Submission date	**09/28/2022**
Each year deadline	**September 30**

Acronym of the R&T project (max 10 characters)

3DUsher

Title of the R&T project (max 100 characters including spaces)

Molecular structure *in situ* of the Usher 2 complex involved in Hearing and Vision

Summary of the R&T project (max 500 characters including spaces)

The Usher 2 complex is essential for the development of hair cells, the sensory cells of hearing. However, its molecular organization and physiopathology are poorly understood. In the 3DUsher, we propose to implement innovative and cutting-edge imaging technologies to characterize *in situ* the Usher 2 complex. The PhD student will use high resolution fluorescence nanoscopy and cryo-electron tomography, two complementary approaches to describe the native Usher 2 complex at nanoscale resolution.

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R&T project (max 2,500 characters including spaces)

1. Background

The sense of hearing requires highly specialized sensory cells (**hair cells**) in the cochlea of the inner ear. At the apical surfaces of hair cells, three rows of actin-filled stereocilia are connected by different types of extracellular links. During hair cell development, the hair bundle harbours transient links called **ankle links**. Most of the proteins that form these anchoring densities are called **Usher 2** proteins, as mutations altering their function cause the Usher syndrome, that is the most common form of hereditary hearing-vision loss in humans. The long extracellular domains of two large transmembrane ADGRV1 and usherin build the core of the ankle links and their cytoplasmic domains are connected to

the scaffold cytoplasmic proteins whirlin and PDZD7, that in turn associate to actin-binding proteins, thus strongly anchoring the system to actin filaments.

2. Hypothesis and scientific question

We propose a dual role of the Usher 2 complex, structural and signaling, that is critical for proper development and ultimately hair cell survival. The genetic and physiologic basis of Usher 2 syndrome are well described. However, its underlying molecular basis is poorly understood. The molecular organization of the long extracellular links formed by the Usher 2 proteins remains uncharacterized and the submembrane interactions have been only investigated *in vitro,* by Team1. The characterization of the molecular organization of the ankle link complex *in situ* would have a potential impact on Usher 2 diagnosis, and a better understanding of the physiopathology.

3. Specific objectives

With this project, we want to address **the general structure *in situ* of the native ankle-links complex,** then define the domains that organize and maintain the functional cohesion of this complex, and then understand the impact of the Usher 2 mutations in pathological processes. We propose to combine innovative imaging approaches using **cryo-electron tomography** (cryo-ET) and **stimulated emission depletion nanoscopy** (STED) on mouse cochlea samples to collect images of ankle links at a resolution of tens of nanometers. STED and cryo-ET provide complementary information knowing that sample preparations are different (fixed entire cochlea, or slices of cochlea after high pressure freezing), with or without fluorescent labelling, and at different resolutions.

R&T added value of the co-direction of this project (max 1,000 characters including spaces)

This project brings together a team of two accomplished researchers with highly complementary expertise to achieve the goals laid out in this proposal. A. Sartori-Rupp has a recognized expertise in experimental cryogenic electron microscopy and tomography development and application acquired in world-class laboratories. She has developed and setup at Institut Pasteur original cryo-CLEM pipelines to address *in situ* structural biology questions, that can be applied both to isolated cells and to more complex multicellular systems. N. Wolff is expert in protein biochemistry and biophysics, is working since many years on the Usher syndrome and the molecular mechanisms of hearing, and has recently developed advanced workflow of cochlea sample preparations for *in situ* characterization by microscopies. Preliminary critical data have been already obtained concerning the two approaches of this project to ensure the smooth running of the PhD program (two color STED images, specific antibodies against Usher 2 proteins, dissection, chemical fixation, high-pressure freezing of cochlea and lift-out procedures). The preparation of samples (cochlea dissection, immunofluorescence, HPF and preparation of tissue blocks), will be supervised by the Wolff's team as well as the STED acquisition (in collaboration with Gaël Moneron and D. DiGregorio team), while the acquisition and the processing of the Cryo-electron tomograms will be done under the supervision of Anna Sartori-Rupp and the NanoImaging Core Facility (NCF). The NCF - the cryo-EM facility of Institut Pasteur - is equipped with cutting edge systems from sample preparation to microscopy imaging that include all the advanced tools necessary to carry out the project. Therefore, the student will benefit from the experimental and theoretical expertises of the two teams that will work in strong synergy and complementarity to tackle this exciting and ambitious PhD project.

Organisation of the project among partners (work packages, roles and communication; max 1 page)

The project 3DUsher is organized in two main work packages. The first one aims at characterising the Usher 2 complex in entire mouse cochlea over time using super resolution fluorescence nanoscopy STED. The second work package is dedicated to the ultrastructural characterization of Usher 2 complex at sub-nanometric resolution in 3D using cryo-ET on cryo-lamellae of cochlea. The two tasks of the project involve euthanasia of animals, which requires specific ethical authorization already validated

(CETEA N°200007). N. Wolff from Team Pasteur is trained as designer for projects on animals, including mice.

Workpackage 1 *In situ* imaging of ankle links by high resolution fluorescence nanoscopy

Although ankle links localization between stereocilia is crucial for the morphogenesis of the hair bundle, the nanoscale distribution of ankle links has not been examined. This is in part due to the limit of resolution of conventional confocal fluorescence microscopy (~200nm), largely used in the field. In order to bridge the gap of spatial resolution between electron microscopy and diffraction-limited confocal microscopy, we will perform multicolor super-resolution imaging of ankle links at ~30 nm spatial resolution on hair bundles of whole mouse organ of Corti explants. STED nanoscopy will be used, maintaining the advantage of the user-friendliness and multi-molecular specificity of fluorescence microscopy.

We have already validated a series of specific antibodies against ADGRV1 and PDZD7. We have also produced several nanobodies (fragments of antibodies (20 kDa) produced by alpagas) specifically directed against different region of ADGVR1; they constitute small fluorescent probes after their labelling to fully benefit from the resolution of STED technology. Promising two color STED images of hair bundles have been collected revealing the distribution of ADGRV1 that provides important insights into the connection patterns between stereocilia from parallel rows, impossible to infer from the confocal counterpart image. Tools will be developped to analyze 2D cross-sections, or 3D stacks, and quantify the locations (distances and angles) of the Usher 2 proteins relatively to the closest stereocilia, along the stereocilia, and as a function of the positions of stereocilia in the hair bundle (**WP1.1**). All optimizations will be first performed on mice organ of Corti samples at P5 (post-natal) stage. We will also explore the evolution of the USH2 complex over time, currently only partially and poorly described, between P5 and P25 stages (**WP1.2**). STED experiments are performed in collaboration with Gaël Monéron, Synapse and Circuit Dynamics Unit, Institut Pasteur (Head: David DiGregorio).

Workpackage 2 Ultrastructural organization of Usher 2 network in cochlea using cryo-ET

The overall goal of this package is to study the (near) atomic-resolution details of the Usher 2 complex within its intact native cellular and tissue environment by employing advanced cryo-electron microscopy (cryo-EM) imaging workflows based on cryo-ET coupled to fluorescent microscopy and high-resolution image processing. These cutting-edge methods, recently established at the cryo-EM facility of Institut Pasteur, will provide invaluable 3D information with sub-nanometer resolution on the structural organization of intact stereocilia and of the Usher 2 complex at the tissue level. The protocol of cochlea preparation for cryo-EM observations has been already established. Pieces of dissected cochlea are chemically fixed, immunolabelled without permeabilization and then rapidly frozen at high pressure and low temperature to preserve cellular ultrastructure. We have controlled the integrity of hair cells and ankle links by freeze substitution/transmission electron microscopy of resin embedded samples and by fluorescence in cryogenic conditions. The novel procedure to cut and isolate the frozen block of tissue of interest in areas targeted by fluorescent labelling of stereocilia components has been already successfully conducted. The throughput of the workflow needs to be increased by refining the targeting precision and including a higher degree of automation (**WP2.1**). Data collection of images for tomographic reconstructions is planned according to established procedures using the Glacios 200kV and Titan Krios 300kV cryo-electron microscopes (**WP2.2**). The images will be then subsequently aligned and reconstructed to generate 3D tomograms. The high-resolution structure of the Usher 2 complex and of its associated partners *in situ* will be determined by aligning and averaging multiple copies of the targeted structures using computational sub-tomogram averaging approaches (**WP2.3**).

R&T candidate background required for developing the project

Ideally, the R&T candidate should seek solid foundation in protein chemistry, cell and molecular biology. Previous hands-on experience in EM and/or cryo-EM will be a plus. A genuine enthusiasm for advanced microscopy techniques will be essential for the successful development of the project.

Selected publications or patents (max 5) of partners

WOLFF PUBLICATIONS

- Colcombet-Cazenave B, Cordier F, Zhu Y, Bouvier G, Litsardaki E, Laserre L, Prevost MS, Raynal B, Caillet-Saguy C, **Wolff N.** (2022) Deciphering the Molecular Interaction Between the Adhesion G Protein-Coupled Receptor ADGRV1 and its PDZ-Containing Regulator PDZD7. *Front Mol Biosci*. Jun 9:923740.
- Caillet-Saguy, C., Durbesson, F., Rezelj, V. V., Gogl, G., Tran, Q., Twizere, J.-C., Vignuzzi, M., Vincentelli, V., **Wolff N**. (2021) Host PDZ-containing proteins targeted by SARS-Cov-2, *FEBS J*., 2256:89-124.
- Giraud E, Del Val CO, Caillet-Saguy C, Zehrouni N, Khou C, Caillet J, Jacob Y, Pardigon N, **Wolff N**. **(**2021) Role of PDZ-binding motif from West Nile virus NS5 protein on viral replication. *Sci Rep.* 11(1):3266.
- Zhu Y, Delhommel F, Cordier F, Lüchow S, Mechaly A, Colcombet-Cazenave B, Girault V, Pepermans E, Bahloul A, Gautier C, Brûlé S, Raynal B, Hoos S, Haouz A, Caillet-Saguy C, Ivarsson Y, **Wolff N.** (2020) Deciphering the Unexpected Binding Capacity of the Third PDZ Domain of Whirlin to Various Cochlear Hair Cell Partners. *J Mol Biol.* 432(22):5920-5937.
- Gautier C, Troilo F, Cordier F, Malagrinò F, Toto A, Visconti L, Zhu Y, Brunori M, **Wolff N** and Gianni S (2020) Hidden kinetic traps in multidomain folding highlight the presence of a misfolded, but functionally competent, intermediate, *Proc Natl Acad Sci U S A*, 18,117(33):19963-1996.

SARTORI-RUPP PUBLICATIONS

- Witwinowski J, **Sartori-Rupp A**, Taib N, Pende N, Tham N, Poppleton D, Ghigo JM, Beloin C, Gribaldo S. (2022) An ancient divide in outer membrane tethering systems in bacteria suggests a mechanism for the diderm-to-monoderm transition. *Nat Microbiol.* 7:411-22. https://doi.org/10.1038/s41564-022-01066-3
- Swistak L, **Sartori-Rupp A**, Vos M, Enninga J. (2021) Micropatterning of cells on EM grids for efficient cryo-correlative light electron microscopy. *Methods in Microbiology*, 48:95-110. https://doi.org/10.1016/bs.mim.2020.11.001
- **Sartori-Rupp A**, Cordero Cervantes D, Pepe A, Gousset K, Delage E, Corroyer-Dulmont S, Schmitt C, Krijnse-Locker J, Zurzolo C. (2019) Correlative cryo-electron microscopy reveals the structure of TNTs in neuronal cells. *Nat Comm.* 10:342-58. https://doi.org/10.1038/s41467-018-08178-7
- Staropoli I, Dufloo J, Ducher A, Commere PH, **Sartori-Rupp A**, Novault S, Bruel T, Lorin V, Mouquet H, Schwartz O, Casartelli N. (2019) Flow-cytometry analysis of HIV-1 Env conformations at the surface of infected cells and virions: role of Nef, CD4 and SERINC5. *J Virol.* pii: JVI.01783-19. doi: 10.1128/JVI.01783-19.
- Kühn S, Lopez-Montero N, Chang YY A, **Sartori-Rupp A**, Enninga J. (2017) Imaging macropinosomes during Shigella infections. *Methods*. 127:12-22.