
Synopsis

Spatio-temporal regulation of GPCR mediated MAPK Transactivation in living cells

Signal transduction is a mode of cellular communication essential for an organism's sustenance and survival. Cell communication in higher organisms is largely executed by two classes of cell surface receptors i.e. G-protein coupled Receptors (GPCRs) and receptor tyrosine kinases (RTKs). Though the activation of each of these receptors have shown to regulate cellular responses through a linear pathway the underlying signaling events are much more complicated. A number of studies have shown that the signaling regulators of the RTK mediated pathway can be cross-activated by GPCRs activation via a process termed as 'transactivation.' However, these studies are limited, as they have largely missed the spatio-temporal dynamics of the signaling regulators involved in the transactivation pathway. With this premise, the present research was structured to investigate the spatio-temporal dynamics of MAPK cascade transactivated by GPCR signalling pathways in live cells. Towards this broad objective, we subdivided the study into three parts:

I. Construction and validation of live cell imaging biosensors for MAPK signalling cascade.

In the initial part of the study, we developed a series of tools (biosensors) for evaluating the activation of MAPK cascade in living cells. These sensors were to be utilized for studying the MAPK signalling events under various environmental perturbations (including but not limited to stimulations of receptors of various classes such as RTKs and GPCRs) using an advanced microscopy setup. The MAPK sensors generated are essentially genetically engineered proteins which contain fluorescent proteins fused to a protein in the MAPK cascade which translocates upon activation. In the validation experiments, these translocation based chimeric reporters were used for recording MAPK signalling events after stimulation of classical RTK signalling pathway. The sensors generated were found to be competent in reporting the spatio-temporal dynamics of various steps of the MAPK signalling cascade. In stage 1, we verified our ability to record dynamics of MAPK signalling in living cells using the developed microscopy based biosensor toolbox. Sensors for detecting activation of Grb2 protein which associates with activated RTK; ERK protein; MMP2 and ERF were successfully generated and functionally validated in living cells.

II. Analysis of spatio-temporal dynamics of GPCR mediated MAPK transactivation.

Using the biosensor toolbox generated in step I, we screened and established a model system which was subsequently used for studying MAPK signalling events transactivated by GPCR signalling pathways. After screening a large panel of GPCR agonists and cell lines, we narrowed down to the detailed analysis of histamine stimulated ERK signalling in HeLa cells for the purpose of studying MAPK signalling events. This was essentially governed by endogenous expression of histamine receptors in HeLa cells, which helped us to avoid overexpression related artefacts, which have largely limited the understanding of existing observations reported in literature. Our subsequent investigations surprisingly revealed the absence of ERK translocation into the nucleus after histamine stimulation, despite ERK being phosphorylated, an event which is known to be tightly associated with its spatial distribution. Based on this atypical observation, the study was divided into two parts, for analysing the different aspects of ERK activation through histamine stimulation.

A. Identification of pathway involved in ERK phosphorylation by histamine stimulation. Our biochemical analysis revealed that histamine stimulation leads to ERK phosphorylation within 5 minutes of stimulation, which resets to basal level within 30 minutes similar to RTK mediated activation. Using receptor specific antagonists and G-protein inhibitors, the activation was found to be routed through H1 receptor. Further using protein specific inhibitors, the involvement of proteins downstream of activated H1 receptor was investigated and pERK generation was found to be mediated by cascade involving PLC β , Ca⁺², PKC δ , Src and MEK proteins. Given that the involvement of MMP or EGFR was found to be minimal, we proposed the existence of a histamine induced RTK independent transactivation mechanism in HeLa cells. Effect of activated pERK was examined on specific downstream responses by transcript expression and phosphorylation analysis of the RSK protein (an ERK cytosolic substrate) and ELK1 (an ERK specific nuclear substrate). While we recorded distinct activation of cytosolic targets, no effect on nuclear target was observed. In agreement to these, no change in cell proliferation (attributed to activated nuclear targets) was found, which supported the cytosolic localization dependent behaviour of pERK. On the whole, the biochemical analysis backed

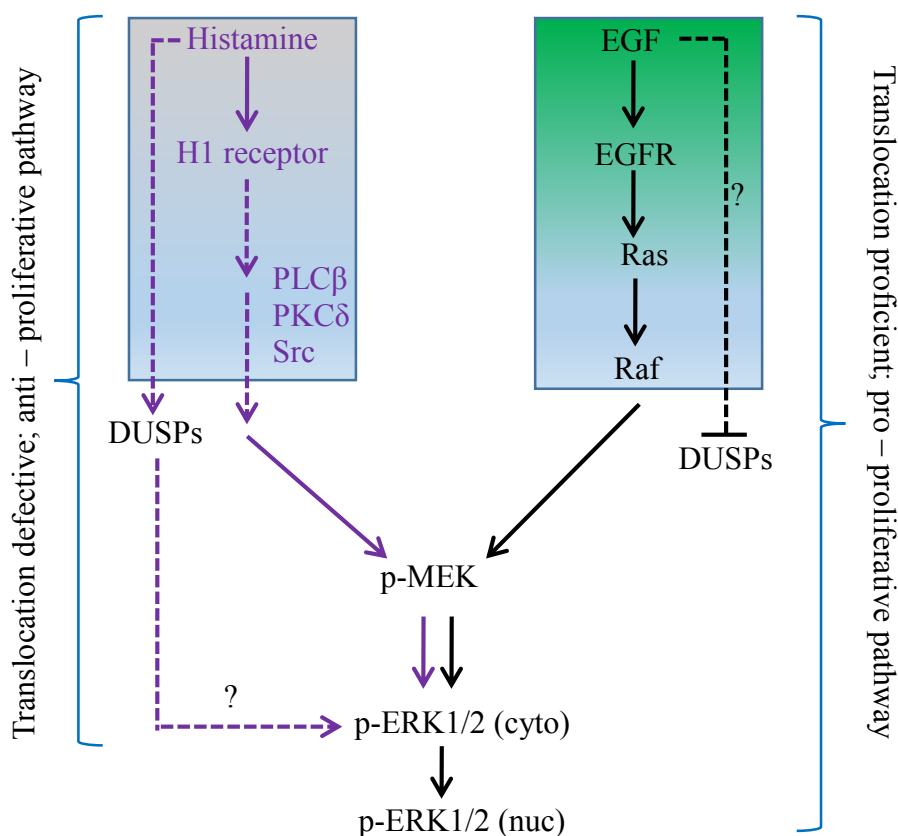
with live imaging analysis yielding a detailed account of the molecular cascade involved in ERK phosphorylation as well as for the downstream responses generated by pERK, in response to histamine stimulation in HeLa cells.

B. Spatio-temporal analysis of histamine activated pERK. Subsequent to pathway mapping, the aberrant behaviour of pERK warranted investigations into the spatial constraints on pERK, generated from histamine as against the one generated through RTK activation. We explored this by using FRAP analysis, with the shuttling and translocation proficient ERK biosensor, which revealed that perhaps histamine signalling activates some cytosolic scaffold, responsible for cytosolic restriction of pERK. Subsequent experiments were primarily aimed to identify this spatio-temporal regulator which allows histamine to activate pERK but restrict it in cytosol, using shRNA mediated gene silencing approaches.

C. DUSPs as spatial regulator of pERK.

For identifying the spatial regulators of histamine stimulated ERK localization, expression of ERK specific DUSPs and β -arrestin was blocked using specific shRNAs in HeLa cells and the localization of GFP-ERK2 was tested in these cells following histamine stimulation under live conditions. The screening revealed a role for DUSP7, a cytoplasmic ERK phosphatase, as a spatial regulator of pERK during histamine stimulation. The FRAP analysis in DUSP7 knockdown cell demonstrated that the DUSP7 does act as cytoplasmic scaffold of pERK. Concomitant to this, change in the pERK location, induction of the ELK1 transcript as well as pro-proliferative changes in the DUSP7 knockdown cells were recorded, reinforcing the role of DUSP7 in spatial regulation of the histamine activated pERK.

Based on these sequences of events and the role of various molecules involved in histamine mediated activation of pERK, the MAPK activation in canonical (EGF) and non canonical mode of activation can be schematically defined as per the figure shown below.



Summary of signalling schematics of ERK activation by histamine or EGF. The flow diagram represents the molecular regulators determined in this study to be involved in histamine mediated phosphorylation of ERK and its spatial localization in cytoplasm (cyto) (left). The sequence of events involve in ERK phosphorylation and its nuclear localization by EGF is shown in the right cascade and point towards the presence or occurrence of two event model for MAPK activation. While both ERK phosphorylation and translocation occurs in response to RTK activation, which leads to pro-proliferative changes, in response to histamine stimulation, ERK phosphorylation happens but no translocation occurs which blocks pro proliferative changes. The involvement of DUSP in the cascades is also shown. This is supported by the role of DUSP7, elucidated in our studies.