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Screening of Ceramide-Dependent Exosomal Cargoes in Terms of their Vesicular Abundance in Colorectal Cancer

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Abstract: ***Colorectal cancer (CRC) is one of the most common malignancies worldwide and a leading cause of cancer-related mortalities. It originates from the epithelial cells of the colon or rectum, evolving through various genetic and epigenetic changes. CRC development is often associated with the adenoma carcinoma sequence, where benign polyps transform into malignant tumors over time. CRC metastasis is largely regulated by the secretome components released from the cancer cells. Neutral sphingomyelinase 2 (nSMase2) is potentially related to secretomes due to its role in exosome biogenesis which include the release of exosomal cargoes. This report highlights the significance of studying ceramide-dependent exosomal cargoes to enhance our understanding of colorectal cancer metastasis via Exosomes. Exosomes, the small extracellular vesicles secreted by cells, transport a variety of molecules, including proteins, nucleic acids, and lipids, that can affect disease progression. Ceramide-dependent exosomal cargoes are those that are reduced at their exosomal level upon blocking nSMase-2. Current study focusses on screening the ceramide-dependent exosomal cargoes that are highly reported in vesicular pools of "Vesiclespedia" database in regards to CRC. The finding of the study underscores the role of selected proteins classes that are involved in mediating intercellular communication between, providing insights into the molecular keystones of metastasis. The study highlights the role of Ceramide-dependent Exosomal Cargoes in promoting CRC metastasis through exosome-mediated intercellular communication and intracellular signaling modulation, suggesting potential therapeutic targets for managing CRC progression. From overall observation we have selected SPRED2 and LAMT as representative understudied proteins, given their minimal or absent documentation in CRC exosome literature. The inclusion of these low-frequency candidates aims to uncover potentially novel roles and functional significance that may have been previously overlooked in CRC pathogenesis.***
Keywords: Colorectal cancer metastasis, Netural sphingomyelinase-2, Ceramide, SPRED2, LAMT.

I. INTRODUCTION

Exosomes are small extracellular vesicles (EVs), typically 30–150 nm in diameter [1] that mediate intercellular communication by transporting bioactive molecules such as proteins, lipids, and nucleic acids [2] These vesicles originate from the endosomal system of cells and are formed through a tightly regulated biogenesis pathway. The process begins with the inward budding of the plasma membrane to form early endosomes, which mature into late endosomes or multivesicular bodies (MVBs). During MVB formation, the endosomal membrane invaginates to produce intraluminal vesicles (ILVs) within the MVB. These ILVs, when released into the extracellular space upon fusion of MVBs with the plasma membrane, are known as exosomes [3]The biogenesis of exosomes involves two major pathways: the ESCRT (Endosomal Sorting Complex Required for Transport)-dependent pathway and the ESCRT-independent pathway. The ESCRT machinery comprises four protein complexes (ESCRT-0, -I, -II, and -III) that are composed of cargo sorting, membrane budding, and vesicle scission [4]. Trajkovic et al. reveal the ceramide-dependent pathway of exosomal generation and find that ceramide has a significant role in multivesicular body (MVB) formation. The pathway was clarified by studying the movement of the proteolipid protein (PLP). The study suggests the presence of sphingolipid-rich microdomains in the endosomal membrane [5] Ceramide, formed from these microdomains, has a cone-shaped structure that induces membrane curvature, leading to PLP sorting and vesicle release into the endosomes [5] Exosomes play a pivotal role in mediating intercellular communication by transferring bioactive molecules between cells. Once secreted into the extracellular environment, exosomes can be taken up by recipient cells through various mechanisms, including endocytosis, phagocytosis, micropinocytosis, or direct membrane fusion [6]. Upon internalization, the cargo within exosomes can modulate gene expression, signaling pathways, and phenotypic behavior of recipient cells [7] Exosomal miRNAs are among the most studied cargoes due to their ability to regulate gene expression post-transcriptionally.

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Tumor-derived exosomal miRNAs can promote angiogenesis, immune suppression, and epithelial-mesenchymal transition (EMT) all of which facilitate tumor growth and metastasis [7], [8]. For instance, exosomal miR-205 been implicated in promoting oncogenic transformation [7] Proteins within exosomes can activate signaling pathways in recipient cells. These may include oncogenic proteins (e.g., EGFRvIII), enzymes (e.g., matrix metalloproteinases), and signaling molecules that prepare the pre-metastatic niche or induce drug resistance. Similarly, lncRNAs and circRNAs in exosomes act as competitive endogenous RNAs (ceRNAs), sequestering miRNAs and modulating the expression of tumor-related genes. These cargoes not only reprogram the behavior of recipient cells but can also alter the tumor microenvironment to support cancer cell survival and dissemination.

Ceramide is a central regulator in the biogenesis of exosomes, particularly within the ESCRT-independent pathway (Trajkovic Katarina, 2008). Its unique structural and biophysical properties enable ceramide to induce membrane curvature and promote the inward budding of endosomal membranes to form intraluminal vesicles (ILVs), which are ultimately released as exosomes upon fusion of multivesicular bodies (MVBs) with the plasma membrane [6] Ceramide is generated from sphingomyelin by the action of neutral sphingomyelinase 2 (nSMase2) [9]Neutral sphingomyelinase 2 (nSMase2) is an essential enzyme involved in the hydrolysis of sphingomyelin to generate ceramide, a key lipid that regulates exosome biogenesis and secretion [9] As a membrane-associated enzyme, nSMase2 operates primarily at the Golgi and plasma membrane but also plays a critical role at the endosomal membranes, where ceramide production facilitates the formation of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs) [10]. Once produced, ceramide aggregates into small lipid microdomains that facilitate the lateral organization of specific cargo molecules within endosomal membranes. These microdomains promote the sorting and inclusion of lipids, proteins, and RNAs into forming ILVs. Ceramide-enriched exosomes often carry stress-response molecules and pro-inflammatory signals, linking ceramide metabolism with immune modulation and cell survival under stress conditions [11], [12], [13]. This suggests a potential role for ceramide-enriched exosomes in regulating immune responses and promoting cell survival during stress.

The presence of these exosomes in colorectal cancer may provide crucial insights into the disease. By analyzing these cargoes, researchers can identify specific markers that may aid in the diagnosis and treatment of cancer. This research has the potential to improve treatment strategies for colorectal cancer and enhance patient outcomes. Investigating ceramide-dependent exosomal cargoes, particularly their vesicular abundance in the context of colorectal cancer, is of considerable importance. This study seeks to explain the role of ceramide in determining the composition and quantity of exosomes, thereby offering insights into the molecular mechanisms that drive colorectal cancer progression and identifying potential therapeutic targets. By examining the abundance of these ceramide-dependent exosomal components, researchers can better understand their influence on tumor biology, paving the way for the development of biomarkers or targeted therapies. This research not only enriches the current understanding of exosomal biology but also underscores the critical role of ceramide in the disease process.

Furthermore, understanding how ceramide-dependent cargoes contribute to tumor microenvironment remodeling and metastatic dissemination could open new avenues for targeted therapeutic interventions. Ceramide-dependent exosomal cargoes play a crucial role in CRC progression by modulating intercellular communication and the tumor microenvironment. In a very recent High-throughput screening proteomics study highlights the significance of ceramide, produced through the action of neutral sphingomyelinase-2 (nSMase-2), in the biogenesis of exosomes. By focusing on ceramide-dependent cargoes, the research identifies a distinct set of proteins that are preferentially secreted, providing insights into the differences between primary and metastatic CRC cell lines. Quantitative proteomic profiling using iTRAQ identified 1781 proteins, with 22.8% and 17.01% depleted in sEVs of treated SW480 and SW620 cells, respectively, representing cargo secreted through sEVCer. CargoCer-SW480 overrepresented integrin signaling pathway members, while CargoCer-SW620 overrepresented integrin and platelet-derived growth factor (PDGF) signaling pathway members, suggesting a possible transfer of metastatic signals via sEVCer [14]. A study identifies common 112 proteins in colorectal cancer cell lines that are crucial for understanding cancer cell communication and their role in pro-metastatic functions. The down-regulation of these proteins after nSMase-2 inhibition suggests they are secreted through the ceramide-dependent exosomal pathway. Screening for these cargoes and analyzing their vesicular abundance, this study will contribute to a deeper understanding of exosome biology and its implications for CRC diagnosis and therapy. The findings may pave the way for novel exosome-based biomarkers and therapeutic interventions, ultimately improving CRC management and patient outcomes.

II. MATERIAL AND METHOD

1) Downloading of supplemental dataset of high throughput screening proteomics data for ceramide-dependent exosomal cargoes.

To download the supplementary dataset of high-throughput proteomics data related to ceramide-dependent exosomal cargoes in colorectal cancer (CRC), begin by locating the original research article that reports on this study [14]. On the article page, navigate

to the "Supplementary Information" section, where downloadable files that are provided in Excel formats. These files often include proteomics data tables listing the all reported protein from SW480 cell line model as well as SW620 cell line model [14].

2) Screening of Ceramide-dependent exosomal cargoes thar are common for metastasis and primary CRC cell

To screen the ceramide-dependent exosomal cargoes in colorectal cancer (CRC) cell line model, we conducted a comparative proteomic screening of exosomes derived from both primary CRC cells (SW480) and their metastatic (SW620) from the downloaded dataset. Upon comparative analysis, we found 112 proteins that were commonly downregulated in both SW480 and SW620 exosomes upon ceramide pathway inhibition. These proteins represent a conserved subset of ceramide-dependent exosomal cargoes in both primary and metastatic CRC cells. The identification of these 112 common proteins is particularly significant, as it suggests their potential involvement in various different protein classes. This dataset provides a valuable resource for identifying candidate biomarkers and therapeutic targets that could disrupt exosome-mediated communication in colorectal cancer [14].

3) Protein Classification

The study conducted a thorough analysis of Gene Ontology (GO) and pathways overrepresentation in the context of exosomal proteins, specifically those preferentially secreted via the ceramide-dependent route in colorectal cancer (CRC) cells. Utilizing the Panther Pathway Analysis tool, the researchers already identified significant enrichments in both Biological Processes (GOBP) and Cellular Compartments (GOCC). The top ten enriched GOBP categories highlighted crucial processes associated with cancer progression, including regulation of extracellular exosome assembly, golgi vesicle transport, regulation of cell-substrate adhesion, cell–cell junction organization, and vesicle-mediated transport. These findings suggest a robust involvement of these processes in cancer metastasis [14]

4) Analysis of Vesicular abundance for each protein classes

To search for potential secretomes associated with cancer metastasis by using "Vesiclepedia" protein repositories, we required a web browser, a computer with internet access, and access to the Vesiclespedia database. Target protein candidates were screened from the repositories by utilizing the search strategy, filtering and sorting results, analyzing annotations and publications, cross-referencing with literature, and prioritizing candidates with experimental evidence. This Vesiclepedia-based approach not only validates the vesicular nature of the identified proteins but also helps prioritize functionally important candidates for further mechanistic or therapeutic investigation [15].

5) Comparative analysis between each protein classes

To perform a comparative analysis between each protein class, first categorize the identified exosomal proteins into functional classes using Gene Ontology. Quantify the number of proteins in each class and assess their relative abundance. Use visualization tools like bar graphs or pie charts to compare representation across classes. Statistical analysis can be applied to identify significantly enriched classes. This comparative approach reveals dominant functional themes and highlights protein groups potentially driving CRC progression via exosomal pathways [14]

III.OBSERVATION

The analysis revealed a total of 112 common proteins between primary (SW480) and metastatic (SW620) CRC cells, categorized into 20 functional protein classes. Metabolic interconversion enzymes, RNA metabolism proteins, and membrane trafficking proteins were the most abundant, reflecting key processes involved in metastasis. Vesiclepedia-based evaluation showed protein-binding activity modulators were reported more frequently than scaffold/adaptor proteins. However, only ~13% of these studies were specific to colon/rectum samples, indicating an underrepresentation of CRC-specific vesicular studies. RAP2A, ARF6, and GPC4 emerged as notable CRC-relevant exosomal proteins worth further exploration. These findings suggest that there is a need for more research focusing on vesicular studies specific to colorectal cancer (CRC) in order to better understand the role of exosomal proteins in the metastatic process. RAP2A, ARF6, and GPC4 are potential targets for further investigation due to their relevance to CRC. By delving deeper into the functions of these exosomal proteins, new insights may be gained that could lead to the development of novel therapeutic strategies for treating metastatic colorectal cancer. Further research into RAP2A, ARF6, and GPC4 could provide valuable information on how these proteins contribute to the spread of CRC and potentially identify new biomarkers for early detection or targeted therapies.

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Understanding the mechanisms by which these exosomal proteins promote metastasis could offer new possibilities for improving patient outcomes and developing personalized treatment approaches. By focusing on vesicular studies specific to CRC, scientists may uncover crucial information that could ultimately lead to more effective strategies for managing advanced colorectal cancer.

IV. RESULTS AND DISCUSSION

1) *Classification of Common Proteins from SW480 and SW620 Cells into Different Protein Classes and Their Distribution in Vesicular Studies*

Metastatic colorectal cancer (CRC) cells undergo significant molecular and proteomic alterations that facilitate their aggressive behavior, including enhanced migration, invasion, and survival. The SW620 cell line, derived from a metastatic site of CRC, serves as a model to study proteins associated with tumor progression and metastasis. A total of 112 proteins were classified into 20 distinct functional categories based on their biological roles, including metabolic processes, structural functions, enzymatic activity, and signaling mechanisms. The categorization of proteins common in SW480 and SW620 cells provides insights into the molecular adaptations of metastatic CRC cells (Figure 1). The most enriched protein classes include: Metabolic Interconversion Enzymes (12 proteins) which is representing the largest group, these enzymes are critical for cellular metabolism, supporting the energy demands and biosynthetic processes essential for metastasis [16]. RNA Metabolism Proteins (11 proteins) are the protein classes that regulate RNA processing, stability, and splicing, which are essential for rapid and dynamic gene expression changes in metastatic cells [17]. Membrane Trafficking Proteins (10 proteins) which highlights proteins involved in vesicular transport, crucial for intercellular communication, exosome-mediated signaling, and metastatic niche formation [18]. Protein Binding Activity Modulators (9 proteins) and Protein Modifying Enzymes (9 proteins) these proteins are involved in post-translational modifications and protein-protein interactions, key processes in oncogenic signaling [19]. Transporters (9 proteins) this group consists of proteins involved in the movement of ions, nutrients, and biomolecules across membranes, which is vital for maintaining the altered metabolic state of metastatic cells [20] Translational Proteins (7 proteins) these proteins contribute to enhanced protein synthesis, a hallmark of highly proliferative and metastatic cancer cells [21] Scaffold or Adaptor Proteins (6 proteins) these proteins facilitate complex signal transduction pathways that regulate metastasis and cell survival [22] Other notable categories include cytoskeletal proteins (5 proteins), transcription factors (1 protein), chromatin-binding proteins (1 protein), and storage proteins (2 proteins), which play roles in maintaining cellular integrity, gene regulation, and stress adaptation in metastatic CRC cells. Furthermore, while screening we found that 11 proteins are there which does not suits in any of the categories so this is named as “unclassified protein category” represents potential novel regulators that have yet to be characterized in the context of CRC metastasis.

By systematically characterizing the proteomic landscape of CRC cells, this study provides a foundation for understanding the metastatic mechanisms of CRC and identifying potential therapeutic targets for intervention.

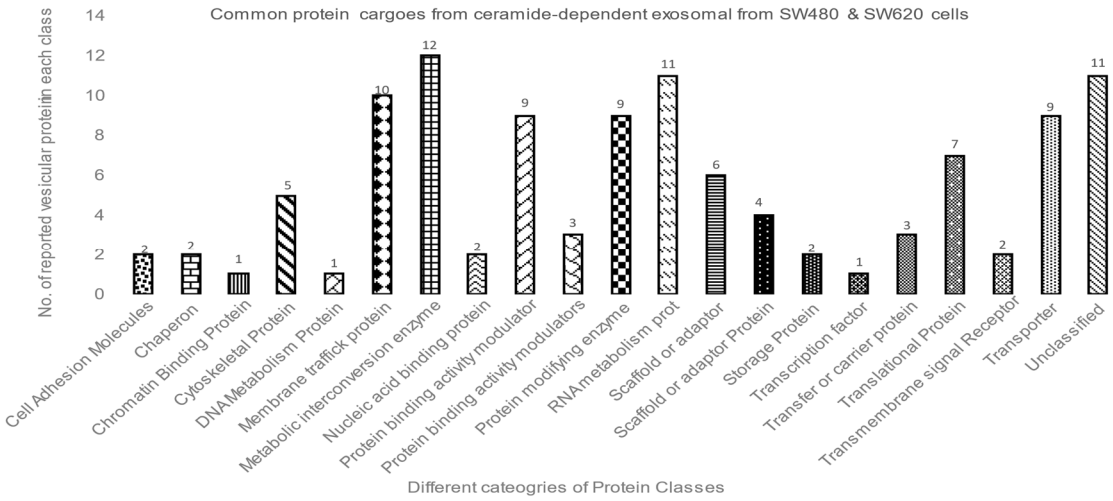


Fig. 1 Common Proteins cargoes from ceramide-dependent exosomal from SW480 and SW620 Cells: Common proteins identified in SW480 and SW620 cells are categorized into various protein classes based on their biological functions. The number of reported vesicular proteins in each category is represented as a bar graph.

2) Analysis of Reported Studies in vesicular pool of different Protein classes across various Sample types

This graph highlights the difference in research focus between various different sample type and colon/ rectum related sample type (CRC)-specific investigations across various protein functional classes. The majority of studies are conducted on various sample types, with few a small fraction of studies focusing on colon/rectum-related samples. This indicates a research gap in understanding the roles of these protein groups specifically in colorectal cancer (CRC). Metabolic interconversion enzymes, protein-binding activity modulators, membrane trafficking proteins, and RNA metabolism proteins show a high representation in general various different sample types studies. These proteins are crucial in cancer progression, influencing metabolic adaptations, signal transduction, and post-transcriptional regulation. Across all protein groups, colon/rectum-related studies account for less than 15% of the total studies. This suggests that while these proteins have been widely studied in cancer biology, their specific implications in CRC progression, metastasis, and exosome-mediated signaling remain largely unexplored. Chaperones, chromatin-binding proteins, DNA metabolism proteins, and transcription factors show minimal CRC-specific studies, despite their known roles in genome stability, stress response, and tumor progression. The transporter and transmembrane signal receptor groups, essential for cell communication and exosomal cargo transfer, also exhibit a research gap in CRC studies. From the above data, we will focus on protein classes that have been reported in at least 15% of Colon/rectum-related studies. These selected protein classes are likely to play crucial roles in CRC progression, metastasis, and tumor microenvironment modulation. This suggests a significant research gap, particularly in areas like transcription factors, transporters, and chromatin-binding proteins, which are crucial for tumor progression, metastasis, and exosome-mediated communication in CRC. The underrepresentation of CRC-related studies in these protein classes highlights the need for targeted research to uncover their potential as biomarkers or therapeutic targets in CRC.

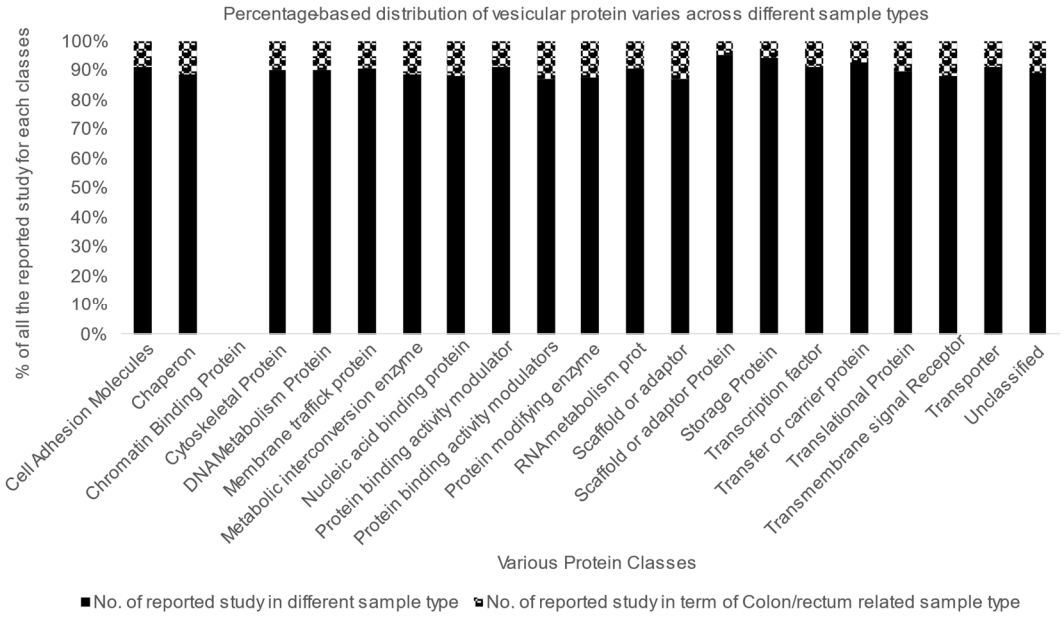


Fig. 2 Percentage-wise distribution of the reported studies involving these protein classes in vesicular research. The black bars represent the number of reported studies involving different sample types, while the patterned bars indicate the number of studies specifically related to colorectal cancer samples.

In our analysis, we prioritized protein classes based on their prevalence in exosomal studies. Specifically, we focused on classes that were either reported in the most sample types or had been studied extensively, with more than 500 reported studies. This selection criterion was applied to identify the most prominent and potentially functionally significant protein classes in CRC exosomes, thereby enabling a targeted investigation of proteins with substantial experimental backing and broader relevance. Based on this approach, we focused on the following protein classes: cytoskeleton proteins, membrane trafficking proteins, translational proteins, transporters, unclassified proteins, metabolic interconversion enzymes, protein-binding activity modulators, and protein-modifying enzymes. Among these, cytoskeleton proteins are pivotal in maintaining cellular integrity and facilitating dynamic cellular processes such as migration, invasion, and cell division all critical hallmarks of metastatic behavior. These proteins, including

ARPC3, PDCD6, TUBG2, KLC4, VPS4A permitting tumor cells to navigate through the extracellular matrix and intravasate into blood vessels. Their involvement in exosomal pathways further accentuates their significance, as exosomes loaded with cytoskeletal components can influence the tumor microenvironment and promote pre-metastatic niche formation. Membrane trafficking proteins, on the other hand, play a vital role in vesicle biogenesis, cargo sorting, and exosome secretion, which are integral for intercellular communication in CRC. These proteins regulate the formation of multivesicular bodies and facilitate the targeted delivery of oncogenic molecules to recipient cells, thereby promoting metastasis, immune evasion, and tumor progression. Their extensive presence in the proteomic profile highlights their centrality in exosome-mediated signaling pathways, making them attractive targets for disrupting metastatic communication networks. Translational proteins, primarily involved in the protein synthesis machinery, are also prominently represented and reflect the heightened proliferative activity characteristic of CRC cells. Components such as ribosomal proteins and translation initiation factors support the increased demand for protein production necessary for rapid tumor growth and adaptation to diverse microenvironments. Their abundance in the exosomal cargo also suggests a potential role in modifying recipient cell behavior, possibly by modulating their translational landscape or signaling pathways. Transporters are another crucial class, mediating the exchange of ions, nutrients, and metabolites across cell membranes. In CRC, the altered expression of specific transporters supports metabolic reprogramming, which is a hallmark of cancer progression. This reprogramming provides the energy and biosynthetic precursors essential for tumor growth and metastasis, especially under hypoxic and nutrient-deprived conditions within the tumor microenvironment. Their presence in exosomes indicates a role in facilitating metabolic adaptation not only within tumor cells but also in neighboring stromal or immune cells. Unclassified proteins, which do not fit into conventional categories, stand out as potential novel regulators in CRC metastasis. Their identification underscores gaps in current understanding and opens avenues for discovering previously unrecognized mechanisms of tumor progression. These proteins may represent unique biomarkers or therapeutic targets specific to CRC, warranting further functional characterization. Metabolic interconversion enzymes are also highly prevalent among the identified proteins, underlining the importance of metabolic plasticity in CRC. These enzymes catalyse reactions within glycolytic pathways, lipid metabolism, and amino acid processing, supporting the tumors adaptability to fluctuating microenvironmental conditions. Their role in exosomal cargo suggests that metabolic enzymes may influence the metabolic state of recipient cells or modulate local immune responses, thereby contributing to metastatic dissemination. Another critical class comprises protein-binding activity modulators, which orchestrate complex protein-protein interactions and signaling cascades. These modulators, including kinases, phosphatases, and scaffold proteins, regulate various oncogenic pathways such as integrin, growth factor signaling, and cell survival. Their widespread representation indicates their influence in fine-tuning cellular responses, maintaining oncogenic signaling loops, and promoting resistance to therapy. Protein-modifying enzymes such as kinases, ubiquitin ligases, and acetyltransferases also feature prominently, reflecting their role in dynamic post-translational modifications that regulate protein stability, activity, and localization. These modifications are essential for sustaining the aggressive phenotype of metastatic CRC cells, modulating pathways involved in proliferation, apoptosis, and immune evasion. The intricate interplay of these enzyme classes facilitates rapid cellular adaptation and is a key driver of tumor heterogeneity and metastatic potential. Collectively, these classes reveal the complexity of metastatic CRC at the proteomic level, emphasizing their interconnected roles in promoting tumor growth, invasion, and immune modulation. The diverse representation of structural (cytoskeleton), trafficking (membrane trafficking), biosynthetic (translational), metabolic (interconversion enzymes and transporters), and regulatory (binding modifiers and enzymes) classes illustrates the multi-layered control mechanisms governing tumor behavior.

Furthermore, the screening context emphasizing some classes is justified by the evidence that they are less studied in CRC-specific exosomal research (~13.8% of studies), representing significant knowledge gaps. Their pivotal roles in exosome-mediated communication and tumor microenvironment modulation make them prime candidates for therapeutic intervention. Targeting scaffold and adaptor proteins may prevent the assembly of metastasis-promoting signaling complexes, while modulating binding activity regulators could disrupt oncogenic signaling pathways at a post-translational level. Thus, the deliberate focus on these two classes stems from their central position in signal transduction, their relevance to tumor metastasis, and the underexplored nature of their roles specifically in CRC. This targeted approach aims to uncover novel mechanisms and identify potential biomarkers or drug candidates that can be translated into effective therapies to inhibit CRC progression and metastasis. The selection of scaffold and adaptor proteins, along with protein-binding activity modulators, as key protein classes in the study is highly strategic and underscores their critical roles in colorectal cancer (CRC) metastasis and exosomal communication. Scaffold and adaptor proteins are essential for organizing and stabilizing signaling complexes; they facilitate specific and efficient signal transduction by bringing together various enzymes, receptors, and other signaling molecules.

Protein-binding activity modulators, which include kinases, phosphatases, and other regulatory proteins, are equally crucial because they fine-tune signaling cascades through post-translational modifications and dynamic interactions. These modulators influence the activity, localization, and stability of key signaling proteins, ensuring that cellular responses are appropriately regulated. Their dysregulation often leads to aberrant signaling, promoting oncogenesis and metastasis. By focusing on these classes, researchers can identify critical nodes within signaling networks that are potentially druggable targets.

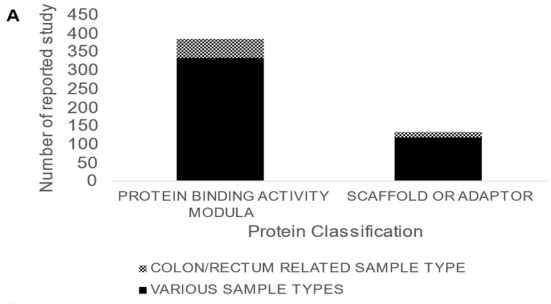
3) Comparative Vesiclepedia-Based Analysis of Ceramide-Dependent Exosomal Protein Classes in Colorectal Cancer

The protein binding activity modulator category, which plays a crucial role in signal transduction, cytoskeletal dynamics, and tumor progression in colorectal cancer (CRC). The number of reported studies for three specific proteins RAP2A, ARF6, and RAS6KA1 across various sample types, including colon/rectum-related studies. RAP2A has been reported in 89 studies, with 22 specifically related to CRC. RAP2A, a small GTPase, is known to regulate cell adhesion and migration by modulating integrin signaling and actin cytoskeleton dynamics. Its significant representation in CRC studies suggests a potential role in tumor cell motility and metastasis. ARF6 has the highest number of reported studies (188), but only 17 are CRC-related. ARF6 is a key regulator of membrane trafficking and cytoskeletal remodeling, influencing cell invasion and exosome-mediated communication in CRC. Despite its broad role across cancer types, its relatively lower CRC-specific representation indicates that its function in CRC progression requires further targeted research. RAS6KA1 has been studied in 56 reports, with 11 focusing on CRC. As a serine/threonine kinase involved in cellular proliferation and survival signaling, RAS6KA1 may contribute to CRC progression by modulating oncogenic pathways like MAPK and PI3K/AKT. From the total dataset, 333 studies cover various sample types, while 50 specifically focus on colon/rectum-related cancers, making up ~13% of the total reports. This aligns with the earlier observation that protein binding activity modulators are widely studied across multiple sample types, but their CRC-specific roles require deeper investigation. Studying these proteins further in CRC could help uncover novel therapeutic targets, especially in the context of tumor cell plasticity, metastasis, and treatment resistance.

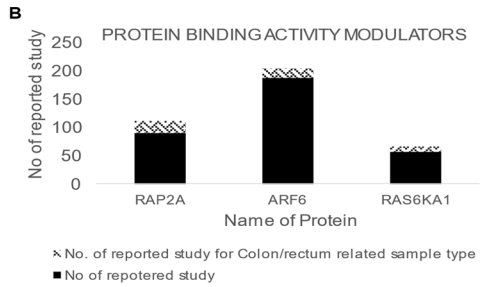
4) *Scaffold or Adaptor Proteins*

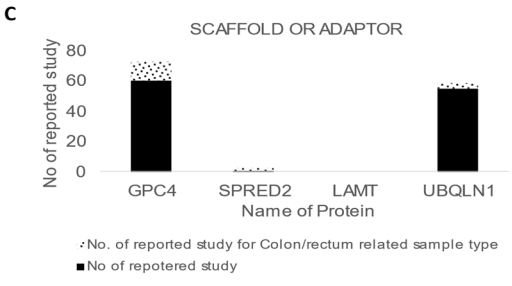
Scaffold and adaptor proteins play a crucial role in signal transduction, protein-protein interactions, and intracellular trafficking, making them key regulators in cancer progression. The selected candidates GPC4, SPRED2, LAMT, and UBQLN1 reported across various cancer studies, with a subset focusing on colorectal cancer (CRC). GPC4 (Glypican 4) has been reported in 60 studies, with 12 specifically related to CRC. Glypicans are heparan sulfate proteoglycans involved in modulating growth factor signaling, such as Wnt and Hedgehog pathways. GPC4 may contribute to CRC progression by facilitating oncogenic signaling and promoting tumor cell survival. SPRED2 (Sprouty-related EVH1 domain-containing protein 2) has been studied only once, but that single study is CRC-related (1/1). SPRED2 is a negative regulator of the Ras/MAPK signaling pathway, which is frequently dysregulated in CRC. Its role suggests a potential tumor-suppressive function in CRC by inhibiting excessive cell proliferation. LAMT has not been reported in any study (0 studies in total), indicating that its role as a scaffold/adaptor protein in CRC is either unexplored or insignificant in the context of current research. UBQLN1 (Ubiquilin-1) has been reported in 55 studies, but only 3 in CRC. UBQLN1 is involved in protein quality control and degradation pathways by linking ubiquitinated proteins to the proteasome. Its low representation in CRC studies suggests that its function in CRC progression might be underexplored, but given its role in proteostasis, it may have implications for tumor cell survival under stress conditions. From a broader perspective, 116 studies have investigated these scaffold or adaptor proteins in various sample types, while only 16 focus on CRC (~13.8% of total studies). This highlights the underrepresentation of scaffold/adaptor proteins in CRC research compared to other cancers. Further exploration of these proteins could provide new insights into potential CRC therapeutic targets. Figure 3. presents a comparative analysis of ceramide-dependent exosomal proteins identified in colorectal cancer (CRC), focusing on two major functional classes: protein binding activity modulators and scaffold or adaptor proteins. Panel A illustrates the total number of reported studies in which proteins from these two classes have been detected in extracellular vesicles, based on data extracted from Vesiclepedia. Protein binding activity modulators are markedly more represented, with 333 studies across various sample types and 50 studies specific to colon/rectum-related samples. In contrast, scaffold or adaptor proteins are reported in 116 studies across various sample types and only 16 colon/rectum-related studies, as summarized in the adjacent table. (Panel B) The protein binding activity modulator category, which plays a crucial role in signal transduction, cytoskeletal dynamics, and tumor progression in colorectal cancer (CRC). The number of reported studies for three specific proteins RAP2A, ARF6, and RAS6KA1 across various sample types, including colon/rectum-related studies. RAP2A has been reported in 89 studies, with 22 specifically related to CRC**.**

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All Reported studied in various sample type	Protein binding activity modulators	Scaffold or adaptor
Various sample type	333	116
Colon/rectum relate sample type	50	16





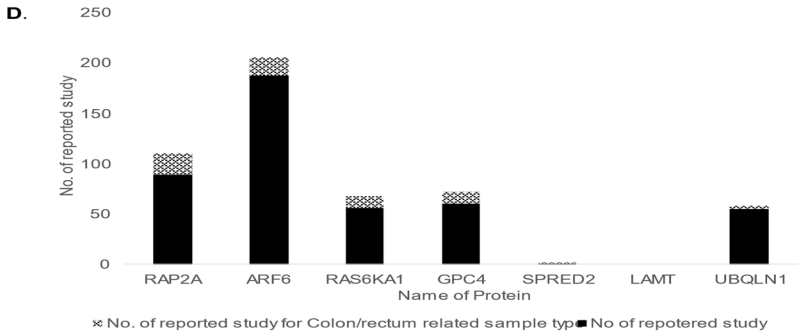


Fig. 3: Comparative analysis of ceramide-dependent exosomal cargoes based on protein classification and Vesiclepedia-reported abundance; A: Bar graph showing the number of reported studies for two major protein classes protein binding activity modulators and scaffold or adaptor proteins according to Vesiclepedia data. The accompanying table summarizes these values numerically. B: Sub-class analysis of the top three protein binding activity modulators (RAP2A, ARF6, and RAB5KA1) found among the 112 ceramide-dependent exosomal proteins. Each bar represents the total number of studies, with colon/rectum-specific reports overlaid. C: Sub-class analysis of representative scaffold or adaptor proteins (GPC4, SPRED2, LAMT, and UBQLN1) showing similar representation with black bars (total studies) and dashed overlays (colon/rectum-specific studies).

V. CONCLUSION

The data collectively reveal that various proteins involved in exosome-mediated processes have been extensively studied in different sample types, their specific roles in CRC remain ineffectively explored. The classification and analysis of proteins identified from CRC primary and metastasis cells (SW480 & SW620) provide critical insights into the molecular landscape of metastatic colorectal cancer (CRC). Our findings highlight significant variations in protein functional classes, highlighting the importance of metabolic interconversion enzymes, RNA metabolism proteins, membrane trafficking proteins in CRC progression. Despite widespread research on these proteins across various sample types, our analysis indicates a significant research gap highly reported study in their specific roles in CRC, particularly in exosome-mediated signaling and tumor microenvironment modulation. Focusing on protein binding activity modulators, RAP2A, ARF6, and RAS6KA1 are notable candidates with documented roles in cell adhesion, cytoskeletal remodeling, and oncogenic signaling. However, CRC-specific studies account for only ~13% of total reports, highlighting the need for targeted investigations into their mechanistic contributions to CRC metastasis. Similarly, scaffold and adaptor proteins such as GPC4, SPRED2, and UBQLN1 play crucial roles in signal transduction and protein-protein interactions, yet they remain understudied in CRC, with only 16 out of 116 total studies (~13.8%) focusing on CRC-related samples. This underscores the necessity for further exploration of these proteins in CRC, particularly in the context of tumor progression, metastasis, and exosome-mediated communication. Understanding their functions could pave the way for identifying novel biomarkers and therapeutic targets. Future research should highlight CRC-specific studies to bridge these knowledge gaps and enhance our ability to develop targeted therapeutic strategies for metastatic CRC.

VI. FUTURE DIRECTIONS

Some of these classification highlights key proteomic signatures of SW620 cells, emphasizing the molecular adaptations that drive CRC metastasis. However, the limited focus on CRC-specific exosomal studies presents a critical research gap. Future studies should prioritize the screening and functional characterization of ceramide-dependent exosomal cargo in CRC, particularly focusing on underexplored proteins such as SPRED2, LAMT, and RAS6KA1. Addressing these gaps could provide valuable insights into CRC progression and metastasis, potentially leading to novel therapeutic targets. Functional studies are needed to Validate the roles of metabolic and RNA metabolism proteins in sustaining metastatic potential. Investigate the contribution of membrane trafficking proteins to exosome-mediated communication. Explore the therapeutic potential of targeting protein modifying enzymes and scaffold proteins to disrupt oncogenic signaling. Importantly, the identification of unclassified proteins highlights critical gaps in current knowledge and presents opportunities for discovering new therapeutic targets. As exosomal pathways are central to cell-cell

communication and metastatic niche formation, targeting these protein classes could disrupt the intricate signaling networks that facilitate CRC metastasis. Overall, the insights gained from this proteomic analysis of circulating and tumor-derived exosomes deepen our understanding of CRC biology and open new avenues for biomarker development and tailored therapeutic strategies aimed at halting disease progression and improving patient outcomes.

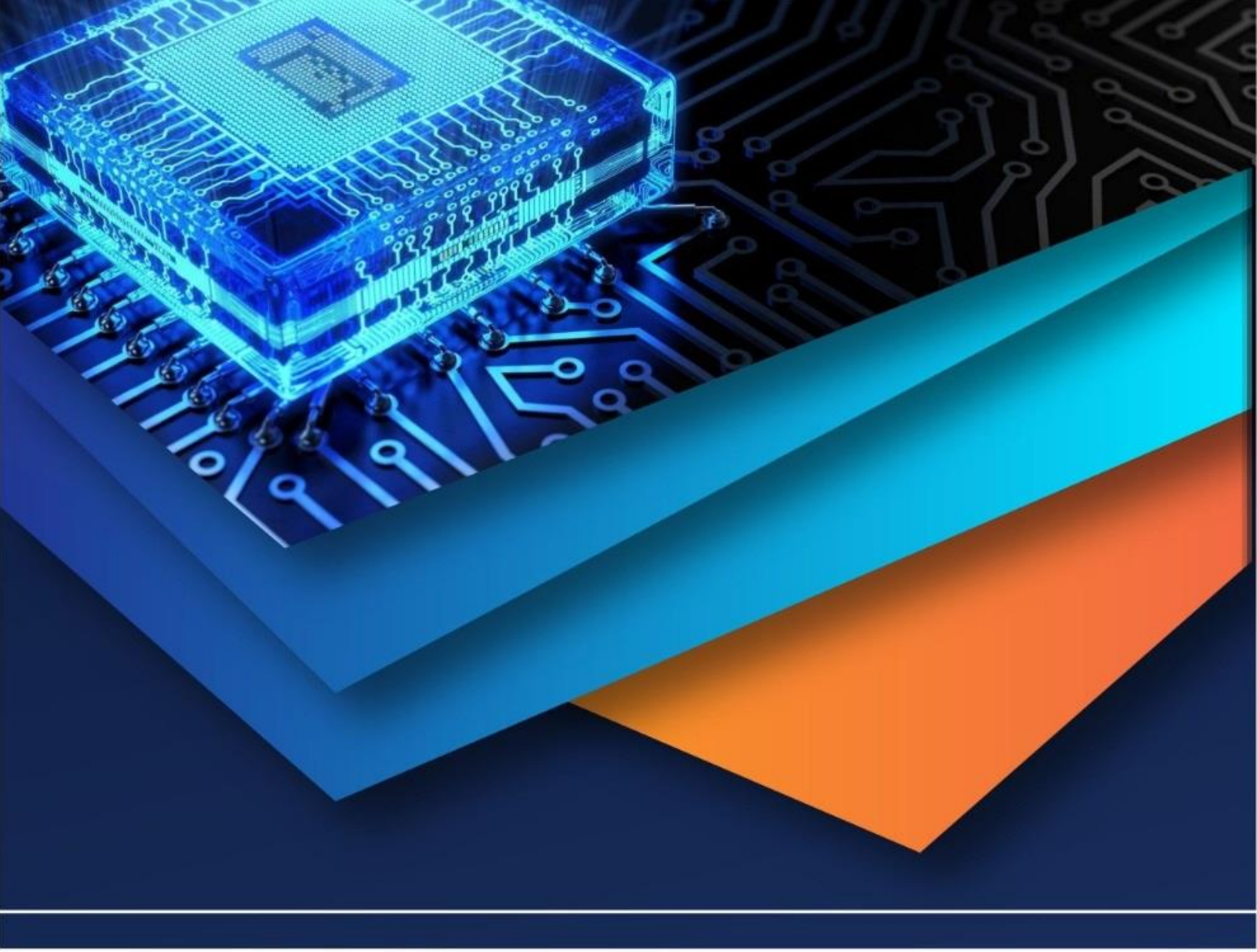
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