futreal A. 2014a. Identification of Double- stranded Genomic DNA Spanning All Chromosomes with Mutated KRAS and p53 DNA in the Serum Exosomes. J Biol Chem 289:3869–3875.
Kahlert C, Melo SA, Protopopov A, Tang J, Seth S, Koch O, Zhang J, Weitz J, Chin L, Futreal A, Kalluri R. 2014b. Identification of doublestranded genomic dna spanning all chromosomes with mutated KRAS and P53 DNA in the serum exosomes of patients with pancreatic cancer. J Biol Chem 289:3869–3875.
Kaiser J. 2018. "Liquid biopsy" for cancer promises early detection. Science (80-) [Internet] 359:259–259. Available from: http://www.sciencemag.org/lookup/doi/10.1126/science.359.6373.259
Kalinich M, Haber DA. 2018. Cancer detection: Seeking signals in blood. Science (80-) [Internet] 359:866–867. Available from: http://www.sciencemag.org/lookup/doi/10.1126/science.aas9102
Kalsbeek D, Golsteyn RM. 2017. G2/M-phase checkpoint adaptation and micronuclei formation as mechanisms that contribute to genomic instability in human cells. Int J Mol Sci 18.
Kamata T, Pritchard C. 2011. Mechanisms of aneuploidy induction by RAS and RAF oncogenes. Am J Cancer Res [Internet] 1:955–71. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3196290&tool=pmcentrez&rendertype=abstract
Kanada M, Bachmann MH, Hardy JW, Frimannson DO, Bronsart L, Wang A, Sylvester MD, Schmidt TL, Kaspar RL, Butte MJ, Matin AC, Contag CH. 2015. Differential fates of biomolecules delivered to target cells via extracellular vesicles. Proc Natl Acad Sci [Internet]:201418401. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1418401112
Karnoub AE, Weinberg RA. 2008. Ras oncogenes: Split personalities. Nat Rev Mol Cell Biol 9:517–531.
Keller S, Rupp C, Stoeck A, Runz S, Fogel M, Lugert S, Hager HD, Abdel-Bakky MS, Gutwein P, Altevogt P. 2007. CD24 is a marker of exosomes secreted into urine and amniotic fluid. Kidney Int Pap Shock protein-containing exosomes mid-trimester amniotic fluids - PubMed - NCBI.pdf [Internet] 72:1095–1102. Available from: http://dx.doi.org/10.1038/sj.ki.5002486
Kim KM, Abdelmohsen K, Mustapic M, Kapogiannis D, Gorospe M. 2017. RNA in extracellular vesicles. Wiley Interdiscip Rev RNA 8.
Kimura K, Hohjoh H, Fukuoka M, Sato W, Oki S, Tomi C, Yamaguchi H, Kondo T, Takahashi

R, Yamamura T. 2018. Circulating exosomes suppress the induction of regulatory T cells via let-7i in multiple sclerosis. Nat Commun [Internet] 9:10–15. Available from: http://dx.doi.org/10.1038/s41467-017-02406-2
Kisurina-Evgen'eva OP, Bryantseva SA, Shtil AA, Onishchenko GE. 2006. Antimicrotubule agents can activate different apoptotic pathways. cell Biophys 51:875–879.
Kisurina-Evgenieva OP, Sutiagina OI, Onishchenko GE. 2016. Biogenesis of micronuclei. Biochem [Internet] 81:453–464. Available from: http://link.springer.com/10.1134/S0006297916050035
Kitanaka C, Kato K, Ijiri R, Sakurada K, Tomiyama A, Noguchi K, Nagashima Y, Nakagawara A, Momoi T, Toyoda Y, Kigasawa H, Nishi T, Shirouzu M, Yokoyama S, Tanaka Y, Kuchino Y. 2002. Increased Ras Expression and Caspase-Independent Neuroblastoma Cell Death: Possible Mechanism of Spontaneous Neuroblastoma Regression. J Natl Cancer Inst [Internet] 94:358–368. Available from: http://jnci.oxfordjournals.org/content/94/5/358.abstract
Knauf JA, Ouyang B, Knudsen ES, Fukasawa K, Babcock G, Fagin JA. 2006. Oncogenic RAS induces accelerated transition through G2/M and promotes defects in the G2 DNA damage and mitotic spindle checkpoints. J Biol Chem 281:3800–3809.
Kops GJPL, Foltz DR, Cleveland DW. 2004. Lethality to human cancer cells through massive chromosome loss by inhibition of the mitotic checkpoint. Proc Natl Acad Sci [Internet] 101:8699–8704. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.0401142101
Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M, Théry C. 2016. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc Natl Acad Sci [Internet] 113:E968–E977. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1521230113
Kumari A, Folk WP, Sakamuro D. 2017. The dual roles of MYC in genomic instability and cancer chemoresistance. Genes (Basel) 8.
Kuttler F, Mai S. 2007. Formation of non-random extrachromosomal elements during development, differentiation and oncogenesis. Semin Cancer Biol 17:56–64.
Labidi B, Gregoire M, Frackowiak S, Hernandez-Verdun D, Bouteille M. 1987. RNA polymerase activity in PtK1 micronuclei containing individual chromosomes. An in vitro and in situ study. Exp Cell Res 169:233–244.
Laird PW, Zijderveld a, Linders K, Rudnicki M a, Jaenisch R, Berns a. 1991. Simplified mammalian DNA isolation procedure. Nucleic Acids Res 19:4293.
Lake D, Corrêa SAL, Müller J. 2016. Negative feedback regulation of the ERK1/2 MAPK pathway. Cell Mol Life Sci [Internet] 73:4397–4413. Available from: http://link.springer.com/10.1007/s00018-016-2297-8
Lamb CA, Yoshimori T, Tooze SA. 2013. The autophagosome: Origins unknown, biogenesis complex. Nat Rev Mol Cell Biol 14:759–774.
Lamichhane TN, Sokic S, Schardt JS, Raiker RS, Lin JW, Jay SM. 2015. Emerging Roles for Extracellular Vesicles in Tissue Engineering and Regenerative Medicine. Tissue Eng Part B Rev [Internet] 21:45–54. Available from: http://online.liebertpub.com/doi/abs/10.1089/ten.teb.2014.0300
Lässer C, Seyed Alikhani V, Ekström K, Eldh M, Torregrosa Paredes P, Bossios A, Sjöstrand M, Gabrielsson S, Lötvall J, Valadi H. 2011. Human saliva, plasma and breast milk exosomes contain RNA: Uptake by macrophages. J Transl Med [Internet] 9:9. Available from:

http://www.translational-medicine.com/content/9/1/9
Lazaro-Ibanez E, Sanz-Garcia A, Visakorpi T, Escobedo-Lucea C, Siljander P, Ayuso-Sacido ??ngel, Yliperttula M. 2014. Different gDNA content in the subpopulations of prostate cancer extracellular vesicles: Apoptotic bodies, microvesicles, and exosomes. Prostate 74:1379–1390.
Leach NT, Jackson-Cook C. 2001. The application of spectral karyotyping (SKY) and fluorescent in situ hybridization (FISH) technology to determine the chromosomal content(s) of micronuclei. Mutat Res - Genet Toxicol Environ Mutagen 495:11–19.
Lee TH, Chennakrishnaiah S, Audemard E, Montermini L, Meehan B, Rak J. 2014. Oncogenic ras-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells. Biochem Biophys Res Commun [Internet] 451:295–301. Available from: http://dx.doi.org/10.1016/j.bbrc.2014.07.109
Lee TH, Chennakrishnaiah S, Meehan B, Montermini L, Garnier D, D'Asti E, Hou W, Magnus N, Gayden T, Jabado N, Eppert K, Majewska L, Rak J. 2016a. Barriers to horizontal cell transformation by extracellular vesicles containing oncogenic H-ras. Oncotarget [Internet]:8–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27437771
Lee TH, Chennakrishnaiah S, Meehan B, Montermini L, Garnier D, D'Asti E, Hou W, Magnus N, Gayden T, Jabado N, Eppert K, Majewska L, Rak J. 2016b. Barriers to horizontal cell transformation by extracellular vesicles containing oncogenic H-ras. Oncotarget [Internet] 7:8–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27437771
Lee TH, Chennakrishnaiah S, Rak J. 2015. Oncogene-dependent survival of highly transformed cancer cells under conditions of extreme centrifugal force - Implications for studies on extracellular vesicles. Cell Mol Biol Lett 20:117–129.
Lee TH, D'Asti E, Magnus N, Al-Nedawi K, Meehan B, Rak J. 2011. Microvesicles as mediators of intercellular communication in cancer--the emerging science of cellular "debris". Semin Immunopathol 33:455–467.
Lengauer C, Kinzler KW, Vogelstein B. 1997. Genetic instability in colorectal cancers. Nature 386:623–627.
Lespagnol A, Duflaut D, Beekman C, Blanc L, Fiucci G, Marine JC, Vidal M, Amson R, Telerman A. 2008. Exosome secretion, including the DNA damage-induced p53-dependent secretory pathway, is severely compromised in TSAP6/Steap3-null mice. Cell Death Differ 15:1723–1733.
Levy S, Shoham T. 2005. The tetraspanin web modulates immune-signalling complexes. Nat Rev Immunol 5:136–148.
Lewis CW, Golsteyn RM. 2016. Cancer cells that survive checkpoint adaptation contain micronuclei that harbor damaged DNA. Cell Cycle [Internet] 15:3131–3145. Available from: http://dx.doi.org/10.1080/15384101.2016.1231287
Li D, Zhu J, Firozi PF, Abbruzzese JL, Evans DB, Cleary K, Friess H, Sen S. 2003. Overexpression of oncogenic STK15/BTAK/Aurora A kinase in human pancreatic cancer. Clin Cancer Res 9:991–997.
Lim JP, Gleeson PA. 2011. Macropinocytosis: An endocytic pathway for internalising large gulps. Immunol Cell Biol [Internet] 89:836–843. Available from: http://dx.doi.org/10.1038/icb.2011.20
Lindberg HK, Falck GCM, Järventaus H, Norppa H. 2008. Characterization of chromosomes and chromosomal fragments in human lymphocyte micronuclei by telomeric and centromeric FISH. Mutagenesis 23:371–376.

Lisanti MP, Martinez-Outschoorn UE, Sotgia F. 2013. Oncogenes induce the cancer-associated fibroblast phenotype: Metabolic symbiosis and “fibroblast addiction” are new therapeutic targets for drug discovery. Cell Cycle 12:2723–2732.
Lobb RJ, Becker M, Wen SW, Wong CSF, Wiegmans AP, Leimgruber A, Möller A. 2015. Optimized exosome isolation protocol for cell culture supernatant and human plasma. J Extracell Vesicles 4:1–11.
Long LC. 2002. Simplified DNA Extraction from Cell or Tissue. J Biol methods.
Lotvall J, Hill AF, Hochberg F, Buzas EI, Vizio D Di, Gardiner C, Gho YS, Kurochkin I V., Mathivanan S, Quesenberry P, Sahoo S, Tahara H, Wauben MH, Witwer KW, Thï¿½ry C. 2014. Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles 3:1–6.
Loughran P, Stolz DB, Basse P, Wang T, Houten B Van. 2013. Inhibiting autophagy during interleukin 2(IL-2) immunotherapy promotes long term tumor regression. Cancer Res 72:2791–2801.
Luzhna L, Kathiria P, Kovalchuk O. 2013. Micronuclei in genotoxicity assessment: From genetics to epigenetics and beyond. Front Genet 4:1–17.
MacKenzie KJ, Carroll P, Martin CA, Murina O, Fluteau A, Simpson DJ, Olova N, Sutcliffe H, Rainger JK, Leitch A, Osborn RT, Wheeler AP, Nowotny M, Gilbert N, Chandra T, Reijns MAM, Jackson AP. 2017. CGAS surveillance of micronuclei links genome instability to innate immunity. Nature [Internet] 548:461–465. Available from: http://dx.doi.org/10.1038/nature23449
Mai S, Fluri M, Siwarski D, Huppi K. 1996. Genomic instability in MycER-activated Rat1A-MycER cells. Chromosom Res 4:365–371.
Maleki SS, Röcken C. 2017. Chromosomal Instability in Gastric Cancer Biology. Neoplasia [Internet] 19:412–420. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1476558617300805
Malloy A. 2011. Count, size and visualize nanoparticles. Mater Today [Internet] 14:170–173. Available from: http://dx.doi.org/10.1016/S1369-7021(11)70089-X
Mamat AMI, Nasir REM, Wisnoe WKW, Ngah Z, Ramly R. 2008. Histological Follow-Up in Patients with Atypical Glandular Cells on Pap Smears. J Mech Eng 5:4–8.
Marzella L, Ahlberg J, Glaumann H. 1981. Autophagy, heterophagy, microautophagy and crinophagy as the means for intracellular degradation. Virchows Arch B Cell Pathol Incl Mol Pathol 36:219–234.
Marzesco A-M. 2005. Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. J Cell Sci [Internet] 118:2849–2858. Available from: http://jcs.biologists.org/cgi/doi/10.1242/jcs.02439
Medvedeva NG, Panyutin I V, Panyutin IG, Neumann RD. 2007. Phosphorylation of histone H2AX in radiation-induced micronuclei. Radiat Res [Internet] 168:493–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17903033
Meehan B, Rak J, Vizio D Di. 2016. Oncosomes - large and small: What are they, where they came from? J Extracell Vesicles 5.
Di Micco R. 2017. Sensing the Breaks: Cytosolic Chromatin in Senescence and Cancer. Trends Mol Med 23:1067–1070.
Milsom CC, Yu JL, Mackman N, Micallef J, Anderson GM, Guha A, Rak JW. 2008. Tissue factor regulation by epidermal growth factor receptor and epithelial-to-mesenchymal

transitions: Effect on tumor initiation and angiogenesis. Cancer Res 68:10068–10076.
Mizushima N. 2007. Autophagy : process and function. Genes Dev.
Mizushima N, Levine B, Cuervo AM, Klionsky DJ. 2008. Autophagy fights disease through cellular self digestion. Nature 451:1069–1075.
Montermini L, Meehan B, Garnier D, Lee WJ, Lee TH, Guha A, Al-Nedawi K, Rak J. 2015. Inhibition of oncogenic epidermal growth factor receptor kinase triggers release of exosome-like extracellular vesicles and impacts their phosphoprotein and DNA content. J Biol Chem 290:24534–24546.
Mortimer RK, Hawthorne DC. 1975. Yeast Cells - David M. Prescott - Google Books. Methods Cell Biol [Internet]. Available from: http://books.google.com/books?hl=en&lr=&id=HoNOcBFnpv0C&oi=fnd&pg=PA221&dq=Genetic+analysis+in+Saccharomyces+cerevisiae+and+Schizosaccharomyces+pombe+In+The+Yeasts+Rose+AH+Harrison+JS+eds&ots=xrgm8PCF7r&sig=PZWAIoeXcIIIN1E9DNhK4IkmVbA
Morvan J, Köchl R, Watson R, Collinson LM, Jefferies HBJ, Tooze SA. 2009. In vitro reconstitution of fusion between immature autophagosomes and endosomes. Autophagy 5:676–689.
Mrakovcic M, Fröhlich LF. 2018. p53-Mediated Molecular Control of Autophagy in Tumor Cells. Biomolecules [Internet] 8:14. Available from: http://www.mdpi.com/2218-273X/8/2/14
Muhsin-Sharafaldine M-R, Saunderson SC, Dunn AC, Faed JM, Kleffmann T, McLellan AD. 2016. Procoagulant and immunogenic properties of melanoma exosomes, microvesicles and apoptotic vesicles. Oncotarget [Internet] 7:56279–56294. Available from: http://www.oncotarget.com/fulltext/10783
Mulcahy LA, Pink RC, Carter DRF. 2014. Routes and mechanisms of extracellular vesicle uptake. J Extracell Vesicles 3.
Müller G, Schneider M, Gassenhuber J, Wied S. 2012. Release of exosomes and microvesicles harbouring specific RNAs and glycosylphosphatidylinositol-anchored proteins from rat and human adipocytes is controlled by histone methylation. Am J Mol Biol [Internet] 2:187–209. Available from: http://www.scirp.org/journal/PaperDownload.aspx?DOI=10.4236/ajmb.2012.23020
Münz C. 2017. Epstein-Barr virus-specific immune control by innate lymphocytes. Front Immunol 8:1–7.
Mure K, Takeshita T, Morimoto K. 1996. Categorization of Micronuclei by Size and Measurement of Each Ratio in Cytokinesis-block and Conventional Cultures of Human Lymphocytes exposed to Mitomycin C and Colchicine. Environ Health Prev Med 1:93–99.
Murrow L, Malhotra R, Debnath J. 2015a. ATG12-ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. Nat Cell Biol 17:300–310.
Murrow L, Malhotra R, Debnath J. 2015b. ATG12-ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. Nat Cell Biol 17:300–310.
Musante L, Tataruch D, Gu D, Benito-Martin A, Calzaferri G, Aherne S, Holthofer H. 2014. A simplified method to recover urinary vesicles for clinical applications, and sample banking. Sci Rep 4.
Nabet B, Ó Broin P, Reyes JM, Shieh K, Lin CY, Will CM, Popovic R, Ezponda T, Bradner JE, Golden AA, Licht JD. 2015. Deregulation of the Ras-Erk Signaling Axis Modulates the Enhancer Landscape. Cell Rep 12:1300–1313.

Nakano I, Garnier D, Minata M, Rak J. 2015. Extracellular vesicles in the biology of brain tumour stem cells--Implications for inter-cellular communication, therapy and biomarker development. Semin Cell Dev Biol 40:17–26.
Nathanson DA, Gini B, Mottahedeh J, Visnyei K, Koga T, Gomez G, Eskin A, Hwang K, Wang J, Masui K, Paucar A, Yang H, Ohashi M, Zhu S, Wykosky J, Reed R, Nelson SF, Cloughesy TF, James CD, Rao PN, Kornblum HI, Heath JR, Cavenee WK, Furnari FB, Mischel PS. 2014. Targeted therapy resistance mediated by dynamic regulation of extrachromosomal mutant EGFR DNA. Science (80-) 343:72–76.
Netea-Maier RT, Klück V, Plantinga TS, Smit JWA. 2015. Autophagy in thyroid cancer: Present knowledge and future perspectives. Front Endocrinol (Lausanne) 6:27–29.
Norppa H, Falck GCM. 2003. What do human micronuclei contain? Mutagenesis 18:221–233.
Nowell PC. 1976. The clonal evolution of tumor cell populations. Science [Internet] 194:23–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/959840
Nussinov R, Jang H, Tsai C-J. 2014. The structural basis for cancer treatment decisions. Oncotarget [Internet] 5:7285–7302. Available from: http://www.oncotarget.com/fulltext/2439
Obe G, Beek B, Vidyadhar Gopal Vaidya. 1975. The human leukocyte test system. III. Premature chromosome condensation from chemically and X-ray induced micronuclei. Mutat Res - Fundam Mol Mech Mutagen 27:89–101.
Ogawa Y, Taketomi Y, Murakami M, Tsujimoto M, Yanoshita R. 2013. Small RNA transcriptomes of two types of exosomes in human whole saliva determined by next generation sequencing. Biol Pharm Bull [Internet] 36:66–75. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23302638
Ojha CR, Lapierre J, Rodriguez M, Dever SM, Zadeh MA, Demarino C, Pleet ML, Kashanchi F, El-Hage N. 2017. Interplay between autophagy, exosomes and HIV-1 associated neurological disorders: New insights for diagnosis and therapeutic applications. Viruses 9:1–21.
Okamoto A, Utani KI, Shimizu N. 2012. DNA replication occurs in all lamina positive micronuclei, but never in lamina negative micronuclei. Mutagenesis 27:323–327.
Orr B, Compton DA. 2013. A Double-Edged Sword: How Oncogenes and Tumor Suppressor Genes Can Contribute to Chromosomal Instability. Front Oncol [Internet] 3:1–14. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2013.00164/abstract
Osswald M, Jung E, Sahm F, Solecki G, Venkataramani V, Blaes J, Weil S, Horstmann H, Wiestler B, Syed M, Huang L, Ratliff M, Karimian Jazi K, Kurz FT, Schmenger T, Lemke D, Gömmel M, Pauli M, Liao Y, Häring P, Pusch S, Herl V, Steinhäuser C, Krunic D, Jarahian M, Miletic H, Berghoff AS, Griesbeck O, Kalamakis G, Garaschuk O, Preusser M, Weiss S, Liu H, Heiland S, Platten M, Huber PE, Kuner T, Von Deimling A, Wick W, Winkler F. 2015. Brain tumour cells interconnect to a functional and resistant network. Nature 528:93–98.
Owens a PI, Mackman N. 2011. Microparticles in hemostasis and thrombosis. Circ Res 108:1284–1297.
Palanisamy V, Sharma S, Deshpande A, Zhou H, Gimzewski J, Wong DT. 2010. Nanostructural and transcriptomic analyses of human saliva derived exosomes. PLoS One 5.
Pampalona J, Roscioli E, Silkworth WT, Bowden B, Genescà A, Tusell L, Cimini D. 2016. Chromosome bridges maintain kinetochore-microtubule attachment throughout mitosis and

rarely break during anaphase. PLoS One 11:1–17.
Pan BT, Johnstone RM. 1983. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: Selective externalization of the receptor. Cell 33:967–978.
Pan BT, Teng K, Wu C, Adam M, Johnstone RM. 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. J Cell Biol 101:942–948.
Papandreou M, Tavernarakis N. 2017. Autophagy and the endo / exosomal pathways in health and disease. Biotechnol J:1–11.
Peace BE, Livingston G, Silberstein EB, Loper JC. 1999. A case of elevated spontaneous micronucleus frequency derived from chromosome 2. Mutat Res - Fundam Mol Mech Mutagen 430:109–119.
Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, García-Santos G, Ghajar CM, Nitadori-Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y, Bromberg J, Lyden D. 2012. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 18:883–891.
Perera D, Venkitaraman AR. 2016. Oncogenic KRAS triggers MAPK-dependent errors in mitosis and MYC-dependent sensitivity to anti-mitotic agents. Sci Rep 6.
Pisitkun T, Shen R-F, Knepper MA. 2004. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA [Internet] 101:13368–13373. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=516573&tool=pmcentrez&rendertype=abstract
Pols MS, Klumperman J. 2009. Trafficking and function of the tetraspanin CD63. Exp Cell Res [Internet] 315:1584–1592. Available from: http://dx.doi.org/10.1016/j.yexcr.2008.09.020
Potapova T, Gorbsky G. 2017. The Consequences of Chromosome Segregation Errors in Mitosis and Meiosis. Biology (Basel) [Internet] 6:12. Available from: http://www.mdpi.com/2079-7737/6/1/12
Putz U, Howitt J, Doan A, Goh CP, Low LH, Silke J, Tan SS. 2012. The tumor suppressor PTEN is exported in exosomes and has phosphatase activity in recipient cells. Sci Signal 5.
Quesenberry PJ, Aliotta J, Deregibus MC, Camussi G. 2015. Role of extracellular RNA-carrying vesicles in cell differentiation and reprogramming. Stem Cell Res Ther 6.
Rajalingam K, Schreck R, Rapp UR, Albert Š. 2007. Ras oncogenes and their downstream targets. Biochim Biophys Acta - Mol Cell Res 1773:1177–1195.
Rajasekharan S, Raman T. 2013. Ras and Ras mutations in cancer. Open Life Sci [Internet] 8. Available from: http://www.degruyter.com/view/j/biol.2013.8.issue-7/s11535-013-0158-5/s11535-013-0158-5.xml
Rak J, Filmus J, Finkenzeller G, Grugel S, Marmé D, Rs K. 1995a. Oncogenes as inducers of tumor angiogenesis . Cancer Metastasis Rev 14.
Rak J, Mitsuhashi Y, Erdos V, Huang S, Filmus J, Kerbel RS. 1995b. Massive Programmed Cell Death in Intestinal Epithelial Cells Induced by Three-dimensional Growth Conditions: Suppression by Mutant. J Cell Biol 131:1587–1598.
Rak J, Yu J, Milsom C. 2009. Oncogene-driven hemostatic changes in cancer. Cancer Invest 27:28–35.
Raposo G. 1996. B lymphocytes secrete antigen-presenting vesicles. J Exp Med [Internet] 183:1161–1172. Available from: http://www.jem.org/cgi/doi/10.1084/jem.183.3.1161

Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, Dvorak P, Ratajczak MZ. 2006. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: Evidence for horizontal transfer of mRNA and protein delivery. Leukemia 20:847–856.
Reggiori F, Komatsu M, Finley K, Simonsen A. 2012a. Autophagy: More than a nonselective pathway. Int J Cell Biol 2012.
Reggiori F, Komatsu M, Finley K, Simonsen A. 2012b. Selective types of autophagy. Int J Cell Biol 2012.
Rello-Varona S, Lissa D, Shen S, Niso-Santano M, Senovilla L, Mariño G, Vitale I, Jemaà M, Harper F, Pierron G, Castedo M, Kroemer G. 2012. Autophagic removal of micronuclei. Cell Cycle 11:170–176.
Richard F, Muleris M, Bernard D. 1994. The frequency of micronuclei with X chromosome increases with age in human females. Mutat Res DNAging 316:1–7.
Robbins PD, Morelli AE. 2014. Regulation of Immune Responses by Extracellular Vesicules. Nat Immunol 14:195–208.
Rubinsztein DC, Bento CF, Deretic V. 2015. Therapeutic targeting of autophagy in neurodegenerative and infectious diseases. J Exp Med [Internet] 212:979–990. Available from: http://www.jem.org/lookup/doi/10.1084/jem.20150956
Rusten TE, Stenmark H. 2009. How do ESCRT proteins control autophagy? J Cell Sci [Internet] 122:2179–2183. Available from: http://jcs.biologists.org/cgi/doi/10.1242/jcs.050021
Saavedra HI, Fukasawa K, Conn CW, Stambrook PJ. 1999. MAPK mediates RAS-induced chromosome instability. J Biol Chem 274:38083–38090.
Saavedra HI, Knauf J a, Shirokawa JM, Wang J, Ouyang B, Elisei R, Stambrook PJ, Fagin J a. 2000. The RAS oncogene induces genomic instability in thyroid PCCL3 cells via the MAPK pathway. Oncogene [Internet] 19:3948–3954. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10951588%5Cnhttp://www.nature.com/onc/journal/v19/n34/pdf/1203723a.pdf
Sagona AP, Nezis IP, Stenmark H. 2014. Association of CHMP4B and autophagy with micronuclei: Implications for cataract formation. Biomed Res Int 2014.
Sahu R, Kaushik S, Clement CC, Cannizzo ES, Scharf B, Follenzi A, Potolicchio I, Nieves E, Cuervo AM, Santambrogio L. 2011. Microautophagy of Cytosolic Proteins by Late Endosomes. Dev Cell 20:131–139.
Sakuno T, Tada K, Watanabe Y. 2009. Kinetochore geometry defined by cohesion within the centromere. Nature [Internet] 458:852–858. Available from: http://dx.doi.org/10.1038/nature07876
Sansone P, Savini C, Kurelac I, Chang Q, Amato LB, Strillacci A, Stepanova A, Iommarini L, Mastroleo C, Daly L, Galkin A, Thakur BK, Soplop N, Uryu K, Hoshinob A, Norton L, Bonafé M, Cricca M, Gasparre G, Lyden D, Bromberg J. 2017. Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. Proc Natl Acad Sci [Internet]:201704862. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1704862114
Sanz-garcia A, Visakorpi T, Escobedo-lucea C, Siljander P. 2014. Different gDNAContent in the Subpopulations of Prostate Cancer Extracellular Vesicles : Apoptotic Bodies , Microvesicles , and Exosomes a n. Prostate 1390.
Schiera G, Di Liegro CM, Puleo V, Colletta O, Fricano A, Cancemi P, Di Cara G, Di Liegro I. 2016. Extracellular vesicles shed by melanoma cells contain a modified form of H1.0 linker histone and H1.0 mRNA-binding proteins. Int J Oncol 49:1807–1814.

Schiera G, Di Liegro CM, Saladino P, Pitti R, Savettieri G, Proia P, Di Liegro I. 2013. Oligodendroglioma cells synthesize the differentiation-specific linker histone H1deg; and release it into the extracellular environment through shed vesicles. Int J Oncol 43:1771–1776.
Schmidt O, Teis D. 2012. The ESCRT machinery. Curr Biol [Internet] 22:R116–R120. Available from: http://dx.doi.org/10.1016/j.cub.2012.01.028
Schmukler E, Kloog Y, Pinkas-Kramarski R. 2014. Ras and autophagy in cancer development and therapy. Oncotarget 5:577–86.
Schmukler E, Wolfson E, Haklai R, Elad-sfadia G, Kloog Y. 2013. Chloroquine synergizes with FTS to enhance cell growth inhibition and cell death ABSTRACT : 5.
Schultz N, Önfelt A. 1994. Video time-lapse study of mitosis in binucleate V79 cells: Chromosome segregation and cleavage. Mutagenesis 9:117–123.
Sears AD, Udden MM. 2012. Howell - Jolly Bodies : A Brief Historical Review. Am J Med Sci 343:407–409.
Sen S, Zhou H, Zhang R-D, Yoon DS, Vakar-Lopez F, Ito S, Jiang F, Johnston D, Grossman HB, Ruifrok AC, Katz RL, Brinkley W, Czerniak B. 2002. Amplification/overexpression of a mitotic kinase gene in human bladder cancer. J Natl Cancer Inst 94:1320–1329.
Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). Cell 88:593–602.
Shelke G, Jang SC, Yin Y, Lässer C, Lötvall J. 2016. Human mast cells release extracellular vesicle-associated DNA. Matters (Zürich) [Internet]. Available from: https://sciencematters.io/articles/10.19185/matters.201602000034
Shimizu N. 2011. Molecular mechanisms of the origin of micronuclei from extrachromosomal elements. Mutagenesis 26:119–123.
Shimizu N, Shimura T, Tanaka T. 2000. Selective elimination of acentric double minutes from cancer cells through the extrusion of micronuclei. Mutat Res Mol Mech Mutagen 448:81–90.
Simpson RJ, Jensen SS, Lim JWE. 2008. Proteomic profiling of exosomes: Current perspectives. Proteomics 8:4083–4099.
Singh R, Cuervo AM. 2011. Autophagy in the cellular energetic balance. Cell Metab [Internet] 13:495–504. Available from: http://dx.doi.org/10.1016/j.cmet.2011.04.004
Siravegna G, Marsoni S, Siena S, Bardelli A. 2017. Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 14:531–548.
Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Curry WT, Carter BS, Krichevsky AM, Breakefield XO. 2008. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 10:1470–1476.
Slater AFG. 1993. Chloroquine: Mechanism of Drug Action and Resistance in Plasmodium Falcipar Um. Pharmac Ther 57:203–235.
Steensel B Van, S., Belmont A. 2017. Lamina-Associated Domains: Links with Chromosome Architecture, Heterochromatin, and Gene Repression. Cell [Internet] 169:780–791. Available from: http://dx.doi.org/10.1016/j.cell.2017.04.022
Stephens PJ, Greenman CD, Fu B, Yang F, Bignell GR, Mudie LJ, Pleasance ED, Lau KW, Beare D, Stebbings LA, McLaren S, Lin ML, McBride DJ, Varela I, Nik-Zainal S, Leroy C, Jia M, Menzies A, Butler AP, Teague JW, Quail MA, Burton J, Swerdlow H, Carter NP, Morsberger LA, Iacobuzio-Donahue C, Follows GA, Green AR, Flanagan AM, Stratton

MR, Futreal PA, Campbell PJ. 2011. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell [Internet] 144:27–40. Available from: http://dx.doi.org/10.1016/j.cell.2010.11.055

Street JM, Barran PE, Mackay CL, Weidt S, Balmforth C, Walsh TS, Chalmers RTA, Webb DJ, Dear JW. 2012. Identification and proteomic profiling of exosomes in human cerebrospinal fluid. J Transl Med [Internet] 10:5. Available from: http://www.translational-medicine.com/content/10/1/5

Stuffers S, Sem Wegner C, Stenmark H, Brech A. 2009. Multivesicular endosome biogenesis in the absence of ESCRTs. Traffic 10:925–937.

Sugihara E, Kanai M, Matsui A, Onodera M, Schwab M, Miwa M. 2004. Enhanced expression of MYCN leads to centrosome hyperamplification after DNA damage in neuroblastoma cells. Oncogene 23:1005–1009.

Sukegawa J, Blobel G. 1993. A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. Cell 72:29–38.

Sun B, Dalvi P, Abadjian L, Tang N, Pulliam L. 2017. Blood neuron-derived exosomes as biomarkers of cognitive impairment in HIV. 1.

Tae Hoon Lee, Montermini L, Meehan B, Guha A, Rak J. 2011. Microvesicles as intercellular messengers carrying oncogenic and tumour suppressory signals. Canadian Cancer Research Conference 2011.

Takahashi A, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S, Takasugi M, Watanabe S, Kanemaki MT, Obuse C, Hara E. 2017. Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. Nat Commun [Internet] 8:1–14. Available from: http://dx.doi.org/10.1038/ncomms15287

Tarapore P, Fukasawa K. 2002. Loss of p53 and centrosome hyperamplification. Oncogene 21:6234–6240.

Terradas M, Martín M, Tusell L, Genescà A. 2009. DNA lesions sequestered in micronuclei induce a local defective-damage response. DNA Repair (Amst) 8:1225–1234.

Terradas M, Martín M, Tusell L, Genescà A. 2010. Genetic activities in micronuclei: Is the DNA entrapped in micronuclei lost for the cell? Mutat Res - Rev Mutat Res 705:60–67.

Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, Zheng Y, Hoshino A, Brazier H, Xiang J, Williams C, Rodriguez-Barrueco R, Silva JM, Zhang W, Hearn S, Elemento O, Paknejad N, Manova-Todorova K, Welte K, Bromberg J, Peinado H, Lyden D. 2014. Double-stranded DNA in exosomes: A novel biomarker in cancer detection. Cell Res 24:766–769.

The Nobel Assembly. 2016. The Nobel Assembly at Karolinska Institutet has today decided to award the 2016 Nobel Prize in Physiology or Medicine.

Théry C, Ostrowski M, Segura E. 2009. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9:581–593.

Thompson SL, Compton DA. 2008. Examining the link between chromosomal instability and aneuploidy in human cells. J Cell Biol 180:665–672.

Thompson SL, Compton DA. 2011a. Chromosomes and cancer cells. Chromosom Res 19:433–444.

Thompson SL, Compton DA. 2011b. Chromosome missegregation in human cells arises through specific types of kinetochore-microtubule attachment errors. Proc Natl Acad Sci [Internet] 108:17974–17978. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.1109720108

Tomas A, Futter CE, Eden ER. 2014. EGF receptor trafficking: Consequences for signaling and

cancer. Trends Cell Biol 24:26–34.
Tusell L, Pampalona J, Soler D, Frias C, Genescà A. 2010. Different outcomes of telomere-dependent anaphase bridges. Biochem Soc Trans [Internet] 38:1698–1703. Available from: http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=21118150&retmode=ref&cmd=prlinks%5Cnpapers3://publication/doi/10.1042/BST0381698
Utani KI, Kohno Y, Okamoto A, Shimizu N. 2010. Emergence of micronuclei and their effects on the fate of cells under replication stress. PLoS One 5.
Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654–659.
Villarroya-Beltri C, Baixauli F, Mittelbrunn M, Fernández-Delgado I, Torralba D, Moreno-Gonzalo O, Baldanta S, Enrich C, Guerra S, Sánchez-Madrid F. 2016. ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins. Nat Commun 7.
Di Vizio D, Kim J, Hager MH, Morello M, Yang W, Lafargue CJ, True LD, Rubin MA, Adam RM, Beroukhim R, Demichelis F, Freeman MR. 2009. Oncosome formation in prostate cancer: Association with a region of frequent chromosomal deletion in metastatic disease. Cancer Res 69:5601–5609.
Vogelstein B, Kinzler KW. 2004a. Cancer genes and the pathways they control Bert Vogelstein & Kenneth W Kinzler. Nat Med 10:789–799.
Vogelstein B, Kinzler KW. 2004b. Cancer genes and the pathways they control. Nat Med 10:789–799.
Vogelstein B, Lane D, Levine AJ. 2000. Surfing the p53 network. Nature 408:307–310.
de Vries JE, Kornips FHAC, Marx P, Bosman FT, Geraedts JPM, Kate J ten. 1993. Transfected c-Ha-ras oncogene enhances karyotypic instability and integrates predominantly in aberrant chromosomes. Cancer Genet Cytogenet 67:35–43.
Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, Pacey S, Baird R, Rosenfeld N. 2017. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nat Rev Cancer [Internet] 17:223–238. Available from: http://dx.doi.org/10.1038/nrc.2017.7
Wang AL, Lukas TJ, Yuan M, Du N, Tso MO, Neufeld AH. 2009. Autophagy and Exosomes in the Aged Retinal Pigment Epithelium: Possible Relevance to Drusen Formation and Age-Related Macular Degeneration. PLoS One [Internet] 4:e4160. Available from: http://dx.plos.org/10.1371/journal.pone.0004160
Wei Q, Huang H. 2013. Insights into the role of cell-cell junctions in physiology and disease. 1st ed. Elsevier Inc. Available from: http://dx.doi.org/10.1016/B978-0-12-407694-5.00005-5
White E. 2015. The role for autophagy in cancer. J Clin Invest [Internet] 125:42–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25654549%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4382247
Wido TM, Myles JL, Pisharodi L, Sawyer T, Kim K, Gohara AF, Howard JM. 1990. Aneuploid DNA content in pancreatic adenocarcinoma. Int J Pancreatol 7:129–134.
Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, Nolte-'t Hoen EN, Piper MG, Sivaraman S, Skog J, Théry C, Wauben MH, Hochberg F. 2013. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles 2:1–25.
Wollert T, Hurley JH. 2010. Molecular mechanism of Multivesicular Body Biogenesis by

ESCRT Complexes. Nature 464:864–869.
Woo RA, Poon RYC. 2004. Activated oncogenes promote and cooperate with chromosomal instability for neoplastic transformation. Genes Dev 18:1317–1330.
Xiaoyan L, Vera ME De, Buchser WJ, Chavez AR de V, Loughran P, Stolz DB, Basse P, Wang T, Houten B Van, Herbert J. Zeh A, Lotze MT. 2012. Inhibiting Autophagy During Interleukin 2 (IL-2) Immunotherapy Promotes Long Term Tumor Regression. Cancer Res 72:2791–2801.
Yang G, Mercado-Uribe I, Multani AS, Sen S, Shih IM, Wong KK, Gershenson DM, Liu J. 2013. RAS promotes tumorigenesis through genomic instability induced by imbalanced expression of Aurora-A and BRCA2 in midbody during cytokinesis. Int J Cancer 133:275–285.
Yang ZJ, Chee CE, Huang S, Sinicrope FA. 2011. The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther [Internet] 10:1533–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21878654%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3170456
Yasui M, Koyama N, Koizumi T, Senda-Murata K, Takashima Y, Hayashi M, Sugimoto K, Honma M. 2010. Live cell imaging of micronucleus formation and development. Mutat Res - Fundam Mol Mech Mutagen [Internet] 692:12–18. Available from: http://dx.doi.org/10.1016/j.mrfmmm.2010.07.009
Yu JL, May L, Lhotak V, Shahrzad S, Shirasawa S, Weitz JI, Coomber BL, Mackman N, Rak JW. 2005. Oncogenic events regulate tissue factor expression in colorectal cancer cells : implications for tumor progression and angiogenesis. Blood 105:1734–1742.
Yuyama K, Yamamoto N, Yanagisawa K. 2008. Accelerated release of exosome-associated GM1 ganglioside (GM1) by endocytic pathway abnormality: Another putative pathway for GM1-induced amyloid fibril formation. J Neurochem 105:217–224.
Zadeh MA, Demarino C, Pleet ML, Kashanchi F. 2017. Interplay between Autophagy, Exosomes and HIV-1 Associated Neurological Disorders: New Insights for Diagnosis and Therapeutic Applications. Viruses 1:1–21.
Zenonos K. 2013. RAS signaling pathways, mutations and their role in colorectal cancer. World J Gastrointest Oncol [Internet] 5:97. Available from: http://www.wjgnet.com/1948-5204/full/v5/i5/97.htm
Zhang H, Freitas D, Sang Kim H,Fabijanic K, Li Z, Chen H, Tesic Mark M, Molina H, Benito Martin A, Bojmar L, Fang J, Rampersaud S, Hoshino A, Matei I, Kenific CM, Nakajima M, Peter Mutvei A, Sansone P, Buehring W, Wang H, Pablo Jimenez J, Cohen-Gould L, Paknejad N, Brendel M, Manova-Todorova K, Magalhães A, Alexandre Ferreira J, Osório H, Silva AM, Massey A, Cubillos-Ruiz JR, Galletti G, Giannakakou P, Maria Cuervo A, Blenis J, Schwartz R, Sue Brady M, Peinado H, Bromberg J, Matsui H, Reis CA, Lyden D. 2018. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. Nat Cell Biol [Internet] 20. Available from: https://www.nature.com/articles/s41556-018-0040-4.pdf
Zhang CZ, Spektor A, Cornils H, Francis JM, Jackson EK, Liu S, Meyerson M, Pellman D. 2015. Chromothripsis from DNA damage in micronuclei. Nature 522:179–184.
Zhang W, Xia W, Lv Z, Ni C, Xin Y, Yang L. 2017. Liquid Biopsy for Cancer: Circulating Tumor Cells, Circulating Free DNA or Exosomes? Cell Physiol Biochem 41:755–768.
Zhou Q, Li M, Wang X, Li Q, Wang T, Zhu Q, Zhou X, Wang X, Gao X, Li X. 2011. Immune-related microRNAs are abundant in breast milk exosomes. Int J Biol Sci 8:118–123.

Zijlstra A, Di Vizio D. 2018. Size matters in nanoscale communication. Nat Cell Biol 20:228–230.
Zöller M. 2009. Tetraspanins: Push and pull in suppressing and promoting metastasis. Nat Rev Cancer 9:40–55.