**University of Leicester**

**BBSRC MIBTP Studentship Project 2025-6 entry.**

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**Section 2 – *Project Information***

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| **Project Title** | Obtaining insights into how a signalling hub protein Ras activates multiple effectors  |
| **Project Summary**  |
| The RAS family of small GTPases act as signalling hubs regulating cell proliferation and differentiation. It is highly conserved from yeast to humans, highlighting its fundamental biological roles. The physiological importance of RAS is underlined by various observations. These include that: (i) in mouse models, *KRAS*, one of the RAS isoforms, is essential, (ii) germline mutations in humans cause disorders termed RASopathies, (iii) human *RAS* genes are frequently mutated in cancers, and (iv) overexpression of oncogenic RAS causes premature senescence.  As a signalling hub, RAS activates multiple downstream pathways by directly interacting with “effector” molecules, which harbour domains termed “RAS binding domain (RBD)” or “RAS associating (RA)” domain, responsible for the RAS-effector binding. Extensive efforts have revealed RAS-RBD and RAS-RA complex crystal structures for prototype effectors, including RBDs of Raf kinases (that lead to ERK activation), RBDs of PI3 kinases (that lead to Akt activation) and RAs of RalGEFs (guanine nucleotide exchange factors for another small G proteins RalA and RalB). Interestingly, affinities between RAS and RBDs/RAs vary; the dissociation constant (KD) values range from 50 nM to a few µM, making almost 100 times the difference between the RBDs/RAs of the highest and the lowest affinity. Whilst it has been widely accepted that RAS activates multiple effectors, how RAS interacts with low-affinity effectors in the physiological setting is under-explored. This PhD project aims to address this question and to establish a novel concept of the mechanism of a signalling hub protein to activate multiple effectors of various affinities. As listed below, we set three working hypotheses, which are not mutually exclusive, and address them during the PhD project. **Working hypothesis 1**: The low-affinity effectors are more abundant in a cell than the high-affinity effectors. To examine this possibility, we will generate a set of isogenic human cell lines where a prototype RAS, KRAS, and prototype high- and low-affinity effectors (BRAF and Rgl2, respectively) are endogenously tagged with a variant of green fluorescent protein, mStayGold, using CRISPR-Cas9 technology. We will use hTERT RPE-1 ER-Cre-ER as the host human cell line, which we recently generated as a human cell culture model where the karyotype is stable and an inducible Cre recombinase can facilitate the gene editing (Hindul et al., 2022, *Biol Open*, **11**: bio059056). The generated cells will be analysed by quantitative live cell imaging to obtain the spatio-temporal signals of mStayGold so that the relative molar ratios of KRAS, BRAF and Rgl2 will be determined. This part will be conducted in collaboration with Dr. Rob Mahen. **Working hypothesis 2**: The full-length effectors show a higher affinity towards RAS than RBDs/RAs only, which can be a low-affinity RAS binder. To examine this possibility, we will prepare bacteria recombinant human KRAS, the full-length BRAF, and the full-length Rgl2 and test the affinities using various biochemical assays including biolayer interferometry (BLI), isothermal titration calorimetry (ITC) and mass photometry. We will also conduct cryo-electron microscopy (cryo-EM) of KRAS and full-length Rgl2 complex. We recently determined the crystal structure of the complex of KRAS and the RA of Rgl2 (Tariq et al (2024) *Life Science Alliance*, **7**, e202302080), and this data will help solve the cryo-EM structure of KRAS and full-length Rgl2 complex. This part of the work will potentially unveil previously unidentified RAS binding elements in the Rgl2 protein.  **Working hypothesis 3:** It has been well-established that RAS-effector interactions occur at the plasma membrane (PM). In the presence of PM, the low-affinity effectors may interact strongly with RAS. To examine this possibility, we will prepare membrane nano-disc and include it in the above-mentioned binding assays.  Techniques that will be undertaken during the projectRecombinant protein production using size-exclusion chromatography, biochemical analyses of protein-protein interactions using biolayer interferometry and mass photometry, gene editing (human culture cells), quantitative cell imaging analyses including super-resolution microscopy, and cryo-electron microscopy.   |
| **References** |
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