Title of the PhD project:

Dissecting glutathione signaling pathways in *Chlamydomonas reinhardtii* using a bioorthogonal chemo-biological strategy

PhD Supervisor

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Overview of the scientific projects of the team

Notre espèce et notre civilisation sont peut-être confrontées aux défis agricoles, environnementaux, industriels et économiques les plus importants et les plus inédits de leur histoire. La biologie synthétique et la biologie systémique offrent de nouvelles possibilités pour répondre à des questions fondamentales en utilisant de nouvelles approches et de nouveaux concepts ou pour créer des systèmes artificiels ayant des applications biotechnologiques et industrielles. Nous considérons qu'il s'agit d'une merveilleuse opportunité pour permettre une deuxième révolution verte à même de relever les défis les plus urgents en tirant parti des microorganismes phototrophes et de leur capacité inhérente à fixer le CO_2 pour produire des molécules organiques par un processus durable et dépendant de la lumière du soleil. S'attaquer à ces grands défis nécessitera une meilleure compréhension des mécanismes de fixation photosynthétique du carbone, des processus permettant l'adaptation aux contraintes environnementales et du réseau de signalisation redox qui contrôle étroitement les deux processus. L'équipe s'intéresse à ces trois thématiques et développe également, d'une part, des outils et concepts de biologie synthétique chez les microalgues et d'autre part des technologies de stockage numérique sur ADN. L'équipe utilise une approche multidisciplinaire combinant la biologie des systèmes, la biologie synthétique, la biochimie, l'ingénierie des protéines, la protéomique, la biologie structurale, la génétique et la biologie cellulaire. Nos études se focalisent principalement sur l'algue verte unicellulaire *Chlamydomonas reinhardtii*, un organisme modèle majeur pour l'étude de la photosynthèse et possédant un fort potentiel biotechnologique.

Main publications since January 1er, 2016

1. Pérez-Pérez ME, Mauriès A, Maes A, Tourasse NJ, Hamon M, **Lemaire SD#, Marchand CH#** (2017) The deep thioredoxome in Chlamydomonas reinhardtii: new insights into redox regulation. *Mol. Plant* **10**, 1107-1125. #Corresponding authors
2. Crozet P, Navarro FJ, Willmund F, Mehrshahi P, Bakowski K, Lauersen KJ, Pérez-Pérez M-E, Auroy P, Gorchs Rovira A, Sauret-Gueto S, Niemeyer J, Spaniol B, Theis J, Trösch R, Westrich L-D, Vavitsas K, Baier T, Hübner W, de Carpentier F, Cassarini M, Danon A, Henri J, **Marchand CH**, de Mia M, Sarkissian K, Baulcombe DC, Peltier G, Crespo J-L, Kruse O, Jensen P-E, Schroda M#, Smith AG#, **Lemaire SD#** (2018) Birth of a Photosynthetic Chassis: A MoClo Toolkit Enabling Synthetic Biology in the Microalga *Chlamydomonas reinhardtii*. *ACS Synthetic Biology* **7**, 2074-2086. #Corresponding authors
3. **Marchand CH**, Fermani S, Rossi J, Gurrieri L, Tedesco D, Henri J, Sparla F, Trost P, **Lemaire SD**, Zaffagnini M. (2019) Structural and biochemical insights into the reactivity of thioredoxin h1 from *Chlamydomonas reinhardtii*. Antioxidants 8, E10
4. Gurrieri L., Del Giudice A, Demitri N, Falini G, Pavel N, Zaffagnini M, Polentarutti M, Crozet P, **Marchand CH**, Henri J, Trost P, **Lemaire SD**, Sparla F, Fermani S. (2019) Arabidopsis and Chlamydomonas phosphoribulokinase crystal structures complete the redox structural proteome of the Calvin-Benson cycle. *Proc. Natl. Acad. Sci. USA.* **116**, 8048-8053
5. Zaffagnini M, **Marchand CH**, Malferrari M, Murail S, Bonacchi S, Genovese D, Montalti M, Venturoli G, Falini G, Baaden M, **Lemaire SD**, Fermani S, Trost P (2019) Glutathionylation primes soluble glyceraldehyde-3-phosphate dehydrogenase for late collapse into insoluble aggregates. *Proc. Natl. Acad. Sci. USA.* **116**, 26057-26065.

93 publications in peer-reviewed journals, 1 patent, 4 book chapters, and 23 invited conferences

Full publication list: **Google Scholar** (https://scholar.google.co.in/citations?user=zuhDqyoAAAAJ)
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Title of the team : Sondes et Modulateurs pour Cibles Biologiques (SMCB)
Team leader *(if different) :*
Doctoral School : Sciences chimiques : Molécules, Matériaux, Instrumentation et Biosystèmes (2MIB, ED521)

Overview of the scientific projects of the team

Ce groupe thématique développe ses activités de recherche autour de plusieurs aspects de la chimie à l'interface de la biologie, notamment l'élaboration de stratégies synthétiques innovantes pour les molécules d'intérêt biologique, ainsi que la conception, la synthèse et l'évaluation primaire de sondes moléculaires pour diverses applications, notamment l'imagerie. Nos projets de recherche étant intrinsèquement interdisciplinaires sont intégrés à l' « Initiative de recherche stratégique » Bioprobe: Rapporteurs innovants sur la chimie cellulaire et la signalisation: des nouvelles sondes aux cliniques, labélisée en 2016 comme programme de recherche prioritaire, et en cours de transformation en Objet Interdisciplinaire structurant par l'Université Paris-Saclay. Nous sommes également impliqués dans des infrastructures de recherche telles que France Life Imaging (FLI), France BioImaging (FBI), les GDR agents d'imagerie moléculaire (AIM) et « RNA as a tool and a target for medicinal chemistry and chemical biology », ainsi que le Réseau Thématique de Chémobiologie en cours de création par le CNRS.

Le marquage métabolique permet la modification de glycanes, par assimilation et incorporation d'un monosaccharide modifié. Cette stratégie trouve de nombreuses applications en biologie fondamentale et appliquée, avec des exemples spectaculaires tels que le marquage *in vivo* de glycanes sur des embryons de poissons zèbres, et plus récemment quelques exemples de marquage *in vivo* de cellules tumorales. Nous avons également montré que cette stratégie pouvait servir à la détection de pathogènes tels que des bactéries.

Nous nous intéressons également au marquage de polysaccharides végétaux, et nous avons montré que nous pouvions marquer efficacement le Rhamnogalaturonane II (RGII), un polysaccharide pariétal contenant du Kdo (en collaboration avec Patrice Lerouge, Univ. Rouen). Enfin nous développons de nouvelles sondes pour la détection rapide de ROS (Formes Réactives de l'Oxygène).

Main publications since January 1er, 2016

1. Gatin-Fraudet B., Ottenwelter R., Le Saux T., Lombès T., Baron A., Durand P., Norsikian S., Doisneau G., Bourdreux Y., Guianvarc'h D., Erard M., Jullien L., Urban D., Vauzeilles B. (2020). Borinic Acids as New Fast-Responsive Triggers for Hydrogen Peroxide Detection, ChemRxiv, doi : 10.26434/chemrxiv.12032670.v1
2. Lesur E., Baron A., Dietrich C., Buchotte M., Doisneau G., Urban D., Beau J.-M., Bayan N., Vauzeilles B., Guianvarc'h D., Bourdreux Y. (2019). First access to a mycolic acid-based bioorthogonal reporter

for the study of the mycomembrane and mycoloyltransferases in corynebacteria. *Chem. Commun.*, *55* (87), 13074-13077.

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4. Berthelot N., Brossay A., Gasciolli V., Bono J.-J., Baron A., Beau J.-M., Urban D., Boyer F.-D. Vauzeilles B. (2017). Synthesis of lipo-chitooligosaccharide analogues and their interaction with LYR3, a high affinity binding protein for Nod factors and Myc-LCOs. *Org. Biomol. Chem.*, 15(37), 7802-7812.
5. Dumont M., Lehner A., Vauzeilles B., Malassis J., Marchant A., Smyth K., Linclau B., Baron A., Mas Pons J., Anderson C. T., Schapman D., Galas L., Mollet J.-C., Lerouge P**.** (2016). Plant cell wall imaging by metabolic click-mediated labelling of rhamnogalacturonan II using azido 3-deoxy-D-manno-oct-2-ulosonic acid. *Plant J*., 85, 437-447.

Doctoral Project

Dissecting signaling pathways of glutathione in the green unicellular microalga *Chlamydomonas reinhardtii* using a bioorthogonal chemo-biological strategy

***Abstract*.**

Glutathione is a crucial tripeptide (γGlu-Cys-Gly or GSH) present in almost all living organisms. GSH can form a disulfide bond on protein cysteines, a redox post-translational modification favored under stress conditions and called S-glutathionylation. This dynamic modification is mainly controlled by glutaredoxins (GRXs) and constitutes a molecular switch with regulatory and signaling roles. By engineering a Chlamydomonas strain able to synthesize metabolically a clickable analog of GSH, we will be able, after Copper(I)-catalyzed Azide-Alkyne Cycloaddition (CuAAC) reaction, to enrich efficiently and selectively proteins undergoing glutathionylation. Combined with quantitative mass spectrometry, this click-chemistry based approach will allow to profile the *in vivo* dynamics of S-glutathionylation. This strategy will allow us to determine the specificities of this redox modification in response to physiological stresses. Selective inactivation in this engineered strain of genes coding for the major GRXs, will allow to decipher their specific roles. Overall, this innovative chemo-biological approach will allow a better understanding of the structural determinants governing both glutathionylation and de-glutathionylation processes and will shed light on the importance and the role of glutathionylation in eukaryotes.

SCIENTIFIC CONTEXT AND OBJECTIVES.

The tripeptide glutathione (γGlu-Cys-Gly) is a crucial metabolite in eukaryotes and many bacteria. Glutathione deficiency leads to human pathologies or organ failure[1,2] and to severe developmental defects in plants[3,4]. Glutathione is the major non-protein thiol, present in the millimolar range in all subcellular compartments[5]. It is synthesized from its constituent amino acids by two ATP-dependent enzymes, glutamate cysteine ligase (GSH1) and glutathione synthase (GSH2)[6]. Glutathione mainly exists in a reduced form (GSH), the disulfide form (GSSG) being continuously recycled into GSH by glutathione reductase using NADPH as electron donor. Glutathione constitutes a dynamic cellular redox buffer and has multiple other functions in plants including sulfur assimilation, glyoxal metabolism, antioxidant functions or tolerance to xenobiotics and heavy metals[6].

GSH binds covalently to diverse classes of endogenous or exogenous molecules. Notably, it can form a mixed-disulfide bond with protein cysteines. This post-translational modification (PTM), termed S-glutathionylation, is favored under stress conditions and by reactive oxygen and nitrogen species[5,7]. This PTM can protect cysteines from irreversible oxidation but can also impact protein functions by altering enzyme activities, interactions or localization. S-glutathionylation constitutes therefore a molecular switch with regulatory and signaling roles and involved in numerous fundamental processes and in a wide range of human diseases[8]. Hundreds of glutathionylated proteins were identified in both animals and photosynthetic organisms and we have contributed to these studies by unraveling glutathionylation dependent regulatory mechanisms for diverse enzymes and by identifying around 200 glutathionylated proteins in Chlamydomonas[9,10] and 383 in Synechocystis[11]. The reverse reaction, deglutathionylation, is mainly catalyzed by glutaredoxins (GRXs) and to a lesser extent thioredoxins (TRXs).

Selective enrichment methods are mandatory to characterize protein PTMs from complex biological samples and a deeper understanding of PTM-based signaling pathways requires also determining their stoichiometry and dynamics. Nevertheless, for redox PTMs, the inherent variability of the current multi-step biotin-switch enrichment methods precludes coupling with a time-resolved quantitative proteomics strategy by mass spectrometry (MS). In this context, the principle of click chemistry introduced by Sharpless and more specifically the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction developed by Bertozzi in biology is particularly promising. CuAAC reaction allows direct, efficient and selective coupling with no cross-reactivity with the different functional groups present in a complex biological sample. Moreover, azide or alkyne groups display a very low steric hindrance and therefore, they allow the biomolecules to which they are bound to be more easily processed by enzymes. **In this project, we will develop a robust and specific click chemistry-based approach to investigate the *in vivo* dynamics of S-glutathionylation.** Similar approaches have been successfully used to profile different PTMs[12-16]. We will employ **a multidisciplinary approach combining chemistry, protein engineering, synthetic biology, genetics and cell biology** to develop a genetic circuit **enabling the biosynthesis of a clickable analog of GSH in a photosynthetic organism.**

METHODOLOGY.

In vivo aspects of this project will be developed in the photosynthetic eukaryotic unicellular green alga *Chlamydomonas reinhardtii*, a major model system well-suited for proteomics[17] and for synthetic biology approaches[18,19]. We will design an artificial GSH2 enzyme engineered to accommodate a clickable amino-acid (azidoalanine, propargylglycine or allylglycine). Validated for human GSH2[15,16], this design is transposable to the Chlamydomonas enzyme based on our preliminary results. The Chlamydomonas nuclear genome will be transformed with genetic circuits expressing the redesigned GSH2 enzyme. In the presence of clickable amino acids in cell cultures, the engineered strain will be able to metabolically synthesize clickable GSH. The presence of a clickable group on GSH will allow either the detection of glutathionylated proteins by in-gel fluorescence and western blot or the direct and selective pull-down of glutathionylated proteins or peptides. After selective elution, glutathionylated peptides and proteins will be identified and quantified by MS using quantitative multiplexing strategies (TMT or iTRAQ) (figure 1).

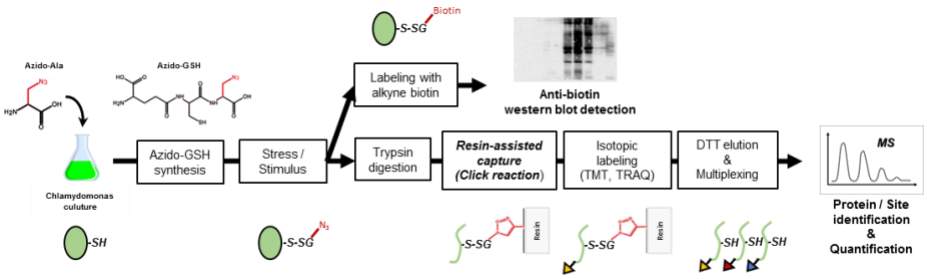

Figure 1. Workflow of the chemo-biological strategy used to unravel the *in vivo* dynamics of S-glutathionylation

This project is organized in two work packages:

Work package 1: All tools mandatory to perform the bioorthogonal strategy will be generated. Clickable analogs of GSH will be synthesized by Solid Phase Peptide Synthesis[20] and clickable GSSG and nitrosoglutathione will be then synthesized using conventional methods[21,22]. Synthesized clickable analogs of glutathione will be used to assess *in vitro* both the tolerance of glutathione-dependent enzymes and their propensity to glutathionylate protein models[23-25]. Chemical parameters of glutathione analogs will be also determined and compared to those of natural GSH. Similarly, clickable analogs of glycine will be used to evaluate their impact at

the level of wild-type and engineered CrGSH2. These results will allow selection of the most efficient designs based on the best compromise between GSH biosynthesis, GSH reactivity and use of clickable glutathione by GSH-dependent enzymes.

The redesigned *gsh2* gene will be expressed in *wt* and *gsh2-kd* strains. Strains exhibiting growths rates comparable to WT cells (under diverse conditions of light, temperature and chemical stress) will be characterized for glutathione content (redox state and total content). WP1 will deliver a clickable GSH producing strain exhibiting, under normal and stress conditions, no major physiological or phenotypical difference compared to the WT strain containing only natural GSH. The selected strain will be further engineered to generate 4 new click-GSH producing strains knocked-out for one of the four major GRXs.

Work package 2: The goal of this work package will consist in analyzing time-resolved samples under diverse stress conditions. The dynamics of glutathionylation will be screened by in-gel fluorescence after CuAAC reaction to focus in-depth proteome wide analyses on the conditions favorable for unraveling the network dynamics. The time-resolved and quantitative proteomic data generated will serve to build a basic framework of the glutathionylation network in *C. reinhardtii.* The specificity and contribution of each GRX will also be unraveled using the four strains deficient for one major GRX. The large amount of data generated will be handled by the MinOmics framework[26], an integrated analysis pipeline for manipulation and analysis of large experimental, multi-omics, and structural datasets but also network and structural modelling.

EXPECTED RESULTS.

This project will provide new insights on 1/ the importance and the role of glutathionylation in eukaryotes, 2/ a large set of target proteins and the physiological conditions triggering their formation *in vivo*, 3/ the processes under the control of glutathionylation pathways and 4/ the chemical and physical features conferring glutathionylation specificity.

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Justification of suitability for *i-Bio*:

This doctoral project aims at unraveling the dynamics of glutathione signaling pathways in a eukaryotic photosynthetic organism. It is clearly at the interface between biology and chemistry as it gathers several concepts and approaches from these two disciplines such as click chemistry, quantitative mass spectrometry, protein engineering, synthetic biology and cell biology. This ambitious project is clearly within the scope of the axis “Initiative for Biology” of Sorbonne University, which aims to promote the exploration of fundamental biological questions through the application of approach and/or concepts from other disciplines.

More importantly, we already obtained preliminary results confirming the feasibility of this doctoral project within 3 years. We previously employed metabolic labeling of the glutathione pool by feeding Chlamydomonas cultures with ^{35}S-cysteine and other amino-acids have been shown to cross membranes either actively or passively. We confirmed that 1/ L-azido-alanine and L-propargylglycine have no cytotoxic effects on Chlamydomonas cultures; 2/ the clickable glutathione where glycine was replaced by L-azidohomoalanine, was tolerated by most GSH-dependent enzymes tested (GR; GRX; GSNOR) and could induce *in vitro* glutathionylation of CrTrxf2 and AtGAPC1 as efficiently as natural glutathione. We produced and purified the S159G/F160A CrGSH2 and activity assays showed that the engineered enzyme can accommodate L-azido-alanine (AzA), L-propargylglycine or L-allylglycine. Finally, the chemical synthesis of γGlu-Cys-AzA is possible at small scale and can be scaled up by optimizing some limiting factors.

Finally, although the tools and glutathione analogs developed in this project won’t be used for this purpose, we envision that they would be also of great added value to unravel other biological functions of glutathione such as GSH conjugation to endogenous or exogenous molecules mediated by glutathione-transferases (GSTs), a biological process facilitating their recycling or intracellular distribution but for which substrate specificities of GSTs remain unclear.

Role of each supervisor / skills provided:

This project will be carried out both in the team “Synthetic and Systems Biology of Microalgae” hosted in LCQB (UMR 7238, IBPS, Sorbonne Université) and leaded by S. Lemaire (DR CNRS), and in the team “Probes and Modulators for Biological Targets” hosted in ICSN (UPR 2301, CNRS, Gif-sur Yvette) and leaded by B. Vauzeilles (DR CNRS). Both teams have a privileged access to complementary technological resources mandatory for this doctoral project (mass spectrometry, peptide synthesizer, HPLC, …).

S. Lemaire and B. Vauzeilles will be both assisted by two team members who will also co-supervise at full-time the PhD student.

Dr. Christophe Marchand (IR CNRS, 44 publications) is leading the project “Redox signaling networks” in the team of S. Lemaire. C. Marchand has strong expertise in proteomics, redox regulation and signaling, PTMs and protein chemistry and he has developed many tools to study redox PTMs by mass spectrometry. He is responsible of the IBPC proteomics platform. C. Marchand and S. Lemaire are collaborating since 15 years; they have co-authored 33 publications and have already co-supervised one PhD thesis and one postdoctoral researcher. Dr. Aurélie Baron (IR CNRS, 16 publications and 6 patents) is a Research Engineer in the *Chemical Biology* department. She has an expertise in chemical synthesis, in particular in glycochemistry and various probes for chemical biology, as well as coupling on iminosugars, and in metabolic labeling of glycans.

Profile of the desired student:
We seek a candidate that should ideally have a solid background both in biochemistry and synthetic chemistry. Notions in plant biology and mass spectrometry would be also highly appreciated.