ABC-transporter activity and eicosanoid-signaling are required for germ cell migration a basal chordate

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Abstract:
The colonial ascidian *Botryllus schlosseri* provides a unique model to study germ cell migration, as long-lived germline stem cells (GSCs) constantly migrate to new germline niches as they develop during repetitive cycles of asexual reproduction. We have previously shown that migration of germ cells in *Botryllus* is directed by sphingosine-1-phosphate (S1P) signaling. Here, we show that the ABC-transporters *abcc1* and *abcb1* are highly expressed in germ cells, and inhibition of ABC-transporter activity leads to a reduced migratory response towards S1P *in vitro* and failure of germ cell homing in vivo. ABC-transporters are highly conserved in the animal kingdom and are involved in the export of lipid-signaling molecules. Phospholipase A2 (PLA2) produces arachidonic acid, which is further metabolized by cyclooxygenases (COX), or by Lipoxygenases (LOX) to eicosanoid signaling molecules. We show that in *Botryllus*, PLA2 activity is required for migration of germ cells towards low concentrations of S1P *in vitro*, as well as homing of germ cells *in vivo*. *Botryllus* lipoxygenase (*bslox*) has homology to the 3 human lipoxygenases 5-LOX, 12-LOX and 15-LOX. In humans, 12-LOX metabolizes arachidonic acid to12-Hydroxyeicosatetraenoic acid (12-S-HETE), which stimulates migration of cancer cells and smooth muscle cells. We have identified a g-protein coupled receptor in *Botryllus* that is closely related to the human receptor for 12-S-HETE, GPR31. *BSgpr31* is expressed in *vasa*-positive germ cells. *In vitro*, an inhibitor of all 3 types of lipoxygenases significantly reduces migration of *Botryllus* germ cells towards S1P, whereas specific inhibitors of COX1, COX2 or 5-LOX have no effect. 12-S-HETE rescues migration towards S1P in the presence of inhibitors of ABCC1, ABCB1, PLA2 or LOX. *In vivo*, inhibition of LOX inhibits homing of germ cells to secondary buds. We conclude that autocrine stimulation by production and ACB-transporter-mediated export of 12-S-HETE is required for migration of germ cells towards a chemotactic gradient of S1P. We hypothesize that signaling of 12-S-HETE through *BSgpr31* enhances migratory activity during chemotaxis induced by low-level activation of the S1P-receptor. This is the first report of an eicosanoid signaling molecule regulating germ cell migration.

Introduction:

Cell migration is a fundamental process of development and maintenance of multicellular organisms and mediates tissue organization, organogenesis, immune response and homeostasis (Vicente-Manzanares, Webb et al. 2005). Regulation of cell migration requires a complex interplay of signaling cascades that influence cell adhesion, polarization and cell motility. Temporal-spatial cues are tightly controlled, as dysregulation of cell migration can have catastrophic consequences for the organism, including developmental defects and cancer.

In many species, germ cells that are specified during embryonic development need to migrate across the embryo to reach the somatic gonad, where they will develop into eggs and testes (Richardson and Lehmann 2010). Germ cell migration is studied in a variety of organisms, and many features are widely conserved. All germ cells seem to undergo an active migration guided by attractive and repulsive cues toward the genital ridges or somatic gonadal precursors of the developing gonad (Barton, LeBlanc et al. 2016).

In addition to being cell membrane components, research in the last two decades has shown that many lipids, now termed “bioactive lipids”, have critical cell signaling functions (Bieberich 2012). Some lipid classes such as lysophospholipids (including sphingosine-1-phosphate), eicosanoids (e.g. prostaglandins), and endocannaboids can signal through receptors in the cell membrane (Bieberich 2012). These lipids activate G-protein coupled receptors (GPCRs) of the Gi, Gq, and G12/13 type can be activated by gradients of bioactive lipids and thus influence cell polarity. Bioactive lipids associated with cell polarity include lysophospholipids (LPLs) and phosphatidylinositolphosphates (PIPs). LPLs are bioactive lipids that can be

generated by hydrolytic cleavage of fatty acid from glycerophospholipids, a reaction catalyzed by phospholipases. Distinct phospholipases cleave off either one of the two (PLA1 and PLA2) fatty acid residues. The biosynthesis of eicosanoids is initiated by the activation of PLA2, leading to the release of arachidonic acid. Arachidonic acid is metabolized to a variety of eicosanoid signaling molecules such as thromboxanes, and leukotrienes (Bieberich 2012).

So far, very little is know about the roles of bioactive lipids in germ cell migration. A potential role for lipid signaling in germ cells was first discovered in Drosophila, in which mutations in the lipid phosphate phosphatases (LPP), wunen and wunen2, disrupt directed migration of germ cells to the gonad (Starz-Gaiano, Cho et al. 2001, Barton, LeBlanc et al. 2016), but the ligand has not been identified. However, this mechanism appears to be conserved, as LPPs also repel germ cells away from nearby somites in the zebrafish (Paksa, Bandemer et al. 2016).

Recently, our group discovered that migration of germ cells is directed by the lysophospholipid Sphingosine-1-phosphate (S1P) in the colonial ascidian *Botryllus schlosseri* (Kassmer SH 2015).

Botryllus is a unique model to study germline biology, because an individual does not grow by increasing in size, but rather by a lifelong, recurring asexual budding process (called blastogenesis) during which entire bodies are regenerated de novo every week. This results in a constantly expanding colony of genetically identical individuals, called zooids, which are linked by a common extracorporeal vasculature (Figure 1). Thus each week, Botryllus regenerates all somatic and germline tissues.

The best understood regenerative process in Botryllus occurs in the germline. Like most metazoans, *Botryllus* sets aside a population of PGCs early in embryogenesis that are responsible for gametogenesis for the life of the organism (Brown, Tiozzo et al. 2009). However, unlike most model organisms, *Botryllus* retains a population of mobile, self-renewing, lineage-restricted adult germline stem cells (GSCs) that retain pluripotency for life of the individual (Sabbadin and Zaniolo 1979). During the weekly development of new zooids, a new germline niche is formed, and GSCs migrate from the old germline niche to the new. A subset of these GSCs settle, differentiate into gametes (zooids are hermaphrodites and make sperm and eggs), while others self-renew and migrate to the next generation. Asexual development is synchronized throughout the colony, and migration occurs over a defined 48 hr. period as GSCs leave the primary bud niche and enter the secondary bud niche via the vasculature joining the two. During this window, GSCs are also found in the colony vasculature and ampullae. GSC migration is controlled by a S1P gradient, which is synthesized in the new niche and binds to the GPCR S1PR1on migrating GSCs (Kassmer SH 2015).

The ATP binding cassette (ABC) are transport proteins that are conserved in all phyla from prokaryotes to humans (Locher 2016). ABC transporters shuttle a variety of hydrophobic lipophilic compounds across the cell membrane in an ATP-dependent manner, including bioactive lipids such as S1P, leukotrienes and prostaglandins (Neumann, Rose-Sperling et al. 2017). Some ABC transporters play roles in cell migration. In Drosophila, a germ cell attractant is geranylgeranylated and secreted by mesodermal cells in a signal peptide-independent manner through an ABCB transporter of the MDR family (Ricardo and Lehmann 2009). Chemotaxis of human dendritic cells to CCL19 requires stimulation with the exogenous leukotriene C4, an eicosanoid transported out of the cell via ABCC1 (Randolph 2001).

In the present study, we show that germ cell in *Botryllus* express ABCC1 and ABCB1, and that their activity is required for migration to S1P and for homing towards developing niches. We further show that an arachidonic acid-derived product of lipoxygenase is required for germ cell migration towards low concentrations of S1P, and identify 12-S-HETE as a stimulant of germ cell migration that can rescue inhibition of ABCC1, ABCB1 and LOX. *Botryllus* germ cells express a homolog of the human 12-S-HETE receptor gpr31. We conclude that low concentrations of S1P lead to activation of LOX and production and export of 12-S-HETE, resulting in autocrine stimulation of migratory activity.

Results:

Vasa-positive cells express ABCC1 and ABCB1

We have shown previously that *vasa*-positive germline stem cells can be prospectively isolated from the blood of *Botryllus schlosseri* by flow cytometry, using a mononclonal antibody against Integrin-alpha-6 (IA6) (Kassmer SH 2015). By quantitative real time PCR, *abcc1* and *abcb1* mRNA are highly enriched in IA6+ cells, with *abcb1* is expressed at higher levels than *abcc1* (6.2 fold enrichment vs 2.2 fold enrichment). We confirmed expression of *abcc1* and *abcb1* in *vasa*-positive cells migrating to secondary buds (Arrows, Figure 2b) by fluorescent in situ hybridization (FISH). To test whether these transporters are functionally active, we tested efflux of Calcein AM, which is a substrate of both ABCC1 and ABCB1, by flow cytometry. Calcein-AM is capable of diffusing across cell membranes, and the resulting fluorescence intensity of the cell(s) is a measure of the activity of the ABC transporters (Hollo, Homolya et al. 1994)[5]. Cells with highly active transporters will display lower fluorescence intensity values because of increased efflux of Calcein-AM. In the absence of inhibitors, Calcein fluorescence is detectable compared to unstained cells (mean fluorescence intensity 1,424 vs 31), but low (figure 2C). An inhibitor of both ABCC1 and ABCB1 transporters (Reversan) blocks efflux of Calcein, increasing the mean fluorescence intensity almost 10 fold to 10,500 compared to 1,424 for the Calcein-only control. Inhibitors that block activity of either ABCC1 or ABCB1, but not both, do not affect Calcein efflux significantly, indicating that both transporters actively export Calcein and act redundantly for this substrate.

ABCB1 and ABCC1 activity is required for migration towards low concentrations of S1P

To test whether inhibition of ABC transporter activity affects migration of Botryllus germ cells we isolated IA6+ cells by flow cytometry and assessed their migratory activity to S1P in our transwell migration assay. In the presence of inhibitors of either ABCC1 or ABCB1, migratory activity to a low concentration of S1P (0.2μM) is significantly reduced ($p \leq 0.05$, figure 3A). An inhibitor of both ABCC1 and ABCB1 reduces migration even further, almost to control levels. In contrast, migration in the presence of a high concentration of S1P (2μM) is not significantly affected by ABC-transporter inhibition (figure 3A).
These results suggest that ABCC1 and ABCB1 export a signaling molecule that enhances migratory activity in the presence of low concentrations of the chemoattractant S1P (Figure 3B). We hypothesize this signaling molecule acts as an autocrine stimulant, binding to a receptor on the GSC surface.. In the presence of high concentrations of S1P, stimulation through the S1P receptor alone is sufficient to stimulate chemotaxis. Synthesis and export of the unknown signaling molecule may or may not be occurring in these conditions.

Phospholipase A2 activity and lipoxygenase activity are required for migration to S1P

Next, we aimed to identify the autocrine signaling molecule that is secreted in response to low S1P. ABC transporters export a variety of substrates. Among these are a variety of lipid signaling molecules, such as phospholipids and derivatives of arachidonic acid (Fletcher, Haber et al. 2010, Neumann, Rose-Sperling et al. 2017). In humans, the cytoplasmic enzyme phospholipase A2 (PLA2, Fig3C) generates the polyunsaturated omega-6 fatty acid arachidonic acid from phospholipids. Arachidonic acid is further metabolized by either lipoxygenases (LOX) or Cyclooxygenases (COX) to generate bioactive lipids such as leukotrienes or prostaglandins, which are exported out of the cytoplasm by ABC transporters (Fig3C) (Fletcher, Haber et al. 2010).

To test whether a derivative of arachidonic acid plays a role in germ cell migration towards low concentrations of S1P, we assessed migratory activity in the presence of an inhibitor of PLA2. Inhibition of PLA2 completely blocked the migratory response to 0.2μM of S1P (Fig3D). These results show that a derivative of arachidonic acid is required for the migratory response to low concentrations of S1P. To assess whether this derivative is a product of cyclooxygenase or lipoxygenase, we tested the migratory response to 0.2μM of S1P in the presence of inhibitors of COX-1 or COX-2 or an inhibitor of lipoxygenases. *Botryllus schlosseri* lipoxygenase (*bslox*) has homology to 5-LOX, 12-LOX and 15-LOX from different organisms, and *bslox* expression is enriched in IA6+ cells (Figure 6A). An inhibitor that blocks the activity of all 3 human lipoxygenases blocks germ cell migration to S1P. COX-inhibitors had no

effect (Fig 4A). These results show that an arachidonic-acid-derived product of lipoxygenase is exported by ABCC1 or ABCB1 and is required for migration towards low concentrations of S1P.

Inhibition of ABCC1, ABCB1, PLA2 or Lipoxygenases reduces migration of germ cells to secondary bud niches in vivo.

To test whether migration of germ cells to secondary buds in vivo requires activity of ABCC1, ABCB1, PLA2 or Lipoxygenase, we allowed *B. schlosseri* colonies to develop in the presence of inhibitors. Drugs were added to the seawater at stage A1, when the new generation of zooids first begin to develop. Following the migration period, when the GSCs s have migrated from old to new germline niches, the colonies where fixed in formaldehyde and germ cell migration analyzed by vasa-FISH (Figure 4B). *Vasa*-positive cells in the new niche were quantified using confocal microscopy. In colonies treated with inhibitors, significantly fewer vasa-positive cells (green) entered into new germline niche (white circles) ($p \leq 0{,}01$, Figure 4C) compared to vehicle treated controls.

Botryllus germ cells express a receptor for the 12-LOX product 12-S-HETE

Different types of lipoxygenases use arachidonic acid as a substrate to generate a variety of downstream signaling molecules, including leukotrienes, lipoxins, and other fatty acids such as different types of hydroperoxyeiocatetraenoic acid (HPETE) and hydroxyicosatetraenoic acid (HETE) (Figure 5B). To assess which lipoxygenase-product is responsible for enhancing migration to S1P, we tested an inhibitor of 5-LOX, as well as a cysteinyl leukotriene receptor antagonist. Neither of these significantly affected migration to S1P (Fig5A). These data suggested that a product of either 12-LOX or 15-LOX is responsible for migration to S1P. 12-lipoxygenase generates 12-S-HETE, a signaling molecule that stimulates migration of cancer cells and smooth muscle cells (Powell and Rokach 2015). In humans, the g-protein-coupled receptor GPR31 is a high affinity receptor for 12-S-HETE (Guo, Zhang et al. 2011). We identified a *Botryllus* homolog of GPR31 in our EST database, and have named it *Bsgpr31*. In humans, effects of 12-S-HETE can also be mediated by Leukotriene B4 receptor 2 (Yokomizo 2015), but we were not able to identify a homolog of this receptor in *Botryllus*. Using qPCR, we found that *Bsgpr31* expression is significantly enriched in IA6+ cells (figure 5C). We also identified a *Botryllus* homolog of the closely related GPCR "Trapped in endoderm" *tre-1,* a fatty acid receptor that is important for germ cell guidance in Drosophila (Kunwar, Starz-Gaiano et al. 2003). This sequence is not enriched in Integrin-alpha-6-positive cells (Figure 5C). We confirmed that *Bsgpr31* mRNA is expressed exclusively in *vasa*-positive cells migrating to secondary buds (figure 5D, white arrows).

The 12-LOX product 12-S-HETE stimulates germ cell migration and rescues inhibition of ABC-transporters, PLA2 and lipoxygenases.

As vasa-positive cells in *Botryllus* express the putative receptor for 12-S-HETE, bsgpr31, we aimed to test whether 12-S-HETE would stimulate migratory activity of IA6+ cells *in vitro*. Astonishingly, we found that 12-S-HETE alone has a very strong stimulating effect on migration of IA6+ cells (Figure 6A). 12-S-HETE alone induces migration nearly to nearly the same extent as 0.2µM S1P. In combination, both molecules induce the same amount of migration as 12-S-HETE alone, strongly suggesting that migration to 0.2µM S1P is entirely dependent on autocrine stimulation by12-S-HETE. Importantly, 12-S-HETE rescues migratory activity to 0.2µM S1P in the presence of inhibitors of either ABCC1, ABCB1, PLA2 or LOX and (Figure 6A). These data suggest that S1P-stimulated generation and export of 12-S-HETE is required for the migratory response to a low concentration of S1P (Figure 6B).

In conclusion, migration of GSC towards low concentrations of S1P requires autocrine stimulation of migratory activity through production of and export of 12-S-HETE. 12-S-HETE production by 12-LOX depends on production of arachidonic acid by PLA2. 12-S-HETE is exported by ABCB1/ABCC1, and the 12-S-HETE-receptor *gpr31* is expressed on migrating germ cells.

Discussion

This is the first report of an eicosanoid signaling molecule affecting germ cell migration, and the first report of 12-S-HETE-stimulated migration of germ cells. The roles of bioactive lipids in germ cell migration are

poorly understood, but there is growing evidence for their importance. In Drosophila germ cell migration, the GPCR Tre1 directs migration through the midgut (Kunwar, Starz-Gaiano et al. 2003). While the ligand for Tre1 still unknown, the closest mammalian homolog, GPR84, binds to medium chain fatty acids. In Drosophila and zebrafish, lipid phosphate phosphatases are required for directed migration of germ cells (Starz-Gaiano, Cho et al. 2001, Paksa, Bandemer et al. 2016). So far, no studies have addressed the role of bioactive lipids in germ cell migration in other species. We have shown that the bioactive lipids S1P and 12-S-HETE regulate germ cell migration in an invertebrate chordate, suggesting that bioactive lipids may play conserved roles in directing germ cell migration across phyla.

Cells undergoing directional migration require environmental guidance cues as well as the ability to initiate and sustain motility. Depending on the organism, migrating germ cells must sustain directed migration for twenty-four to forty-eight hours (Barton, LeBlanc et al. 2016). In *Botryllus*, migration of germ cells to new, germline niches occurs during a defined 48 h period (Langenbacher and De Tomaso 2016). It is common for a migrating cell to require more than one signal to induce this type of directed migration and motility. In mice, the chemokine SDF-1 provides the guidance cue to migrating primordial germ cells, whereas signaling of SCF through the receptor c-kit enhances motility (Barton, LeBlanc et al. 2016). On the same cells, signaling of Wnt5a through Ror2 and are required for migration, suggesting that Ror2 activation is needed for a robust response to SCF (Barton, LeBlanc et al. 2016). In migrating germ cells, the response to guidance cues is often mediated by GPCRs or receptor tyrosine kinases (RTKs). In *Botryllus*, we previously showed that the GPCR S1PR1 mediates migration to secondary buds along a chemotactic gradient of S1P. Here, we show that migration of *Botryllus* germ cells to the S1P gradient formed by the developing germline niche requires an additional, autocrine signal that facilitates the migratory response. The phenomenon of directed migration being regulated by more than one signal has also been observed in other contexts. For example, chemotaxis of human dendritic cells to CCL19 requires stimulation with the exogenous leukotriene C4, an eicosanoid transported out of the cell via ABCC1 (Randolph 2001). Thus, we speculate that in migrating *Botryllus* germ cells, S1P is the guidance cue, and 12-S-HETE is an autonomous motility factor that acts downstream of S1P signaling. Based on the data presented here, we hypothesize that activation of the S1P-receptor by S1P activates 12-LOX, which generates 12-S-HETE. 12-S-HETE is subsequently exported from the cytoplasm by ABCC1 and ABCB1 and binds to *bsgpr31* on the cell surface. Signaling of 12-S-HETE through *BSgpr31* enhances migratory activity during chemotaxis induced by low-level activation of the S1P-receptor (Figure 6B). It is possible that other factors are involved in regulating migration of germ cells to secondary bud niches, and we are currently investigating this question.

12-S-HETE **has also** been shown to stimulate cell migration in human cell types. Specifically, it induces migration of cancer cells on laminin (Szekeres, Trikha et al. 2000). This is interesting, since *Botryllus* germ cells also migrate on laminin (Kassmer SH 2015). 12S-HETE induces PKC-dependent cytoskeletal rearrangements in tumor cells, resulting in increased motility (Powell and Rokach 2015) and stimulates aortic smooth muscle cell migration (Nakao, Ito et al. 1983). Interestingly, upregulation of 12-LOX induces a migratory phenotype in cancer cells (Klampfl, Bogner et al. 2012) , suggesting autocrine stimulation by 12-S-HETE plays a role in metastasis. In a carcinoma cell line, activation of beta-4 integrin induces translocation of 12-LOX to the membrane and upregulates its enzymatic activity (Tang, Cai et al. 2015).
We show that 12-S-HETE stimulates germ cell migration in an autocrine fashion, acting on the same cells that secrete it. Similarly, in ovarian cancer cells, activation of beta1 integrin by laminin induces production of lysophosphatidic acid (LPA) and binding of LPA to its g-protein coupled receptor, resulting in enhanced migration. In *Botryllus* germ cells, integrin-alpha-6 signaling activated by low concentrations of S1P and laminin may induce activity of 12-LOX and production of 12-S-HETE. It is also possible that 12-LOX translocates to the leading edge and that 12-S-HETE secretion is spatially controlled to enhance the chemotactic cue.

In addition to its effect on cell motility and migration, 12-S-HETE may enhance cell survival. In cancer cells, inhibition of 12-LOX resulted in cell cycle arrest and apoptosis (Powell and Rokach 2015). S1P, the molecule that attracts germ cells to secondary buds, can regulate anti-apoptotic and pro-survival responses (Rutherford, Childs et al. 2013). In Drosophila, the lipid phosphate phosphatase Wunen-2 (Wun2) regulates PGC survival in a dose-dependent manner (Slaidina and Lehmann 2017), showing that lipid signaling and

germ cell survival may be linked. Therefore, we think it likely that 12-S-HETE and/or S1P signaling play roles in regulating germ cell fitness in *Botryllus*.

While ABC transporters play known roles in dendritic cell and cancer cell migration (Fletcher, Haber et al. 2010), only one study has so far reported a requirement of an ABC transporter in germ cell migration. MDR49 regulates the export of farnesyl-modified mating factors in yeast and is expressed in the drosophila mesoderm. MDR49 mutants have defects in PGC migration (Ricardo and Lehmann 2009). Here, we show that in *Botryllus*, germ cell migration depends on activity of ABCB1 and ABCC1 and the export of an eicosanoid signaling molecule. We hope that future studies will reveal conserved roles of ABC-transporters in germ cell migration in other species.

Materials and Methods:

Animals

Botryllus schlosseri colonies used in this study were lab-cultivated strains, spawned from animals collected in Santa Barbara, CA, and cultured in laboratory conditions at 18-20 °C according to (Boyd HC 1986). Colonies were developmentally staged according to (Lauzon, Ishizuka et al. 2002).

Cell Sorting

Genetically identical, stage matched animals were pooled, and a single cell suspension was generated by mechanical dissociation. Whole animals were minced and passed through 70 µm and 40 µm cell strainers in ice-cold sorting buffer (filtered sea-water with 2% horse serum and 50mM EDTA). Anti-Human/Mouse-CD49f–eFluor450 (Ebioscience, cloneGoH3) was added at a dilution of 1/50 and incubated on ice for 30 min and washed with sorting buffer. Fluorescence activated cell sorting (FACS) was performed using a FACSAria (BD Biosciences) cell sorter. Samples were gated IA6 (CD49f)-positive or –negative based on isotype control staining (RatIgG2A-isotype-control eFluor450, Ebioscience). Analysis was performed using FACSDiva software (BD Biosciences). Cells were sorted using a 70 µm nozzle and collected into sorting buffer.

Quantitative RT PCR

Sorted cells were pelleted at 700g for 10min, and RNA was extracted using the Nucleospin RNA XS kit (Macherey Nagel), which included a DNAse treatment step. RNA was reverse transcribed into cDNA using random primers (Life Technologies) and Superscript II Reverse Transcriptase (Life Technologies). Quantitative RT-PCR (Q-PCR) was performed using a LightCycler 480 II (Roche) and LightCycler DNA Master SYBR Green I detection (Roche) according to the manufacturers instructions. The thermocycling profile was 5 min at 95, followed by 45 cycles of 95 °C for 10 sec, 60 °C for 10 sec. The specificity of each primer pair was determined by BLAST analysis (to human, *Ciona* and *Botryllus* genomes), by melting curve analysis and gel electrophoresis of the PCR product. To control for amplification of genomic DNA, 'no RT'-controls were used. Primer pairs were analyzed for amplification efficiency using calibration dilution curves. All genes included in the analysis had CT values of <35. Primer sequences are listed in Supplemental table 1. Relative gene expression analysis was performed using the $2^{-\Delta\Delta CT}$ Method. The CT of the target gene was normalized to the CT of the reference gene *actin* : $\Delta C_T = C_{T\,(target)} - C_{T\,(actin)}$. To calculate the normalized expression ratio, the ΔC_T of the test sample (IA6-positive cells) was first normalized to the ΔC_T of the calibrator sample (IA6-negative cells): $\Delta\Delta C_T = \Delta C_{T(IA6\text{-}positive)} - \Delta C_{T(IA6\text{-}negative)}$. Second, the expression ratio was calculated: $2^{-\Delta\Delta CT}$= Normalized expression ratio. The result obtained is the fold increase (or decrease) of the target gene in the test samples relative to IA6-negative cells. Each qPCR was performed at least three times on cells from independent sorting experiments gene was analyzed in duplicate in each run. The ΔC_T between the target gene and *actin* was first calculated for each replicate and then averaged across replicates. The average ΔC_T for each target gene was then used to calculate the $\Delta\Delta C_T$ as described above. Data are expressed as averages of the normalized expression ratio (fold change). Standard deviations were calculated for each average normalized expression ratio. Statistical analysis was performed using Student's T-test.

In Situ Hybridization

Whole mount *in situ* hybridization was performed as described in [(Langenbacher, Rodriguez et al.)]. Briefly, *B. schlosseri* homologs of genes of interest were identified by tblastn searches of the *B. schlosseri* EST database (http://octopus.obs-vlfr.fr/public/botryllus/blast_ botryllus.php) using human or Ciona (when available) protein sequences. Primer pairs were designed to amplify a 500-800 bp fragment of each transcript (Primer sequences in Supplemental table 1). PCR was performed with Advantage cDNA Polymerase (Clontech, 639105) and products were cloned into the pGEM-T Easy vector (Promega, A1360). *In vitro* transcription was performed with SP6 or T7 RNA polymerase (Roche, 10810274001, 10881767001) using either digoxigenin or dinitrophenol labeling. HRP-conjugated anti-digoxigenin antibody (Roche, 11207733910) or HRP-conjugated anti-dinitrophenol antibody (Perkin Elmer, FP1129) were used to detect labeled probes by fluorophore deposition (Fluorescein or Cyanine 3) using the TSA Plus System (Perkin Elmer, NEL753001KT). Nuclei were stained with Hoechst 33342 (Life Technologies). Imaging of labeled samples was performed using an Olympus FLV1000S Spectral Laser Scanning Confocal.

Calcein assay of ABC transporter activity

Cells were isolated as described above for cell sorting and filtered through a 10uM cell strainer. Cells were incubated with Calcein-AM (1/1000, Thermo Fisher) in seawater for 30 minutes at room temperature in the presence of 10uM CP 1000356 hydrochloride (ABCB1-inhibitor), 10uM Probenecid (ABCB1 inhibitor) or 10uM Reversan (ABCB1 and ABCC1 inhibitor). Controls were incubated in Calcein only. Unstained controls were incubated in seawater only. Live cells were gated using forward and side scatter properties. Mean fluorescence intensity (MFI) of Calcein fluorescence was analyzed using a FACSAria (BD Biosciences) cell sorter and FACSDiva software (BD Biosciences).

Transwell Migration Assay

Transwell filters with 8µm pore size inserted in a 24 well plate (Corning) were coated with laminin over night at 4°C and briefly air dried before adding 50,000 sorted cells, resuspended in 100µl filtered seawater with 10% DMEM and 1% FBS. The bottom of the well contained filtered seawater with 10% DMEM /1% FBS. Sphingosine-1-phosphate (0.2 - 20µM, Echelon), 12(S)-Hydroxy-(5Z,8Z,10E,14Z)-eicosatetraenoic acid (80nM, Sigma-Aldrich) and 10uM CP 1000356 hydrochloride (ABCB1-inhibitor), 10uM Probenecid (ABCB1 inhibitor), 10uM Reversan (ABCB1 and ABCC1 inhibitor),10uM AACOCF3 (inhibitor of phospholipase A2), 0.5uM 2-TEDC (inhibitor of 5-,12- and 15-lipoxygenase), 10uM Zileuton (inhibitor of 5-lipoxygenase), 1uM BAY-u 9773 (Cysteinyl leukotriene receptor antagonist), 10uM (S)-(+)-Ibuprofen (Cox-1 inhibitor), SC 236 (Cox-2 inhibitor) (all from Tocris) were added to the bottom chamber where applicable. After 2 hours incubation at room temperature, nuclei were stained with Hoechst 33342 and counted in images taken at 3 random locations of the bottom well (at 100x magnification) using the "cell counter" plugin in FIJI software. All assays were performed in triplicates with cells from 3 independent sorts. Statistical analysis was performed using Student's t-test.

Small Molecule Inhibitor Treatment

Small colonies were incubated in 5 ml of seawater containing 25µM Reversan (ABCB1 and ABCC1 inhibitor), 100µM Probenecid (ABCB1 inhibitor) 20µM CP 1000356 hydrochloride (ABCB1-inhibitor) or 25 µM 2-TEDC (inhibitor of 5-,12- and 15-lipoxygenase) or 14 µM AACOCF3 (inhibitor of phospholipase A2). Controls were incubated in seawater. Treatment was started at stage A2, and animals were fixed at stage B2 and analyzed by *in situ* hybridization as described above. Each treatment was performed on 3 genetically identical colonies. Buds containing *vasa*-positive cells were counted on each treated and untreated colony under an epifluoresence microscope. Statistical analysis was performed using Student's t-test.

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Figures:

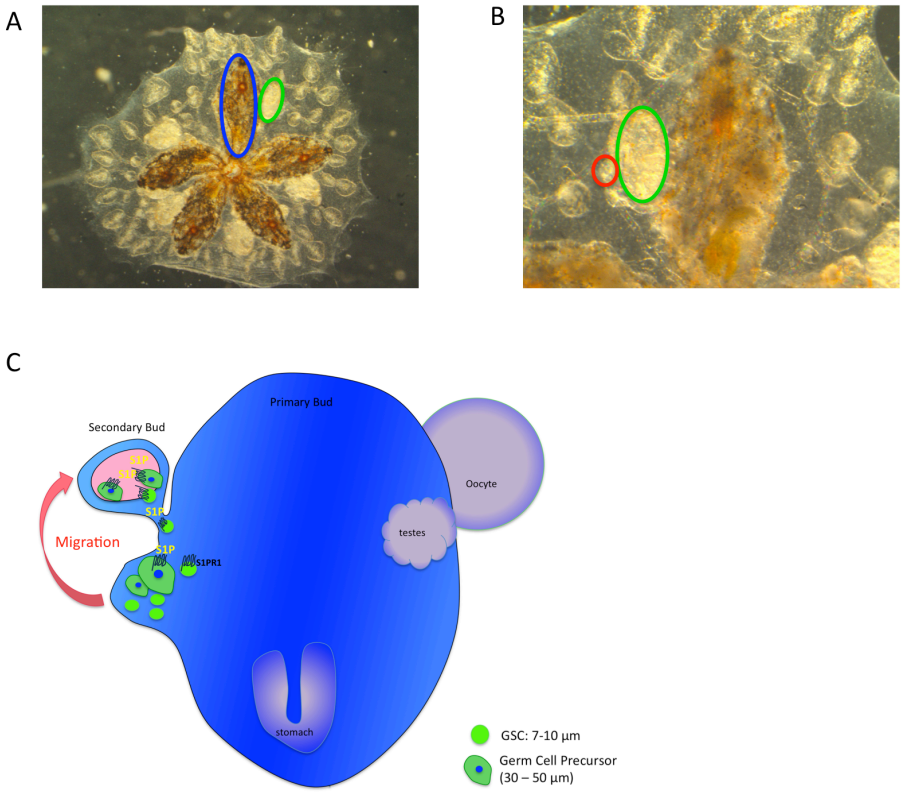

Figure 1: Morphology of Botryllus schlosseri colony, germ cell migration to secondary buds.

A and B: colony morohology and asexual reproduction. All individual bodies within the colony are embedded in an extracellular matrix known as the tunic and share an extracorporeal vasculature. Adult zooids (blue outline) asexually reproduce by giving rise to primary (green outline) and secondary buds (red outline). C: Migration of germ cells to secondary buds. Secondary buds begin as small protrusions of the body wall of primary buds, and later form a closed double vesicle. This vesicle later grows and undergoes invaginations and tissue differentiation, completing development into the adult form. Germline stem cells and (GSC, 7-10µM) and germ cell precursors (30-50µM) migrate from the primary bud into the secondary bud at the time when the double vesicle forms. When the double vesicle is fully formed, germ cells have completed migration. Migration into the secondary bud is directed by a chemotactic gradient of sphingosine-1-phosphate, which is secreted within the secondary bud and detected by sphingosine-1-phosphate-receptor-1 expressed by the migrating germ cells (Kassmer et al 2015).

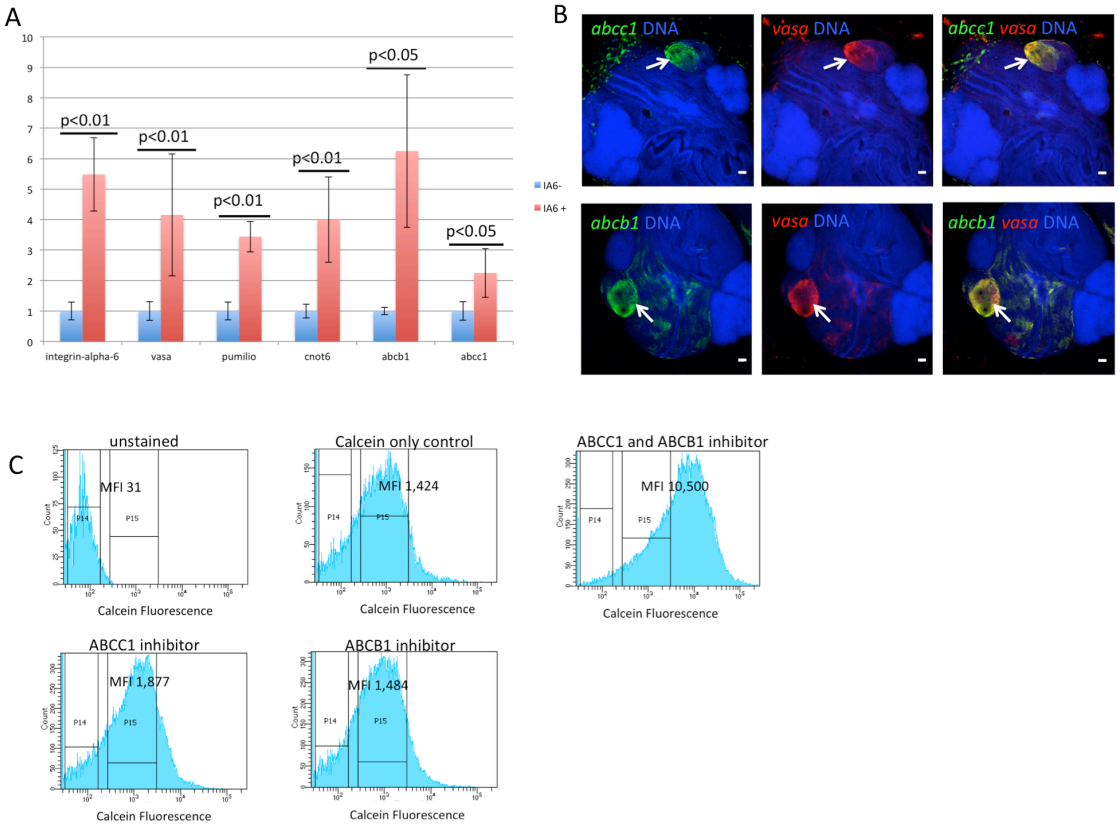

Figure 2: Botryllus germ cells express *abcb1* and *abcc1*. A: *Abcb1* and *abcc1* are expressed in Integrin-alpha-6-positive (IA6+) germline stem cells. IA6-positive and-negative cells were isolated by flow cytometry and expression of germ cell marker genes (*vasa, pumilio, cnot6*) and ABC-transporters (*abcb1* and *abcc1*) was assessed by quantitative real time PCR. Relative quantification was performed using the $2^{-\Delta\Delta CT}$-method, with *actin* as control gene. Data are expressed as averages of the relative expression ratio (fold change), normalized to IA6-negative cells. Standard deviations were calculated for each average expression ratio (n=3). Statistical analysis was performed using Student's *t*-test. B: Representative examples of double-labeled fluorescent in situ hybridization showing expression of *abcc1* and *abcb1* in *vasa*-positive cells. All *vasa*-positive (red) germ cell precursors (arrows) co-express *abcc1 and abcb1* (green, arrows). Red and green channels are shown individually with nuclear counterstaining (blue), and merged images on the right show co-expression of both genes (yellow) Scale bars = 20µm. C: Flow cytometry based functional analysis of ABC transporter activity. Histograms show Calcein fluorescence intensity (within the live cell gate) for unstained cells, cells stained with Calcein, and cells stained with Calcein in the presence of inhibitors of ABC transporters. Mean fluorescence intensity values are shown for each histogram. In the presence of an inhibitor of both Abcc1 and Abcb1, Calcein fluorescence is significantly increased.

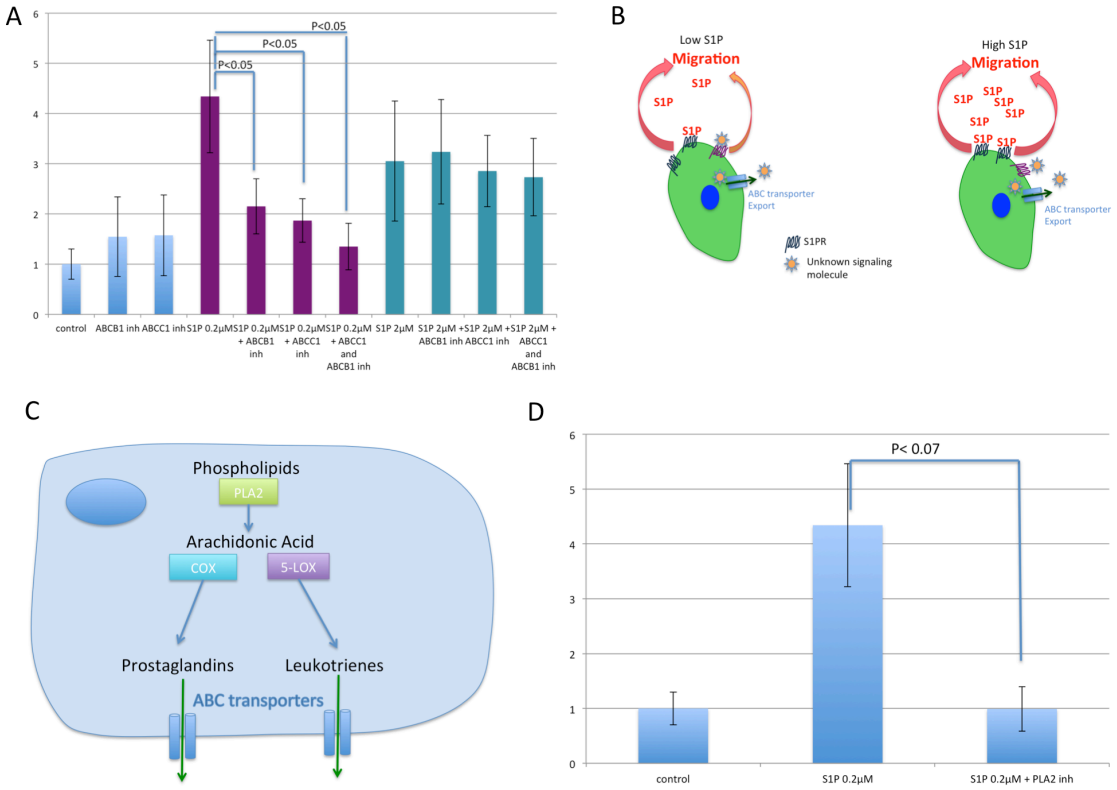

Figure 3: Migration of Integrin-alpha-6-positive germ cell precursors towards low concentrations of sphingosine-1-phosphate (S1P) depends on ABC-transporter activity. Migration assay of IA6-positive cells in response to different concentrations of S1P, with or without inhibitors of ABC-transporters, as indicated. No S1P was added to control wells. IA6+ cells were added to the upper chamber of a transwell system coated with laminin, and after 2h, migrated cells in the lower chamber were counted. Data are expressed as fold changes of numbers of migrated cells, normalized to unstimulated controls (n=4). Statistical analysis was performed using Student's *t*-test. B: Hypothetical model of ABC-transporter mediated export of an unknown autocrine signaling molecule. Migration to low concentrations of S1P depends on ABC-transporter activity. Low-level stimulation of the S1P-receptor might induce production and ABC-transporter mediated secretion of an unknown signaling molecule, which provides an autocrine signal to enhance migratory activity. High concentrations of S1P are sufficient to stimulate migratory activity, and ABC-transporter-mediated export of an autocrine signal is not required. C: The cytoplasmic enzyme phospholipase A2 (PLA2) generates arachidonic acid from phospholipids. Arachidonic acid is further metabolized by either lipoxygenases (LOX) or Cyclooxygenases (COX) to generate bioactive lipids such as leukotrienes or prostaglandins, which are exported out of the cytoplasm by ABC transporters. D: migration assay of IA6+ cells in response to 0.2uM of S1P the presence of an inhibitor of PLA2. Inhibition of PLA2 completely blocked the migratory activity to 0.2uM of S1P. Data are expressed as fold changes of numbers of migrated cells, normalized to unstimulated controls (n=4). Statistical analysis was performed using Student's *t*-test.

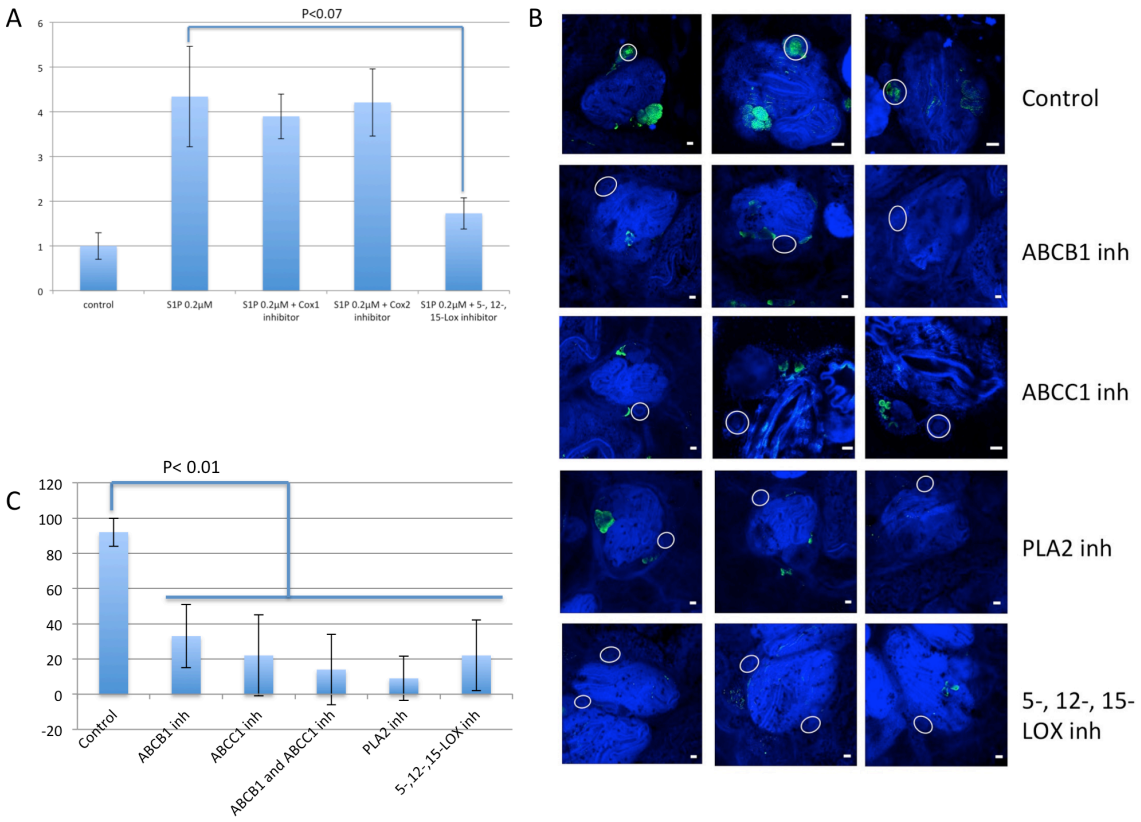

Figure 4: Migration to low concentrations of S1P depends on activity of Lipoxygenase. Homing of germ cells in vivo depends on activity of PLA2 and LOX. A: Migration assay of IA6-positive cells in response to 0.2µM S1P, with or without inhibitors Cox1, Cox2 or 5-,12-, and 15-LOX, as indicated. No S1P was added to control wells. IA6+ cells were added to the upper chamber of a transwell system coated with laminin, and after 2h, migrated cells in the lower chamber were counted. Data are expressed as fold changes of numbers of migrated cells, normalized to unstimulated controls (n=4). Statistical analysis was performed using Student's *t*-test. B and C: Animals were treated with inhibitors of ABC transporters, PLA2 or 5-,12-, and 15-LOX for 3 days, starting at stage A1, and fixed at stage B2, when the secondary bud forms a closed double vesicle (n=4). Controls were left untreated. *Vasa*-FISH was performed on fixed animals, and the number of secondary buds containing germ cells were counted by confocal microscopy. Nuclei were counterstained with Hoechst 33342 (blue). Scale bars = 20µm. In control animals, *vasa*-positive germ cells (green) homed into the double vesicle stage secondary buds (circles). All 4 inhibitors significantly reduced migration of vasa-positive cells to secondary buds. Graph in C shows the percentage of double vesicle stage secondary buds containing *vasa*-positive cells for each treatment. Error bars represent the standard deviation for each average (n=4). Statistical analysis was performed using Student's *t*-test.

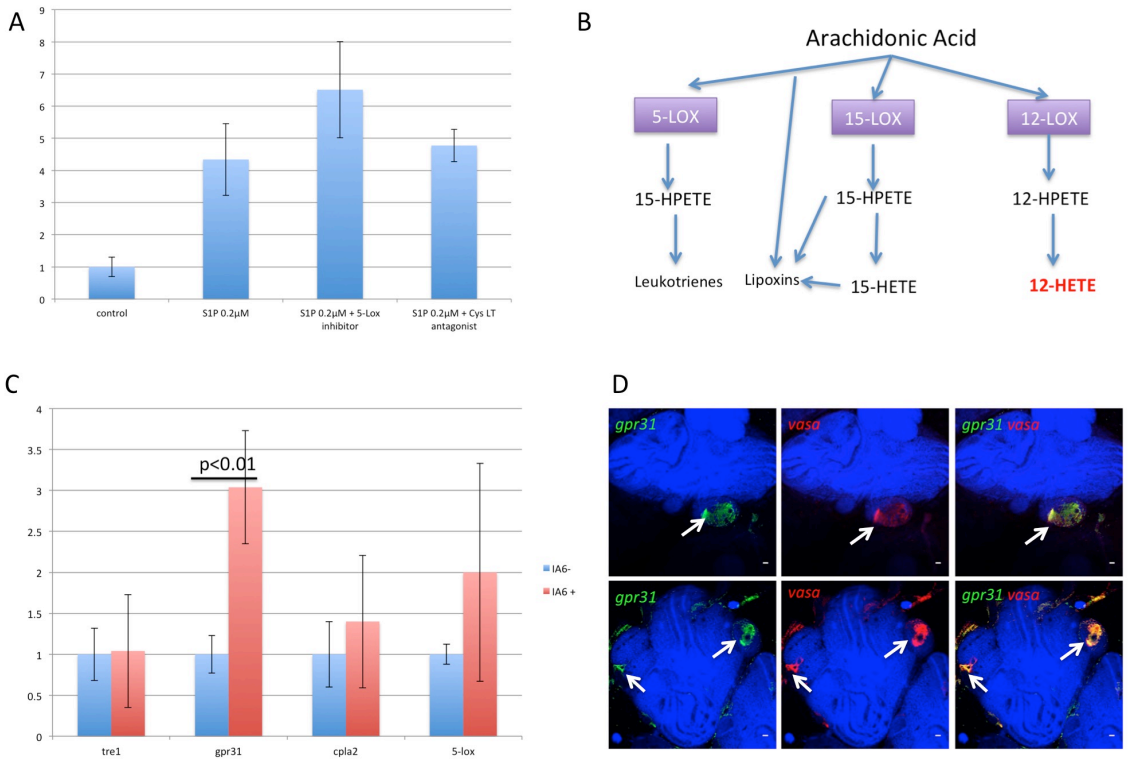

Figure 5: Migration of IA6+ cells to S1P does not depend on 5-LOX or leukotrienes. Botryllus germ cells express the 12-S-HETE receptor *Gpr31*. A: Migration assay of IA6-positive cells in response to 0.2µM S1P, with or without inhibitors 5-LOX or Cysteinyl Leukotriene Receptor, as indicated. No S1P was added to control wells. IA6+ cells were added to the upper chamber of a transwell system coated with laminin, and after 2h, migrated cells in the lower chamber were counted. Data are expressed as fold changes of numbers of migrated cells, normalized to unstimulated controls (n=4). Statistical analysis was performed using Student's *t*-test. B: In humans, 3 different types of lipoxygenase metabolize arachidonic acid to various signaling agents. 12-Lox metabolizes arachidonic acid to 12(S)-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12(S)-HpETE). 12(S)-HpETE is rapidly reduced to 12(S)-HETE by ubiquitous cellular peroxidases, such as Glutathione peroxidases. C: IA6-positive and- negative cells were isolated by flow cytometry and expression of *tre1, gpr31, cytosolic phospholipase a2, cpla2*) and ABC-transporters (*abcb1* and *abcc1*) was assessed by quantitative real time PCR. Relative quantification was performed using the $2^{-\Delta\Delta CT}$-method, with *actin* as control gene. Data are expressed as averages of the relative expression ratio (fold change), normalized to IA6-negative cells. Standard deviations were calculated for each average expression ratio (n=3). Statistical analysis was performed using Student's *t*-test. D: Representative examples of double-labeled fluorescent in situ hybridization showing expression of *gpr31* in *vasa*-positive cells. All *vasa*-positive (red) germ cell precursors (arrows) co-express *gpr31* (green, arrows). Red and green channels are shown individually with nuclear counterstaining (blue), and merged images on the right show co-expression of both genes (yellow) Scale bars = 20µm.

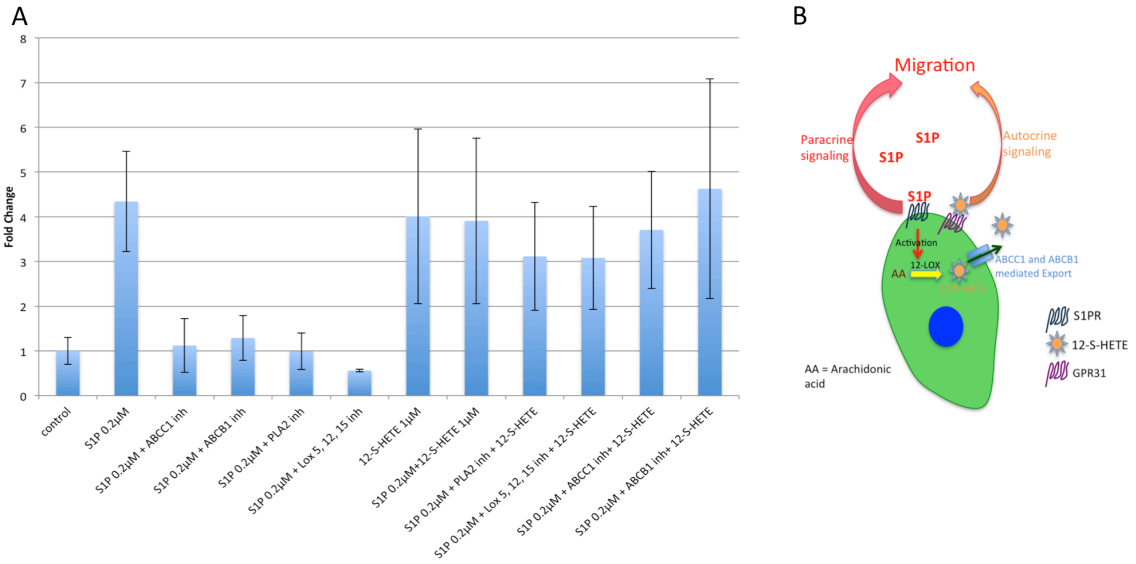

Figure 6: Migration to low concentrations of S1P depends on 12-S-HETE production: A: Migration assay of IA6-positive cells in response to 0.2µM S1P and/or12-S-HETE, with or without inhibitors of ABC-transporters, PLA2 or 5-,12-, and 15-LOX, as indicated. No S1P was added to control wells. IA6+ cells were added to the upper chamber of a transwell system coated with laminin, and after 2h, migrated cells in the lower chamber were counted. Data are expressed as fold changes of numbers of migrated cells, normalized to unstimulated controls (n=4). Statistical analysis was performed using Student's *t*-test. B: Hypothetical model of ABC-transporter mediated autocrine signaling by 12-S-HETE. Migration to low concentrations of S1P depends on ABC-transporter activity. Low-level stimulation of the S1P-receptor might induce activity of 12-LOX and production of 12-S-HETE. 12-S-HETE is exported by ABCC1 and ABCB1 and binds to GPR31 expressed on the same cells, provides an autocrine signal to enhance migratory activity.