Ligand-directed two-step labelling: a new technology to map the trafficking and interactome of GLUT4

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Project outline

Background: GLUT4 plays a vital role in maintaining glucose homeostasis by promoting glucose uptake into adipocytes and skeletal muscle upon stimulation by insulin or exercise. Disruption in GLUT4 trafficking is a hallmark of type 2 diabetes and is linked to obesity.[1] Current techniques for investigating GLUT4 largely rely on the expression of GFP-GLUT4 fusion proteins[2] or the use of antibodies.[3] Nonetheless, GFP fusion proteins are unsuitable for investigating subpopulations of GLUT4, and antibody-based methods suffer from specificity issues and are often restricted to fixed tissues. The lack of tools to label endogenous GLUT4 and identify its interaction partners in individual compartments hinders the understanding of its trafficking and regulation, and limits the development of strategies to modulate its distribution for therapeutic purposes.

Ligand-directed two-step labelling offers a platform to label endogenous GLUT4 with exquisite specificity while retaining its function (**Fig. 1a**). This method allows labelling of subpopulations of a protein of interest (POI) and has previously been employed to investigate AMPA receptor trafficking in neurons.[4] Our lab has extensive experience in the design, synthesis, and application of similar ligand-directed labelling probes. We propose to employ this technology to label endogenous GLUT4 to investigate its trafficking and map its interactome.

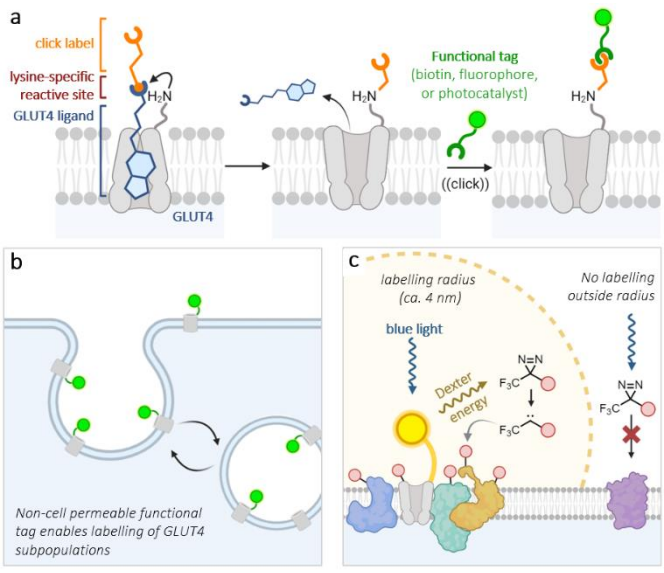

Fig 1.a Ligand-directed two-step labelling of GLUT4. **b** Measurement of trafficking rates of GLUT4 subpopulations *via* fluorophore conjugation. **c** Mapping of the GLUT4 interactome *via* Dexter energy transfer.

Objectives: ***O1.*** To design and synthesise a toolbox of selective GLUT4 probes for *in situ* two-step labelling. ***O2.*** To characterise real-time rapid GLUT4 trafficking in response to diverse physiological cues, including insulin and exercise-activated pathways. ***O3.*** To monitor context-specific compartmentalization in specialized pools responsive to particular stimuli. ***O4.*** To map the interactome of GLUT4 at different stages to identify the molecular determinants of GLUT4 trafficking and compartmentalisation.

Approach: ***O1.*** Ligand-directed two-step labelling is based on the proximity-induced conjugation of a POI with a click label, facilitated by the reversible binding of a ligand (**Fig. 1a**). After ligand release and recovery of the POI function, a biorthogonal click reaction can be performed to attach a functional

probe to the POI. This two-step process inherently ensures high specificity. We will design and synthesise a toolbox of probes consisting of a GLUT4 ligand conjugated to a click label with a lysine-reactive tether, and structure-matched negative controls. Probe design will be informed by previous structure-activity-relationship data.[5-6] ***O2.*** The probes will then be applied to human adipocytes in the presence of insulin, which will allow conjugation of endogenous GLUT4 with the click label. Subsequent treatment with a tetrazine-containing reagent will promote the second labelling step to attach a functional tag onto the previously labelled GLUT4. A biotin tag will be used to assess labelling efficiency and selectivity through pull-down analysis in competition experiments with a reversible GLUT4 ligand and in knockout cell lines. Normal GLUT4 distribution will be verified through live cell imaging after labelling with a fluorophore, and 2-deoxy-D-glucose or 3H-glucose uptake (the latter in collaboration with Prof. Lisa Heather's lab) will be used to confirm the functional integrity of the modified GLUT4. ***O2, O3.*** Once validated, this labelling technique will be used to measure the trafficking rates and compartmentalisation of GLUT4 in adipocytes and skeletal myotubes in response to insulin or AMPK activation (**Fig. 1b**). ***O4.*** Finally, a photocatalytic tag will be conjugated to the click label to investigate the interactome of GLUT4 *via* Dexter energy transfer (**Fig. 1c**).[7] This proximity-based technology allows labelling of proteins surrounding the POI (within *ca.* 4 nm radius), and their enrichment and analysis by proteomics. Parallel labelling experiments on cells with inducible GLUT4 knockout will be used to distinguish off-target labelling from genuine interactome labelling. This will allow identification of proteins involved in the trafficking of GLUT4 in disease-relevant cells with high spatiotemporal resolution.

Impact: This molecular toolkit will provide fundamental insight into elusive aspects of GLUT4 trafficking, such as its endocytosis and context-specific compartmentalisation. This technology will identify molecular determinants of GLUT4 distribution in adipocytes and skeletal myotubes, and help elucidate the differences and similarities between GLUT4 trafficking induced by insulin and exercise. Such insights will inform the development of GLUT4-based therapeutics to alter its distribution for the treatment of metabolic diseases.

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