Horizon Europe (HORIZON)
Marie Skłodowska-Curie Actions
Doctoral Networks (MSCA-DN)

GLYCOprotein N-glycosylation from non-life to eukaryotes:

a Doctoral Network to expand the knowledge on a ubiquitous posttranslational modification of proteins

GLYCO-N

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INDIVIDUAL RESEARCH PROJECTS

Fellow DC1	Host institution UNINA	Enrollment in the doctoral schoo UNINA
Project Title: N-glycosylation in the extreme halophile *Haloquadratum walsbyi.* **Supervisor:** Prof Antonio Molinaro **Relation to the GLYCO-N program**: DC1 will conduct a **doctorate in bioinformatics and structural Glycoscience**. The project fits within the **multidisciplinary** program scope through connections with **structural and biochemical characterization** of **glycans and glycoprocessing enzymes** of archaeal origin.		
Objectives: *Haloquadratum* (*Hqu*) *walsbyi* (an archaea in the *Halobacteria* class) is noted for its flat, square-shaped cells, and its unusual ability to survive in extremely high concentrations of sodium chloride and magnesium chloride. *Hqu* is a strictly aerobic bacterium, with an optimal growth temperature of 37-40 °C, and has a unique cellular structure that resembles a flat-shaped quadratum. Currently, two strains are sequenced and available commercially, and DC-1 will focus on the type-strain, named C23, due to the simpler construction of its cell-wall, consisting of a cell membrane with one S-layer on the top, while the other strain has two S-layers; both archaea have some (sulfo)glycolipids in addition. The C23 genome encodes (by prediction) 20 GTs with three likely non-functional and several proteins able to manipulate sugars at different levels (Kandiba, doi: 10.1111/1574-6968.12193). Notably, *Hqu* encodes two of the enzymes involved in the biosynthesis of neuraminic acid (NeuA), albeit no experimental study has addressed the functionality of these enzymes or the presence of this monosaccharide, as well as of other glycans, on its surface. Despite this lack of information, the S-layer protein (code CCC39200.1) is predicted to be highly glycosylated, with 16 N-glycosylation sites in canonical sequences of which 6 rated with high probability of substitution, and 24 potential O-glycosylation sites (NetNGlyc-1.0 and NetOGlyc-1.0 servers, respectively, available at https://services.healthtech.dtu.dk/). Thus, the first goal of DC-1 will consist of the isolation and the structure elucidation of *Hqu* glycans by merging chemical, NMR and MS approaches. This first goal will resolve the conundrum about the presence of NeuA, and about how glycans impact on the strong salt-tolerance of this archaeon. These achievements will pave the way to the second goal, the biosynthetic studies. Homology study of the annotated proteins will be in collaboration with Bernard Henrissat (DTU) to make an educated guess on the GT that acts in the formation of the detected glycosidic linkages, and on the enzymes that synthesize the activated monosaccharides. Then, the candidate proteins will be expressed in recombinant form to proceed with their characterization and functional studies in collaboration with UDE while their evolutionary location will be analyzed in collaboration with DTU.		
Expected results: *Training*: *Hqr* growth, isolation and structure determination of N-glycans, bioinformatics on enzyme identification; protein expression and purification; enzyme functional assay and characterization. *Research*: functional characterization of new archaeal carbohydrate active enzymes for biotechnological purposes: discovery of enzymes involved in N-glycosylation; providing enzymes stable in salt-rich media for biotechnological application; contribute to the understanding of the evolution of archaeal GTs.		
Planned secondments: **UDE**. Timing: M8; duration 4M. **Purpose**: biochemical analysis of archaeal neuraminidases; **AB**, Timing, M20, duration, 2M, **Purpose**: large scale algae protein production		

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Fellow DC2	Host institution DTU	Enrollment in the doctoral school DTU
Project Title: Study of the glycosylation machinery of archaea: a new perspective to unravel the evolutionary history of the tree of life. **Supervisor:** Prof. Bernard Henrissat **Relation to the GLYCO-N program**: DC2 will conduct an **interdisciplinary doctorate** on **structural**, **biochemical** and **computational Glycoscience**. The project fits within the **multidisciplinary** program scope through connections with **glycan-related biotechnology**.		
Objectives: The goal of this doctoral project is to study the glycosylation system of different microbial archaea, with the aim to understand on the one hand how the glycosylation machinery evolved among prokaryotes, archaea and eukaryotes and on the other hand to shed light on the evolutionary history of these organisms from the N-glycosylation perspective. These studies are required because: 1) the structures of the glycans are largely undiscovered; 2) the enzymes responsible for the production, manipulation and assembly of sugars are yet to be fully discovered and classified; and 3) the similarities /differences with the archaea, prokaryotes and eukaryotes can provide essential elements for understanding the origin of the glycosylation machinery. In detail, DC-2 will identify and characterize carbohydrate active enzymes (CAZymes) by screening fully sequenced archaea genomes in search of the glycan-related genes. For this a bioinformatics approach will be taken that pays attention specifically to those genes involved in nucleotide-sugar synthesis. Once good candidates have been identified, these will be cloned, expressed, structurally and functionally annotated. In the second part DC2 will evaluate phylogenetic distances between the enzymes and their analogous enzymes in bacteria, archaea and eukaryotes to understand where they are located in the evolutionary tree.		
Expected results: *Training*: Bioinformatics on enzyme activities; protein expression and purification; protein structure determination. *Research*: discovery of new carbohydrate active enzymes for biotechnological purposes: discovery of new glycans structures; understanding evolutionary history of eukaryogenesis.		
Planned secondments: **UNINA**. Timing: M8; duration 4M. **Purpose**: GT/GHs from archaea structure and function determination; **AMP**, Timing, M20, duration, 2M, **Purpose**: cloning and expression of selected CAZymes		

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Fellow DC3	Host institution UDE	Enrollment in the doctoral school UDE

Project Title: Study of the function of glycosyltransferases in the crenarchaeal model organism *Sulfolobus acidocaldarius* to unravel their potential for biotechnological application.
Supervisor: Prof Bettina Siebers
Relation to the GLYCO-N program: DC3 will conduct an **interdisciplinary doctorate** on **structural**, **biochemical** and **synthetic glycosciences**. The project fits within the **multidisciplinary** program scope through connections with **glycan-related bioinformatic, structural analysis (glucan and protein)** and **biotechnology**.

Objectives. The aim of the doctorate project is to study the function of glycosyltransferases in the thermoacidophilic, crenarchaeal model organism *Sulfolobus acidocaldarius* with optimal growth at pH 2-3 and 80 °C. These studies are required because: 1) *Saci* possesses 29 annotated glycosyltransferases and their possible function in N-glycosylation still needs to be addressed; 2) the effect of available carbon source as well as stress conditions such as starvation on N-glucan structure is not known; 3) detailed enzymatic studies are still missing for most GTs thus hampering their use in biotechnological applications; and 4) functional characterization of glucosyltransferases is essential for phylogenetic analysis to retrieve evolutionary implications. In detail DC3 will grow *Saci* under different growth conditions and in collaboration with UNINA changes in N-glycan structure and composition of the S-layer protein will be studied. Focussed GT-omics involving transcriptomic, proteomic and chemical biology such as ABPP approaches (for instance, RT PCR as well as full proteomics) will be used to follow changes in GT expression and activity. To follow the integration of carbohydrates into the N-glycan we will use metabolic labelling with azide-modified carbohydrates. Possible candidates will be determined based on bioinformatic analysis in close collaboration with DTU. In addition, available GT mutants as well as of the flippase will be studied in regard to changes in glucan composition and structure. GTs involved in N-glycosylation will be expressed either in *Escherichia coli* or in *S. acidocaldarius*, purified and the enzymes analyzed in respect of their enzymatic properties. The *Saci* expression system will be also available for other partners to express thermophilic bacterial/archaeal enzymes that cannot be produced in the common mesophilic expression hosts. Enzyme assays will be designed in close collaboration with UNINA (synthesis of glucan-substrates, analysis of glucan structure) and the protein structure will be resolved or modelled together with BIO.

Expected results: *Training*: Bioinformatics on enzyme activities; protein expression (in bacteria and archaea) and purification; enzyme characterization, chemical proteomics. *Research*: functional characterization of new archaeal carbohydrate active enzymes for biotechnological purposes: discovery of enzymes involved in N-glycosylation and possible changes in N-glucan structure; providing novel, robust extremozymes or enzyme cascades for biotechnological application; contribute to the understanding of the evolution of archaeal GTs by providing functional information.

Planned secondments: **UL**. Timing: M8; duration 4M. **Purpose**: ABPP design and synthesis; **SAM**, Timing, M20, duration, 2M, **Purpose**: large scale archaeal protein production

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Fellow DC4	Host institution BIO	Enrollment in the doctoral school UPV/EHU
Project Title: Structure and molecular recognition of viral N-glycoproteins. **Supervisor:** Prof. Jesùs Jiménez Barbero **Relation to the GLYCO-N program**: DC4 will conduct an **interdisciplinary doctorate** on **structural** and **biophysical Glycoscience** and **glycan-related biomedicine**. The project fits within the **multidisciplinary** program scope through connections with **synthetic Glycoscience**.		
Objectives: To disentangle the fine details of the interaction of the viral N-glycans with the key glycosyltransferases and the glycomimetics with the essential glucosidases at the maximum possible level of resolution. The specific epitopes and molecular recognition features of the viral N-glycans and N-glycosylated proteins from *Hau*, isolated by UNINA (DC1), *versus* at least one of the key GTs from *Sulfolobus acidocaldarius*, identified by UDE (DC3), will be studied by a combination of NMR and X-ray methods, assisted by biophysical techniques (BLI, ITC), and molecular modelling protocols (docking and molecular dynamics). Additionally, the interaction of at least one of the selected glycomimetics developed by UL (DC5) with the key ER-alpha-glucosidase I (ER-I) and II (ER-II) will be deciphered by using ligand-based NMR methods and X-Ray crystallography. Specifically, DC4 will address the following objectives: 1) The key glycan epitopes in the viral N-glycan and the glycoprotein will be deduced by NMR, using the ^{13}C-labelling protocols developed by the group. 2) The X-Ray structures of the selected GT and glycomimetic/glycosidase complexes will be unraveled using X-ray crystallography. 3) The molecular recognition features of the interaction between the selected N-glycans and GTs and glycomimetics/glycosidases will be deciphered using a synergic combination of NMR and X-Ray crystallography, assisted by MD simulations. Depending on the stoichiometry and size of the generated complexes, cryo-EM methods will be applied. 4) The kinetic and thermodynamic parameters of the corresponding interactions will be also quantitatively assessed by using biophysical techniques including isothermal titration calorimetry (ITC) and biolayer interferometry (BLI).		
Expected Results: *Training*: recombinant glycoprotein and GT production, including stable isotope labelling; structural characterization of protein/carbohydrate complexes, hands on NMR, X-Ray, cryoEM, and molecular modelling; biophysical methods to establish ligand/receptor interaction. *Research*: Fine structural details of N-glycans, N-glycoproteins, and GTs		
Planned secondments: **UNINA**. Timing: M8; duration 4M. **Purpose**: GT selection; **IC**, Timing, M20, duration, 2M, **Purpose**: synthesis of selected glycans		

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Fellow DC5	Host institution UL	Enrollment in the doctoral school UL
Project Title: Designer molecules to interfere with host and viral N-glycosylation processing. **Supervisor:** Prof Hermen Overkleeft **Relation to the GLYCO-N program**: DC5 will conduct an **interdisciplinary doctorate** on **synthetic Glycoscience** and **glycan-related biomedicine**. The project fits within the **multidisciplinary** program scope through connections with **biochemical and computational Glycoscience**.		
Objectives: Interference with maturation of host N-glycans on viral capsid proteins is an established therapeutic strategy that however has not led to clinically applied antiviral drugs. Arguably, potency and/or selectivity of the glycoprocessing enzyme targeting glycomimetics studied to date precludes their clinical use, this while interference with host-derived viral N-glycoproteins on mature virions shows the validity of the strategy: targeting (with Tamiflu or Relenza) influenza neuraminidase that is essential for liberating budding virions from the infected host cells preceding their infection of surrounding tissue. At the same time, the involvement of glycosidases in maturation of virally encoded N-glycans in giant viruses is not known yet. Aim of DC5 is therefore two-fold: 1) the design (jointly with UB) of improved glycomimetics targeting the essential ER N-glycoprotein folding glycosidases, ER-alpha-glucosidase I (ER-I) and II (ER-II); and 2) in later stages of the GLYCO-N program and once giant virus glycosidases are identified (UNINA), transferring inhibitor designs to these enzymes. DC5 is complementary to DC9 in which glycomimetic inhibitors are designed to target host/viral glycosyltransferases and designs and synthesis schemes will be selected to feed into both DCs. Up until today small molecule glycomimetics targeting host/viral glycoprocessing enzymes were designed as competitive inhibitors, this while arguably (and as evidenced from the field of antibiotics) mechanism-based covalent and irreversible inhibitors would be more effective. Aided by enzyme reaction mechanism modulation (UB) candidate-mechanism-based inhibitors will be designed, both for the inverting glycosidase, ER-I, and the retaining one, ER-II. Candidate-inhibitors will be assayed on recombinant enzymes as well as in cells and cell extracts. For this purpose, fluorogenic substrate assays that are commercially available (ER-II) and that has been developed at UL (ER-II) will be employed. As well, the mechanism-based inhibitors will be transformed into activity-based probes, allowing ABPP/chemical proteomics studies to identify (off)-targets and to establish target engagement in relevant cell models in later stages of the project.		
Expected Results: *Training*: Glycomimetic design and organic synthesis; recombinant enzyme production and purification; enzyme activity assay development; activity-based protein profiling methodology development. *Research*: Development of candidate-antivirals; establishment of chemical proteomics assays to identify viral N-glycan processing enzymes and the effect of inhibitors on their action.		
Planned secondments: **UB**. Timing: M8; duration 4M. **Purpose**: CAZymes reaction mechanism and modelling; **ZB**, Timing, M20, duration, 2M, **Purpose**: cell biology of CAZymes and their inhibitors		

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Fellow DC6	Host institution URN	Enrollment in the doctoral school URN
Project Title: Unraveling the xylosylation pathway of N-glycoproteins in microalgae. **Supervisor:** Prof. Muriel Bardor **Relation to the GLYCO-N program**: DC6 will conduct an **interdisciplinary doctorate** on **biochemical Glycoscience** and **glycan-related biotechnology**. The project fits within the **multidisciplinary** scope of the program through connections with **structural and computational Glycoscience**.		
Objectives: N-glycans from *Chlamydomonas reinhardtii*, a green microalga, are maturated in the Golgi apparatus by multiple xylosyltransferases (XylT) to give rise to methylated *N*-glycans carrying xylose residues on different mannose including one linked in β(1,2) to the core of the *N*-glycans. Five genes encoding for putative XylT candidates are predicted in the *Chlamydomonas* genome while only scarce information regarding their activity is available. In this context, the first objective of the doctoral project is to biochemically decipher the function of these XylT candidates in the *N*-glycan pathway of *Chlamydomonas*. This will be achieved by 1) the expression of soluble XylT candidates in tobacco leaves, their purification and the monitoring of their XylT specificity by testing various glycan substrates; 2) the complementation of plant mutants impaired in *N*-glycan xylosylation with *Chlamydomonas* XylT candidate genes; and 3) jointly with BIO perform structural studies on candidate proteins. In addition, the cell localization in the *Chlamydomonas* secretory system will be performed by expressing, in *Chlamydomonas*, the XylT candidates fused to a reporter fluorescent protein and analysis by confocal microscopy and Transmission Electron Microscopy (TEM). Finally, as soon as the specificity and cell localization of XylT candidates will be established, bioinformatic identification of orthologous XylT in the microalga kingdom will be carried out in order to understand how xylosylation of *N*-glycans evolved within the different phyla of the tree of life.		
Expected Results: *Training*: Recombinant protein production and purification; enzyme activity assay development; protein structural biology, correlated light and electron microscopy (CLEM). *Research*: Structure and function of N-glycoprocessing enzymes essential for *Chlamydomonas*, filling in gaps in the microalgae N-glycosylation map.		
Planned secondments: **BIO**. Timing: M8; duration 4M. **Purpose**: structural analysis of microalgae glycans; **AB**, Timing, M20, duration, 2M, **Purpose**: large scale algae protein production		

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Fellow DC7	Host institution UB	Enrollment in the doctoral school UB
Project Title: Modeling of mechanisms in wild type and engineered archaeal and microalgal glycoprocessing enzymes for the efficient synthesis of (neo)-glycans. **Supervisor:** Prof. Carme Rovira **Relation to the GLYCO-N program**: DC7 will conduct a **doctorate** on **computational Glycoscience**. The project fits within the **multidisciplinary** program scope through connections with **structural and biochemical characterization** of **N-glycans and glycoprocessing enzymes**.		
Objectives: Glycosyltransferases (GTs) are indispensable tools for the chemo-enzymatic construction of well-defined glycans of various nature and complexity. With the aid of GTs, glycan arrays representing the complexity and diversity of mammalian cell N-glycans, or mother milk glycans, have become available in recent years for screening of, for instance, interacting proteins (antibodies in the context of immune-reactive glycans). The use of GTs for synthetic purposes at industrial scale can be boosted by the discovery of their molecular mechanisms of action. This will also enable design of inhibitors for GTs associated to glycosylation-related diseases, for which structural information is lacking. This project will survey in an unbiased approach both GTs and GHs for the preparation of individual glycans on scale, and for broadening the scope of glycan arrays to encompass also neo-glycans. The focus will be on fucosylations and sialylations, being the glycosides that currently are most efficiently introduced enzymatically. Candidate-GTs and GHs (TGs) will be selected and their mode of action (catalytic reaction coordinates of sugar transfer) will be obtained by state-of-art multiscale approaches based on molecular dynamics (MD) and quantum mechanics/molecular mechanics (QM/MM) methods. In the lack of structures, we will benefit from the wide survey of structures through AlphaFold, followed by the binding of ligands (donor and acceptor) using docking approaches. *In silico* point mutations, i.e., within the active site region, will be introduced and their effect on sugar transfer activity and selectivity predicted. Candidate-mutant enzymes will be cloned and expressed and their glycosylation efficiency using their respective (GT/GH) substrates on glycan precursors evaluated (secondment at UL).		
Expected results: *Training*: computational analysis of GT/GH reaction coordinates; in silico enzyme engineering; inhibitor design; cloning and expressing of mutant enzymes; enzyme activity assays; glycan product analysis. *Research*: new GTs and/or GHs/TGs for the large-scale synthesis of commercially relevant glycans and for the construction of high-diversity glycan arrays.		
Planned secondments: **UL**. Timing: M8; duration 4M. **Purpose**: glycomimetic inhibitor design and synthesis. **AMP**, Timing, M20, duration, 2M, **Purpose**: large scale GT protein production		

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Fellow DC8	Host institution UL	Enrollment in the doctoral school UL
Project Title: Inhibitors and activity-based probes targeting host and viral glycosyltransferases. **Supervisor:** Prof Hermen Overkleeft **Relation to the GLYCO-N program**: DC8 will conduct an **interdisciplinary doctorate** on **synthetic Glycoscience** and **glycan-related biomedicine**. The project fits within the **multidisciplinary** program scope through connections with **structural** and **computational Glycoscience**.		
Objectives: Glycosyltransferases (GTs) constitute one of the most desirable targets in drug discovery because of their involvement in many human pathologies including cancer, infectious diseases and inherited diseases. Yet and with the exception of glucosylceramide synthase (GCS, inhibition of which is at the basis of the substrate reduction therapy for treatment of Gaucher disease patients) no effective, selective and bioavailable GT inhibitors exist. In this project, and based on the experience that candidate inhibitors have not been found using contemporary methods (screening large collections or designing substrate analogues), they will be designed *in silico*. DC8 complements DC5, in that, while in the latter host respectively viral glycosidases are targeted consecutively, here at first a host and later a viral transferase are subject of study. In the first instance we will select one of mammalian α2,3-, α2,6- and α2,8-sialyltransferases involved in N-glycan biosynthesis of influenza capsid proteins. In collaboration with UB a reaction coordinate map will be established based on which the first generation of candidate inhibitors will be designed and synthesized. The inhibitors will be tested in in vitro assays for which purpose truncated N-glycan substrates will be synthesized, and in in vivo assays in healthy cells with as readout N-glycan structure and diversity. For the purpose of the latter bump-hole sialyl transferases and their corresponding artificial Leloir substrates will be generated in collaboration with ICL. In the second stage and guided by findings at UNINA (DC9) the methodology will be translated towards giant virus transferases that install sialic acid or related sugars onto viral N-glycoproteins.		
Expected results: *Training*: inhibitor design and synthesis; design of in vitro and in cellulo enzyme activity/inhibition assays. *Research*: inhibitors selective for GS; new therapeutic lead compounds for Pompe disease.		
Planned secondments: **ICL**. Timing: M8; duration 4M. **Purpose**: bump hole sialyltransferase system design; **IC**, Timing, M20, duration, 2M, **Purpose**: large scale GT/GH inhibitor synthesis		

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Fellow DC9	Host institution UNINA	Enrollment in the doctoral school UNINA

Project Title: Mining chlorovirus PBCV-1 genome to decipher the N-glycosylation biosynthetic pathway.
Supervisor: Prof. Antonio Molinaro
Relation to the GLYCO-N program: DC9 will conduct a **doctorate** in between **bioinformatic and structural Glycoscience**. The project fits within the **multidisciplinary** program scope through connections with **structural and biochemical characterization** of **glycans and glycoprocessing enzymes**.

Objectives: Chloroviruses are a group of viruses in the Phycodnaviridae family, characterized by having dsDNA encoding for about 400 proteins with various functions, including an almost complete glycosylation machinery. Indeed, chloroviruses are almost independent from the host resources, and their glycans do not resemble those of the host, the microalgae Chlorella, and exhibit unique structural features.[2] PBCV-1 encodes 8 GTs, with the activity of A064R is fully defined, and that of A111/114R and A075 supported by preliminary biochemical data. The activity of the other GTs is not determined yet and constitute the aim of DC9.[2] In the first instance, DC9 will address the question about how the first monosaccharide is activated, namely with a nucleotide or with Dol-P, which in turn would imply the existence of a Dol-P-Glc synthase. Next and in collaboration with DTU bioinformatic analysis will be performed on the candidate GTs to evaluate their homology with known enzymes and to evaluate their likely glycosyl donors. This information will be matched with those of the viral N-glycans to identify which glycosidic linkage the GT assembles, in order to synthetize an opportune acceptor to be used in the functional enzymatic assay. The experimental work will prioritize the study of the GTs for which the bioinformatic search will suggest a donor with high level of confidence. The recombinant proteins will be expressed in *E. coli* or other suitable bacteria, and the purification strategy, including the choice of the tag, will depend on the predicted GT features, namely if it is a soluble or transmembrane enzyme. Functional assays will be monitored by HPLC and/or appropriate assay like the UDP-GLO, while an in-depth NMR study will be carried out to define the structure of the reaction products. NMR epitope mapping studies to define the mode of interaction between the GT and the donor or the acceptor, and molecular modelling studies, like docking of the substrates with the GT built with AlphaFold, will furnish a first insight about how the molecules interact together and will be performed in collaboration with BIO.

Expected results: *Training*: bioinformatic analysis (homology searching, gene alignment), cloning and expression of enzymes; enzyme activity assays; glycan product analysis. *Research*: new biosynthetic pathways, new GT(s) for the synthesis of glycoproteins with glycans in non-canonical sequons, GTs for the construction of high-diversity glycan arrays.

Planned secondments: **DTU**. Timing: M8; duration 4M. **Purpose**: chlorovirus N-glycan biosynthesis machinery bioinformatics
ZB, Timing, M20, duration, 2M, **Purpose**: cell biology of viral N-glycans.

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Fellow DC10	Host institution UDE	Enrollment in the doctoral school UDE
Project Title: Development of chemical probes for studying N-glycosylation from bioactive metabolites. **Supervisor:** Prof. Markus Kaiser **Relation to the GLYCO-N program**: DC10 will conduct an **interdisciplinary doctorate** on **synthetic**, **biochemical** and **biological glycosciences**. The project fits within the **multidisciplinary** program scope through connections with **biochemical and computational Glycoscience**.		
Objectives. The aim of the doctorate project is to develop novel chemical tools for studying N-glycosylation in various organisms, such as the thermoacidophilic, crenarchaeal model organism *Sulfolobus acidocaldarius*, chloroviruses or various microalgae, including *Chlamydomonas reinhardtii*. To this end, we aim to exploit validated inhibitors of N-glycosylation, e.g. natural products with known molecular mechanisms in N-glycosylation processes or previously developed small molecules, as starting points for targeted rational chemical probe design. These probes will then be applied by UDE, UNINA or URN for 1) characterizing the mode-of-action of these probes in diverse organisms, 2) for functional probing N-glycosylation pathways. To this end, diverse target structures for natural product-derived probe design will be pursued. For example, we will investigate the potential of Cyclopeptolide 1 (SDZ 90-215), an inhibitor of N-glycosylation, for probe design. To this end, diverse Cyclopeptolide 1 analogues will be synthesized, including the generation of photoaffinity probes by incorporation of photoreactive groups and alkyne tags, and functionally characterized. Additionally, we will explore sulfamate analogues of uridine-5-diphosphate sugars, previously established as antiviral N-glycosylation inhibitors. The project will benefit from close collaboration with UL for (carbohydrate) chemical synthesis and chemical proteomics, including ABPP, UB for rational design and UDE, UNINA and URN for in vivo characterization.		
Expected results: *Training*: Chemical synthesis, chemical proteomics for target identification, structure-guided drug design, protein purification and functional biochemical assays. *Research*: establishment of chemical probes for functional probing of N-glycosylation; identification of novel enzymes and protein factors involved in N-glycosyaltion by a chemical proteomics application of the probes.		
Planned secondments: **UL**. Timing: M8; duration 4M. **Purpose**: synthesis and chemical proteomics application of photoaffinity ABPP probes from natural products; **SAM**, Timing, M20, duration, 2M, **Purpose**: chemical proteomics		

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Fellow DC11	Host institution ICL, CRICK**	Enrollment in the doctoral school ICL

Project Title: Unraveling how viral glycosylation machineries affect host glycoproteins.
Supervisor: Dr. Benjamin Schumann
Relation to the GLYCO-N program: DC11 will conduct an **interdisciplinary doctorate** on **structural** and **synthetic Glycoscience** paired with **protein engineering** and **glycoproteomics**. The project fits within the **multidisciplinary** program scope addressing the interactions between host and virus. DC11 will carry out the doctoral thesis at the Crick Institute London.

Objectives: The finding that certain viruses re-program host glycosylation suggests a fundamental importance of the ensuing glycan structures, and an intricate interplay between viral and host N-glycosylation. One striking example is the *N*-acetylglucosaminyltransferase Bo17 from bovine herpesvirus 4 that originally evolved from a host GT but massively expanded its substrate promiscuity: Bo17 displays at least three different GlcNAc transferase activities, including for N-glycans, within the same active site. Within the viral replication cycle, it is unclear whether Bo17 is required for glycosylation of glycoproteins from host, virus, or both. We will employ cutting-edge chemoproteomics to unravel the protein substrates of Bo17 in molecular detail. In a tactic termed bump-and-hole engineering, the active site of Bo17 will be engineered (containing a “hole”) to accommodate a bioorthogonal tag (“bump”) in a synthetic nucleotide-sugar substrate that is not accepted by wild-type Bo17 or any other cellular GlcNAc transferase. The AlphaFold structure of Bo17 serves to identify suitable amino acids (for instance, Leu, Ile, Phe) that will be mutated to smaller ones (Ala, Gly) to introduce the “hole”. We already have a collection of 10 synthetic, bioorthogonal nucleotide-sugars, and DC11 will be trained to expand this collection by approx. 5 novel compounds. They will be further trained in protein engineering and expression as well as enzymology to define optimal enzyme-substrate pairs and measure their catalytic constants. We have established a method for cellular delivery of bioorthogonal nucleotide-sugars, allowing to establish a fully functional Bo17 bump-and-hole system through transfection of known bovine cell lines with engineered Bo17. Incorporation of the bioorthogonal allows for attachment of fluorophores (for microscopy) or biotin (for glycoprotein profiling by mass spectrometry-glycoproteomics). We will establish which proteins from host and virus are glycosylated throughout infection with live virus, defining a new dimension of host-virus interaction. This method will also allow us to produce bioorthogonally tagged virions that allow following the viral glycocalyx throughout the infection process.

Expected Results: *Training*: Chemo-enzymatic synthesis of bioorthogonal nucleotide-sugars; protein engineering, expression and isolation; enzyme kinetics; cell biology, fluorescence microscopy and chemical glycoproteomics. *Research*: Understanding how glycosylation shapes the basic principles of virus-host interaction.

Planned secondments: **UDE**. Timing: M8; duration 4M. **Purpose**: Design and synthesis of advanced glycosyltransferase substrates, development of improved bioorthogonal chemistries. **SAM**, Timing, M20, duration, 2M, **Purpose**: recombinant GT production.
** 36 months of recruitment are covered by ICL and not by EU funding

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