PSD lab achievements (2015-2020)

Resorting to disease-mediated molecular networks as entry points, we continue to explore how the hearing and vision organs are tuned to the specific needs of corresponding sensory modalities, and how that might have impacted organ subcellular structures & disease-linked mechanisms (Geleoc G & El-Amraoui A, *Hear. Res.* 2020; https://www.sciencedirect.com/science/article/pii/S0378595519304733; Delmaghani S. & El-Amraoui A. *J. Clin. Med.* 2020; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7408650/).

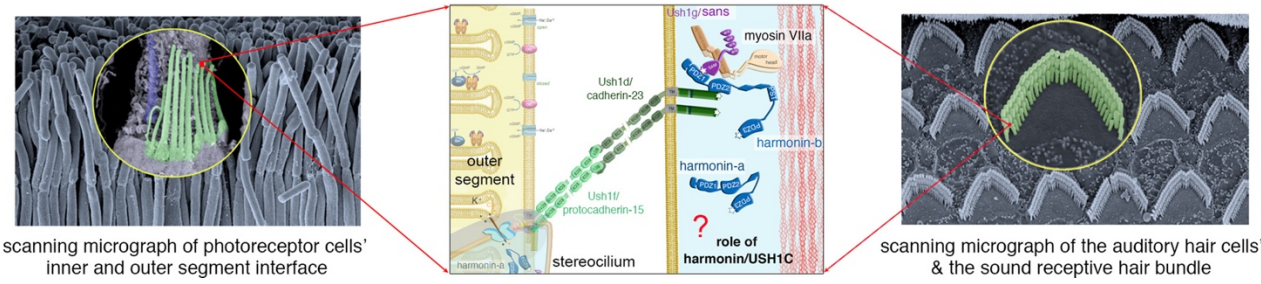
VISION IN HEALTH AND DISEASE: FROM GENES TO PATHOGENESIS

Over the years, we aimed to characterize the function of Usher proteins in the eye, and identify the cause of Usher vision loss in USH1 patients (Bonnet C & El-Amraoui A, *Curr. Opin. Neurol.* 2012, https://www.researchgate.net/publication/51879019_Usher_syndrome_sensorineural_deafness_and_retinitis_pigmentosa_Pathogenesis_molecular_diagnosis_and_therapeutic_approaches; El-Amraoui, A. & Petit, C., *C. R. Biol.* 2014 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2827896. In a first step, we showed that this myosin is present in the sensory cells: hair cells in the inner ear, pigment epithelium and photoreceptor cells in the retina (El-Amraoui et al., 1996). Search for myosin VIIa ligands have led us to characterize the molecular complexes through which this actin-based motor protein sets the melanosomes into motion in the pigmentary cells (El-Amraoui et al., *EMBO Rep.* 2002) and initiates a tension at different adhesion sites (Kussel-Andermann P. et al. EMBO J. 2000). We later showed that spectrin βV was cytoplasmic in photoreceptor cells, linking the components of the phototransduction machinery to the actin- and microtubule-based motors, and mediating their transport to the photoreceptor outer segment. A failure of the myosin VIIa-spectrin-opsin complex probably accounts for the opsin transport delay observed in myosin VIIa defective mutants (Papal et al., 2013). Overtime, comparative studies have been conducted in both the eye and the inner ear to obtain an integrative view of the role of Usher proteins in different sensory systems (Geleoc G & El-Amraoui A, *Hear. Res.* 2020).

• **Mechanisms of Usher vision loss?**

Until recently, the origin of Usher 1 retinal dystrophy has remained elusive, mostly because none of the Usher1 mice display a visual defect. In 2012, we showed that this lack of a retinal phenotype in mice is due to the absence in rodent photoreceptor cells of the calyceal processes, microvilli-like structure that surround the outer segment disks, the site of phototransduction (Sahly I., J. Cell Biol. 2012; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3471240/). We later confirmed the key role of these structures in *X. tropicalis* using knockdown of Usher1 genes, which lead to absent calyceal processes, and defective morphogenesis of the light sensitive outer segment membranous discs (Schietroma C. *et al. J Cell Biol* 2017; http://jcb.rupress.org/content/216/6/1849.long).

The USH1-mediate molecular complex in the eye (photoreceptors) and the inner ear (hair cells) ?



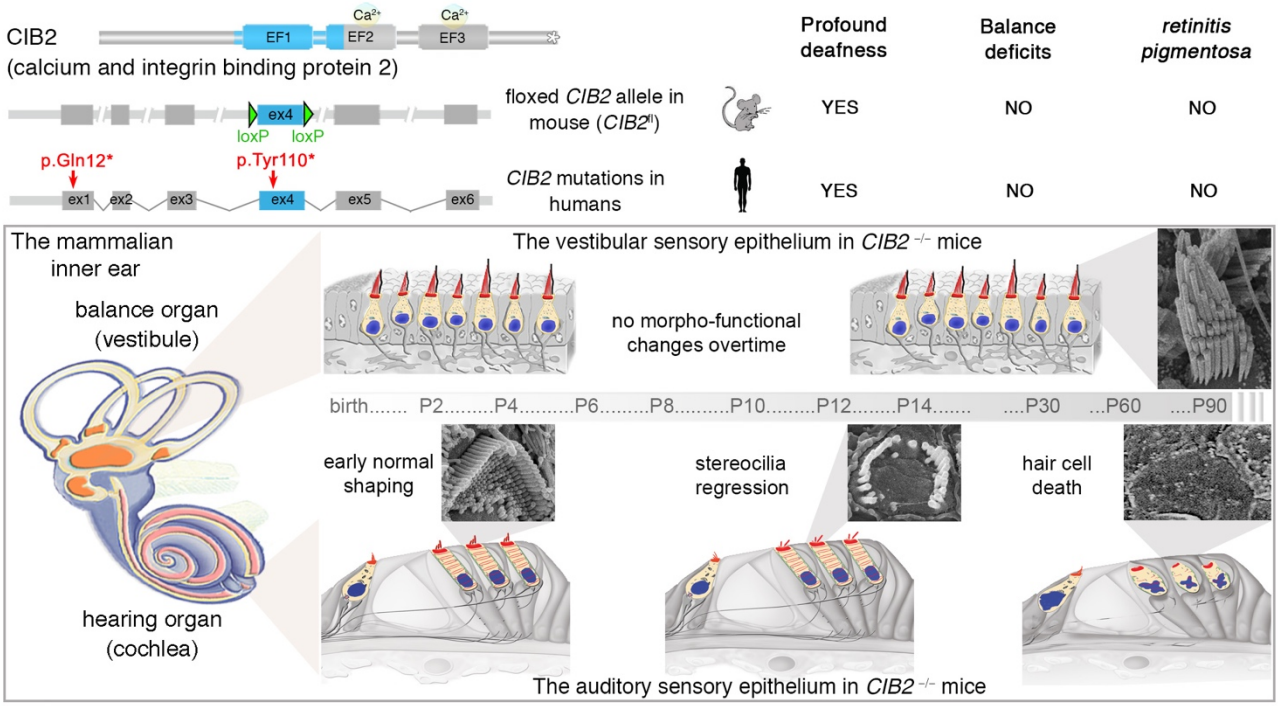
scanning micrograph of photoreceptor cells' inner and outer segment interface

scanning micrograph of the auditory hair cells' & the sound receptive hair bundle

USH1 proteins form a molecular complex. Likewise, in the stereocilia, myosin VIIa (USH1B), along with Sans (USH1G), probably plays a key transport and adaptor role in anchoring cadherin-23 (USH1D) and protocadherin-15 (USH1F)-mediated links to the core of actin filaments in the calyceal processes. Harmonin (USH1C) and Sans (USH1G) act as scaffolding proteins, linking sub- and transmembrane proteins to the underlying cytoskeleton, and related motor proteins.

• **Is CIB2/USH1J an Usher protein, and if so, what does it do?**

The most recently identified Usher gene, *USH1J*, encodes the CIB2 protein. We used CIB2-deficient mice to study the function of this putative new Usher 1 protein. Interestingly, our results show that, unlike the other Usher 1 proteins, CIB2 is required for normal hearing, but a deficiency of this protein does not result in balance or visual defects in mice or humans. It has become clear that a loss of CIB2 function, even in humans, does not necessarily lead to Usher syndrome. This result has important implications for genetic counseling and molecular diagnosis for patients carrying mutations of the CIB2 gene *(Michel V. et al. EMBO Mol. Med. 2017,* http://embomolmed.embopress.org/content/9/12/1711.long*; Booth K. et al., Clin Genet. 2018;* https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5851821/).



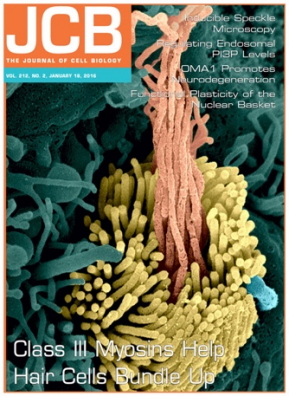
CIB2 is key to hearing in both humans and mice. Two novel loss-of-function CIB2 mutations are identified in patients with severe to profound deafness, with no vestibular or retinal phenotype. In mutant mice, albeit dispensable for early structural development of the hair bundle, total loss of CIB2 leads to complete disruption of Mechanoelectrical transduction in auditory but not vestibular hair cells. Lack of CIB2 results in rapid postnatal loss of auditory hair bundles, followed by rapid cell death

HEARING IN HEALTH AND DISEASE: FROM GENES TO PATHOGENESIS

- **Auditory hair bundle architecture is molded by Class III myosins**

Cellular actin-rich protrusions (e.g. brush border microvilli, growth cones, hair bundle) are critical to a wide range of biological functions, thanks to controlled variations in the dimensions, dynamics and positioning of these actin structures within cells. In the inner ear, the sound mechano-sensitive hair bundle, is made up of arrays of mechano-sensitive microvilli-like protrusions, stereocilia, exhibiting a precise graded spatial arrangement and size, vital for converting mechanical stimuli-like sound to neural signals (see Fig. 1A). The importance of actin-based cytoskeleton for hearing is reflected in the increasing number of disabling mutations in several actin-binding proteins (espin, eps8, erzin, fascin, fimbrin) and the actin-based myosin motor proteins (MyH9, MYH14, MYO3, MYO6, MYO7, or MYO15) causing deafness in human and mice (see http://hereditaryhearingloss.org/). Myosin IIIa (defective in the late-onset deafness form DFNB30) and its paralog myosin IIIb are both expressed at the stereocilia tips of the developing hair bundle, but their precise role and underlying pathogenic mechanisms have remained elusive.

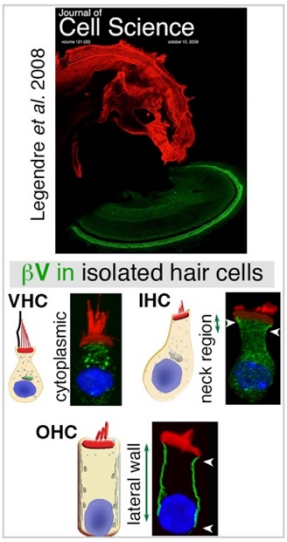
Together with A. Lelli (postdoctorant), we studied the hearing abilities of single (*Myo3a*$^{-/-}$, and *Myo3b*$^{-/-}$) and double knockout mice. We found that, unlike the situation in previous *in cellulo* studies on filopodia, the actin-crosslinking protein espin-1 is properly targeted to stereocilia tips *in vivo*, even in the absence of the two myosins. The Myo3a$^{-/-}$Myo3b$^{-/-}$ mice were profoundly deaf.



Albeit Myo3a$^{-/-}$Myo3b$^{-/-}$ cochlear hair bundles display robust mechano-electrical transduction currents with normal kinetics, their morphology is severely affected starting at embryonic stages, with abnormal structural features that are highly dynamic: these include abnormally tall and numerous stereocilia, ungraded stereocilia bundles, and bundle rounding and closure (instead of V-like shape). On the other hand, Myo3b$^{-/-}$ Myo3a-cKO mice that lack myosin IIIb and lose myosin IIIa postnatally exhibited normal hearing. **We conclude that the two class III myosins are required for size and shape control of the hair bundle. Unexpectedly, instead of promoting growth, as previously thought, the two myosins act redundantly to limit the elongation of stereocilia and of subsequently regressing microvilli; this contribution, essential for bundle shaping, is necessary only at the very early stages of bundle morphogenesis** (Lelli et al., 2016). http://jcb.rupress.org/content/212/2/231.long

• A giant spectrin defies convention in auditory and visual sensory cells

Over the years, we have been interested to evolution and emergence of new functions, notably exploring similarities in function between species and differences between systems. In this context, I have shown how Usher proteins and related partners adapt in substructures seemingly involved in different cell-specific processes pertaining to different sensory modalities. For instance, the auditory outer hair cells (OHCs) are sensory cell types unique to mammals; they are key to sound amplification in the inner ear.



By analogy to membrane deformations in red blood cells, their unique ability of to shorten and elongate was for many years thought to be due to the presence of conventional spectrins, the βII and αII subunits. Surprisingly though, we showed that out of the five known β spectrins, the giant non-conventional spectrin βV, which is twice the length of conventional β spectrins (βI, βII, βIII and βIV), was the only one detected over the entire lateral wall of OHCs. It was probably recruited to the LPM as an adaptation to the strong mechanical constraints on OHCs and their need for greater flexibility (Legendre *et al.* 2008; https://jcs.biologists.org/content/121/20/3347). Using phylogenetic analyses of spectrin sequences (including βV, formerly identified as a partner of myosin VIIa that is involved in deaf-blindness in humans), we recently unveiled strong signatures of adaptive evolution at multiple sites along βV amino acid sequence in the lineage leading to mammals, accompanied with substantial differences in the subcellular location of this protein between the frog and the mouse vestibular (VHC) and cochlear (IHC & OHC) hair cells. This illustrates how evolution shape proteins and networks to adapt to specific needs of sensory modalities (Cortese *et al. PNAS* 2017; http://www.pnas.org/content/114/8/2054.long).

➢ **The Usher proteins as key players in the development and functioning of the sensory hair cells**

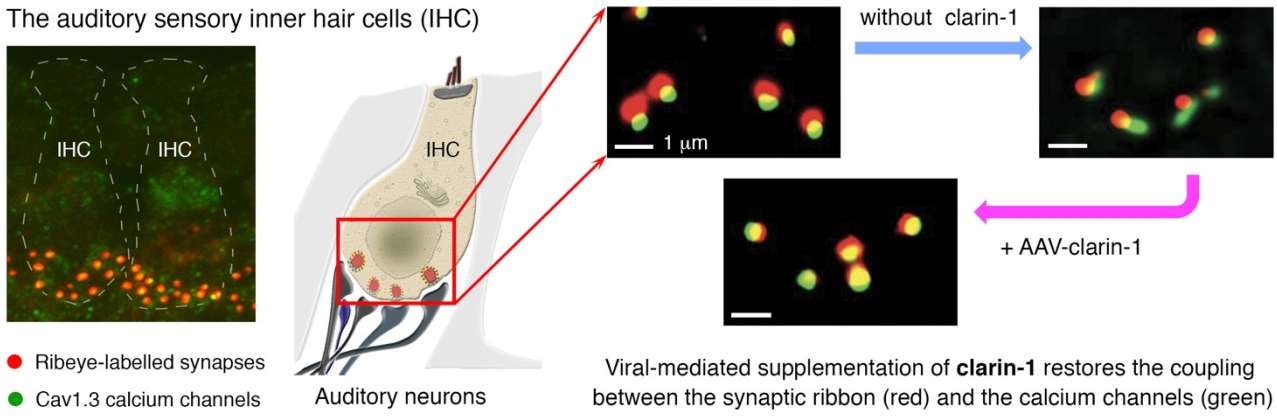
The Usher Syndrome (USH) is the first cause of deafness blindness in humans. Three USH clinical types (USH1-3) are defined according to the severity of the sensorineural hearing impairment, the presence or absence of vestibular defects, and age of onset of retinal degeneration.

• Disease mechanisms of USH1 and USH2 hearing loss?

By studying the properties of the deafness defective proteins and their molecular networks, and by performing physiological, morphological, and molecular analyses of the corresponding animal models, we have showed that the USH1 and USH2 proteins are required for hair-bundle development and functioning (Geleoc G. & El-Amraoui A. 2020). A defect in the organization of the hair bundle actin-filled stereocilia, which probably accounts for the congenital profound deafness encountered in USH1/2 patients. We have indeed shown that the actin-based motor protein myosin VIIa (USH1B), the PDZ protein harmonin (USH1C) and cadherin 23 (USH1D) interact together, forming a key molecular complex at the stereocilia tips. We proposed a model that explains hair-bundle fragmentation in USH1 mice, which was extended to new USH proteins as they were identified: Sans (USH1G) and protocadherin-15 (USH1F) (Weil *et al*. 2003, Adato et al. 2005a). In 2005, we showed that Usherin, a USH2 protein defective in a milder form of Usher syndrome and known to be secreted from basal membranes, is a key transmembrane component of another subset of transient links, the ankle-links, which connect the bases of the stereocilia, and that, like USH1-mediated interstereocilium links, these links also guide the normal shaping of the differentiating auditory hair bundle (Adato *et al*. 2005b).

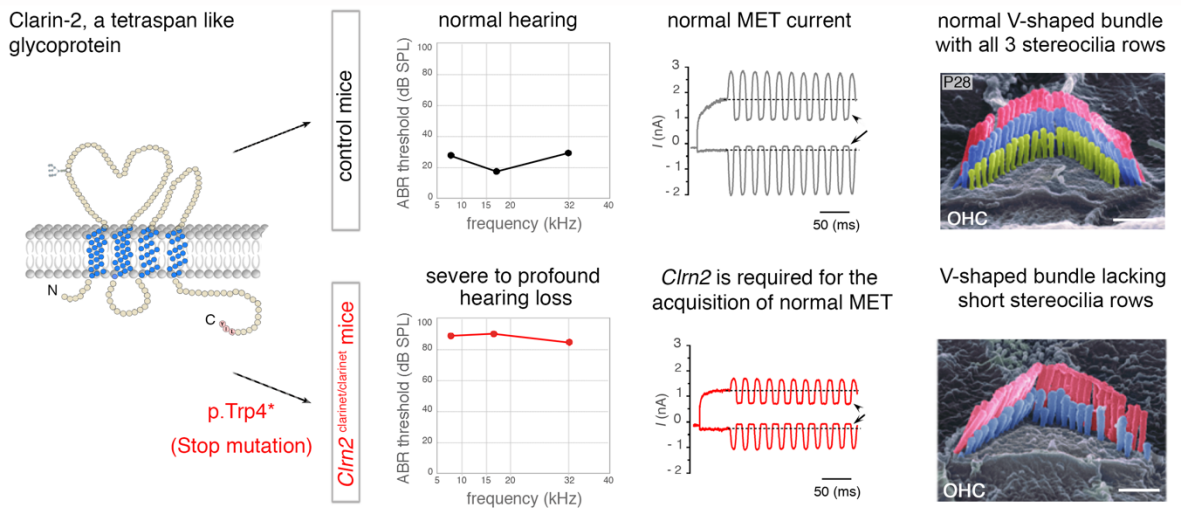
- **Origin and therapy of Usher 3 hearing loss?**

So far, only one USH3 gene has been identified, *CLRN1*; it encodes clarin-1, a four-transmembrane protein expressed in the auditory hair cells and their primary auditory neurons. We have actively contributed to the study of the possible role of clarin-1 in the inner ear, by generating and characterizing two clarin-1–deficient mouse models: one with an early ubiquitous deletion of *Clrn1* (*Clrn1*$^{-/-}$) and the other with a hair cell–specific & postnatal deletion (*Clrn1*$^{fl/fl}$ *Myo15-Cre*$^{+/-}$). Besides hair bundle defects, comparative morpho-functional analyses of the two clarin-1–deficient mice showed that clarin-1 is also essential for the structural organization and function of the presynaptic $Ca_v1.3$ Ca^{2+} channels at the inner hair cell (IHC) ribbon synapse. Finally, viral-mediated transfer of the intact *Clrn1* into the cochlea durably prevented synaptic defects and occurrence of the hearing loss in *Clrn1*$^{fl/fl}$ *Myo15-Cre*$^{+/-}$ mice *(Dulon D. et al. J. Clin. Invest. 2018;* https://www.jci.org/articles/view/94351).



Clarin-1 therapeutics: *Viral gene transfer of clarin-1 durably rescued the inner ear hair cell synaptic defects and preserved hearing in this mouse model of Usher III syndrome.*

Our work led to identification of another member of the clarin family, clarin-2, the absence of which leads to hearing loss. The *Clrn2*$^{clarinet/clarinet}$ mice (Trp4Stop mutation) have a progressive, early-onset hearing loss. Utilizing data from the UK Biobank study we could show that *CLRN2* is involved in non-syndromic progressive hearing loss in both human (Vona B. et al. 2020; https://www.biorxiv.org/content/10.1101/2020.07.29.222828v1) and mice (Dunbar L. et al. 2019. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6728604/).



***Clarin-2 findings**. Our findings identify CLRN2/Clrn2 as the latest deafness gene required in sensory cochlear hair cells for the maintenance of transducing stereocilia. The lack of clarin-2 leads to early-onset hearing loss in mice, probably due to mechanoelectrical transduction defects accompanied by loss of the transducing short stereocilia row of the hair bundle.*

The in-depth morphological, molecular and functional investigations established that, while clarin-2 is not required for initial formation of cochlear sensory hair cell stereocilia bundles, it is critical for maintaining normal bundle integrity and functioning. In the differentiating hair bundles, lack of clarin-2 leads to significant decrease of mechano-electrical transduction in the auditory hair cells, followed by selective progressive loss of the transducing short row stereocilia. Together, our findings demonstrate a key role for clarin-2 in mammalian hearing, providing insights into the interplay between mechano-electrical transduction and stereocilia maintenance (Dunbar L. et al. 2019; Vona B. et al. 2020).

Further elucidation of the mechanisms through which the two clarins interact, and the importance of such interactions in the vestibular and cochlear systems is underway.