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# **GPRASP/ARMCX protein family: potential involvement in health and diseases revealed by their novel interacting partners**

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## Abstract

GPRASP (GPCR-associated sorting protein)/ARMCX (ARMadillo repeat-Containing proteins on the X chromosome) family is composed of 10 proteins, which genes are located on a small locus of the X chromosome except one. They possess at least two armadillo-like repeats on their carboxyl-terminal homologous sequence, but they can be subdivided on specific sequence features. Subfamily 1 (GPRASP1, GPRASP2, GPRASP3, ARM CX4 and ARM CX5) displays additional repeated motifs while a mitochondrial targeting transmembrane domain is present in subfamily 2 (ARM C10, ARM CX1, ARM CX2, ARM CX3 and ARM CX6). Although their roles are not yet fully understood, the recent identification of several interacting partners have shed new light on the processes in which GPRASP/ARM CX proteins are implicated. Among the interacting partners of proteins from subfamily 1, many are GPCRs. GPRASP1 binds trafficking proteins such as Beclin2 and the Dysbindin-HRS-Gas complex to participate in GPCR post-endocytic sorting. Moreover, *in vitro* as well as *in vivo* experiments indicate that GPRASP1 is a critical player in the adaptive responses related to chronic treatments with GPCR agonists. GPRASP2 seems to play a key role in the signalling of the hedgehog pathway in the primary cilium through a Smoothed-GPRASP2-Pifo complex. Its deletion in mice causes neurodevelopmental alteration and affects mGluR5 regulation, reflected by autism-like behaviour. Several members of subfamily 2, in complex with TRAK2 and MIRO, are involved in the trafficking of mitochondria in axons and on the regulation of their size and division, influencing the cell cycle. The essential role of GPRASP/ARM CX proteins in the cellular physiology is supported by human cases of deletions, causing male neonatal lethality by pulmonary delayed development, dysmorphic face and psychiatric and intellectual impacts.

Keywords: pharmacology, signalling,  $\alpha$ -solenoid scaffolding proteins, membrane trafficking, mitochondria dynamics, ciliopathies, neurite outgrowth

## Introduction

To participate actively in the transmission of a message in a signalling cascade, the protein involved, whether the receptor at the plasma membrane or all the subsequent effectors of the response, must be in the right place within the cell and at the right time. It must therefore be correctly addressed in the appropriate cell compartment and this proper targeting is tightly regulated. Proper targeting of organelles themselves can be determinant for efficient signalling particularly in polarized cells such as neurons. Accessory proteins that regulate the dynamics of receptor targeting or that serve as scaffold are the proteins that help fulfilling this goal of assembling all the partners in a nanocompartment in order to constitute an efficient functional signalosome.

In this special issue “Receptors in health and diseases” we will review a specific family of proteins that gathers the G Protein-coupled Receptor-Associated Sorting Proteins (GPRASP) and the ARMadillo repeat-Containing proteins on the X chromosome (ARMCX). This family is composed of ten members convincingly implicated either in G protein-coupled receptors (GPCR) trafficking and signalling or in mitochondria trafficking. We will focus on the discoveries of these past ten years suggesting a scaffolding role for these proteins.

Evidence that GPRASP1 (alias GASP1, KIAA0443 and PIPS), GPRASP2 (also named GASP2 and HIP15), GPRASP3 (also known as GASP3, p60TRP and bHLHB9), ARMCX4 (GASP4), ARMCX5 (GASP5), ARMCX3 (GASP6 and ALEX3), ARMCX1 (GASP7 and ALEX1), ARMC10 (GASP8 and SVH), ARMCX2 (GASP9 and ALEX2) and ARMCX6 (GASP10) belong to a unique protein family has been reviewed by us previously [1;2]. Briefly, these 10 proteins share a conserved and unique carboxyl-terminal domain of around 250 residues (with a minimum of 20% sequence identity with GPRASP1) that contains at least 2 armadillo-like repeats (Figure 1). Except for ARMC10, they are each encoded by a single coding exon. *ARMC10* is considered the common ancestor of the *GPRASP/ARMCX* gene family [3] since *ARMC10* homologs are present in all vertebrates exclusively, as early as in the teleost fish *Danio rerio*, while no homologs are found for the other *GPRASP/AMRCX* genes outside mammals. ARMC10 gene is located on the human chromosome 7 and is encoded by several exons (seven coding exons; [2]) with up to 6 possible isoforms, 4 having been detected [4;5]. A single coding exon for ARMC10 exists

on the human chromosome 3 but is referred to as a pseudogene in Pubmed Gene ID389137. It is not yet clearly established which of the two *ARMC10* copies gave rise to the gene family. Likewise, a pseudogene of human *ARMCX6* was proposed to serve as a redundant copy for facilitating gene conversion events to its neighbouring *ARMCX6* gene [6]. Except for *ARMC10*, *GPRASP/ARMCX* genes are arrayed in tandem on the locus Xq22.1-q22.2 of the human X chromosome, in a region unique to Eutherian placental mammals [6]. Deletions in this region in humans have been linked to intellectual disabilities and others syndromes [7;8;9] that will be discussed below. Although *GPRASP/ARMCX* proteins seem ubiquitously expressed, they are all highly or predominantly expressed in the central nervous system [1] as it is often the case for proteins encoded on the X chromosome [10]. Since the 2.3 Mb region Xq22.1-q22.2 is an innovation of placental mammals, it was speculated that *GPRASP/ARMCX* proteins, together with the other proteins encoded in this region, could be involved in the adaptive evolution of the neocortex that is also unique to mammals [6]. Interestingly with respect to the neocortex emergence, the 5'UTR of the three gene families repeated in tandem in the Xq22.1-q22.2 locus, *GPRASP/ARMCX* (nine members), *BEX* (Brain-Expressed X-linked, five members) and *TCEAL* (Transcription Elongation Factor A protein-Like also known as *WEX* for WWbp5-like X-linked, Seven members) contain a highly similar DNA motif named BGW (for *BEX/GASP/WEX*) with no significant alignments outside mammalian sequences nor outside mammalian X chromosome. The exon coding regions of these 3 gene families have rapidly diverged probably from the *ARMCX6* gene that unlike other *GPRASP/ARMCX* genes, contains a three exon structure, similarly to *BEX* and *WEX* genes, with the third exon encoding the entire protein [6]. The three protein families share functions in signalling but the function of this particular BGW element has not been yet investigated.

In the following chapters, we will first discuss the reasons why *GPRASP/AMRCX* are probably scaffolding proteins with multiple repeated motifs that fold into structured domains (Armadillo-like repeats) or into Intrinsic Disordered domains. We will list exhaustively the protein partners of the *GPRASP/ARMCX* and when identified, the interacting domains involved. Secondly, the strong evidence for *GPRASP1* and *GPRASP2* involvement in GPCR sorting from endosomes and to primary cilium, respectively, will be presented with evidence based on knockout mice and novel interacting partners. These results have functional implications in adaptation to chronic activation of G-

protein coupled receptors, in hematopoietic stem cell repopulation and in ciliopathies. We will finally describe the experiments that have led to the discoveries of the mitochondrial ARMCX proteins as effectors of WNT signalling and as metabolic sensors implicated in axon regeneration and survival via regulation of mitochondrial dynamics. Their putative roles in cancer and age-related neurological disorders will be discussed.

## **I. Sequence analysis of the GPRASP/ARMCX protein family.**

GPRASP1, GPRASP2, GPRASP3, ARM CX-1 to -6 (Armadillo repeat-containing X-linked proteins) and ARMC10 have been originally classified as “GASP” family members by our team based on the sequence homology of their carboxyl-terminal (C-ter) 250 amino-acids ranging from 20 to 77% identity with GPRASP1 [1]. We had further delineated 2 additional domains in these proteins, their amino-terminal part (N-ter) and a central region. For GPRASP1, we decomposed the protein as N-ter residues 1-379, GASP1-middle as residues 380-1073 and C-ter residues 1074-1395 (Figure 1). For GPRASP2, we decomposed the protein based on homologies with GPRASP1 as N-ter residues 1-353, GPRASP2-middle as residues 354-520 and GPRASP2 C-ter residues 521-838. In our previous work, we have proposed to split the family into two-subfamilies based on the presence of a repeated motif only in the central region of subfamily 1 (GPRASP1, GPRASP2, GPRASP3, ARM CX4 and ARM CX5) that we named GASP motif (Figure 1; [1; 14]).

For the purpose of this review, we have revisited the sequence similarities between the ten members of the human GPRASP/ARM CX proteins with the motif-based sequence analysis tools MEME Suite (for Multiple Expectation Maximization for Motif Elicitation; [11]). *MEME* discovers novel, *un-gapped* motifs that occur repeatedly in a group of related sequences. Individual MEME motifs do not contain gaps. Patterns with variable-length gaps are split by MEME into two or more separate motifs. With this method, we could strengthen the similarities between GPRASP1 and GPRASP2 and with bHLHB9 that we propose to rename GPRASP3 (Figures 1 and 2). ARM CX4 belongs clearly to the GPRASP subfamily 1 due to its GASP motifs content but in fact it falls in between the two subfamilies. Its function has however never been studied so far. MEME represents motifs as position-dependent letter-probability matrices that describe the

probability of each possible letter at each position in the pattern. The different motifs found in proteins of the GPRASP/ARMCX family are depicted in Figure 2.

### **I-A. Armadillo-like repeats in the C-ter domain of all GPRASP/ARMCX proteins**

Convergent indications suggest that the conserved 250 residues C-ter domain of GPRASP/ARMCX proteins is composed of several armadillo-like repeats. This was not originally detected for the ten members of the family hence the name ARM (Armadillo repeat-containing proteins) for only ARMCX4, ARMCX5, ARMCX3, ARMCX1, ARM10, ARMCX2 and ARMCX6 [1;2].

The superfamily of armadillo repeat proteins are involved in various fundamental cellular processes, including vesicular transport and molecular signalling. Proteins within the different ARM subfamilies display a conserved architecture and provide a scaffold for the assembly of protein complexes with various functions [12]. Each armadillo repeat is composed of three alpha-helices and tandem armadillo (ARM) repeats form superhelical domains, which stack together and wrap around the interacting region of its partner [13]. While identification of tandem repeats with high sequence similarities is easy, detecting Armadillo-repeats is more difficult and the level of detection by major domain databases was relatively poor when the GPRASP/ARMCX proteins were described. Nevertheless, biocomputing have now established that GPRASP/ARMCX proteins all share a common ARM-like domain at their C-termini falling into the criteria of the PFAM Domain of Unknown Function DUF634 now renamed Armadillo-like Arm\_2 (PF04826): while some articles proposed 3 armadillo repeats others have identified up to 6 armadillo repeats. In a recent review, it was proposed that GPRASP1, GPRASP2, GPRASP3 and ARMCX6 encode proteins with five ARM repeats, the remaining members having an extra ARM repeat at the end of the C-ter region [13]. MEME software detects 2 to 3 Armadillo-like Arm\_2 (PF04826) PFAM motifs of around 50 amino-acids each in the C-ter domain of all GPRASP/ARMCX proteins (Figures 1 and 2A).

### **I-B. The “GASP” motifs in the central part of GPRASP/ARMCX subfamily 1**

Sequence analysis of the GPRASP/ARMCX proteins with the software MEME suite [11] confirms the multiple occurrences of the GASP motif and defines a consensus sequence of twenty one residues, the tetrapeptide SWFW forming a hydrophobic kernel

surrounded by acidic residues, the fifteen most conserved residues form a core corresponding to the previously defined GASP motif. The GPRASP/ARMCX family can be divided into two subfamilies depending on the presence of the GASP motif [2;14]. The central part of GPRASP1, GPRASP2, GPRASP3, ARM CX4 and ARM CX5 contains 22, 7, 3, 12 and 2 GASP motifs, respectively (Figures 1 and 2B). Two GASP motifs are sometimes overlapping as the limits of acidic residues are shared with two close SWFW tetrapeptides, it happens twice in GPRASP1 and once in GPRASP3 and ARM CX5 (see Figures S1, S3 and S5).

To gain insight into the function of the GASP motifs, the orthologous mouse, rat and human proteins were aligned using Clustal software: the three GASP motifs of GPRASP3 (Figure S3) and the two in ARM CX5 (Figure S5) are highly conserved suggesting a crucial functional role, whereas in GPRASP1, GPRASP2 and ARM CX4 (Figures S1, S2 and S4, respectively) where there is more GASP motif repeats, some of them are very conserved as well, while others are missing or divergent from the consensus sequence.

### **I-C. Armadillo-like and GASP repeats of GPRASP/ARMCX proteins are involved in protein/protein interactions**

The C-ter domain of GPRASP1 was originally identified from a yeast two-hybrid screen (2-HS) by the group of Mark Von Zastrow and by our group as an interacting partner of the delta-opioid receptor (DOR; [1;15]). The bait used in the two studies was the carboxyl-terminal intracellular tail (C-tail) of the human DOR. The identified binding domain was called cGASP and encoded residues 899-1395 of GPRASP1 ([15]; Figure 1), while the domain identified by our 2-HS encompassed residues 924 to 1395 of GPRASP1 [1]. Almost 30 GPCRs and/or GPCR C-tails were shown to interact with GPRASP1 (Table S1; [1; 2; 14; 16]). cGASP that contains 3 arm2 PFAM motifs but also 4 GASP motifs was shown to bind to these receptors (GST pull down; affinity capture assays) and was proposed to compete as a dominant negative mutant to disrupt GPRASP1-GPCR complexes [15]. As cGASP shares 62% identity with the C-ter region of GPRASP2, it was proposed that GPRASP1 and GPRASP2 may have similar functions [16]. Accordingly, 7 GPCRs and/or GPCR C-tails were identified to interact with GPRASP2 *in vitro* (Table S1; [1;2;16]). Later on, we showed by deletion analysis and site directed mutagenesis that both the GASP motifs and the carboxyl-terminal domain containing putative armadillo

repeats of GPRASP1 and GPRASP2 are critical for the interaction with C-tails of several GPCRs ([14]; Table S1). We further showed that the central part of GPRASP1 (380–1073) interacts on its own *in vitro* with several GPCR C-tails ( $\beta$ -adrenergic receptors ADRB1 and ADRB2, DOR, cannabinoid receptor type 2 CNR2 and muscarinic M1R) as well as in cellular context with the overexpressed full-length receptors. In addition, we have demonstrated that the purified central domain of GPRASP1 interacts with purified full-length solubilized ADRB2 and CNR2 with an apparent affinity of 10 to 100nM [14] and that a small synthetic peptide of 20 amino acids derived from the GASP motif is capable of disrupting interactions between GPRASP1 or GPRASP2 and several GPCR C-tails ([1;14]; Table S1). When the two conserved GASP motifs of GPRASP2 were mutated, the interaction of GPRASP2 with GPCRs C-tails (ADRB1, M1, and CALCR) was lost as shown by GST-pull down experiments. In the same study, we also showed that GPRASP3, ARM CX5 and ARM CX1 can display significant interaction with several GPCRs C-tails. Although the region(s) in contact with these GPCRs remains to be discovered, the most likely candidates are the conserved carboxyl-terminal and the GASP repeats present in GPRASP3 and ARM CX5 [2;14].

GPRASP1 was also found to interact with other proteins than GPCRs including proteins implicated in GPCRs sorting at endosomes. Several fragments of GPRASP1 were identified as binding partners of the first 1-88 residues of the human Beclin2 protein in a yeast 2-HS: fragments 723-1011, 808-1069, 880-1167, 931-1190, 934-1211 and 1051-1324 of GPRASP1 [18]. Although it was not discussed in the study, the lack of overlap between fragment 723-1011 that is exclusively localised in the central part of GPRASP1 and contains several GASP motifs and fragment 1051-1324 that covers mainly the ARM repeats extremity of GPRASP1, suggests to us that the interaction could take place at least with the two domains. The interaction was further confirmed by co-immunoprecipitation with either the cGASP fragment or the endogenously expressed GPRASP1. Despite the degree of identity between their C-ter domains, GPRASP2 was not found to co-immunoprecipitate with Beclin2 contrary to GPRASP1 (Table S1; Figure 3; [18]).

GPRASP1 was also identified in a two hybrid screen as an interacting partner of the transcription factor period1 (Table S1; [19]). Several regions located within the N-ter and the central domains of GPRASP1 were shown to be critical for the interaction with

this protein. The involvement of the GASP motif in this interaction has not been investigated so far.

The arm2 repeats containing C-ter domain of ARM CX3 was shown to be required for the interaction with the microtubule adaptor MIRO/TRAK2 complex by co-immunoprecipitation since the N-ter (1-106 residues) of ARM CX3 fused to GFP are not sufficient to interact with MIRO [3]. As ARM CX1, ARM CX2 and ARM CX10 also interact with the MIRO/TRAK2 complex, it is most likely that the interaction is taking place within their homologous C-ter domain although it has not been clearly found to be a direct interaction. Its C-ter domain also mediates the interaction of ARM CX3 with the 100 first residues of the transcriptional factor Sox10. Indeed, a truncated Myc-tagged N-ter (1-109 residues) ARM CX3 does not co-immunoprecipitate with Sox10 anymore [20].

To conclude this part, Armadillo-like and GASP repeats appear to represent essential regions of GPRASP/ARM CX proteins for their interaction with multiple partners.

#### **I-D. The mitochondrial targeting signal peptide and transmembrane domain of ARM CX10, ARM CX4, ARM CX1, ARM CX2, ARM CX3 and ARM CX6 proteins**

MEME detects a motif homologous in ARM CX10, ARM CX1, ARM CX2, ARM CX3, ARM CX4 and ARM CX6 amino extremities from residues 1 to 33 (Figures 1 and 2C). This motif encodes a transmembrane TM domain from residues 7 to 29 [3;4;5;20;21;22]. Murine ARM CX5 was originally proposed to contain a N-ter transmembrane domain as well [3] but at least in human ARM CX5, it is not reported in uniprot, bioinformatics tools do not find a TM domain (example search with Constrained Consensus TOPology prediction server CCTOP) and there is no homology between the first residues of human ARM CX5 and those of the other ARM CX proteins.

Strong evidence indicates that the first residues of ARM CX10, ARM CX1 and ARM CX3 serve as a targeting signal and transmembrane anchor to the outer membrane of mitochondria. Indeed, three independent groups have found ARM CX10 to be localised to the mitochondria [23;24;22;25]. The 4 different isoforms of ARM CX10 were first described as Endoplasmic Reticulum ER proteins based on co-localisation with calnexin of the C-terminally tagged ARM CX10 with GFP. Looking back at the images of co-localisation with the knowledge of its mitochondrial localisation, it is probable that the co-localisation between ARM CX10 and calnexin was a misinterpretation [4].

Alternatively, maybe the co-localisation is taking place at ER-mitochondria contact sites [26]. Myc-tagged overexpressed ARMC10 in HEK293 cells co-localises with the MitoTracker dye staining for mitochondria [3]. Using the engineered ascorbate peroxidase system, ARMC10-V5-APEX2 overexpressed in HEK293T, HeLa, and U2OS cells was found to co-localise with the Mito-BFP protein in confocal immunofluorescence images and to be enriched in the outer membrane of mitochondria with most of the protein facing the cytosol [25]. Finally, the transmembrane TM domain of ARMC10 (residues 5-27) was found necessary for co-localisation of the overexpressed ARMC10 with the mitochondrial marker TOM20 in HEK293T cells [22].

Myc-tagged ARMCX3 overexpressed in mouse olfactory bulb OBL21 cell line but not the ARMCX3 deleted from its residues 19 to 75, co-localises with mitochondrial protein COXIV. Myc-tagged ARMCX3 was found in the mitochondrial purified fraction together with COXIV [20]. Specific polyclonal antibodies against the last ten residues of ARMCX3 (369-379 AA) detect a punctate staining of the endogenous protein in hippocampal neurons at 15 DIV that partially co-localises with the MitoTracker dye and a MitDSRed fusion protein in confocal immunofluorescence images. It is detected as well in spots in the nucleus. Overexpressed ARMCX3 in HEK293 cells co-localises with the MitoTracker and is found by electronic microscopy at the mitochondrial membranes [3]. A GFP-tagged ARMCX3 deleted from its extreme 1-12 residues was found in the nucleus, but the position of the GFP at the beginning of the fusion protein might have hindered the mitochondrial targeting [3]. More convincingly, the first 1 to 30 residues of ARMCX3 followed by a fused GFP, are sufficient to target the GFP to mitochondria as seen by co-localisation with MitoTracker [24].

Myc-tagged overexpressed ARMCX1 in HEK293 cells co-localises with the MitoTracker dye staining for mitochondria [3]. The first 40 residues are necessary for ARMCX1-HA-tagged to co-localise with MitoDsRed marker in mouse cortical neurons [21].

Since the N-ter extremities of ARMC10, ARMCX1 and ARMCX3 but also the ones of the less characterized ARMCX2, ARMCX4 and ARMCX6 proteins are highly similar, it is probable that these 6 proteins are all targeted to the outer mitochondrial membrane via sequences within and/or around their transmembrane domain. Hence, it was shown

that Myc-tagged overexpressed ARM CX2 and ARM CX6 in HEK293 cells also co-localise with the MitoTracker [3].

Additional evidences for mitochondrial localisation are that ARM C10, ARM CX3 and ARM CX1 directly interact with the mitochondrial protein MIRO1 and MIRO2 (Table S1; Figure 3), ARM C10 was found to interact with a number of other peripheral or integral proteins of the outer mitochondrial membrane (Table S1; Figure 3). Finally, ARM CX2 co-immunoprecipitates with the mitochondrial marker TOM22 as ARM CX3 does (Table S1; Figure 3; [27]).

### **I-E. Nuclear localisation signal (NLS) in GPRASP/ARM CX proteins**

Several GPRASP/ARM CX proteins have been proposed to interact with transcriptional factors (ARM CX3 with Sox10, GPRASP1 with Period-1, GPRASP2 with TCF25, ARM C10 and ARM CX5 with p53, ARM CX1 with Myc; see Table S1) but no additional recent information on this subject are available (see review [2]) apart that ARM CX1, ARM CX2 and ARM CX3 affinity-purified with EZH2 and SUZ12 of the polycomb complex ([28]; Table S1). Our bioinformatics analysis led us to re-investigate the putative nuclear localisation signal previously identified in the different members of GPRASP/ARM CX family and to confront them with experimental data regarding nuclear localisation of the proteins. To this purpose we have used NLStradamus and SeqNLS [29;30].

The proteins that contain one or several putative NLS are: GPRASP1 (in human, residues 313-337 in the N-ter domain and 372-394 in the central domain), GPRASP3 (in human, residues 35-47), ARM CX1 (in human, residues 158-180), ARM CX2 (in human, residues 35-49 and 279-301), ARM CX3 (in human, residues 89-108) and ARM CX4 (in human, residues 526-532). Human GPRASP2, ARM C10, ARM CX5 and ARM CX6 have no detectable NLS using the two softwares. Murine ARM CX5 was originally proposed to contain a NLS on the basis of sequence analysis but we did not detect it in human ARM CX5 [3]. Although the absence of a putative NLS does not preclude concentration of a protein into the nucleus via interacting partners, ARM C10 has never been described in the nucleus. Indeed, SFB-ARM C10 and ARM C10-GFP were not observed in the nuclei of HEK293 and QSG-7701 cells, respectively [22;4]. Similarly ARM CX1 was not observed in the nuclei of mouse cortical neurons [21]. ARM CX3 has been clearly localised in the nucleus by several studies. Using myc-tagged-ARM CX3, a mutant lacking the TM domain

was found in the nucleus of OBL21 cells [20]. Although using ARM CX3-GFP and different mutants in HEK293T cells, Serrat and colleagues did not describe ARM CX3 as being in the nucleus [5]. However, a GFP-delta1-12 ARM CX3 was localised in the nucleus of HEK293 cells and in chicken embryos spinal cord [3;31] and anti-ARM CX3 detected endogenous ARM CX3 also in nuclei of cultured hippocampal neurons [3]. Finally, GPRASP2 was detected in nuclei extract of HeLa cells and was shown to be phosphorylated [32]. The phosphorylated residue in GPRASP2 is conserved in GPRASP1 and Period-1 was originally proposed to promote the translocation of GPRASP1 to the nucleus in cultured cells [19].

### **I-F. Other motifs in GPRASP/ARM CX proteins**

#### *I-F-1. A homologous motif in the extreme N-ter region of GPRASP1, GPRASP2 and GPRASP3*

Our sequence analysis of GPRASP/ARM CX proteins with MEME algorithm, allowed us to show that the first residues of GPRASP2 and GPRASP3 (1-29 amino acids) display 72.4 and 41.4 % identity with GPRASP1, respectively (Figure 2D). This sequence is well conserved between human, mice and rat orthologs (Figures S1, S2 and S3). The analysis with the SeqNLS software indicated that this sequence could serve as NLS, although with low probability. The function of this well conserved N-ter extremity has not been yet studied.

#### *I-F-2. A homologous repeated motif in the N-ter domain of GPRASP1 and GPRASP2.*

It is interesting to note that GPRASP1 and GPRASP2 share another conserved motif in their N-ter part with more than 47.4% identity. Composed of 19 residues, it is repeated in tandem three times in GPRASP1 and seven times in GPRASP2 (Figure 2E). No functional data have been assigned to this motif and it is unique to GPRASP1 and GPRASP2. The software SeqNLS detected with low probability that it could serve as NLS. In a large interactomic approaches, the TNF receptor-associated factor TRAF4 protein fished out the N-ter of human GPRASP1 (129-388) but it does not include these repeats ([33]; Table S1 and Figure S1).

#### *I-F-3. Two repeated motifs unique to the central part of ARM CX4*

Finally ARM CX4, the largest protein of the family that has not been yet studied by any research group, contains two additional motifs. According to MEME one is

composed of 29 residues and repeated in tandem 21 times (Figure 2F). It precedes the GASP repeats in the central region of ARM CX4. Another motif, localised together with the GASP motifs is present 13 times and is 21 residues long (Figure 2F). Some of these repeats are conserved between human, mice and rat orthologs (Figure S4).

### **I-G. Perspectives: a scaffolding role for GPRASP/ARM CX proteins**

GPRASP/ARM CX proteins through their homologous C-ter domain belong to the alpha-solenoid proteins. Alpha-solenoids are elongated flexible structural domains formed by alpha-helical repeats that can be sensitive to mechanical stretch forces and can mediate multiple interactions at the same time serving as scaffolding proteins [34;35]. In GPRASP/ARM CX proteins, this domain, which is their most common feature, has been established to mediate some protein-protein interactions. It will be interesting to determine its three-dimensional structure in addition to pursue research on its functional properties, particularly because in some studies its contribution to the binding to partner proteins is mixed with the contribution of the GASP motifs.

Our previous review had subdivided the GPRASP/ARM CX protein family into two groups: those that contain the GASP motifs (subfamily 1: GPRASP1, GPRASP2, GPRASP3, ARM CX4 and ARM CX5) and those that don't (subfamily 2: ARM C10, ARM CX1, ARM CX2, ARM CX3, ARM CX6). This classification continues to hold a functional meaning with respect to GPCRs interaction as reviewed in this closing chapter and in the following chapter II, with ARM CX4 that somehow overlaps the boundary between the two subfamilies: ARM CX4, like the other members of the subfamily 2, contains a transmembrane domain that might target the protein to the outer mitochondrial membrane. The main functional studies on the subfamily 2 concern mitochondrial trafficking and dynamics and will be described in chapter III.

While traditionally, protein function was viewed as dependent on defined folded three-dimensional structure, it appears that the N-ter and central domains of the GPRASP/AMRCX protein subfamily 1 are not folded into alpha-helices or beta-strands (if one run a secondary structure analysis of the proteins). These domains thus probably fall into the definition of Intrinsic Disordered Regions or IDRs. Disordered regions are often subjected to post-translational modifications and can expose short linear motifs available for protein-protein interactions [36]. Interestingly, in GPRASP1, GPRASP2, GPRASP3 and ARM CX4 and ARM CX5, these IDRs are composed of both novel repeats of

unknown function (Figures 1 and 2) and of the GASP repeated motif that we have found to be necessary for interactions with GPCRs and probably with beclin2 as well. The number of GASP repeats varies between orthologs with vast number of repeats (GPRASP1, GPRASP2, ARM CX4), indicating that rapid loss or gain of repeats occurred in evolution. At least in the subfamily 1 of GPRASP/ARM CX proteins, not only the carboxyl-terminal domain serves as scaffold but probably the repeats in their amino-terminal and central domains as well. It remains to explore further the functional properties of these multiple kind of repeats that are unique to this subfamily of proteins. For example, it would be interesting to pinpoint which domains of GPRASP1 can mediate the interaction with the subunit  $G\alpha S$  of heterotrimeric G protein [37] and with the ESCRT protein HRS [38;37], and which domain of GPRASP2 are responsible for direct interaction with the primary cilium located Pifo protein (Table S1 and Figure 3; [39]).

The interactome studies that have identified GPRASP/ARM CX proteins as binding partners have not generally gone more into details: from these plethora data one can extract few useful information but maybe one: the interactants that are common to at least two proteins of the family (Table S1). Hence GPRASP1 and GPRASP2 interact in 2-HS with the microtubule binding protein Stathmin-2 [40;41], GPRASP2 and ARM CX5 were affinity purified with the kinase RIPK4 [42]. GPRASP1 and ARM CX3 can both bind the subunit  $G\alpha S$  of heterotrimeric G protein [37;43] and finally, ARM CX10 and ARM CX5 seem to bind the tumour suppressor and transcriptional factor p53 [44;45]. Again, it would be informative in the future to identify the residues that mediate these protein-protein interactions. Of note, homo and hetero-dimerization between members of the GPRASP/ARM CX protein family have barely been explored experimentally although some interactomic studies suggest it (Table S1).

## **II. GPRASP/ARM CX subfamily 1 in GPCR trafficking**

The main functions of GPRASP/ARM CX subfamily 1 that have been studied are linked to GPCRs trafficking in order to regulate signalling and adaptation to GPCRs activation (Figure 3). Since the last reviews on the subject [2;46;47], very interesting results on GPRASP1 and GPRASP2 and their novel interacting partners together with phenotypes in knockout mice as well as genetic mutations in humans have been reported that shed new light on the functions of GPRASP/ARM CX subfamily 1 (Table 1).

As described in chapter I, many interacting proteins of GPRASPs are G protein-coupled receptors (GPCRs). There are no less than 799 verified sequences of human GPCRs and they can be activated by an extraordinary diversity of ligands, including amino acids, Ca<sup>2+</sup> ions, peptides, lipid-like substances and large glycoprotein hormones [48]. The activated GPCR acts as a GTP exchange factor on the G $\alpha$  subunit triggering the intracellular response cascades [49]. In order to have their large variety of action and a precise regulation of the cellular response, once synthesized, GPCRs must be correctly addressed to the plasma membrane or in specialized membrane compartments such as primary cilium, synapses or mitochondria [50;51]. At the cell surface, upon stimulation by agonists, most GPCRs undergo rapid desensitization and internalization into clathrine-coated pits due to post-translational modifications (phosphorylations, ubiquitinations) leading to recruitment of  $\beta$ -arrestins. Following endocytosis in early endosomes, they can be either recycled back to the cell surface or directed towards multivesicular bodies MVB and lysosomes for degradation. A number of intracellular GPCR binding proteins contributes to this regulation cycle of GPCRs signalling [52]. *In vitro*, GPRASP1 has the largest spectrum of GPCRs interaction, almost as large as the one of  $\beta$ -arrestins (Table S1). But contrary to  $\beta$ -arrestins, in cells, the interaction of GPRASP1 with GPCRs is detected at steady state with scarce report of co-localisation with GPCRs. The recent literature however has brought convincing evidence that GPRASP1 and GPRASP2 participate in sorting of GPCRs from the endosomes and to the primary cilium, respectively.

### **II-A. Role of GPRASP1 in GPCR trafficking and in vivo consequences**

This function of GPRASP1 has been the topics of the reviews published so far [2;17;46;47]. Most studies have evaluated the degradation of tagged-GPCRs overexpressed in cells using a plasma membrane protein biotinylation assay followed by a chase in order to trace the amount of receptors after several minutes or hours of activation with agonists. Radiolabelled binding studies have also been used in different studies. The implication of GPRASP1 has been evaluated mostly by overexpression of the truncated cGASP protein, considered as a dominant negative form of GPRASP1. Some more recent studies have used down-regulation of endogenous GPRASP1 using siRNA or shRNA. Finally, two research teams including ours have compared the down-regulation of GPCRs after chronic stimulation in WT versus GPRASP1 knockout mice.

*II-A-1. Interaction of GPRASP1 with delta-opioid receptor (DOR) together with dysbindin, HRS, Gαs and Beclin2: in vitro effects on activated DOR targeting for degradation*

The cGASP truncated protein was first used in Mark Von Zastrow's laboratory in the seminal work on GPRASP1 in interaction with the delta-opioid receptor DOR [15]. cGASP was proposed to be a dominant-negative form of GPRASP1 based on the fact that it competes with GPRASP1 for DOR interaction both in GST-pull down assays using the C-tail of DOR and in co-immunoprecipitation experiments with cell lysates. cGASP was also found to have the opposite effect to overexpressed GPRASP1 on activated DOR down-regulation as measured by binding assays. Whistler and collaborators showed that in HEK293 cells overexpressing FLAG-tagged-DOR, in immunofluorescence assays, after 3h of agonist application, DOR was observed in the perinuclear region, widely co-localising with the lysosomal markers LAMP1 and LAMP2. HEK293 cells overexpressing cGASP together with FLAG-DOR responded to the agonist by the internalization of DOR, in the same way than in the absence of cGASP: the internalization of the receptor was thus unaffected. But following the washout of the agonist after 30 min, the immunoreactivity of the FLAG-tag was higher at the plasma membrane in cells expressing cGASP. Using a biotin degradation assay, the amount of DOR was maintained in cGASP expressing cells 3 hours after agonist treatment, whereas the receptor was fully degraded in cells without cGASP expression. This indicated that cGASP expression inhibited the degradation of activated DOR [15]. GPRASP1 was thus proposed to be involved in promoting activated DOR degradation.

Other proteins have since been implicated *in vitro* to target DOR towards the lysosomes and interestingly three of them interact directly *in vitro* with GPRASP1: HRS, GαS and Beclin2. In addition dysbindin, a component of the biogenesis of lysosome-related organelles complex-1, co-immunoprecipitates with GPRASP1 (see Table S1 and Figure 3). DOR is degraded via the endosomal sorting complexes required for transport (ESCRT) composed of 5 cytosolic complexes, with multiple proteins (ESCRT-0, -I, -II, -III and the Vps4 complex), which realize successively clear tasks. The ESCRT-0 complex is a heterodimer of HRS (HGF-regulated tyrosine kinase substrate) and STAM (Signal Transducing Adaptor Molecule). The ESCRT-0 localises to endosomal membrane and recognizes the mono-ubiquitin signals to lead the membrane protein target towards the

intraluminal vesicles of the multivesicular bodies MVB and lysosomes [53;54]. While some GPCRs are directly ubiquitinated for recognition by the ESCRT-0, such as the chemokine receptor CXCR4, the ubiquitination of DOR is not required for its degradation [55] and it is proposed that other ubiquitinated proteins binding to DOR serve as adaptors for the receptor to be recruited by the ESCRT machinery [47]. Dysbindin was first proposed to be such an adaptor protein for DOR targeting towards the ESCRT complex, it is the target for ubiquitination by the ubiquitin ligase TRIM32 [56]. Dysbindin is mutated in the Hermansky-Pudlak syndrome with a loss of lysosome-related organelles such as melanosomes causing hypopigmentation and bleeding troubles. The role of dysbindin could be more complex in neurons, with participation to the maturation of neurites and dendritic spines as well as exocytosis of GPCRs [57]. Concerning DOR regulation, dysbindin depletion up-regulated total FLAG-DOR level, as well as cell surface localised one. Finally, its depletion slightly slowed-down the degradation of activated FLAG-DOR after 4 hours [56]. In addition, HA-tagged-dysbindin was found to co-immunoprecipitate with EGFP-GPRASP1 and with myc-tagged HRS in HEK293 cells. It was also shown to co-localised with Cherry-tagged-HRS on intracellular organelles probably endosomes [56].

Christine Lavoie's team has since proposed that GPRASP1 forms a complex with HRS/dysbindin and surprisingly with the heterotrimeric  $G\alpha_s$  subunit [37]. Indeed, among other GPCRs, the flag-tagged DOR cell surface expression was increased in  $G\alpha_s$  knock-down cells depleted by siRNA. Of note, several of the GPCRs sensitive to  $G\alpha_s$  depletion (DOR, CXCR4, D2R) are not primarily coupled to  $G\alpha_s$  signalling and the effect of  $G\alpha_s$  was found independent on its activation state [37]. While treatment by agonists induced degradation of DOR, knock-down of  $G\alpha_s$  reduced its degradation and DOR accumulated in early endosomes instead of lysosomes 2h after activation. Using a constitutively active Rab5 mutant that triggers enlargement of endosomes, it was possible to determine that there was less DOR in endosomal lumen of the MVB in  $G\alpha_s$  knock-down cells as compared with control siRNA transfected cells. The authors concluded that  $G\alpha_s$  is crucial for the sorting of DOR in the intraluminal vesicles of MVB for lysosomal degradation. In addition, in COS transfected cells, mCherry-tagged-GPRASP1 was mostly localised in the cytoplasm. But it could sometimes be detected on intracellular vesicles in co-localisation with both GFP- $G\alpha_s$  and endogenous HRS. In

addition Myc-tagged dysbindin was found to co-localise with G $\alpha$ s-GFP and endogenous HRS on endosomal structures as well. G $\alpha$ s directly interacted in GST-pull down assays with GPRASP1 and dysbindin [37]. Moreover, GPRASP1 was found to directly interact with G $\alpha$ s and HRS. Therefore, GPRASP1 seems to participate to a multicomponent complex involved in DOR sorting to MVBs. Dr. Lavoie's team has lately proposed that G $\alpha$ s contains an Ubiquitin-Interacting Motif (UIM) necessary for its role with the ESCRT machinery [58]. Interestingly, G $\alpha$ s has also been proposed to regulate sorting of other receptors than GPCRs such as the EGFR [59;60;61].

In an independent study, Beclin2, another binding partner of GPRASP1, has been implicated in DOR degradation [18]. Beclin2 is homologous to Beclin1, a protein known to function in autophagy. Beclin2 is also taking part in autophagy and binds to several partners of Beclin1 implicated in autophagy [18]. GPRASP1 was identified using the first unique 88 residues of Beclin2 as bait in a yeast 2-HS, prompting the team to investigate the role of Beclin2 in GPCRs sorting together with GPRASP1. In Beclin2 knock-down cells, DOR was no more degraded after stimulation but stayed in the intracellular compartment. Next, Beclin2 I80S was identified as mutant of Beclin2 that does not bind GPRASP1 anymore in co-immunoprecipitation experiment. Very elegantly, using complementation assay of siRNA depleted Beclin2 cells, overexpression of WT Beclin2 could rescue degradation of activated DOR but not overexpression of the Beclin2 I80S mutant. This suggested that Beclin2 interaction with GPRASP1 is necessary for targeting DOR towards the lysosomes. cGASP expression reduced DOR degradation to the same extent as siRNA against Beclin2 in this study. The authors also showed that GPRASP1 does not take part in the autophagy process together with Beclin2 ([18]; Table S1 and Figure 3).

If the importance of the GPRASP1/HRS/ G $\alpha$ s /dysbindin complex on one hand and GPRASP1/Beclin2 complex on the other hand is established in cells for activating DOR sorting to lysosomes, it is not yet known whether Beclin2 interacts with other proteins of the complex and the spatio-temporal organization of the interactions in the complexes remains to be determined (Figure 3 and Table 1). In addition, the effect of GPRASP1 on DOR signalling *in vivo* has not been studied so far.

*II-A-2. Interaction of GPRASP1 with dopaminergic D2 receptors together with dysbindin and G $\alpha$ S : in vitro and in vivo effects on activated D2R targeting for degradation*

Concerning the response to agonist treatment, the role of GPRASP1 in the adaptations of the dopaminergic receptors has been widely studied in this last decade. In a first study, Jennifer Whistler's team put in light that GPRASP1 was involved in the degradation of the dopaminergic D2R [62]. They showed that activated D2R were degraded in HEK293 cells but not D1R and that this correlated with the stronger affinity of GPRASP1 for D2R versus D1R. cGASP overexpression facilitated recycling of D2R. These data were confirmed independently in neuronal SH-SY5Y cells transfected with D2R tagged with GFP at its C-tail. cGASP was used to study the implication of GPRASP1 in the down-regulation of D2R after an endocytosis provoked by 12 or 24h of agonist treatment. While recycling of the receptor was delayed by overexpression of full-length GPRASP1, it was accelerated by the dominant negative cGASP [63]. SiRNA against GPRASP1 also confirmed these data: the D2 and D3 receptors, but not D4 receptors bind GPRASP1 in co-immunoprecipitation assays and a prolonged treatment with an agonist causes a degradation of the receptors. Stably expressed in HEK293 cells, D2R, D3R and D4R N-terminally flagged fusion proteins have the same endocytic properties after 45 min of agonist treatment [64]. Yet D2R and D3R are more degraded after endocytosis than D4R. A reduction of degradation was shown for activated D2R and D3R in GPRASP1 knock-down cells as compared with WT cells. If it seems to indicate a participation of GPRASP1 to degradation of D2 and D3 receptors after internalization, the data revealed with flow cytometry that GPRASP1 absence did not promote the recycling of the receptors to the cell surface [64]. Finding that GPRASP1 and D2R were co-expressed in rat brain, patch-clamp experiments were carried out on dopaminergic neurons of the ventral tegmental area. They revealed a rapid desensitization of the D2R receptor to its agonist. Using an antibody directed against GPRASP1 to inhibit the interaction with the D2R on the cerebral slices by injection through the patch-clamp pipette, the anti-GPRASP1 antibodies were shown to increase the recovery of D2R signalling after prolonged agonist treatment [62]. Of note, no difference was observed in the acute response to agonist with anti-GPRASP1 antibodies.

At the molecular levels, two partners of GPRASP1, dysbindin and G $\alpha$ s, have been implicated in D2R sorting to lysosomes: siRNA against dysbindin selectively affected total cell surface of D2R but not of D1R, in absence of activation in cells. Upon activation, while internalization kinetics of D2 receptors were not modified by siRNA against dysbindin, proteolysis of D2R was slowed down [38]. In addition, cell surface expression

of the flag-tagged D2R was increased in  $G\alpha s$  knock-down cells depleted by siRNA although no experiments on activated D2R receptors were conducted in this study [37]. The role of Beclin2 in D2R sorting to lysosomes has not been investigated yet but the brains of Beclin2 heterozygous (+/-) mice had increased amount of D2R as measured using western blot against endogenous D2R [18].

Finally, Dr. Whistler's team and independently our team, have generated GPRASP1 knockout mice, allowing us to investigate activated receptors down-regulation *in vivo* [65;66]. Although a similar behavioural phenotype was observed in these two GPRASP1 knockout lines following repeated administration of cocaine (see II-A-5), opposite results were reported concerning dopaminergic receptors degradation. In these two studies, two slightly different models of cocaine administration were used in order to activate dopaminergic receptors internalization (15mg/kg cocaine intraperitoneal daily administration for 5 days [65]; 1mg/(kg\*infusion) for 10 consecutive days in self-administration experiment [66]). Cocaine is an inhibitor of monoamine reuptake, including dopamine and acetylcholine [67;68], which leads to an increased concentration and residence time of monoamines in the synaptic cleft resulting in a sustained activation of their receptors. In the Thompson and collaborator's study, there was no significant difference in D1R quantities between GPRASP1-KO and WT mice striatum, whether in saline or in cocaine administration but there were significant differences between the amounts of D2 receptors [65]. Intriguingly, GPRASP1-KO mice had a lower quantity of D2R at steady state. Then the cocaine treatment, instead of causing a decrease, as in WT mice, revealed a significant increase of the striatal D2 receptors labelled in radioligand binding assays in GPRASP1-KO mice, correlated with an increase of the D2R:D1R ratio. Yet, we have studied the number of D1R, D2R and muscarinic receptors in response to chronic cocaine treatment in GPRASP1-KO mice and have found the opposite [66]. In our hands, in the striatum and other brain areas, after a chronic self-administration of cocaine, the level of dopamine and muscarinic receptors were more down-regulated in GPRASP1-KO mice than in WT littermates. Of note, we did not detect differences between WT and GPRASP1-KO mice in the total amount of receptors before activation contrary to the other study. Our data are in agreement with a role of GPRASP1 in post-endocytic sorting of D2R, but in their recycling rather than in their degradation [66].

*II-A-3. Interaction of GPRASP1 with cannabinoid receptor CB1R together with Beclin2 : in vitro and in vivo effects on activated CB1R targeting for degradation.*

The role of GPRASP1 in activated cannabinoid receptors CB1R sorting has also been studied up to the *in vivo* level. Initially, Martini and collaborators showed in HEK293 cells expressing CB1R that cGASP expression diminished the co-localisation of FLAG-CB1R with lysosomal markers LAMP1 and LAMP2 and delayed the receptor degradation after agonist treatment with WIN55,212-2 agonist during 30 to 180 min, suggesting a participation of GPRASP1 in the targeting to lysosome [69].

In primary culture of spinal neurons, where the cGASP protein was infected thanks to SVF virions, only a minor decrease of the cell surface CB1-GFP tagged receptor was observed after a prolonged agonist treatment (24 h) while there was a full loss of CB1R in the absence of cGASP overexpression. In the central nervous system of wild-type mice, the CB1-GFP receptor co-immunoprecipitated with GPRASP1 as observed *in vitro*, confirming the interaction of the two partners *in vivo* [70]. Selective expression of cGASP in the superficial lamina of the dorsal horn of the spinal cord using AAV caused less diminution of CB1R binding sites as measured by radiolabelled ligand after 14 days of WIN55,212-2 agonist treatment than in WT mice. It was proposed that this decreased degradation of the CB1R was due to the impairment of the interaction between CB1R and GPRASP1, implicating again GPRASP1 in promoting degradation. Finally, the amount of cannabinoid receptor binding sites in the spinal cord and cerebellum of WT and GPRASP1 knockout mice was compared using radiolabelled ligand [71]. After repeated treatment with the WIN55,212-2 agonist, there was a significant diminution of the number of CB1R in the spinal cord in WT mice. In GPRASP1-KO mice there was no difference in receptors number after the treatment in the spinal cord. The authors concluded that GPRASP1 promoted activated CB1R down-regulation *in vivo*. Intriguingly, before activation, there was a reduced number of CB1R in the spinal cord of GPRASP1-KO mice as compared with WT mice [71].

One of the interacting partners of GPRASP1, Beclin2, has been implicated in CB1R homeostasis *in vivo* [18;72]. Indeed, in brains of Beclin2 heterozygous (+/-) mice, there was an increased amount of CB1R at steady state as measured using western blot against endogenous CB1R [18]. In a second paper, the team of Dr. He showed that Beclin2 +/- mice had a decrease of the activated CB1R receptor degradation [70]. This

result was confirmed by knock-down of Beclin2 using siRNA in cells transfected with CB1R, where the receptor stayed in the endosome (co-localisation with early endosomal marker EEA1) instead of being addressed to the lysosomes (marked by LAMP1) after 1 hour of agonist stimulation, confirming the role of Beclin2 in the post-endocytic trafficking of CB1R [72].

*II-A-4. Interaction of GPRASP1 with other GPCRs: recent studies indicating a role in sorting to lysosomes and in signalling*

(i) The chemokine receptor CXCR4

The fascinating role of GPRASP1 but also of its homologs GPRASP2 and GPRASP3 in regulation of hematopoietic stem and progenitor cells (HSPC) has been discovered by the team of Dr. Shannon McKinney-Freeman [73;74]. In a first study, the goal of the team was to identify proteins regulating hematopoietic stem and progenitor cells transplantation. Indeed, HSPC graft is the only curative treatment available for many forms of leukemia [75]. Databases of HSPC gene expression and the results of RT-qPCR experiments with bone marrow samples were analysed. The information of gene expression variation were extracted and correlated to a phenotype of bone marrow repopulation *in vivo* thanks to associated shRNA-transfected HSPCs transplanted in irradiated mice. Among the 17 gene regulators of HSPC repopulation identified in this screen, there were two proteins of the GPRASP/ARMCX family: ARM CX1 and GPRASP2. In addition, they were the only two genes for which knock-down increased the HSPC bone marrow repopulation instead of decreasing it [73].

The role of GPRASP2 was further investigated in a study that included its closest homolog GPRASP1 in the experimental set-up leading to identical results for both genes (ARM CX1 was not further studied). The phenotype of HSPC bone marrow repopulation was confirmed for both genes independently [74]. The authors observed an increase in survival of HSPCs in GPRASP1 or GPRASP2 knock-down cells [73;74]. When both genes were silenced together, there was no further increase in bone marrow repopulation by the transplanted HSPC. Nevertheless, very importantly, there was compensation in each knockout cells with overexpression of their homolog GPRASP3. Down-regulation of GPRASP3 had a cumulative effect in increasing further bone marrow repopulation by either GPRASP1-KO HSPCs or GPRASP2-KO HSPCs without effect on WT HSPCs. Next, the authors decided to investigate if the endogenously expressed chemokine CXCR4

receptor was the target of the negative effect of GPRASPs on the HSPC bone marrow repopulation, because this GPCR is known to be implicated in HSPCs homing. Flow cytometry was used to look at the amount of endogenous CXCR4 by immuno-labelling at the cell surface and in intracellular compartment thanks to permeabilization. The results are complex but the most convincing data are that total CXCR4 level is increased 72h after knock-down of either GPRASP1 or GPRASP2 in HSPC as compared to control and that this seems to be due to a diminution of constitutive CXCR4 degradation (slightly longer half-life of CXCR4 measured in presence of cycloheximide [74]). Finally, the team of Dr McKinney-Freeman showed that GPRASP genes deletion in CXCR4 knockout HSPCs did not lead to an increase in survival and no enhancement effect in bone marrow repopulation by the transplanted HSPCs as compared with control cells. This result strongly suggests that CXCR4 is a key player in the mechanism of implantation modulated by GPRASP1 or GPRASP2 [74;75].

Interestingly, in *in vitro* experiments CXCR4 degradation has been shown to be dependent of  $G\alpha_s$  [37] but independent of Beclin2 [18]. The interaction between GPRASP2 and GPRASP3 with  $G\alpha_s$  or HRS has not been investigated so far but GPRASP2 does not bind Beclin2 [18].

(ii) The serotonin receptor 5HT7

cGASP has still being used in recent studies to investigate GPRASP1 in GPCRs degradation. The serotonin receptor 7 (5HT7) is targeted by psychiatric treatments such as clozapine, which surprisingly causes an inhibition of the G protein signalling pathway and simultaneous internalization of the receptor followed by its degradation. In cGASP transfected HEK293 cells, the normal clozapine-induced down regulation of the 5HT7 receptor after 24h of treatment was prevented, as if GPRASP1 was needed to induce its degradation [76]. However this team could not sufficiently knock-down GPRASP1 in HEK293 using siRNA to confirm and conclude about the effect of GPRASP1 deficiency. The use of cGASP as the only tool to decipher the role of GPRASP1 in GPCR degradation is questionable because first, it is usually expressed at a much higher level than the endogenous GPRASP1, second, it is not excluded that it can interfere with the protein-protein interactions between other GPRASPs and third, due to its size, it might cause steric hindrance blocking the interaction of the GPCRs with other proteins necessary for their proper post-endocytic targeting.

(iii) The cannabinoid related receptor GPR55

More convincingly, in HEK93 cells overexpressing a flag-tagged GPR55 that co-immunoprecipitated with GPRASP1, GPRASP1 shRNA did not influence the internalization of the receptor, but it promoted its recycling to the plasma membrane instead of its degradation after 45min of agonist treatments [77]. This result suggests that GPRASP1 plays a role in the sorting of the endocytosed GPR55 towards its degradation.

(iv) The Virus chemokine receptor US28

The human cytomegalovirus produces 4 GPCRs, including the chemokine-like receptor US28. When expressed in cells, this receptor is constitutively active and accumulates in late endosomes and lysosomes following internalization. The US28R can bind GPRASP1 [78]. In cGASP transfected cells or GPRASP1-knock-down cells transfected with flag-tagged US28R, the lysosomal degradation of the receptor was slowed-down. In addition, there was a modification of the constitutive signalling pathway of US28R in cGASP transfected cells or in the absence of GPRASP1, with unexpectedly, a decrease of the  $G\alpha_q$  pathway (decrease in production of inositol phosphate) and of further downstream signalling events (decrease in NF-KB and CREB transcription factors target expression). Inversely, overexpression of GPRASP1 increased constitutive signalling of US28R and could rescue the decrease in signalling in GPRASP1 shRNA infected cells [78]. These data suggest that GPRASP1 could participate to intracellular signalling in addition to targeting GPCRs towards degradation.

(v) The glucagon-like peptide receptor GLP1R

One of the latest studies looked at another GPCR partner of GPRASP1: the glucagon like peptide receptor 1 [79]. When activated, this receptor causes insulin release by pancreatic  $\beta$  cells but as for many other GPCRs, its activation induces its endocytosis. That is why the team looked particularly after the proteins already known to participate in GPCR trafficking. They tested knock-down effect by RNAi of proteins implicated in post-endocytosis GPCRs trafficking using a functional assay: an incretine-stimulated insulin secretion test to evaluate the response of the overexpressed GLP1R in  $\beta$  pancreatic cells. Surprisingly, knock-down of GPRASP1 induced a lower insulin

secretion, suggesting that like for US28R GPRASP1 is involved in GLP1R-associated signalling [79].

#### *II-A-5. GPRASP1 in the adaptations associated with chronic stimulation of GPCRs in vivo*

Two teams have studied the behavioural consequences in GPRASP1 knockout mice. From the cellular studies with GPRASP1 knock-down, one might have expected to detect an effect on some GPCRs signalling already after acute activation [74;78;79] but with more pronounced effects after repeated activation when the GPCRs will be submitted to several cycles of “activation, endocytosis, recycling or degradation” [62]. Experimental models that were selected for the studies were chronic stimulations of GPCRs for which GPRASP1 was implicated in the degradation process *in vitro* and for which repeated activation by exogenous agonist administration *in vivo* were described to lead to adaptive responses including tolerance to anti-nociception or sensitization to hyperlocomotion [65;71]. In all cases, GPRASP1-KO mice showed a clear decrease in these adaptive responses (Table 1).

(i) Attenuated analgesic tolerance to chronic CB1R stimulation in GPRASP1-KO mice.

The development of analgesic tolerance is well known in the case of chronic stimulation of mu-opioid receptor with opiates. At the behavioural level, it corresponds to a decrease of the analgesia obtained by the same dose of the agonist molecule after several injections [80;81]. Activation of CB1R with agonists also leads to anti-nociception and to the development of tolerance upon chronic activation. In the central nervous system, GPRASP1 is expressed in the dorsal horn of the spinal cord, as well as CB1R, and highly co-expressed with this receptor in the dorsal root ganglia neurons, especially in the non-peptidergic small diameter nociceptive neurons, suggesting an involvement in analgesic modulation [71]. The role of GPRASP1 in CB1R analgesia and tolerance was tested by injecting the CB1R agonist WIN55,212-2 for seven consecutive days in GPRASP1 knockout mice. On day 1 and day 7, behavioural tests were performed to measure thermal anti-nociception, motor incoordination and hypothermia. On the first day, WIN55,212-2 induced similar effects in WT and GPRASP1-KO mice [70;71], except that the decrease of body temperature following agonist administration was very slightly reduced in GPRASP1-KO mice [71]. Thus, acute response to CB1R agonist was

almost unchanged in the absence of GPRASP1. After repeated agonist administration, tolerance to the WIN55,212-2 was detected in all behavioural tests in WT animals while there was no tolerance to the agonist in thermal analgesia and motor incoordination in GPRASP1-KO mice. This phenotype was correlated with the absence of degradation of the CB1R in GPRASP1 deficient animals (see II-A-3). GPRASP1 thus appeared as a key player in the mechanism of endogenous adaptation following chronic activation of CB1R. It is worth to note that tolerance to hypothermia still developed in GPRASP1-KO mice.

Very interestingly, Beclin2 heterozygous  $^{+/-}$  mice had also a decrease of the CB1R agonist tolerance correlated with a decrease in receptor degradation as measured by 14 days WIN agonist injection in a thermal anti-nociception assay [72]. Elegantly, the authors reasoned that if Beclin2 forms two distinct functional complexes, one with GPRASP1 that triggers lysosomal sorting of CB1R and one that participates in autophagy, one could manipulate GPRASP1/Beclin2 complex levels *in vitro* and *in vivo*. By using small compounds that they discover to induce early initiation of autophagy, the GPRASP1/Beclin2 complex was diminished as measured by co-immunoprecipitation in HEK293 cells and in mouse brain [72]. Finally, co-administration of these molecules, inducers of autophagy together with the CB1R agonist, had a reducing effect on the WIN-induced antinociceptive tolerance. These results suggest that one could reduce tolerance by inducing autophagy.

*(ii) Attenuated sensitized hyperlocomotor response to chronic cocaine treatment in GPRASP1-KO mice*

Drugs of abuse cause long lasting alterations in dopaminergic neurotransmission. Repeated exposure to psychostimulants promotes a progressive and stable enhancement of drug-induced hyperlocomotor stimulation. This phenomenon of “behavioural sensitization” is thought to underlie aspects of addiction in which dopamine receptors play an essential role. The other neuronal receptors which have been evaluated in GPRASP1-KO mice were therefore the dopaminergic receptors [65;66]. In the first study, hyperlocomotor activity induced by an acute cocaine administration was significantly reduced in GPRASP1-KO as compared to WT mice [66] while no difference was observed in the second study [65]. Conversely, both studies observed that upon chronic administration of cocaine, the sensitization to hyperlocomotor activity was significantly reduced in GPRASP1-KO as compared to WT

animals [66;65]. In a second model of cocaine self-administration we observed that the rate of acquisition of self-administration was significantly impaired in the absence of GPRASP1 but the reinforcing effects of cocaine were equivalent between WT and deficient mice that reached the threshold of acquisition [66].

The role of GPRASP1 on the D2R signalling response following repeated cocaine treatment was next explored by electrophysiological recordings on ventral tegmental area neurons [82]. Interestingly, the daily administration of cocaine for 5 days caused a desensitization of D2 receptors in the VTA neurons of WT mice but not in GPRASP1-KO mice. Therefore D2R signalling is restored in GPRASP1-KO mice [82].

Overall, if we consider the results of the different *in vivo* studies that were conducted with GPRASP1 deficient mice, it is tempting to speculate that inhibiting GPRASP1 could be beneficial to lower some of the adverse effects associated with chronic administration of GPCR agonists. While one might have expected some redundant effects between GPRASP1 and GPRASP2 due to their high sequence identity and some common identified protein partners, the GPRASP1-KO mice display a clear phenotype. In addition, GPRASP2 was not found overexpressed in GPRASP1 deficient mice excluding a putative compensation effect [66]. Rather unexpected functions for GPRASP2 in GPCR trafficking have recently come to light.

## **II-B. GPRASP2 in GPCR trafficking and *in vivo* functions**

*II-B-1. Interaction of GPRASP2 with Smoothed/Pifo: in vitro and in vivo effects on Smoothed targeting to the primary cilium.*

The primary cilium is a structure present in almost all mammalian cells including neurons [83]. It is a microtubule-based organelle that is erected from the plasma membrane with the basal body composed of the mother centriole of the centrosome. The primary cilia are not mobile and work as mechano-, osmo- and chemo-sensory units. Their role in the development and the maintenance of homeostasis is critical. Their dysfunction leads to different pathologies, called ciliopathies, which can lead to skeletal, neurodevelopmental and cognitive deficits, as well as to obesity. This vast array of potential phenotypic deficits is most probably due to the fact that signals passing through the primary cilium are numerous and necessary for the development of the cell and the organs. Several signalling pathways take place at the primary cilium such as Hedgehog and its receptor Smoothed, Wnt and its receptor Frizzled, PDGFR, Notch,

TGF- $\beta$ , mTOR. A number of GPCRs have been found targeted to the primary cilium as well [84]. For example, dopaminergic D1, D2 and D5 receptors, galanin GalR2 and GalR3, neuropeptide FF NFFR1, QRFPR, PRLHR and Kiss1R, serotonin 5-HT6R [85]. The role of GPCRs in the cilium activity and development and viability is crucial [86;87;88]. For example, serotonin receptor 5-HT6 and the dopaminergic receptors are needed for the primary cilium homeostasis.

The group of Dr. Heiko Lickert has identified GPRASP2 as a binding partner for Pifo (or Pitchfork; [39]). Having before discovered that Pifo is implicated in primary cilium disassembly with consequences on embryonic development [89], the authors conducted an interactome study of Pifo using co-precipitation followed by mass-spectrometry analyses in order to characterize further its molecular function. Pifo interacted and was co-localised with  $\alpha$ ,  $\beta$ ,  $\gamma$ -Tubulin and microtubule-dependent motor proteins. In addition, GPRASP2 was identified as a putative partner and the interaction was confirmed by GST pull-down assay ([39]; Table S1 and Figure 3). Since the Cilium Targeting Motif of some GPCRs has been identified as the cytoplasmic helix VIII, the same domain where GPRASP1 is proposed to bind to the C-tail of GPCRs, the authors tested whether Smoothened targeting to primary cilium was dependent on a GPRASP2/Pifo complex. HA-tagged GPRASP2 could co-precipitate Strep-His-tagged-Smoothened and inversely tagged-Smoothened co-precipitated GPRASP2. A mutant Smoothened receptor defective in ciliary targeting with mutations in its helix VIII C-tail 545W/546R did not co-precipitate with GPRASP2. Activation of Smoothened by SonicHedgehog (SHH) slightly increased GPRASP2 co-precipitation with the receptor. Pifo was only detected in co-precipitation with Smoothened if GPRASP2 was overexpressed and the interaction was increased in presence of SHH. The conditional Pifo knockout or GPRASP2 knock-down in ciliated mouse primary limb bud cultures did not affect their primary cilia morphology and assembly. But in Pifo knockout cells, rapid Smoothened targeting to the primary cilium upon SHH activation was reduced as seen by quantitative live-cell imaging of primary ciliated cells expressing RFP-Smoothened. In RNAi knock-down GPRASP2 cells, there was a reduction both in Smoothened and in Pifo targeting to the primary cilium. In conclusion, GPRASP2 bridges Smoothened and Pifo to target them in the cilium ([39]; Table S1 and Figure 3). Experiments also suggested post-translational modifications of GPRASP2 and Pifo upon Smoothened activation that stabilized the proteins and increased rapidly their quantities.

In a parallel study, Jung and collaborators reported the identification of ten small molecules already in clinical or pre-clinical development using a high-content screening for inhibitors of Smoothened targeting to the primary cilium [90]. All compounds were found to inhibit SHH signalling. Eight out of ten inhibitors were characterized as interfering with the Smoothened/GPRASP2 interaction. This was first seen by NMR studies to verify the direct interaction between GPRASP2 and C-tail Smoothened peptides (residues 539 to 552). The negative control peptide contained residues 545W/546R mutated that reduced GPRASP2 binding affinity. Then it was confirmed in cells by co-immunoprecipitation. These inhibitors have been proposed to have potential to treat drug-resistant Smoothened derived cancer forms as tested in pancreatic primary tumour cells [90].

Interestingly, a mutation in GPRASP2 was found in a five-generation Chinese family with an X-linked recessive syndromic hearing loss (SHL). The mutation (2 bp missense mutation c.1717\_1718GC>AA, p.A573N) was present in four hemizygous male patients and two heterozygous female carriers [91]. The male patients suffer from hearing loss with an abnormal auricular shape, dilated internal auditory canal and incomplete separation of cochlea basal turn from the fundus. They also display facial dysmorphism with bilateral ptosis (Table 1). GPRASP2 mRNA was expressed in numerous structures of the mouse cochlea. The authors suggested that the GPRASP2 deleterious mutation could be the cause of the disability. Since a number of ciliopathies present hearing loss (Usher syndrome, Bardet-Biedl syndrome, Alström syndrome) and even facial dysmorphisms (Joubert syndrome), and Hedgehog signalling is important for hair cell differentiation in the cochlea development for hearing ability [92], it would be interesting to test whether the GPRASP2 A573N mutation causes an alteration in the interaction with Pifo or with Smoothened. Of note, the residue A573 is conserved in the C-ter domain of GPRASP1 but not in the ones of GPRASP3 and ARM CX5 that share similarities in the peptidic sequence surrounding A573 of GPRASP2.

Since, Smoothened is not the only seven-transmembrane domain receptor targeted to the primary cilium, it would be interesting to analyse the function of GPRASP in this specific membrane trafficking pathway. Interestingly Pifo, like ARM C10, the ancestor of the GPRASP/ARM CX protein family, has appeared in evolution with chordates [89]. Of note as well, beta-arrestins are proposed to target Smoothened to the primary cilium

[93] but several redundant pathways seems to exist since the  $\beta$ -arrestin1 or  $\beta$ -arrestin2 KO mice do not appear to have Smoothened signalling-defective phenotypes.

*II-B-2. Behavioural consequences of GPRASP2 deletion in mice: autism spectrum disorder-like phenotype.*

In addition to the GPRASP2 A573N mutation that might be the cause of hearing loss in human, other GPRASP2 single nucleotide mutations have been identified in schizophrenic or autistic patients ([94;95]; Table 1). Considering that GPRASP2 interacts with a number of GPCRs *in vitro* (Table S1), GPRASP2-KO mice have been generated and investigated for neuro-developmental phenotypes [96].

At P20, GPRASP2 knockout mice did not seem to have mRNA compensation for any *GPRASP/ARMCX* genes in the brain (no amplification of *ARMCX5* mRNA). Behavioural evaluation of GPRASP2 deficient mice highlighted learning and memory deficits in those animals, as well as a decrease of preference index for social interactions and an increase of stereotypical behaviours (grooming, digging, scrabbling), corresponding to Autistic Spectrum Disorders (ASD) and Intellectual Disabilities (ID) like behaviours [96].

At the protein level, GPRASP2 was found highly expressed in hippocampus and hypothalamus. In hippocampus of GPRASP2 knockout mice, the CA1 pyramidal neurons morphology was altered with a reduction in dendritic arborisation and a decrease in total dendritic length in their distal regions. Considering the fact that (i) the metabotropic glutamate receptor mGluR5 contributes to synaptic plasticity and circuit development, (ii) in animal models of ASD and ID there is abnormal mGluR5-mediated synaptic plasticity and (iii) the deletion of the scaffolding proteins anchoring mGluR5 in the synapse caused perturbation of synaptic communication and ASD as well [97], Edfawy and colleagues have studied if mGluR5 can be found in complex with GPRASP2 and if a perturbation of mGluR5 signalling contributes to the phenotypes observed in GPRASP2-KO mice [96]. First, the effect of cellular variation of expression was studied in the mouse hippocampal neuronal HT-22 cell line. GPRASP2 and mGluR5 actually co-immunoprecipitated, and this association was stable after 30min of mGluR5 agonist treatment. Interestingly, 5 min agonist treatment increased the co-localisation of GPRASP2 with the lysosomal marker LAMP1 [96]. Second, when GPRASP2 was knock-down by shRNA in primary cultures of rat hippocampal neurons, neuronal complexity and total dendritic length were significantly reduced and it was accompanied by an

increase in mGluR5 cell surface expression as measured by immunolabeling. Using an mGluR5 antagonist on GPRASP2-deficient neurons, the reduction of arborisation was inhibited and the spine density recovered. After half an hour of agonist treatment, while there was significant degradation of mGluR5 levels in WT cells, there was no significant difference in total mGluR5 in shRNA-GPRASP2 primary rat hippocampal neurons. They concluded that a loss of GPRASP2 perturbs normal mechanism of receptor degradation upon agonist-mediated activation. Inversely, GPRASP2 overexpression increased neuronal dendritic arborisation and dendritic length and it was accompanied by a reduction in cell surface mGluR5 [96]. In hippocampal slices, patch-clamp observations revealed an enhanced mGluR-long-term depression in GPRASP2-KO mice versus WT littermates [96].

Altogether, these data suggest that the absence of GPRASP2 is linked to neurodevelopmental abnormalities, by increasing the surface availability of mGluR5 and reducing dendritic complexity, spine density and synaptic maturation. More generally there was a decrease in the post-synaptic density thickness and in the amplitude of the excitatory post-synaptic potentials [96].

### **II-C. Human Xq22.1 1.1Mb deletion encompassing several *GPRASP/ARMCX* genes : phenotype and mice models**

#### *II-C-1. Mental retardation and congenital abnormalities in Xq22.1 deletion in human*

A human deletion of 1.1Mb on the X chromosome was described to encompass four genes of the ARCMX/GPRASP protein subfamily 1. A mother and her daughter had a missing sequence of 12 genes *NXF5*, *ZMAT1*, *TCEAL2*, *TCEAL6*, *BEX5*, *NXF2*, *TMSB15A*, *NXF4*, *ARMCX5*, *GPRASP1*, *GPRASP2*, and *GPRASP3* [7]. An interesting point is that a son had died 15 days after birth from a breathing failure. The microdeletion was associated with many physical and behavioural symptoms. The patients were described with short stature, facial dysmorphisms (small forehead, smooth philtrum and low ears) brachycephaly, coarse face, hypertrichosis, distal muscle atrophy and severe muscle hypotonia. At the neurological level, the mother showed mental retardation, epilepsy and borderline personality disorder while the daughter had ASD with stereotypic movements and self-mutilation behaviour. The daughter showed also asphyxia at birth and a cleft palate was surgically corrected at 1 year of age. The authors suggested a role

of NXF5 gene in the symptoms but recognized that other deleted genes might contribute as well.

Next, a similar deletion of 17 genes, of which seven genes belong to the ARM CX/GPRASP/ARM CX protein family, was identified in a 25-weeks old male infant (*ARM CX6*, *ARM CX3*, *ARM CX2*, *NXF5*, *ZMAT1*, *TCEAL2*, *TCEAL6*, *BEX5*, *NXF2*, *NXF2B*, *TCP11X2*, *TMSB15A*, *NXF4*, *ARM CX5*, *GPRASP1*, *GPRASP2*, and *GPRASP3*, [98]. He had dysmorphic features, hypotonia with spasticity, enlargement of frontal temporal lobe, but also respiratory failure with thoracic insufficiency syndrome. The mother of the patient had intellectual disability that could not be further examined.

*II-C-2. Mice models of the human Xq22.1 1.1Mb deletion: implication of ARM CX5, GPRASP1, GPRASP2 and GPRASP3 in epilepsy, respiratory failure, postnatal development delay and cleft palate.*

To elucidate the genes responsible for the abnormalities observed in the human patients with the Xq22.1 1,1mb deletion, similar deletions were characterized in mice [99;100]. A first deletion, called DelA, was designed to correspond to the entire Xq22.1 1.1Mb deletion between *NXF2* and *NXF3* gene described by Grillo and collaborators with 20 genes deleted. A major obstacle encountered was that the males were not viable. Neonatal death due to an acute respiratory failure was caused by a delayed in lung development. Another specificity observed on male fetuses was a cleft palate [100]. Concerning females, only heterozygous survived, with a ratio of 45% of neonatal lethality and only a quarter survived to childhood. A general growth delay compared to wild-type littermates was observed after birth and epilepsy. A rare part exhibited a cleft palate. To narrow down the critical genes giving these phenotypes, two deletions were further generated, DelB that encompasses *Nxf2* to *Tmsfb15a* and a 0.35Mb deletion DelC, which includes *ARM CX5*, *GPRASP1*, *GPRASP2* and *GPRASP3* genes and four genes with no corresponding orthologs in the human Xq22.1 deletion: *Arxes2*, *Arxes1*, *Bex2*, and *Nxf3*. The DelB mice showed a normal viability and no epilepsy or cleft palate, whereas the DelC males had respiratory failure, neonatal lethality and cleft palate and heterozygous female develop epilepsy, partial post-natal lethality, post-natal developmental delay and cleft palate in a similar ratio than the Xq22.1 human mimicking deletion DelA [100]. The variability of phenotypes in individual DelA and DelC heterozygous females is likely attributed to skewed X inactivation. The authors concluded that *ARM CX5*, *GPRASP1*,

*GPRASP2* and *GPRASP3* were the putative genes for which deletion causes these defects observed in human patients as well [7;98].

Considering that *GPRASP1*-KO mice, in a global aspect, had no abnormalities and a normal male for female ratio, that histological studies revealed no alteration of the nervous system and normal behaviour in home cage conditions [66], *GPRASP1* is most likely not involved in the phenotypes observed in the *DelC* deletion. Only a slight impairment in procedural memory tasks was described [101]. In their model of *GPRASP1*-KO mice, Thompson and collaborators did not observe any difference either in locomotor response, in motor coordination or other motor functions compared to wild-type littermates [65]. In the case of *GPRASP2*-KO mice, the mutant mice were viable and had a normal sexual ratio [96]. On the other hand, the ASD observed in human could be associated to *GPRASP2* since *GPRASP2*-KO mice display such syndromes [96]. Cleft palate has not been reported for the *GPRASP2*-KO mice despite the fact that both Hedgehog signalling [102] and Pif0 [103] have been hypothesized to be implicated in palate development. There are no *ARMCX5*-KO or *GPRASP3*-KO mice described so far. The team of Dr. Klaus Heese reported as unpublished data that only female of *GPRASP3*-KO mice had been obtained [104]. As neither *GPRASP1*-KO nor *GPRASP2*-KO have been reported to display deficit in lung development, it would be interesting to study the role of *ARMCX5* and *GPRASP3* in this process.

#### **II-D. Perspectives for *GPRASP/ARMCX* subfamily 1 : targeting GPCRs in the right place at the right time**

*GPRASP1* and *GPRASP2* together with their novel protein partners are the two proteins of *GPRASP/ARMCX* subfamily 1 that have been best characterized. Cellular and animal models, displaying molecular and behavioural consequences, gave very strong evidence of their role in GPCRs targeting to different organelles, endosomal sorting for both proteins and Smoothed targeting to primary cilium concerning *GPRASP2*.

In large interactomics studies, GPCRs have not been found in interaction with *GPRASP/ARMCX* proteins (Table S1). The main reasons could be technical: GPCRs are not abundant membrane proteins and could therefore fall under the threshold defined to select major binders using mass spectrometry approaches. In addition, the interactions might be with low affinity but high avidity due to multiple binding sites of the interacting repeats. One could generalize and expect that *ARMCX/GPRASP* subfamily

1 might be part of several multiprotein complexes with all their interactants not being much enriched in the pool identified by the criteria used in mass spectrometric analysis. The interactions of GPRASP/ARMCX proteins with GPCRs seem constitutive and not much change has been reported upon signalling activation. Only GPRASP2 has been described to become stabilized due to an uncharacterized post-translational modification upon Smoothened activation and enriched at the lysosomal membrane [39]. Clearly, further studies on the regulation of proteins of the GPRASP/ARMCX subfamily 1 are needed.

It has been firmly established that GPRASP1 contributes to the development of some or most adaptive processes following chronic activation of at least two GPCRs *in vivo*, D2R (sensitization to hyperlocomotion) and CB1R (tolerance). It probably does so in serving as a scaffolding protein that assembles heterotrimeric G $\alpha$ S subunit together with the ESCRT-O complex protein HRS and at least dysbindin (for D2R) or Beclin2 (for CB1R) around the GPCR to be targeted to the lysosome. Nevertheless, while it seems probable that GPRASP1 contributes to lysosomal targeting after activation in cells overexpressing GPCRs, when receptors are endogenously expressed, the effect of the lack of GPRASP1 on the total amount of receptor or at the cell surface is less convincing. Indeed, at steady state in GPRASP1-KO mice, total number of D2R in the striatum were either diminished [65] or unaffected [66] and total CB1R amount in the spinal cord were diminished but not in the cerebellum [71]. After chronic activation total striatal D2R were less degraded [65] or more degraded [66], total CB1R were less degraded [71]. Concerning endogenously expressed CXCR4 in cells, depletion of either GPRASP1 or GPRASP2 increased steady state CXCR4 cell surface expression [74]. On the other hand, although the amount of endogenous GLP1R at steady state was not measured, its signalling was decreased upon knock-down of GPRASP1 suggesting a diminution of active GL1PR. Since it has been discovered during the last decade that GPCR signalling can also come from intracellular compartment including endosomes [105], it would be interesting to investigate whether GPRASP1 also serves as a scaffolding protein for signalling at the level of endosomes.

GPRASP2-KO mice have autistic spectrum disorder-like phenotype at least partly due to an increase of mGluR5 cell surface expression in hippocampal neurons. Since GPRASP2 acts as a scaffold to connect Smoothened to Pifo and directs the receptor to the

primary cilium, it would be interesting to investigate whether GPRASP2 could be implicated in targeting some GPCRs to other organelles or plasma membrane specialized compartments than to primary cilium. Since a number of ciliopathies causes ID, one could also test if targeting of Smoothed or other GPCRs to the primary cilium is altered in these GPRASP2-KO mice.

If human pathologies seem to put in light a major involvement of the other members of the subfamily 1, ARMCX5 and GPRASP3, further studies are limited by the lack of knockout mice. Yet, the putative lethality of GPRASP3 constitutive deletion in mice is a clue for a major implication of this protein in normal developmental processes.

### **III. GPRASP/ARMCX subfamily 2: ARMC10-AMRCX proteins and their associated proteins Miro/Trak2 motor complex and MFF/Fis1/DRP1 fission complex in mitochondrial trafficking**

The main functions of ARMC10-ARMCX proteins that have been studied are linked to mitochondrial trafficking along microtubules in complex with mitochondrial Rho-GTPase proteins MIRO associated with TRAK2 (Figure 3) in order to regulate neuronal proliferation and survival and neurite outgrowth (Table 2).

#### **III-A. Mitochondria dynamics**

Mitochondria within the cells play important roles as producer of ATP, controlling intracellular calcium, mediating apoptosis and being the centre of many metabolic pathways. Mitochondria are dynamic and can adopt multiple shapes, being remodelled by both fusion and fission events governed by GTP-dependent dynamin related proteins. While fusion is proposed to be protective and anti-apoptotic probably by allowing the mixing of components in between damaged and healthy mitochondria, fission of damaged mitochondria precede their degradation by mitophagy and can precede apoptosis.

The proteins Mitofusin MFN-1 and MFN-2 form homo- and hetero-dimers, and their conformations are changed by GTP hydrolysis to fuse the outer mitochondrial membrane [106].

The regulator of mitochondrial fission is Dynamin-related Protein (DRP1). In a dynamin-like manner, DRP1 forms a spiral around the fission site, and GTP hydrolysis

reduces helical pitch and occurs at the endoplasmic reticulum (ER)-mitochondria contact sites. There are four DRP1 adaptor proteins that have been identified on the outer mitochondrial membrane: mitochondrial fission factor (MFF), mitochondrial dynamics proteins of 49 kDa and 51 kDa (MiD49 and MiD51) and Fission 1 (FIS1; [106]).

Mitochondria also need to be targeted to different parts of the cell for example during mitosis or where energy is most needed [107;108;109]. In neurons, pre- and post-synaptic domains, the axon initial segment, nodes of Ranvier and growth cones are enriched in mitochondria. Their movements occur along the microtubules for long distances or along the actin cytoskeleton [110]. In mature cultured neurons, one third of axonal mitochondria are mobiles. The mobile mitochondria can move bi-directionally [111]. Axonal microtubules have their plus end towards the distal part and dendritic microtubules have mixed polarities. MIRO proteins are specific integral mitochondrial adaptors from the outer membrane that recruit microtubules kinesin plus-end motors via their binding to the kinesin binding homologs TRAK1 and TRAK2 [112;113;114]. There are two MIRO family members in mammals, MIRO1 and MIRO2 that share around 60% identity. These proteins are composed of two Rho-GTPase domains that surround two EF-Hand calcium-binding domains. TRAK1 and TRAK2 interact with the first GTPase domain of MIRO. The mitochondria transport can be regulated mainly by ATP/ADP levels and by calcium. Increase in calcium can stop mitochondrial movements and the EF-hand domains of MIRO play probably a major role in this regulation.

There is a strong cross-talk between mitochondria transport and their fission-fusion dynamics and obviously, these mechanisms are finely tuned by extracellular signals such as neurotransmitters and growth factors. Mitochondrial dynamics also coordinate cell differentiation and growing evidence demonstrates that mitochondria could act as a centre of intra and extracellular signals to coordinate signalling pathways, such as Notch, Wnt, and YAP/TAZ during development [109;115].

### **III-B. Role of MIRO/TRAK2 motor complex and DRP1/Fis1/MFF fission complex with ARMC10, ARMCX3 and ARMCX1 on mitochondrial dynamics**

MIRO1 in complex with TRAK2 interacts with ARMC10 [5], ARMCX3 [3] and ARMCX1 ([21]; Table S1). The interaction, at least for ARMCX3, is lost at high calcium concentration (2mM) and a mutant MIRO1-myc-tagged lacking its two EF-Hands calcium binding motifs still co-immunoprecipitates with ARMCX3-GFP but this

interaction becomes insensitive to intracellular calcium level, thus confirming a regulation by calcium ions [3].

The seminal article on the function of ARM CX proteins at the outer mitochondrial membrane comes from the group of Dr. Eduardo Soriano who studies new genes involved in brain development and neuronal plasticity ([3]; Table 2 and Figure 3). Two main phenotypes on mitochondria were found upon expression of ARM CX proteins: perinuclear aggregation and effect on the motile pool of mitochondria in neurites.

Upon myc-tagged ARM CX3 (but also ARM CX1, ARM CX2, ARM CX6 and ARM CX10) protein overexpression in HEK293 cells and in primary hippocampal neurons, the mitochondrial network was systematically altered. The mitochondria were clustered and for 30 % of the HEK293 cells and 23% of neurons expressing ARM CX3, mitochondria converged in one big perinuclear aggregate [3]. The severity of the phenotype was correlated with the level of expression of ARM CX3. Mitofusin1 overexpressing cells have been reported to display this phenotype as well. In neurites, mitochondria were also longer and larger. Nevertheless, no change in the rate or dynamic of mitochondria fusion was detected using photoactivable mitofusin1 in cells overexpressing or with down-regulation of ARM CX3. The aggregation phenotype was also found to be independent on the interaction with MIRO1, since down-regulation of MIRO1 did not block the phenotype [3] and since the overexpression of the N-ter extremity of ARM CX3 alone (1-30 AA) was sufficient to trigger mitochondrial aggregation [24].

A second phenotype was found in neurons overexpressing ARM CX3: a reduction of the percentage of moving mitochondria, in their velocity and distance covered, both in anterograde and retrograde transport [3]. ShRNA against AMRCX3 in neurons caused smaller mitochondria in neurites, a reduction in number of moving mitochondria both in their anterograde and retrograde transport, without affecting the velocity or the distance run by each individual mitochondria. The reduction in the motile fraction of mitochondria is thus observed in both overexpression and down regulation conditions. In this initial work, it was not reported if the effect on the mobile fraction of mitochondria was dependent on MIRO/Trak2 complex [3].

Very importantly, other mitochondrial functions were tested in this experimental model of overexpression of myc-tagged ARM CX3: no effect was found on oxygen

consumption or DNA copy number in HEK293 cells, and on COX/citrate synthase ratio and mitochondrial membrane potential in neuronal cells. Using mitochondria-targeted aequorin/GFP fusion protein to measure mitochondrial calcium concentration, no effect of ARMCX3 overexpression was found on calcium uptake into mitochondria triggered by calcium released from ER stock or from digitonin permeabilized plasma membrane [3]. Since the elevation of calcium triggers the loss of interaction of ARMCX3 with MIRO, it is maybe difficult to test the effect of ARMCX3 on mitochondrial calcium homeostasis.

The same research group published similar but weaker phenotypes concerning the ARMC10 protein overexpression ([5]; Table 2 and Figure 3): mitochondrial aggregation was strong in HEK293 cells but weaker in neurons as compared with the previous reported effect of overexpressing ARMCX3. However, the level of expression of the two proteins was not compared between the studies. The mitochondria were also slightly larger in ARMC10 overexpressing cells. Concerning the percentage of motile mitochondria in axons of ARMC10 overexpressing hippocampal neurons, as for ARMCX3, there was a marked decrease. Velocity of the moving mitochondria were not modified contrary to overexpression of ARMCX3 despite the fact that ARMC10 also interacts with the MIRO/Trak2 complex. Again, overexpression and downregulation of ARMC10 lead to the same phenotype of mitochondria motile fraction: shRNA against all the messengers encoding ARMC10 also reduced the pool of motile mitochondria in axons [5].

Independently, in an elegant study in HEK293T and U2OS cells, ARMC10 was found to play a role in mitochondria dynamics but the results and the protein partners described are somehow the opposite ([22]; Table 2 and Figure 3). Although an aggregation phenotype was observed, the effect of the most abundant form of wild-type ARMC10 (the short isoform B of 308 residues) overexpression was found to drastically increase mitochondrial fission. The protein partners of ARMC10 identified are the crucial fission complex proteins DRP1, its recruiting protein FIS1 and its regulator MFF. Although an interactome of ARMC10 was conducted in HEK293T cells with a BioID2-tagged ARMC10 and identification by BioID2-mass spectrometry, apart from the mitochondrial fission complex, the entire results have not been disclosed. Therefore, the interaction with the MIRO/TRAK2 complex cannot be confirmed from this article [22]. The fraction of motile mitochondria were not analysed in this study either. The

difference between the two studies is that it is not the same ARMC10 isoform that was used for the functional studies: full-length isoform A [5] or ARMC10 isoform B that lacks residues 47-81 from the NH<sub>2</sub>-terminal region [22]. Concerning the aggregation phenotype however, the two studies led to convergent results reporting a small fraction of mitochondrial aggregation in overexpressing ARMC10 U2OS cells. Chen and collaborators analysed it further by looking at mitophagy upon perturbation of cellular energy homeostasis [22]. Indeed, degradation of mitochondria by autophagosomes could occur in steps, first of fission, then aggregation leading to mitophagy. Using microtubule-associated protein 1 light chain LC3 staining for autophagosomes, LC3 puncta were co-localised with the mitochondrial TOM20 marker only in ARMC10 overexpressing U2OS cells and not in ARMC10 deleted cells [1]. The authors concluded that ARMC10 might play a role in the transition to mitophagy.

Finally, ARMCX1 overexpression in explant cultures of adult mice retinal ganglion cells and in cultured embryonic cortical neurons was found to increase the mitochondrial motile fraction and the moving frequency of the entire pool of mitochondria probably by recruiting stationary mitochondria into the motile pool [21]. This effect required ARMCX1 localisation to the organelle since the overexpression of ARMCX1 lacking its TM targeting domain did not show any effect.

ARMCX1 seems thus to have the opposite effect than ARMC10 and ARMCX3 on the motile mitochondria dynamics in neurites when overexpressed. To our knowledge, experiments have not been performed to investigate whether this opposite effect is due to differences in the calcium dependency of the interaction between ARMC10 or ARMCX1 with the MIRO proteins for example.

### **III-C. ARMC10/ARMCX proteins are effectors of receptor signalling converging on mitochondria in order to regulate neurites outgrowth, cell proliferation and survival.**

In neurons, where ARMC10/ARMCX proteins are particularly enriched, mitochondria are transported along the axon, sometimes for long distances, in order to provide energy and calcium buffering. The mitochondria concentrate into the active growth cone when the axon is growing, implicating that mitochondrial transport is regulated in order to sustain axonal growth [116].

In the context of nerve injury, researchers have developed model of neuronal cell cultures with high axonal regenerative capacity. This is the case for the group of Dr Zhigang He [117;118]. Comparing the gene expression profile between injured adult retinal ganglion cells (RGCs) with poor regenerative ability or those mutated in PTEN/SOCS3 that have high regenerative ability, none of the genes previously known to regulate mitochondrial transport such as MIRO proteins were found to be modulated. But ARM CX1 was upregulated in PTEN/SOCS3 RGCs three days after injury [118;21]. In the subsequent in depth study, ARM CX3, ARM CX5 and ARM CX6 were not found to be upregulated in these conditions [21]. In addition to promoting mitochondrial movement, mitochondrially localised ARM CX1 triggered an increase in the number of axons growing out of adult retinal PTEN<sup>-/-</sup> explants as assessed by immunolabelling of axons with the Tuj1 labelling (not observed with non mitochondrial ARM CX1 deleted from its TM domain). ARM CX1 overexpression also significantly increased the average length of the longest neurites of embryonic cortical neurons. In a model of axon and neuronal regeneration from wild-type adult RGCs after an optic nerve crush injury, mitochondrially localised overexpressed AMRCX1 induced a significant higher number of regenerating axons after 15 days. A 45% increase in neuronal survival was found with ARM CX1 wild-type but not ARM CX1 deleted from its TM domain ([21]; Table 2). Finally, ARM CX1 knock-down reduced axon regeneration and neuronal survival in the model where it was found overexpressed (i.e. in injured adult retinal ganglions cells RGCs mutated in PTEN/SOCS3 that have high regenerative ability). This phenotype was correlated with the effect on reduced motile mitochondria [21].

Several proteins regulating mitochondria dynamics are mutated in familial forms of neurological degenerative diseases (Charcot Marie-Tooth disease, Parkinson, Alzheimer and Huntington diseases). Mutations in mitofusin Mfn2 protein are the most common cause of the axonal form of the neurological disorders observed in Charcot-Marie-Tooth disease, triggering an increase in mitochondrial fusion [106]. Loss of Parkin or its partner PINK1 results in an early-onset form of hereditary Parkinson's disease. The E3 ligase Parkin ubiquitinates the mitofusins MFNs that are then degraded by p97 in a proteasome-dependent manner. This is required for mitophagy. Alteration of mitophagy could then contribute to the loss of dopaminergic neurons in Parkinson diseases [106]. The amyloid beta fragment of Alzheimer disease has been described to induce mitochondrial fission that is believed to contribute to neuronal dysfunction and

death. While indeed, treatment of hippocampal neurons in culture with 2 $\mu$ M of Abeta induced mitochondrial fragmentation, overexpression of isoform A of ARMC10 completely abolished this effect and induced a significant reduction (66%) in the number of neuronal cell death [5].

ARMC10 has also been convincingly found to be a substrate of the 5'AMP-activated protein kinase AMPK to regulate mitochondrial dynamics [22]. AMPK is a heterotrimeric kinase (twelve possible combination of isoforms) that is a sensor of AMP/ATP or ADP/ATP ratios and thus of cell energy level. Upon activation, it switches on the production of ATP and switches off its consumption. It can also be regulated by several signalling pathways receiving positive inputs, for example, from Liver-kinase B1 (LKB1) and negative inputs for example upon increase of intracellular calcium via the phosphatase PP2A or of cAMP via the Protein Kinase PKA [119;120]. In condition of prolonged energy stress, AMPK triggers mitochondria fission and then mitophagy. In a search for novel AMPK substrate, the global phosphoproteome was compared between wild-type HEK293 cells and double knockout for the two isoforms of the catalytic subunits of the AMP kinases, AMPK $\alpha$ 1 and AMPK $\alpha$ 2. Treatment with the activator of AMPK, triggered more mitochondria fission in WT HEK293 cells than in double KO AMPK1 $\alpha$ 1/2 cells confirming that AMPK stimulates mitochondria fission. 109 phosphosites with higher phosphorylation in WT cells after treatment with the AMPK activator A769662 as compared with double-KO cells were identified, 32 of which were within an AMPK consensus motif suggesting direct phosphorylation by AMPK. Two different peptides with phospho-Serine 45 of AMRCX10 were detected and the significance of this phosphorylation was further studied on mitochondrial fusion/fission dynamics ([22]; Table 2 and Figure 3).

Upon transient expression either of the most abundant form of wild-type ARMC10 (the short isoform B of 308 residues) or of an ARMC10B S45E mimicking S45 phosphorylation, mitochondria fission was more increased with the ARMC10B S45E than with the WT-ARMC10B [22]. In U2OS cells lacking ARMC10 via CRISPR-Cas9 technology, the treatment with the AMPK activator A769662 failed to increase mitochondria fission, the fission phenotype could be rescued by overexpression of either WT-ARMC10B or S45E mutation but not by overexpression of ARMC10B S45A. Surprisingly, this absence of mitochondria fission in ARMC10 knockout HEK293 or U2OS

cells contributed to a defect of cell survival after glucose starvation, rescued by ectopic expression of WT-AMRC10B but not of ARMC10B S45A. Phosphorylation of ARMC10B by AMPK that triggers mitochondrial fission thus seems necessary for cell survival upon glucose starvation [22]. It would be interesting to study the effect of the ARMC10B S45 phosphorylation on the regulation of mitochondrial motile fraction in neurons, since ATP/ADP levels can regulate mitochondria movements.

Of note, in the original study of the discovery of ARMC10 isoforms, this particular isoform B of AMRC10 has been implicated in cancer progression [4]. An up-regulation of this variant by RT-qPCR was detected in 60% of the patient samples with hepatocellular carcinomas (28 out of 46). Overexpression of ARMC10B in human liver cell line QSG-7701 seemed to trigger an accelerated growth rate and tumorigenicity in nude mice although no statistics was given neither the numbers of mice tested. Finally, depletion of ARMC10 by antisense oligonucleotides in hepatoma cell line BEL-7404 induced apoptosis ([4]; Table 2). Therefore, there could be a link between cell growth rate and mitochondrial dynamics regulation by ARMC10 localised at the outer mitochondrial membrane. Since other ARMCX protein levels are dysregulated in cancer [121;122;123] their mitochondrial functions could be involved.

Wnt activation of the seven-transmembrane receptor Frizzled regulates organ development and aberrant signalling of this ligand/receptor pair triggers tumorigenesis. Several papers have started to look at the possible roles of mitochondrial ARMC10 and ARMCX3 in neuronal development but these attempts need further analysis. Briefly, the non-canonical Wnt, through PKC signalling, has been proposed to regulate ARMCX3 degradation leading to altered mitochondrial dynamics. The half-life of ARMCX3 in presence or absence of Wnt over-secretion was not measured but total overexpression of myc-tagged ARMCX3 was reduced upon co-expression with Wnt-1 ligand or its receptor Frizzled2 in HEK293 cells [5]. The authors observed that activation of the PKC kinase by TPA blocks the Wnt effect of diminishing total ARMCX3 overexpression but no direct phosphorylation of the protein was tested.

To further analyse the potential roles of ARMC10/ARMCX proteins in early neural development, the group of Dr. Soriano chose the chick embryo spinal cord developmental model ([124]; Table 2). Birds including chicken, express only the ARMC10 ancestor protein. According to the Hamburger and Hamilton 46 chronological

stages of chick embryo development, ARMC10 was found to be expressed in the chick embryo using in situ hybridization at HH12 (low expression), at HH19 (preferential expression in the dorsal regions) and HH24 stages in mitotically active neural precursor cells. The riboprobe for hybridization used did not distinguish between the isoforms. ShRNA directed against ARMC10 were electroporated at HH12 embryo's stage and, in the neural tubes only a slight decrease of 30% in cells dividing was found, as labelled by incorporation of BrdU, suggesting that ARMC10 isoforms are required for neuronal cell progenitor proliferation [124]. Then, comparison was performed between overexpression of the full length murine ARMC10 or ectopic overexpression of murine ARMCX3: forced ARMC10 or ARMCX3 expression reduced progenitor cells proliferation while only forced ectopic ARMCX3 expression but not ARMC10 expression promoted neural maturation. Expression of ARMCX3 deleted of its 12 first residues necessary for mitochondria localisation had no effect showing that ARMCX3 mitochondrial localisation is required to control progenitors proliferation and neuronal differentiation. The data suggest that tight regulation of the amount of ARMC10 can guarantee normal progression through the cell cycle. The authors also reported an inhibitory effect of overexpression of murine ARMC10 or ARMCX3 on the WNT/beta-catenin-induced Tcf/LEF transcriptional activity and inversely an activation effect upon down-regulation of endogenous ARMC10. Nevertheless, the effect was observed both on basal activity and following beta-cateninCA induction, so it is not an effect directly at the transcriptional level and it is not clear what the mechanism is [124].

Of note, ARMCX3 was found convincingly to interact with the developmental regulatory transcription factor Sox10 ([20]; Table 2 and Figure 3), which is implicated in neuronal differentiation. These two proteins are co-expressed in mouse brain and spinal cord, in OBL21 cells, in C6 rat glioma and in Hela cells while their mRNA is not expressed in Neuro-2A cells. The interaction that takes place between the C-ter domain of ARMCX3 and the first 100 residues of Sox10 was found to have two main effects *in vitro*. Ectopic overexpression of myc-tagged wt-ARMCX3 in Neuro-2A cells increased the mitochondrial localisation of Sox10. This was not the case when myc-tagged ARMCX3 deleted from its C-ter domain was expressed. In addition, the transcriptional activity of Sox10 as measured with a reporter promoter was increased only when the ARMCX3 interacting domain with Sox10 was present [20].

Since the interaction with Sox10 occurs via the conserved C-ter domain of GPRASP/ARMCX proteins, it might seem interesting to study further the potential interactions between the two families of proteins with the reserves that the first 100 residues of Sox10 that interact with ARM CX3 are localised before the conserved High Mobility Group HMG domain of the Sox proteins.

#### **III-D. Perspectives for GPRASP/ARMCX subfamily 2: under studied regulators of mitochondria dynamics**

ARMC10, ARM CX1, ARM CX3 are the three proteins of the subfamily 2 together with their mitochondrial protein partners that have been best characterized. The aggregation of mitochondria that has been reported upon their overexpression could be linked to mitophagy but it needs further study and at least in the case of AMRCX3, it is independent of MIRO1 interaction (although MIRO2 role in depleted MIRO1 cells was not investigated).

While overexpression of ARMC10 and ARM CX3 leads to a diminution of the pool of mitochondrial motile fraction, inversely, overexpressed ARM CX1 increases this pool with implication in axon regeneration. This phenotype is probably linked to interaction with the MIRO/TRAK microtubules adaptor complex. Interestingly, the roles of MIRO1 and MIRO2 have been recently challenged since deletion of MIRO1 alters retrograde mitochondrial movement but co-deletion of MIRO1 and MIRO2 does not alter kinesin-TRAK-directed mitochondrial movement along microtubule (anterograde movement), it affects coupling of mitochondria to the actin cytoskeleton [114]. Further studies on GPRASP/ARMCX subfamily 2 are needed to better characterize their function and their putative redundancy in regulating mitochondria dynamics.

Interestingly, MIRO clusters were recently shown to link cristae organization to the mitochondrial transport machinery but also to regulate ER-mitochondria contact sites where fission takes place [26]. MIRO proteins are also implicated in mitotic redistribution of the mitochondrial network to regulate cell proliferation [112;114]. A role in the regulation of mitochondrial fission could at least partly explain why subfamily 2 has been implicated in cell division and cancer progression. ARM CX1, ARM CX2 and ARM CX3 were originally named ALEX for ARM-protein-Lost-in-Epithelial-Cancers-on-chromosome-X [121;122;123]. The ARMC10B isoform, implicated in cancer progression, seems to recruit directly mitochondrial fission proteins in addition to

associate with the MIRO complex [4;22]. A striking post-translational modification of ARMC10B in response to energy level through the AMPK has been precisely described and it is likely that other signalling pathways converge on ARMC10/ARMCX proteins to regulate mitochondria dynamics [22].

Can mitochondrially targeted proteins of GPRASP/ARMCX subfamily 2 bind to GPCRs like subfamily 1 does? Although GPCRs can be found in this organelle [125;126;127;128] they are proposed to have the ligand-binding site facing the cytosol and their C-tail facing the inter-membrane space of the mitochondria. Clearly, AMRCX4 belongs to subfamily 1 and contains GASP motifs implicated in GPCRs interaction together with the carboxyl-terminal domain but it shares with subfamily 2 a transmembrane-targeting domain that, considering its sequence homology, probably function as a signal for mitochondrial localisation. One could thus speculate that, even transient, contact sites between mitochondria and other organelles could allow some interaction between the mitochondria localised GPRASP/ARMCX proteins and GPCRs, in the proper orientation with their C-tail available in the cytoplasm. Moreover, ARM CX1 was shown to display low interaction with the C-tail of some GPCRs in *in vitro* GST-Pull down assay [14].

Interestingly, ARM CX1 together with GPRASP2 was shown to be implicated in the regulation of hematopoietic stem and progenitor cells [73]. At the opposite of most of the other targets, a knock-down of ARM CX1 or of GPRASP2 increased the HSPC repopulation of the bone marrow. But the functions of the two proteins could be distinct because, as reviewed, the lack of GPRASP2 was further shown to contribute to enhanced cell survival while in contrast, knock-down of ARM CX1 had no significant effect on cell cycle or apoptosis [73].

Despite the fact that mitochondria dynamics can be altered in diseases such as age-related neurodegeneration, there is so far no mutation described in human families related to subfamily 2 of GPRASP/ARMCX proteins. Interestingly, in the male infant reported with the Xq22.1 1,1Mb deletion by Cao and collaborators *ARM CX6*, *ARM CX3*, *ARM CX2* from subfamily 2 were among the 17 deleted genes [98]. Hypoglycemia, umbilical hernia, tracheomalacia, and laryngomalacia are abnormalities that were not described in the mother and daughter carrying the 1.1Mb deletion with intact *ARM CX6*, *ARM CX3* and *ARM CX2* genes [7].

## **FINAL CONCLUSION**

In conclusion, GPRASP/ARMCX proteins formed two distinct subfamilies with GPRASP1/GPRASP2 from subfamily 1 clearly implicated in GPCRs membrane trafficking and ARMC10/ARMCX3/ARMCX1 from subfamily 2 implicated in mitochondria trafficking and dynamics. Future work is needed at the molecular, cellular and animal levels to understand better their implication in pathophysiological conditions such as ciliopathies, cancer, neuronal development, age-related neurological development and chronic treatment with drugs activating GPCRs.

### **List of Abbreviations**

5-HT<sub>6</sub>, 5-HT<sub>7</sub> = serotonin receptor 6 , 7

AAV = adeno-associated virus

ADRB1 = beta1 adrenergic receptor,

ALEX = ARM-protein-Lost-in-Epithelial-Cancers-on-chromosome-X

AMPK = 5'AMP-activated protein kinase

ARMC = armadillo repeat-containing protein

ARMCX = Armadillo repeat-containing X-linked proteins

ASD = Autistic Spectrum Disorders

ATP = Adenosine triphosphate

BEL-7404

BEX = Brain-Expressed X-linked

bHLHB9 = basic helix-loop-helix domain containing class B, 9

BLOC-1 = biogenesis of lysosome-related organelles complex-1

C-tail = cytoplasmic carboxyl terminal domain of GPCR

C-ter = Carboxyl-terminal

CALCR = calcitonin receptor,

CB1 = CNR1 = cannabinoid receptor type 1

CB2 = CNR2 = cannabinoid receptor type 2

CCTOP= Constrained Consensus TOPology prediction server

cGASP = domain from residues 899 to 1395 of GPRASP1

COX = cyclooxygenase

CREB = cAMP response element-binding protein  
CXCR4 = C-X-C chemokine receptor type 4  
D1R = dopaminergic receptor type 1  
D2R = dopaminergic receptor type 2  
D3R = dopaminergic receptor type 3  
D4R = dopaminergic receptor type 4  
D5R = dopaminergic receptor type 5  
DNA = deoxyribonucleic acid  
DOR = delta-opioid receptor  
DRP1 = Dynamin-related Protein 1  
EEA1 = early endosome antigen 1  
ER = endoplasmic reticulum  
ESCRT = endosomal sorting complexes required for transport  
FIS1 = Fission 1  
FZ4 = frizzled 4 receptor,  
GalR2 , GalR3 = galanin receptor 2, 3  
Gas = stimulatory alpha G protein  
GFP = Green fluorescent protein  
GLP1R = glucagon-like peptide receptor 1  
GPCR = G protein coupled receptor  
GPR55 = cannabinoid related receptor  
GPRASP = GPCR associated sorting protein  
GST = Glutathione S-transferase  
GTP = Guanosine triphosphate  
GTPase = guanosine triphosphate hydrolase  
HEK293 = human embryonic kidney cells  
HRS = HGF-regulated tyrosine kinase substrate, part of the ESCRT-0 complex  
HSPC = Hematopoietic Stem and Progenitor Cells  
ID = Intellectual Disability  
IDRs = Intrinsic Disordered Regions  
KO = knockout  
LAMP1, LAMP2 = lysosomal-associated membrane protein 1, 2  
LKB1 = Liver-kinase B1

M1 = muscarinic 1 receptor,  
Mb = Megabase  
MEME = Multiple Expectation Maximization for Motif Elicitation  
MFF = mitochondrial fission factor  
MFN-1 , MFN-2 = Mitofusin 1, 2  
mGluR5 = metabotropic glutamate receptor 5  
MIRO = mitochondrial rho GTPase  
mRNA = messenger RNA  
MVB = multivesicular bodies  
*Myc = Myelocytimatosi protooncogene*  
NF-KB = nuclear factor kappa-light-chain-enhancer of activated B cells  
NFFR1 = neuropeptide FF receptor 1  
NLS = nuclear localisation signal  
N-ter = amino-terminal  
PFAM = protein families database  
Pifo = Pitchfork  
PINK1 = PTEN-induced kinase 1  
PKA = Protein Kinase A  
PTEN = phosphatase and tensin homolog  
RGC = retinal ganglions cells  
RT-qPCR = reverse transcription-quantitative polymerase chain reaction  
SHH = SonicHedgehog  
shRNA = short hairpin RNA  
siRNA = small interfering RNA  
Smo = Smoothened receptor  
SOCS3 = Suppressor of cytokine signalling 3  
Sox10 = SRY-related HMG-box  
TAZ = Transcriptional co-activator with PDZ-binding motif  
TCEAL= Transcription Elongation Factor A protein-Like  
TCF25 = transcription factor 25  
TM = transmembrane  
TPA = Tissue plasminogen activator  
TRAF4 = TNF receptor-associated factor

TRAK2 = Trafficking kinesin-binding protein 2  
Tuj1 = Neuron-specific class III  $\beta$ -tubulin protein  
TXA2 = Thromboxane A2 receptor  
U2OS = Human bone-osteocarcinoma epithelial cells  
UIM = Ubiquitin-Interacting Motif  
UTR = un-transcribed region  
WEX = WWbp5-like X-linked  
WT = wild-type  
YAP = Yes-associated protein

### **Conflict of Interest**

The authors declare that they have no conflict of interest

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### **Figure legends**

**Figure 1. Schematic representation of the ten members of the GPRASP/ARMCX proteins family with the motif locations as a result of sequences analysis with MEME SUITE.**

Motif-based sequence analyses were performed with all ten full-length human sequences from the Uniprot database (accession numbers in the Table S1) and the MEME SUITE web application 5.1.1 [129]. The classic discovery mode was selected for six motifs with site distribution option set to any number of repetitions (motifs from 6 to 50 amino acids width). Each motif is indicated as a coloured block, the height of which is proportional to the  $-\log(p\text{-value})$  indicating the overall strength of motif matches. The statistical significance of the 6 motifs (A, B, C and F) was confirmed as their E-value was

very low and smaller than  $1.7 \times 10^{-70}$  and p-values below  $2.87 \times 10^{-7}$  (p-value corresponds to the probability that an equal or better site would be found in a random sequence of the same length conforming to the background letter frequency). The family segregates in two subfamilies of five members each according to the presence or not of the previously described GASP motif (motif B)[2]. The ARM CX4 that stands in between both groups was not considered for further analysis of subfamily 1. Using the same procedure the E motif emerged with sufficient statistical E-value of  $4.4 \times 10^{-20}$  and p-values *below*  $8.62 \times 10^{-9}$ . Motif D was identified by comparing the N-ter extremities of GPRASP/ARM CX proteins and was characterized by MEME SUITE when the classic discovery mode was selected for 25 motifs instead of 6 of them. The sequence of each motif is presented as a letter-probability matrix in Figure 2. The location of the original GPRASP1 cGASP domain described in [15] is shown with dotted lines.

**Figure 2. Sequences logos corresponding to the consensus amino-acid sequences determined by MEME SUITE analysis of the GPRASP/ARM CX family.**

The relative sizes of the letters indicate their frequency in the sequences.

**Figure 3. Scheme of the cellular functions of the GPRASP/ARM CX proteins.**

Molecular complexes in which GPRASP/ARM CX proteins are engaged in membrane trafficking of GPCRs (targeting of Smoothed in the primary cilia and GPCRs sorting from the endosomes) or in mitochondria trafficking and dynamics. ER: endoplasmic reticulum, P: phosphorylation, site S45, MVB: Multivesicular bodies, Smo: Smoothed receptor.

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**Table 1**

**Summary of the phenotypes observed in mice, in humans or in cells upon variation of expression of WT or mutant GPRASP/ARMCX proteins from subfamily 1 and their most characterized binding partners**

<b>Subfamily 1 members</b>	<b>Knock-out Mice Phenotype</b>	<b>Human mutation &amp; phenotype</b>	<b>Human deletion &amp; phenotype</b>	<b>Overexpression in cell or <i>animal</i></b>	<b>Down-regulation in cell or <i>animal</i></b>	<b>Interacting proteins potentially implicated</b>
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<p><b>GPRASP1</b></p> <p>(GASP1, KIAA0443, PIPS)</p>	<p>○ <u>KO GPRASP1 : normal conditions</u></p> <ul style="list-style-type: none"> <li>- ↓ spontaneous vertical locomotion [66]</li> <li>- No impairment in locomotor response, coordination or other motor functions [65]</li> <li>- Impaired memory processes : delayed acquisition/expression of complex tasks [66;101]</li> </ul> <p>○ <u>KO GPRASP1 : chronic GPCRs activation</u></p> <ul style="list-style-type: none"> <li>- Loss of CB1receptor analgesic tolerance [71]</li> <li>↓ total CB1R amount</li> <li>↓ degradation of activated CB1R [71]</li> <li>- ↓ hyperlocomotor activity after repeated treatment with cocaine. [66;65]</li> <li>- Absence of cocaine-induced NMDA and AMPAR potentiation [82]</li> </ul> <p><b>Controversy</b> : down-regulation of GPCRs <i>in vivo</i></p> <ul style="list-style-type: none"> <li>- ↑ degradation of dopaminergic and muscarinic receptors in the striatum upon chronic cocaine treatment [66]</li> <li>- ↓ degradation of activated D2R in the striatum upon chronic cocaine treatment [65]</li> </ul> <p>- Deletion Xq22 (see GPRASP2) [100]</p>	<p>○ <u>GPRASP1 E1164G and K352*</u>: whole exome sequencing : missense or stop mutations in papillary thyroid cancer (3/11) [130]</p>	<p>○ <u>Xq22.1: 1.1Mb deletion</u> [7]: see GPRASP2</p> <p>○ <u>Xq22.1: 1.1 Mb deletion</u> [98]: see GPRASP2</p>	<p>○ <u>In vitro</u></p> <ul style="list-style-type: none"> <li>- ↑ constitutive signalling of US28R [78]</li> </ul>	<p>○ <u>In vitro</u> :</p> <ul style="list-style-type: none"> <li>- ↓ constitutive degradation of US28 and ↓ production of inositol phosphate [78]</li> <li>- ↑ recycling of activated GPR55 [77]</li> <li>- ↓ degradation of activated dopaminergic D2 and D3 receptors [64]</li> <li>- ↑ CXCR4 at plasma membrane &amp; delay in its degradation after activation [74]</li> <li>Overexpression of GPRASP3</li> <li>- ↓ incretin dependent insulin secretion / signalling by GLP1R : ↓ of active GLP1R? [79]</li> </ul> <p>○ <u>In vivo</u></p> <ul style="list-style-type: none"> <li>- ↑ hematopoietic stem cells implantation [74]</li> </ul>	<p>○ <u>GPCRs</u></p> <ul style="list-style-type: none"> <li>- DOR [15]</li> <li>- CB1R [69;70;71]</li> <li>- D2R [62;66;65;63]</li> <li>- US28 [78]</li> <li>- GPR55 [77]</li> <li>- 5HT7 [76]</li> <li>- GLP1R [79]</li> <li>- CXCR4 [74]</li> </ul> <p>○ <u>Trafficking complexes</u></p> <ul style="list-style-type: none"> <li>-DOR and D2R/dysbindin [56]</li> <li>-DOR and CXCR4 /Gas/HRS/dysbindin complex [37]</li> <li>-DOR and CB1R /Beclin2 [18;72]</li> </ul>
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<p><b>GPRASP2</b> (GASP2, HIP15)</p>	<p>○ <u>KO GPRASP2</u> : <b>Intellectual disability and Autism Spectrum Disorder like behaviour</b> [96] - Viable and born with Mendelian ratio - No significant alteration in motor function or anxiety but slight increase of time spent in the center of the open field. - <b>Learning and memory impairments.</b> - <b>Social impairments</b> [96] - Obesity with age</p> <p>- Alter hippocampal neuronal morphology: ↓ in dendritic arborization, ↓ total dendritic length in distal region of pyramidal neurons. - Striking effect on apical neuronal arborization - ↓ field excitatory post synaptic potentials in CA1 hippocampus area with no alteration of presynaptic functions - ↓ amplitude of AMPA receptors-mediated miniature excitatory postsynaptic currents - ↓ density of mature spines in apical regions [96]</p> <p>- ↓ mGluR5 amount [96]</p> <p>○ <u>Deletion Xq22</u> [100] <u>DeLa</u> <b>Male</b> : - Neonatal lethality by acute respiratory failure - Cleft palate. - Lung development delay <b>Female heterozygous:</b> - 45% Neonatal lethality</p>	<p>○ <u>GPRASP2 A573N</u> [91] c.1717_1718GC&gt;AA, p.A573N : <b>Missense mutation</b> four hemizygous male patients and two heterozygous female carriers family - <b>Hearingloss</b> - Abnormal auricular shapen bilateral ptosis, - Abnormally dilated internal auditory canal, - Incomplete separation of cochlea basal turn from the fundus - <b>Facial dysmorphism with bilateral palpebral ptosis</b></p> <p>○ <u>GPRASP2 I791K</u> Potentially involved in schizophrenia [131]</p> <p>○ <u>Missense mutation</u> Intellectual disability and Autism Spectrum Disorder: [132] 3 genes mutated in one female: - GPRASP2 R812C, - SEMA3F R679W, - SCN11A frameshift</p>	<p>○ <u>Xq22.1: familial 1.1Mb deletion</u> : <b>Mental retardation behavioural disorders</b> in female patients [7] 12 genes deleted: <i>NXF5, ZMAT1, TCEAL2, TCEAL6, BEX5, NXF2, TMSB15A, NXF4, ARMCX5, GPRASP1, GPRASP2</i> and <i>GPRASP3</i>. <b>mother &amp; daughter:</b> - Short stature, brachycephaly, coarse face, - epilepsy &amp; borderline personality disorder. <b>Son</b> : spontaneous death at 15 days <b>Daughter</b> : - Asphyxia and cleft palate at birth, microbrachycephaly, general hypotonia, unspecific periventricular white matter lateration Hypertrichosis, distal muscle hypotrophy, scoliosis and facial dysmorphisms (small forehead, thick lips, smooth philtrum and low ears) - ASD with stereotypic movements and self-mutilation</p> <p>○ <u>Xq22.1: 1.1Mb deletion</u> [98]: 17 genes of <i>ARMCX6, ARMCX3, ARMCX2, NXF5, ZMAT1, TCEAL2, TCEAL6, BEX5, NXF2, -NXF2B</i></p>	<p>○ <u>In vitro</u> - ↑ dendritic arborization and length, - ↑ spine density, length and head diameter - ↑ mature dendrites</p> <p>- ↓ of mGluR5 at plasma membrane [96]</p>	<p>○ <u>In vitro</u> - ↑ CXCR4 at plasma membrane &amp; delay in its degradation after activation. Overexpression of GPRASP3 [73]</p> <p>- ↓ spine density, neuronal complexity and total dendritic length Reversed by co-expression of mGPRASP2 in Primary neuronal cell culture - Abnormal internalization of activated mGluR5, mGluR5 Antagonist (MPEP) prevents reduction in neuronal arborization and recovers spine density, increase of mGluR5 surface and blocks DHPG-mediated changes in receptor levels [96]</p> <p>○ <u>In vivo</u> - ↑ hematopoietic stem cells implantation [74]</p>	<p>○ <u>GPCRs</u> - mGluR5 [96] - CXCR4 [73]</p> <p>- Smoothened/Pifo complex [39;90]</p>
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	<ul style="list-style-type: none"> <li>- Rare cleft palate,</li> <li>- Postnatal growth delay and</li> <li>- Spontaneous seizures, epilepsy</li> </ul> <p><u>DelC Xq22 0.35Mb</u>: 8 genes deleted [100]</p> <p><b>ARMCX5, GPRASP1 GPRASP2</b> and <b>GPRASP3</b> (orthologs in the Xq22.1 human deletion, Grillo et al. 2010, Cao et al. 2016) + <b>ARXES1, ARXES2, BEX2, NXF3</b> (no human orthologs in the Xq22.1 human deletion)</p> <p><b>Male :</b></p> <ul style="list-style-type: none"> <li>- Neonatal lethality by respiratory failure,</li> <li>- Cleft palate</li> </ul> <p><b>Female :</b></p> <ul style="list-style-type: none"> <li>- Rare cleft palate,</li> <li>- Epilepsy</li> </ul>		<p>(paralog), <b>TCP11X2, TMSB15A, NXF4, ARM CX5, GPRASP1, GPRASP2</b> and <b>GPRASP3</b></p> <p><b>- male infant:</b></p> <p>failure to thrive, hypoglycemia, subtle dysmorphic features, umbilical hernia, hypotonia with spasticity in the lower extremities, focal enlargement of frontal temporal lobe on cerebral MRI, and respiratory failure with thoracic insufficiency syndrome, tracheomalacia, and laryngomalacia</p>			
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<p><b>GPRASP3</b></p> <p>(GASP3, bHLHB9, p60TRP)</p>	<ul style="list-style-type: none"> <li>○ <u>Deletion Xq22</u> (see GPRASP2) [100]</li> </ul>	<ul style="list-style-type: none"> <li>○ <u>Alzheimer disease</u> [133]: - ↓ GPRASP3 in patients' brain with sporadic AD - Influence on NNT1 (new-neurotrophin-1) and p48ZnF (p48-zinc-finger-protein) signalling</li> </ul>	<ul style="list-style-type: none"> <li>○ <u>Xq22.1: 1.1Mb deletion</u> [7] : see GPRASP2</li> <li>○ <u>Xq22.1: 1.1 Mb deletion</u> [98] : see GPRASP2</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i> : - ↑ neurogenesis [134]</li> <li>○ <i>In vivo</i> : - Affects neuroprotective capacities - ↑ synaptogenesis Inhibiting Bace1 &amp; Psen - Improve cognitive functions [134]</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i>: - Modulates the expression and cleavage of pivotal CNS regulator proteins. - activation of apoptosis (↑ activation of Caspase 3 and ↑ annexin-V-PI cells ) [134]</li> </ul>	
<p><b>ARMCX4</b></p> <p>(GASP4)</p>	<p>No KO mice</p>	<p>- <i>Patients : whole exome sequencing</i> : Passenger gene in endometrial cancer c.6167C&gt;A , p.P2056H [135]</p>				
<p><b>ARMCX5</b></p> <p>(GASP5)</p>	<p>- <u>Deletion Xq22</u> (see GPRASP2) [100]</p>		<p>- <u>Xq22.1: 1.1Mb deletion</u> [7]: see GPRASP2</p> <p>- <u>Xq22.1: 1.1 Mb deletion</u> [98]: see GPRASP2</p>			

↑ : increase ; ↓ : decrease ; AD : Alzheimer disease ; CNS : central nervous system.

**Table 2**

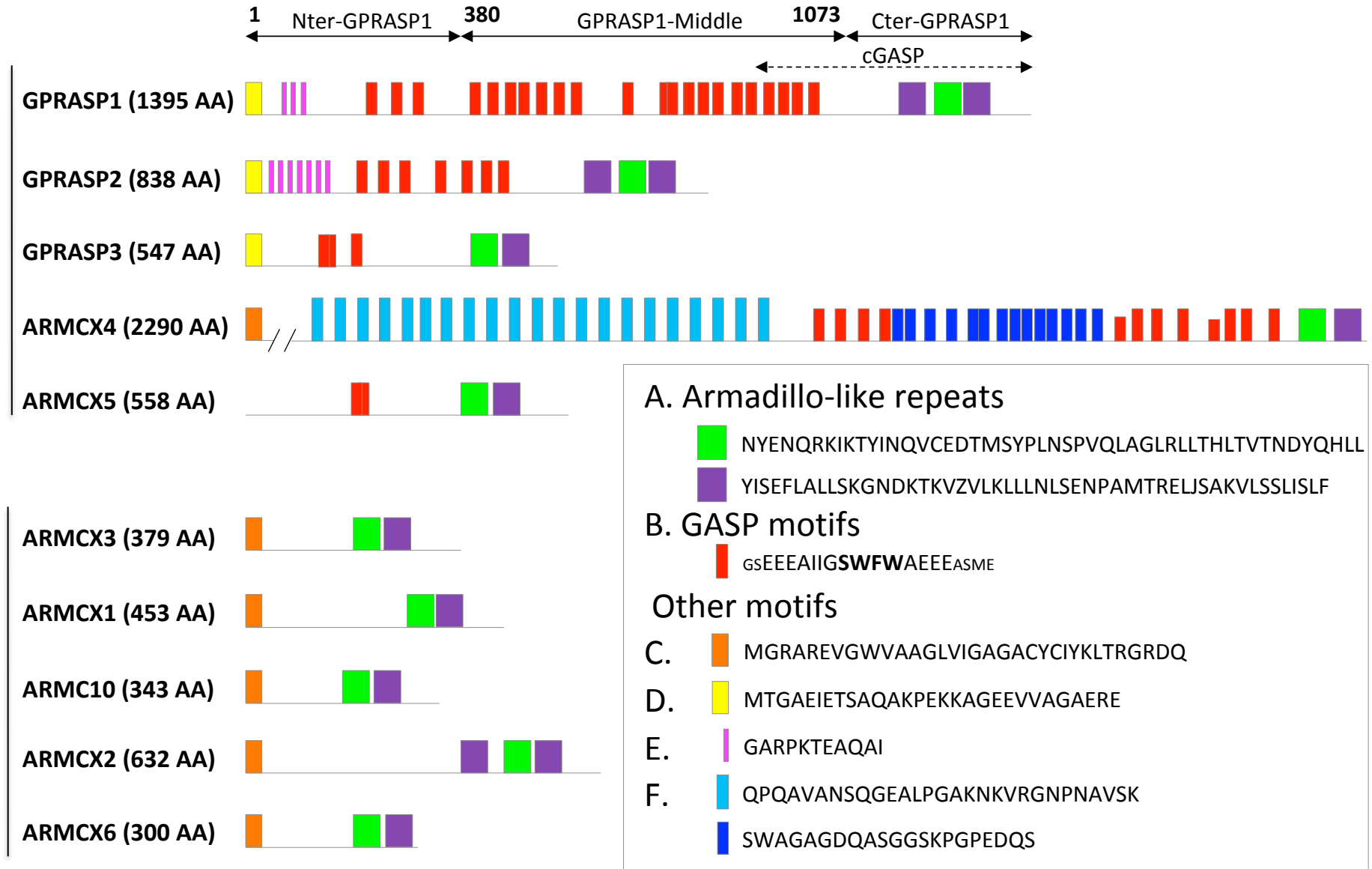
**Summary of the phenotypes observed in mice, in humans or in cells upon variation of expression of WT or mutant GPRASP/ARMCX proteins from subfamily 2 and their most characterized binding partners.**

subfamily 2 members	Human mutation & phenotype	Human deletion & phenotype	Overexpression in cell or <i>animal</i>	Down-regulation in cell or <i>animal</i>	Interacting proteins potentially implicated
<b>ARMCX3</b>  (GASP6, ALEX3)	<ul style="list-style-type: none"> <li>○ <u>Variation of expression in cancer</u> [123]</li> <li>- ↓ of expression : development of non-small cell lung cancer, adverse clinical outcome</li> <li>- ↑ E-cadherin</li> </ul>	<ul style="list-style-type: none"> <li>○ <u>Xq22.1: 1.1Mb deletion</u> [9]: 17 genes of <b>ARMCX6, ARMCX3, ARMCX2, NXF5, ZMAT1, TCEAL2, TCEAL6, BEX5, NXF2, - NXF2B</b> (paralog), <b>TCP11X2, TMSB15A, NXF4, ARMCX5, GPRASP1, GPRASP2</b> and <b>GPRASP3</b></li> <li>- <b>male infant:</b> failure to thrive, hypoglycemia, subtle dysmorphic features, umbilical hernia, hypotonia with spasticity in the lower extremities, focal enlargement of frontal temporal lobe on cerebral MRI, and respiratory failure with thoracic insufficiency syndrome, tracheomalacia, and laryngomalacia</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i> <b>Two mitochondrial dynamics phenotypes:</b></li> <li>1- Perinuclear aggregates</li> <li>2- Decrease in the pool of moving mitochondria</li> <li>- ↓ velocity [3]</li> <li>○ <i>In vivo</i> <b>In chicks embryo</b></li> <li>- ↓ progenitor cells proliferation in neural tubes[31]</li> <li>- ↓ Wnt signalling [31]</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li>1- Small mitochondria [3]</li> <li>2- ↓ pool of moving mitochondria</li> <li>- No effect on velocity [3]</li> <li>○ <i>In vivo</i></li> <li>- ↓ 30% of dividing neural tube cells by ShRNA in chicks embryo HH12 [31]</li> </ul>	- Miro1/Miro2/T RAK2 complex [3]
<b>ARMCX1</b> [21]  (GASP7, ALEX1)		None reported	<ul style="list-style-type: none"> <li>○ <i>In vitro</i> <b>One mitochondrial dynamics phenotype:</b></li> <li>- ↑ pool of moving mitochondria</li> <li><b>Neurons:</b></li> <li>- ↑ pool of axon growing</li> <li>- ↑ length of axons</li> <li>- 45% increase in neuronal survival after injury</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i> <b>Neurons:</b></li> <li>- ↓ axon regeneration and neuronal survival after injury</li> </ul>	- Miro1 [21]
<b>AMRCX1</b> [122]	<ul style="list-style-type: none"> <li>○ <u>Variation of expression in cancer</u></li> </ul>		<ul style="list-style-type: none"> <li>- Rescues the cell apoptosis induced by miR-106b inhibitor</li> <li>- ↑ phosphorylation of JAK1 and STAT3</li> </ul>		

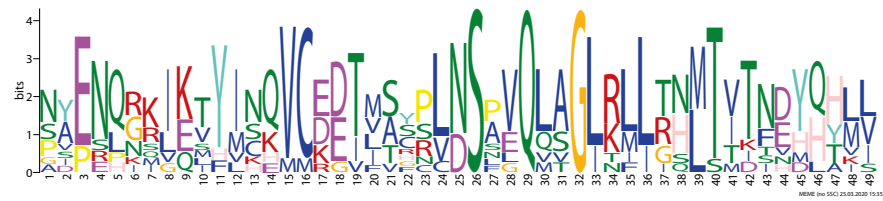
<b>ARMC10</b> (GASP8, SVH, ARMC10A)		None reported	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li><b>Two mitochondrial dynamics phenotypes:</b> [26]</li> <li>1-Perinuclear aggregates less in neurons than in HEK293 cells</li> <li>2- ↓ moving mitochondria</li> <li>-Velocity unchanged</li> <li><b>Neurons :</b></li> <li>- ↓ amyloid beta mitochondrial fragmentation and neuronal cell death [136]</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li>- ↓ moving mitochondria [26]</li> </ul>	- Miro1/Miro2/T RAK2 complex [26]
<b>AMRC10</b> [20]			<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li>- ↑ Sox10 at the mitochondria</li> <li>- ↑ transcriptional activity of Sox10</li> </ul>		
<b>ARMC10B</b> [22] (GASP8B, SVH-B) <b>ARMC10B Ser45E</b>		None reported	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li><b>Two mitochondrial dynamics phenotypes:</b></li> <li>- Mitochondrial aggregation</li> <li>- ↑ mitochondrial fission</li> <li> </li> <li>- Transition to mitophagy ? [22]</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li>- No effect of AMPK activator on mitochondrial fission [22]</li> </ul>	- DRP1/Fis1/MF F complex [22]
<b>ARMC10B</b> [4]			<ul style="list-style-type: none"> <li>○ <i>In vivo</i></li> <li>- Accelerated growth rate and tumorigenicity in nude mice</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li>- Apoptosis</li> </ul>	
<b>ARMCX2</b> (GASP9, ALEX2)	<ul style="list-style-type: none"> <li>○ <u>Variation of expression in cancer</u> [121]</li> <li>○ <u>Hypermethylation of CpG</u> in cisplatin resistant ovarian cancers [121]</li> </ul>	<ul style="list-style-type: none"> <li>○ <u>Xq22.1: 1.1Mb deletion</u> [9]: see GPRASP2 and ARMCX3</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li>- Altered mitochondrial network (data not shown; [3])</li> <li> </li> <li>○ <i>In vivo</i></li> <li>- Damaged liver sample, correlated with degree of damage [137]</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vivo</i></li> <li>- Ovarian differentiation [116]</li> </ul>	
<b>ARMCX6</b> (GASP10)	None reported	<ul style="list-style-type: none"> <li>○ <u>Xq22.1: 1.1Mb deletion</u> [9]: see GPRASP2 and ARMCX3</li> </ul>	<ul style="list-style-type: none"> <li>○ <i>In vitro</i></li> <li>- Altered mitochondrial network (data not shown; [3])</li> </ul>		

↑ : increase ; ↓ : decrease ; AD : Alzheimer disease ; CNS : central nervous system

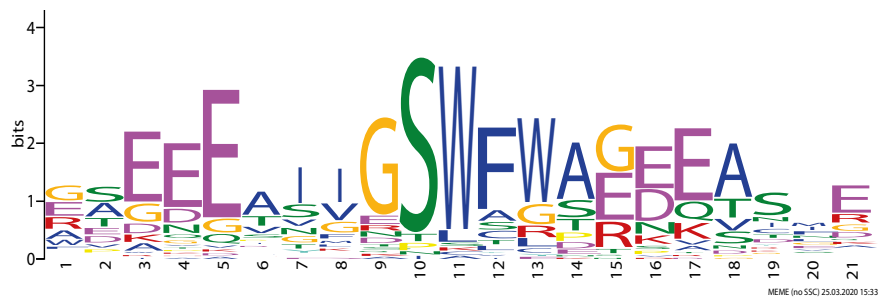
# Figure 1



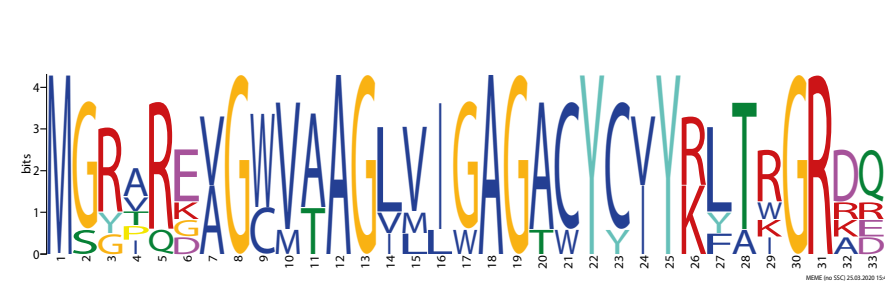
# A FIGURE 2



# B



# C



# D



# E



# F

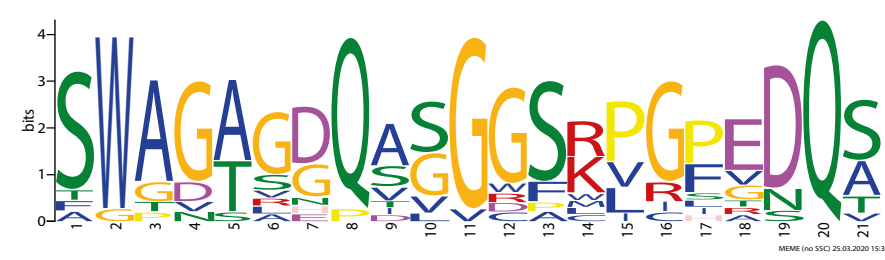
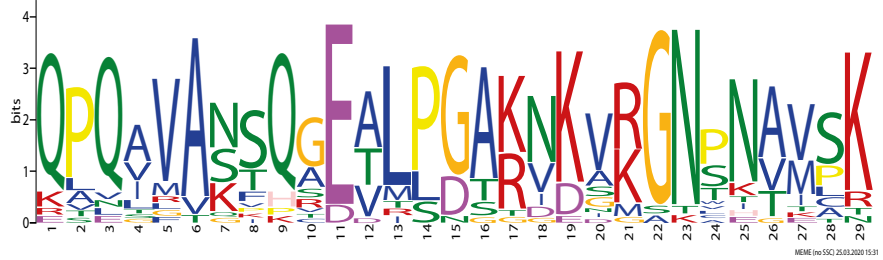


Figure 3

