SECTION V

DISCUSSION
PIP5K is mainly a cytosolic enzyme that has been well studied for its role in membrane dynamics and regulation of actin cytoskeleton including cell migration (Falkenburger BH et al., 2010; Logan MR & Mandato CA., 2006). Recent reports have associated PIP5K with poor prognosis in prostate cancer (Semanas J et al., 2014). Additionally, γ-isoform of the kinase has been implicated in anchorage-independent growth of tumor cells (Thapa N et al., 2013). PIP5K was also shown to be spatially organized to “nuclear speckles” that are distinct from known membrane structures (Boronenkov IV et al., 1998). Studies from several other laboratories have also demonstrated nuclear localization of PIP5K and implicated a range of functions for it (Anderson RA et al., 1999; Barlow CA et al., 2010; Chakrabarti R et al., 2013). Nuclear targeting of other phosphoinositide metabolizing enzymes is achieved through various mechanisms. While some possess a bona fide nuclear localization signal (NLS), others like PIPKIIβ are targeted by a “kinase insert region” (Barlow CA et al., 2010). Interestingly PIP5K neither has a NLS nor any “kinase insert region”. The nuclear pool of several cytosolic proteins like the Golgi protein p115 (Mukherjee S et al., 2009) and Actin (Hofmann WA et al., 2009) have been shown to be modified by SUMOylation. The present study elucidates the role of SUMOylation in mediating nuclear targeting of PIP5K in various patho-physiological conditions in cultured mammalian cells. The nuclear form of PIP5K has been shown to be modified by SUMO-1 (Chakrabarti R. et al., 2013). We have shown that modification at K244 is essential for its nuclear transport (Chakrabarti R. et al., 2015). Moreover, induction of apoptotic stress not only gives rise to the formation SUMO-2-ylated PIP5K but also poly-SUMO-2ylated adducts that interact with RNF4, a polySUMO2 specific E3 ubiquitin ligase. Reports from other laboratories have shown that the degradation of proteins like PML is induced by its modification with poly-SUMO-2 and its subsequent interaction with RNF4 (Tatham MH et al., 2008). Poly-SUMO-2 conjugated PIP5K was found in the 24 hour apoptotic cells; while it was
almost absent in untreated and early apoptotic (12 hr stage) cells. Interestingly our cell viability assay results also revealed that HEK-293 cells over-expressing PIP5K are resistant to apoptotic death for up to 12 hours, while the extent of viable cells after 24 hours are highly comparable to that of their un-transfected counterparts (Chakrabarti R. et al., 2013). Thus, a decrease in viability of HEK-293-FLAG-PIP5K cells at 24 hour stage could be due to a concurrent degradation of nuclear PIP5K (modified by poly-SUMO-2) by its interaction with RNF4. Thus, the result indicates a correlation between poly-SUMO-2 conjugation of PIP5K and its probable degradation by RNF4, during mid to late apoptosis. Indeed, our data corroborates a previous finding that demonstrated the role of PIP2 and PIP5K to block caspase mediated apoptosis (Mejillano M. et al., 2001).

Most of the studies involving nuclear phosphoinositide signaling have stressed on the role of PIP2 in modulating various nuclear functions (Shah ZH et al., 2013; Barlow CA et al., 2010; Osborne SL et al., 2001). However, it was shown that PIP5K interacts with Star-PAP (Speckle Targeted PIPKIα Regulated-Poly(A) Polymerase) independent of PIP2 to regulate pre-mRNA processing (Mellman DL et al. 2008). The nuclear PIP2 had been shown to be regulated by cell cycle stages (York JD & Majerus PW., 1994), and interact with pol I and UBF in the nucleolar cap region (Yildirim S et al., 2013). Interestingly, our study also revealed differential nuclear localization pattern of PIP5K in asynchronous cells (Fig. R12) implicating that the observed phenotype might be governed by the various stages of the cell cycle. Indeed, an enhanced accumulation of PIP5K in the nucleolar cap region during G1/S arrested in multiple cell types gave rise to speculations over its role in modulating the biosynthesis of rRNA. The ChIP experiments revealed that nuclear expression of Wt-PIP5K is associated with its high occupancy throughout the rDNA gene loci along with the repression signature H3K9me3 and HP1-α.
SUMOylation of PIP5K at K490 plays a major role in mediating its association with H3K9me3 and HP1-α. The recruitment of PIP5K was lowest at the promoter region and highest at the intergenic spacer region (IGS/H27) indicating that PIP5K might well be implicated in maintaining the overall epigenetic state of rDNA genes as intergenic transcripts have been shown to regulate the same (Mayer C et al., 2006). Considerable fold enrichment in the Sequential-ChIP assays coupled with a direct physical association of PIP5K between the proteins of the heterochromatin complex namely H3K9me3 and HP1-α in a DNA dependent manner suggests that at least a tripartite complex forms on the rDNA comprising of PIP5K, H3K9me3 and HP1-α.

Interestingly, the decreased association of PIP5K with rDNA loci in the early S phase was quite opposite to its enhanced nucleolar localization in the same phase. This discrepancy in enhanced nucleolar localization and simultaneous reduced association with rDNA of PIP5K during G1/S phase is partly clarified by our results which showed that in the early S phase, synthesis of both 18S and 45S pre-rRNA in cells expressing Wt-PIP5K are de-repressed to a level similar to that in un-transfected HEK-293 cells. This corroborates the ChIP data which showed decreased recruitment of PIP5K on the rRNA genes during the early S phase. The result signifies that PIP5K though enriched in the nucleolus during the G1/S phase had reduced association with the rRNA genes. It has been shown that while the transcription of rRNA genes is activated during late G1 to G2 phases, it is repressed in the M and most of G1 phase (Klein J and Grummt I., 1999). Thus these results suggest that PIP5K, H3K9me3 and HP1-α followed a definite cyclical pattern of occupancy (at mostly G1 phase) and release (S phase) from the rDNA loci throughout the cell cycle.
Based on the fact that PIP2 binds histones (Yu H et al., 1998), one might argue that the PIP5K interaction with the silencing complex proteins is PIP2 mediated. Our *in-vitro* studies have clearly demonstrated that PIP5K can bind to core histones, albeit weakly. Moreover, PIP5K-K490A mutant fails to bind to both H3K9me3 and HP1-α. Even PIP5K did not interact with H3K4me3. Thus, it can be ascertained that PIP5K binding to histones is irrespective of presence of any PIP2 and depends on its SUMOylation status at K490.

While most of the asynchronous cells displayed nuclear staining of PIP5K, some of the cells, mainly at pro-metaphase, cells showed a highly reduced association of PIP5K with the nuclear chromatin material (Fig R12). Following up this observation we saw that PIP5K is indeed not associated with the nuclear material of the cells in the various phases of mitotic division and re-enters into the naïve nuclei at the telophase which is accompanied by its concomitant SUMOylation. Notably, throughout the divisional phase, PIP5K remained associated with the mitotic spindles. Our studies revealed that de-SUMOylation of PIP5K precedes its nuclear exit during S/G2 phase and it enters the newly formed nuclei in a SUMOylated condition during telophase. Several studies have documented that SUMOylation plays a major role in regulating mitotic proteins (Yang F. et al., 2012, 2015). Studies have shown that pre-ribosomal RNA species and nucleolar proteins like B23 arrange in the peri-chromosomal regions during telophase (Dundr M et al., 2000). A similar spatial distribution of PIP5K during the telophase in conjunction with its regulatory role in the rRNA synthesis (Chakrabarti R. et al., 2015) suggests that it might play an important role in post-mitotic reassembly of the nucleoli. Interestingly, during telophase, PIP2 staining is remarkably confined to a limited number of very bright structures that remain cytoplasmic even when the DNA has re-localized to the newly formed nuclei of the two daughter cells (Osborne SL et al., 2001). While PIP2 remains associated with the dividing chromatin (Sobol
our data shows that PIP5K is excluded from it. Thus, PIP5K accumulation at the peri-chromosomal region during telophase suggests a speculative and yet unexplored kinase-independent activity of PIP5K at the prescribed location.

Unlike its nuclear and sub-nuclear targeting, SUMOylation plays no part in mediating localization of PIP5K to the centrosomes, the non-membranous organelles that occupy a tiny volume near the center of the cell, is divided once per cell cycle to produce bipolar spindle and ensure proper chromosome segregation (Doxsey S., 2001). PIP5K is mapped onto the centrosomes in all stages of its division and is present at the mitotic spindle poles which are indicative of its intricate involvement in the centrosome biology. In fact, considering its co-localization with B23 in the mitotic spindle poles coupled with a resemblance of their staining pattern during the telophase, it can be speculated that PIP5K and nucleophosmin might function in sync regulating the centrosome biology.

Though the SUMO-mutant PIP5K(s) localized in the centrosomes, interestingly, our data showed that expression of PIP5K_K490A caused considerable enlargement of the centrosome and its fragmentation. Some of the cells even showed aggregate like accumulation of PIP5K-K490A at and proximal to the centrosomes. Results from different laboratories have stressed on centrosomes to be unique location where proteasome machinery is concentrated. Under instances of high levels of misfolded proteins, the cell responds by increasing the diameter of the centrosomes to about twofold (Wigley WC. et al., 1999). In the light of these observations, it is likely that the cytosolic but not the nuclear pool of PIP5K_K490A might be considerably misfolded thereby resulting in an increased centrosomal dimensions and formation of aggregate like structures.

Additionally, our studies revealed that cells expressing PIP5K-K490A not only had enlarged and fragmented centrosomes, but also failed to remain viable. Though Flow Cytometric analysis did
not reveal any potent cell cycle block, immunofluorescence analysis revealed an increased presence of p-His (ser10) nuclear foci formation in these cells, which is a hallmark of G2/M transition. An increasing number of cancer related proteins have been shown to reside in or traffic in and out of centrosomes in which their role in G2/M checkpoint is being thoroughly investigated (Kramer A. et al., 2004; Hollander MC. et al., 1999; Shinmura K. et al., 2007). The G2/M checkpoint in particular is an area of focus for cancer research (Takai N. et al., 2005; Stewart ZA. et al., 2003). Abnormal expression of several centrosome-associated regulators of the G2/M checkpoint like cyclin B/cdk1 (Blethrow JD. et al., 2008), Aurora A, Aurora B, Plk1, Nek2A (Schmit TL. and Ahmad N., 2007), p53 (Shinmura K. et al., 2007) and BRCA1 (Sankaran S. et al., 2006) have been detected in human tumors. Thus, one or more of these proteins in the centrosomes might be deregulated in cells expressing PIP5K-K490A resulting in G2/M arrest and subsequent cell death. Identification of these interaction(s) would open new avenues in the regulation of centrosome associated G2/M checkpoint. Moreover, targeted delivery and expression of PIP5K_K490A in specific cancer cells might be an efficient procedure for inducing death in cancer cells.