

## Synopsis

Developmental pathways such as Notch are known to regulate self-renewal and cell fate decisions in embryonic and adult tissue-specific stem cells, while deregulation of Notch signalling is associated with malignant transformation. Hypoxia develops in solid tumours due to rapidly dividing cancer cells, leaky blood vessels, and increasing distance from the nearest blood vessel. Tumour hypoxia is considered as malice, aiding cancer progression and hindering successful therapy by promoting EMT, stemness and drug resistance.

Therefore, understanding signalling mechanisms regulated by hypoxia is therapeutically important. Notch pathway signalling is implicated in the maintenance of cancer stem/progenitor cells in a hypoxic tumour-microenvironment. Another protein activated in hypoxic condition is AMP-activated protein kinase (AMPK), a major player in energy homeostasis. Work done in the lab has implicated AMPK in regulating breast cancer stemness and drug resistance. Although independent studies have reported higher levels of activated AMPK and activated Notch1 in breast cancer, and hypoxia, which is prevalent in breast cancer, is shown to activate both pathways, a relationship between AMPK and Notch in breast cancer is yet to be established. These considerations led us to investigate whether AMPK facilitates cancer progression through the Notch pathway under hypoxic condition.

The transmembrane Notch receptor is activated when exposed to membrane bound Jagged and Delta family ligands expressed on neighbouring cells. Ligand-receptor interaction results in two proteolytic cleavage events, producing Notch transmembrane (NTM) and release of Notch intracellular domain (NICD) from the plasma membrane. NICD is transported to the nucleus, where it forms a complex with the CSL DNA-binding protein and transcriptional co-activators. Nuclear NICD then activates expression of specific target genes. Our study revealed that activating AMPK through pharmacological agents or genetic approaches increased the accumulation of cleaved Notch1 protein (NTM and NICD) in invasive breast cancer cell lines such as MDA-MB-231, BT-474 and HCC-1806. An increase in cleaved Notch1 levels led to enhanced activation of Notch downstream target genes. Inhibiting AMPK activity or depleting AMPK reduced cleaved Notch1 levels in cell lines *in-vitro* and in BT-474 xenografts. Moreover, we show that AMPK positively modulates cleaved Notch1 protein levels by increasing its stability. Previous studies have demonstrated hypoxia-induced increase in cleaved Notch1 protein levels as well as stability, however, the mechanisms leading to this stabilization has remained unknown. We show that in hypoxia, activated AMPK is required for an increase in cleaved Notch1 stability. Mechanistically, we identified a reduction in interaction of Notch1 with its ubiquitin ligase Itch/AIP4, upon AMPK activation in hypoxia, thus, bringing about reduced ubiquitination and degradation of cleaved Notch1. Further, we identified AMPK-induced tyrosine phosphorylation of the ubiquitin ligase Itch/AIP4 under hypoxic conditions that disrupted Itch/AIP4-Notch1 interaction. The specific tyrosine kinase responsible was Fyn, a Src-family kinase member. Finally, we found that inhibition of AMPK in hypoxic cells affected Notch-mediated self-renewal adversely. In addition, we uncovered that inhibition of AMPK under hypoxia affected the hypoxia mediated increase in drug resistance. Together, these data suggested the involvement of hypoxia-AMPK-Notch1 axis in imparting self-renewal properties and drug resistance to breast cancer cells

We have investigated the relevance of the signalling mechanism in patient derived breast cancer cells and interestingly, the hypoxia-AMPK-Itch/AIP4-Notch signalling axis is well conserved. More importantly, we assessed the concurrent activation of AMPK and Notch1 pathway in clinical breast cancer tissues which revealed that the activation of both these pathways correlated in large proportion breast cancer. Altogether, our study sheds light on the context-specific oncogenic role of AMPK, in reinforcing Notch1 signalling under hypoxia, to facilitate breast cancer progression. Based on our study, we propose that inhibition of AMPK can alleviate stem-like properties of breast cancer cells, thus rendering them susceptible to existing anti-cancer agents.