9. CONCLUDING REMARKS

The three manuscripts presented collect the outcome of the research work whose lines I contributed to develop during my PhD and represent a comprehensive analysis of the biological function and regulation of Diacylglycerol Kinase alpha. Starting from data indicating that Dgkα activity is regulated downstream of tyrosine kinase receptors in a Src-dependent manner, and that it play a role in the signal transduction leading to cell migration, these manuscripts unravel different aspects of this topic.

In the first paper, Dgkα activity is demonstrated to be necessary for HGF-induced invasiveness and anchorage-independent growth of the breast cancer carcinoma cell line MDA-MB-231. Thus Dgkα results to be a key enzyme in highly relevant cellular processes toward cancer progression, albeit in an *in vitro* model. Moreover, our data suggest that Dgkα might represent a novel target for the development of molecular strategies to selectively target cancer progression.

In order to follow this purpose, it is necessary to identify both the molecular determinants of Dgkα regulation and the transduction cascades regulated by it, in order to become aware of what would be expected in case of selective target of Dgkα signaling. On the other hand, it is equally significant to add novel plugs to the puzzle of our present knowledge about the fine tuning of cellular behaviour both in physiological and pathological conditions.

In the second paper inhere presented, Dgkα is shown to be required for a specific subset of molecular events induced by HGF stimulation in epithelial cell and leading to cell migration, namely for early protrusion of membrane ruffles and for the proper formation of new focal adhesions. In particular, Dgkα emerges as an upstream regulator of Rac small GTPase signaling, whose role in cell migration is presently well established.

Finally, in the third paper, tyrosine 335 of Dgkα is identified as the main phosphorylation site of Dgkα upon growth factors stimulation. Dgkα phosphorylation on Tyr^{335}, mediated by Src in physio-pathological conditions or by oncogenic Src in a cancer context, is shown to mediate its enzymatic activation and to be required for HGF-induced cell migration. In addition, both Tyr^{335} and proline-rich sequence of

Dgkα result critical for Dgkα phosphorylation, ability to interact with Src and proper targeting to the plasma membrane upon HGF stimulation.

However, though extensively investigated, a comprehensive understanding of Dgkα regulation and precise role in signal transduction is far from being exhausted. In particular, the exact meaning and function of its tyrosine phosphorylation, the immediate downstream target(s) and the role in *in vivo* tumor progression still remain to be assessed.

Concerning the role of Dgkα tyrosine phosphorylation, particularly relevant and still unexplained are the observation that both Y335F and ΔP mutants of Dgkα, which can not be either phosphorylated or activated, are associated to intracellular vesicles (rather than being diffuse in the cytosol) and, upon HGF stimulation, cannot be targeted to the plasma membrane. This observation, together with the accumulation of both wild-type Dgkα and Src in intracellular vesicles upon cell treatment with a Golgi-disrupting agent, lead to the intriguing hypothesis that phosphorylation of Dgkα Y335 by Src may be required to couple Dgkα to the vesicular transport from the inner cytoplasm to the plasma membrane. Indeed, upon growth factor stimulation, Src itself is recruited from the perinuclear area to the plasma membrane through Rab11-dependent endosomal traffic [40]. These observations allow to speculate that SH3- and SH2-mediated interaction with Src may couple Dgkα to the endosomal traffic machinery responsible for Src targeting from the perinuclear region to the plasma membrane. Moreover, the biological relevance of Dgkα plasma membrane recruitment in conveying growth factors-induced migratory signal is underscored by the findings that constitutive recruitment of Dgkα at the membrane provides intracellular signalling sufficient to trigger spontaneous cell motility.

The challenge for the future remains to discover the direct target(s) of the DAG/PA balance operated by Dgkα. The data presented in the second manuscript strongly indicate that Dgkα play its role in the pathway linking RTKs to Rac, and maybe to other members of the Rho family of small GTPases. Indeed, an increasing body of evidence suggests that Dgks regulate small GTPases, including Rac, through multiple mechanisms. Rac targeting and GTP loading are regulated by a complex signaling network involving the recruitment of distinct Rac-regulating proteins to multiple molecular complexes at the leading edge of migrating cells. Several plausible

candidates may regulate Rac activation in a Dgkα-dependent manner, but no one of them has presently been clearly identified.

β2-chimaerin has been described as a DAG-activated Rac GAP, implying that Dgkα may enhance Rac activation through subtracting DAG to this Rac GAP [41,42,43]. On the other hand, Dgkγ has been shown to negatively regulate PDGF- and EGF-induced Rac activation and membrane ruffling, by enhancing the activity of β2-chimaerin [44,45], highlighting that the role of either DAG or PA in membrane recruitment and activation of β2-chimaerin is still controversial.

Another putative target for direct regulation by Dgk activity is the PI(4)P-5 Kinase, whose activity is positively modulated by PA [46]. Dgkζ and PI(4)P 5-kinase co-localize with F actin at lamellipodia protrusions in epithelial cells [47], where Dgk-generated PA is required for full activation of PI(4)P 5-kinase activity, consistently with a role of both lipid kinases in positive regulating Rac function. Interestingly, a Dgk and a PI(4)P 5-kinase activities were found to associate in a complex with Rac and RhoGDI [48].

RhoGDI forms a complex with Rac, keeping it in a cytosolic inactive GDP-bound form, and, upon Rac activation, it contributes to Rac targeting to specific sites at the plasma membrane [28]. As Rac targeting implies the displacement of the interaction between Rac and RhoGDI, the finding that *in vitro* PA and $PI(4,5)P_2$ impair RhoGDI affinity for Rac [49,50], raises the hypothesis that activation of the RhoGDI-associated Dgk may allow the release of Rac from RhoGDI, and leads to speculate that also Dgkα may regulate Rac activation through this mechanism.

The Rho GAP p190 negatively regulates both Rac and Rho signaling, by enhancing their ability to hydrolyze GTP to GDP. PA modulate the specifity of this potent GAP by inhibiting its effectiveness specifically toward Rho [51]. Many other GEFs or GAPs or upstream regulators of Rho small GTPases, namely Vav2, DOCK180/Elmo, βPIX, Tiam1, Arf6 and Arf GAPs, might be regulated by Dgk activity and specifically by Dgkα, but presently any consideration can not be other than a speculation and a framework for future investigation. Moreover, the regulation operated on small GTPases is not simply the net result of different signaling pathways leading to their activation/inactivation, but is a complex coordination of their dynamic cycling of GDP/GTP loading, thus suggesting that even apparently contradictory contributions to the regulation may act in concert.

In conclusion, the data presented in the three reported paper constitute a outstanding advance in the knowledge of Dgkα regulation and function, especially in a perspective of a putative role in cancer progression, and lay the foundation for the future research directions.