SUMMARY

Proteins present at the plasma membrane (PM) and in the extracellular milieu (ECM) constitute an important component of the total cellular proteome. Molecules present in these extracellular locations are involved in a variety of cellular functions such as, regulation of cell signaling, as a physical scaffold and regulation of cellular homeostasis. Due to the wide variety of roles played by the extracellular proteins, any shortcoming in their transport machinery can results in development of pathological conditions. A major portion of the proteins translocated to PM and ECM are known to follow the classical pathway of protein secretion. In this, proteins are first translocated to ER with the help of an N-terminal signal sequence. After passing through various quality control steps and proper folding, they are targeted to Golgi with the help of COPII vesicles. Form Golgi, proteins designated for extracellular transport are trafficked out with the help of secretory vesicles. The primary requisite for secretion via the classical pathway is the presence of an N-terminal signal sequence that directs the nascent protein to enter into ER. However, a number of proteins lacking any signal sequence are also found in ECM. In the last decade, various mechanisms have been demonstrated to be involved in extracellular trafficking of signal sequence lacking proteins. Collectively these pathways are known as non-classical or unconventional mechanisms of protein secretion.

Previous studies from our lab have shown the novel localization of the moonlighting protein glyceraldehyde-3-phospate dehydrogenase (GAPDH) on the mammalian cell surface. Apart from its unconventional surface localization, GAPDH is also actively secreted by cells. Enhanced secretion of GAPDH into the extracellular fluid has been observed in the case of diverse disease conditions like cancer and infection. It has now been revealed that this surface exposed and secreted GAPDH functions as a receptor for iron carrier proteins transferrin and lactoferrin. Our group has also documented that under conditions of iron excess cells translocate a distinct isoform of GAPDH to the PM in order to facilitate iron egress from cells. Despite these vital roles played by GAPDH in extracellular space, its mechanism of membrane trafficking has remained an unanswered question.

The present study was aimed at the detailed characterization of the GAPDH export mechanism. For this, we first began with investigating any role for the classical pathway in GAPDH membrane trafficking. Inhibition of classical pathway by brefeldin A (BFA)

does not affect membrane trafficking of GAPDH. This is in accordance with the fact that GAPDH doesn't contain any signal sequence for its targeting to ER-Golgi pathway. We next went on to look at the cytosolic localization of GAPDH in J774 and CHO-TRVb cells. Localization studies of both native, as well as heterologously expressed GAPDH, showed the presence of GAPDH in vesicles apart from its free diffuse cytosolic localization. For any membrane trafficking event to occur, secretory vesicles should be able to move and fuse with the plasma membrane. To check for the mobile nature of the GAPDH containing vesicles, we carried out live cell imaging. Live cell imaging of cells expressing GAPDH-EGFP confirmed the mobile nature of the GAPDH containing vesicles. We also observed that they moved in both anterograde and retrograde directions. Despite the mobile nature of the GAPDH containing vesicles, their involvement in GAPDH trafficking cannot be conclusively established. To find out the possible role of GAPDH containing mobile vesicles for its membrane trafficking, we disrupted cellular microtubules using nocodazole and vinblastine to prevent vesicular movement and found a significant inhibition in the membrane trafficking of GAPDH. FRAP analysis of the GAPDH-EGFP expressing cells revealed that GAPDH can directly translocate from the cytoplasm into these vesicles.

The next question that we addressed was to determine the identity of these vesicles. Confocal based co-localization analysis revealed that the GAPDH containing vesicles belong to the endocytic machinery of the cell. The presence of native GAPDH in purified organelles of endocytic machinery was also confirmed by western blotting against GAPDH.

As presence of GAPDH was found in the lysosomes during co-localization and western blotting analysis, we hypothesized for the possible role of the lysosomal secretory pathway in GAPDH membrane trafficking. For this, we looked for the effect of various lysosomotropic drugs on membrane as well as ECM trafficking of GAPDH. Treatment with agents that promote lysosomal exocytosis demonstrated an enhanced membrane as well as ECM trafficking of GAPDH. On the other hand, treatment with agents preventing lysosomal exocytosis resulted in a decreased membrane trafficking of GAPDH. Cytosolic distribution of lysosomes after various treatments was also assessed by confocal microscopy. These experiments demonstrated the role of the lysosomal exocytosis pathway in membrane trafficking of GAPDH. Previously, we have shown that iron

depletion results in enhanced membrane trafficking of GAPDH. To check the effect of iron depletion on the lysosomal secretory pathway, we first looked at the cytosolic distribution of lysosomes in iron-depleted cells as compared to control cells. Localization studies upon cellular iron depletion demonstrated redistribution of lysosomes towards cellular periphery in both J774 as well CHO-TRVb cells. Lysosomes were not only found to be redistributed towards the periphery but also their enhanced fusion was observed upon depletion of iron.

Previously the presence of GAPDH has been detected in exosomes secreted out of cells. As the exosomal secretory pathway is also an important mechanism of non-classical secretion, we next went on to characterize the role of the exosomal secretory pathway in membrane and ECM trafficking of GAPDH. Depletion of exosomes from cell culture supernatants showed that a significant but not major portion of the extracellular GAPDH is associated with the exosomes. This accounted for about 35% of total extracellular GAPDH secreted by the cells. To further characterize this pathway, we treated cells with monensin. It has been shown that treatment with monensin significantly enhances multivesiclular body (MVBs) formation, resulting in enhanced exosomal biogenesis and exocytosis. As reported earlier, treatment with monensin resulted in enhanced MVBs formation in J774 cells. Later we found that enhancement in MVBs formation by monensin resulted in up-regulated trafficking of GAPDH to PM and ECM. Similar to the effect observed in case of lysosomal exocytosis pathway, we found up-regulation of MVBs formation as well as exosomal exocytosis upon iron depletion. Iron depletion not only enhanced exosomal exocytosis but also resulted in enhanced transport of GAPDH into exosomes. Earlier modulation in the intracellular calcium levels have shown to regulate both, exosomal as well as lysosomal exocytosis. To understand the mechanism of iron depletion induced exosomal exocytosis and lysosomal exocytosis, we looked at the intracellular calcium levels. Confocal microscopy and flow cytometric analysis of intracellular calcium showed significant increase in cellular calcium levels. These results suggest that iron deprivation promotes mobilization of calcium probably through intracellular calcium reservoirs to promote lysosomal and exosomal exocytosis

Lysosomes are cellular organelles involved in degradation and recycling processes in cells. Recently they have also been recognized to play a role in membrane trafficking of proteins. Up-regulation of lysosomal biogenesis and exocytosis has been demonstrated to

promote cellular clearance of protein aggregates. In our current investigation, iron deprivation has been shown to promote lysosomal exocytosis, which has previously been demonstrated to promote cellular clearance of the huntingtin protein aggregates. Considering these observations, we thought to analyze the effect of iron deprivation on aggregate formation. Our analysis revealed that, cells where enhancement of lysosomal exocytosis (by depletion of cellular iron) had been effected, presented with significantly less huntingtin protein aggregates as compared to control cells. We also observed that not only the numbers of aggregates were decreased but also, the volume of these aggregates was significantly reduced in iron depleted cells as compared to control cells.

In summary, for the first time, we present evidence that membrane trafficking of the higher order moonlighting protein GAPDH involves multiple pathways of unconventional secretion. We demonstrate that direct translocation of GAPDH into endosomes marks the start point of its membrane trafficking. Subsequently, it is delivered to the exosomal and lysosomal arms of the non-classical secretory pathways. To the best of our knowledge, this is the first report demonstrating that exosomal and lysosomal secretory pathways do not operate in isolation, instead, they can exchange their content to traffic physiologically important molecules via multiple routes. A major portion of the GAPDH trafficked to PM and ECM was found to be mediated via the lysosomal pathway of secretion. We also demonstrate enhanced lysosomal and exosomal exocytosis under conditions of low cellular iron. For the first time we also showed that the desferoxamine treatment can be utilized to prevent protein misfolding via up-regulating lysosomal exocytosis. We propose that this enhancement in exosomes and lysosomal exocytosis could be a strategy adopted by cells to up-regulate transport of GAPDH to surface and ECM, in order to internalize more TF/LF bound iron **(Fig.2.32)**.

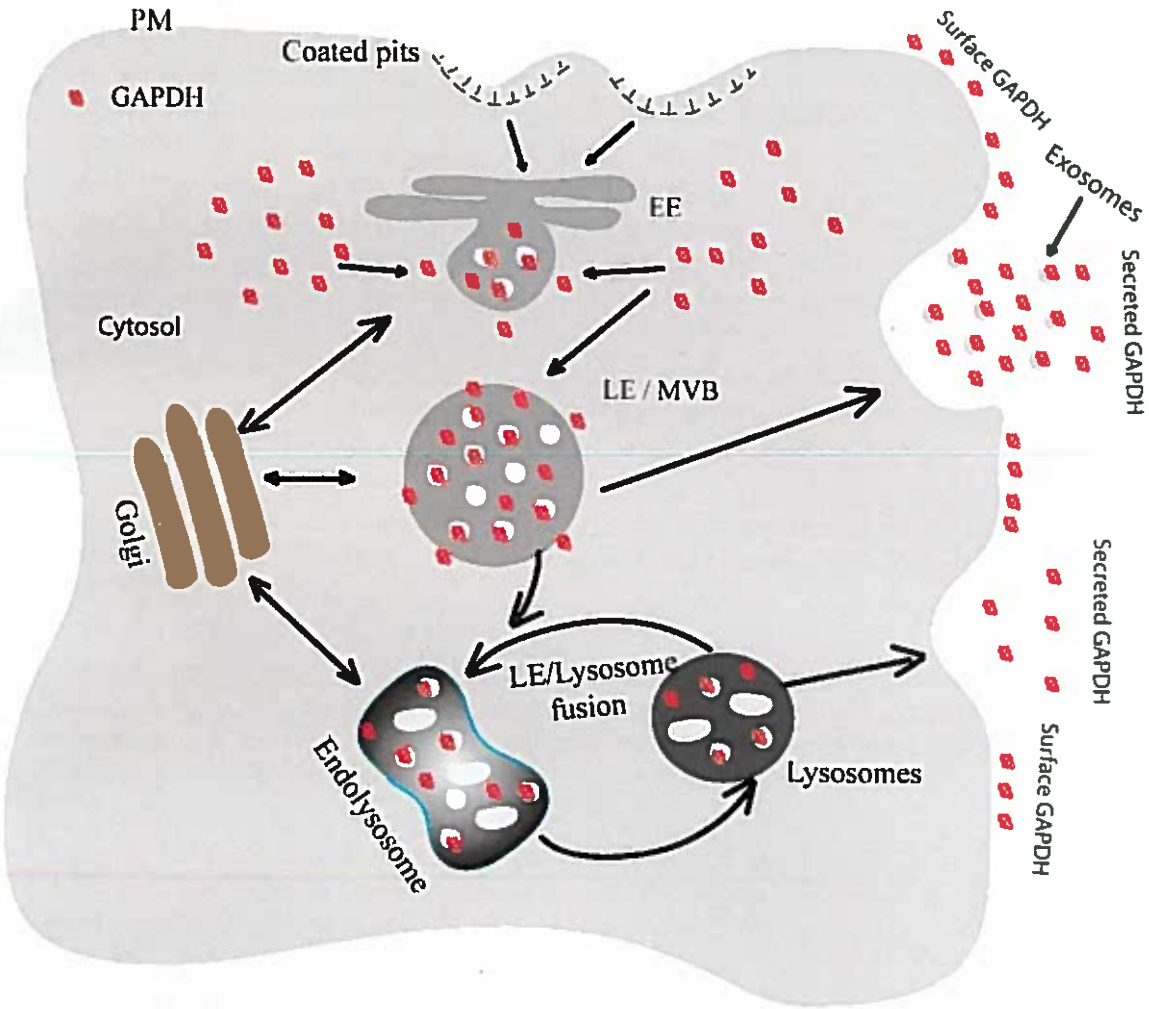

Fig.2.32 Model depicting mechanism of GAPDH trafficking to plasma membrane and extra cellular milieu.