Chapter 7: Conclusion and Discussion

Increasing evidences suggest that the plasma membrane is laterally segregated into domains that form hotspots for performing important cellular functions such as sorting and signaling (Simons and Sampaio, 2011; Mayor and Rao, 2004). Further studies using different biochemical and biophysical tools have identified an enriched pool of specific lipids namely cholesterol, sphingolipids and lipid tethered proteins like GPI anchored proteins (GPI-APs) (Gaus et al., 2003; Munro, 2003) in these domains. Since then, a plethora of studies have utilized GPI-APs to define the size and to dissect the mechanism of formation of these domains (Benting et al., 1999; van Zanten et al., 2009; Sharma et al., 2004).

GPI-APs are lipid-tethered proteins present in all eukaryotic cells and located at the exoplasmic leaflet of the plasma membrane. Studies from our and other laboratories have shown that the GPI-AP's are organized into nanoclusters at the plasma membrane (Sharma et al., 2004; van Zanten et al., 2009). This organization is important for GPI-AP endocytosis and for the functional consequence of the protein (Ritter et al., 1995; Varma and Mayor, 1998). Hence it becomes important to understand the mechanism of formation of these GPI-AP nanoclusters. Several evidences suggest that the GPI anchor of GPI-AP has a role in the nanoclustering of GPI-APs (Varma and Mayor, 1998; Kametaka et al., 2007). The study reported in this thesis has focused on unraveling the structural contribution of GPI anchor in the formation of GPI-AP nanoclusters. For this purpose, fluorescent-tagged GPI analogues were synthesized.

The salient findings from my study are stated below:
1) A new synthetic strategy to synthesize fluorescently tagged GPI analogues was designed and modified at 3 different steps to obtain better yield of the product (Saikam et al., 2011).

2) A novel method of lipid incorporation using commercially available lipofectamine reagent (Saha et al., 2015) was optimized.

3) GPI analogues with long and saturated acyl chains formed cholesterol and actin dependent nanoscale clusters at the plasma membrane, a behavior similar to GPI-APs.

4) GPI analogue with a bulky Boc protected amine group [GlcN(Boc)PI-NBD] also formed cholesterol dependent nanoscale clusters like the free amine containing GlcNPI-NBD, suggesting that the free amine at glucosamine has no role in GPI-AP nanoclustering.

5) Since the GPI analogues that formed nanoclusters were disaccharides instead of the native pentasaccharide, the study directly suggests that neither the protein nor the mannoses are required for GPI-AP nanoclustering.

6) GPI analogues with long and saturated acyl chains when incorporated into PS mutant cells were found to undergo nanoclustering only if PS was synthesized in the cell; thereby confirming that PS is the inner leaflet candidate for transbilayer coupling to actin.

7) F-DHPE formed cholesterol dependent nanoscale clusters in PS mutant cells only under conditions when PS was synthesized in the cell, confirming that the transbilayer coupling occurs via inner leaflet PS and that any lipid in the outer leaflet containing long and saturated acyl chains can be interlinked to actin through this mechanism to form nanoclusters.
8) In a different context, GPI analogues were utilized to test the nanoclustering capability of the membrane in PGAP2/3 mutants. Additionally these GPI analogues were utilized as control to test the PI-PLC sensitivity of non-hydrolysable GPI analogues (Yadav et al., 2014).

In essence, the study reported in this thesis has contributed towards understanding the structural contribution of GPI anchor in formation of GPI-AP domains at the plasma membrane. The use of various synthetic structural analogues of GPI and its incorporation into the live cell membrane has been a challenge throughout the study. Though previous studies have utilized structural variants of GPI-AP to determine its diffusion properties (Paulick et al., 2007) and mechanisms of endocytic sorting (Bhagatji et al., 2009), it is the first time that such analogues have been used to study the mechanism of formation of GPI-AP nanoclusters at the cell surface. This work provides evidences to the proposal that GPI-AP nanoclusters are dictated by actin machinery via transbilayer acyl chain interactions with an inner leaflet lipid (Figure 7.1). The known actin binding inner leaflet lipids, phosphatidylserine (PS) and phosphatidylinositol (PI) were screened for its ability to nanocluster GPI-APs (work done by Anupama Ambika in the laboratory) (Raghupathy et al., 2015). The results from this work suggest that PS is the key-coupling agent to link GPI-AP to actin. Theoretical calculations in conjugation with experimental evidences have previously reported the existence of short actin filaments that can form asters, which in turn could regulate the clustering of GPI-APs at the outer leaflet (Gowrishankar et al., 2012). The existence of a transbilayer coupling between inner leaflet and outer leaflet lipids is also reported from previous in vitro studies facilitated by the presence of lipids containing long and saturated acyl chains in both outer and inner leaflet (Chiantia and
London, 2012; Benting et al., 1999). The transbilayer coupling therefore is dependent on the availability of lipids with long and saturated acyl chains, cholesterol levels at the outer and inner leaflet and the binding of inner leaflet lipid to actin. Though the longest acyl chains for GPI-APs in mammalian cells are C16:0 and C18:0, C22:0 or C24:0 is observed for other lipids such as sphingolipids. Possibly sphingolipids like sphingolmyeloin also undergo similar transbilayer interactions to form nanoclusters.

Logical explanation of the previous findings suggested that GPI anchor is important for GPI-AP nanoclustering. A crucial finding in this respect was the report that the GPI-AP carrying one unsaturated lipid tail at the GPI anchor (in PGAP2/3 mutant (Kametaka et al., 2007)) is unable to associate with DRM fractions (a method used to identify raft association). We further tested the nanoclustering ability (Raghupathy et al., 2015) of the defective GPI-APs in the PGAP2/3 mutant cell line and found that they are less efficiently clustered compared to the parent cell line. This result indirectly suggested that the GPI anchor acyl chains are important for GPI-AP nanoclustering. Hence it was decided to systematically unravel the contribution of each of the structural elements of the GPI anchor in nanocluster formation. The GPI analogues were also utilized as a tool to test the membrane clustering ability of these mutant cells.

Utilizing fluorescently tagged GPI analogues (GlcNPI instead of 3Man-GlcNPI), the study reported in this thesis indicates that the GPI anchor requires only long and saturated fatty acid chains (C16:0 or C18:0) to form cholesterol and actin dependent nanoclusters similar to GPI-APs. Utilizing the GlcNPI-NBD and its N-Boc protected version, the requirement for glucosamine free amine in GPI nanoclustering is ruled out. This study also indicates that neither the protein nor the GPI glycans of a GPI-AP is required for its nanoclustering.
This result implied that any lipid containing long and saturated acyl chains could ideally couple to PS to form nanoclusters. This idea was tested using fluorescein tagged dihexadecanoyl phosphatidylethanolamine (F-DHPE) in PS mutant cells and found that F-DHPE also form cholesterol sensitive nanoclusters only when PS is present at the inner leaflet. This result confirms that the PS dependent nanoclustering of outer leaflet molecules can be a general mechanism for forming nanoclusters at the plasma membrane. Therefore in this study synthetic fluorescent GPI analogues and lipids were utilized to unravel the structural role of GPI-AP membrane organization and conclude that the mechanism of GPI-AP clustering may hold true as a general principle for the formation of nanoscale domains at the plasma membrane. The efficiency of this lipid sorting is dependent on the availability of cholesterol, affinity of acyl chain interactions with PS and its serine head group to actin binding interactors. The levels of cholesterol at the outer and inner leaflet are different and this may have implications in tight regulation of clusters at the outer leaflet (Mondal et al., 2009). The identification of the actin-binding partners remains elusive and needs further scrutiny.

Thus the cell actively regulates the spatiotemporal segregation of proteins and lipids at the membrane to prepare them for specific functions. Any defect that prevents a protein to form nanoclusters may perturb the functioning of these platforms. For example, cells expressing a chimeric construct of folate receptor containing a transmembrane domain instead of GPI anchor (FR-TM) was unable to nanocluster (Varma and Mayor, 1998) and deliver folic acid to the cell (Ritter et al., 1995). Additionally, PGAP3 deficient mice known to produce GPI-AP containing defective GPI anchor (retains one unsaturated acyl chain) was shown to exhibit increased immune response compared to the wild type
(Murakami et al., 2012). On the other hand, a mutation in the same PGAP3 gene results in a subtype of Hyperphosphatasia causing mental retardation in Humans (Howard et al., 2014). These evidences clearly indicate that the right functioning of the protein is dependent on its inclusion into nanoclusters, that act as platforms for sorting and signaling at the plasma membrane.
**Figure: 7.1**

Transbilayer coupling of GPI-AP to PS: This cartoon depicts transverse section of the plasma membrane and represents our current view of formation of GPI-AP nanoclusters. GPI-AP nanoclustering is driven by short actin filaments juxtaposed to the bilayer and this transbilayer coupling is brought about by phosphatidylserine (PS) in the lower leaflet.