Molecular mechanisms of asymmetric divisions in mammary stem cells

Angela Santoro[†], Thalia Vlachou[†], Manuel Carminati, Pier Giuseppe Pelicci^{*} & Marina Mapelli^{**}

Abstract

Stem cells have the remarkable ability to undergo proliferative symmetric divisions and self-renewing asymmetric divisions. Balancing of the two modes of division sustains tissue morphogenesis and homeostasis. Asymmetric divisions of *Drosophila* neuroblasts (NBs) and sensory organ precursor (SOP) cells served as prototypes to learn what we consider now principles of asymmetric mitoses. They also provide initial evidence supporting the notion that aberrant symmetric divisions of stem cells could correlate with malignancy. However, transferring the molecular knowledge of circuits underlying asymmetry from flies to mammals has proven more challenging than expected. Several experimental approaches have been used to define asymmetry in mammalian systems, based on daughter cell fate, unequal partitioning of determinants and niche contacts, or proliferative potential. In this review, we aim to provide a critical evaluation of the assays used to establish the stem cell mode of division, with a particular focus on the mammary gland system. In this context, we will discuss the genetic alterations that impinge on the modality of stem cell division and their role in breast cancer development.

Keywords asymmetric cell divisions; asymmetric cell division assays; breast cancer; mammary stem cells

DOI 10.15252/embr.201643021 | Received 7 July 2016 | Revised 4 October 2016 | Accepted 25 October 2016 | Published online 21 November 2016

EMBO Reports (2016) 17: 1700–1720

See the Glossary for abbreviations used in this article.

Introduction

Stem cells are defined by their capacity of long-term self-renewal coupled with the ability to generate differentiated progeny, both of which enable them to sustain morphogenetic programs and tissue homeostasis. Self-renewal and differentiation are accomplished through a single mitosis, in which at least one of the two daughters retains stemness (asymmetric divisions; ACD). Alternatively, stem cells can undergo symmetric proliferative divisions (SCD) generating two stem cells (Fig 1A). In multicellular organisms, to prevent

aberrant growth or loss of tissue, the balance between asymmetric and symmetric mitoses must be exquisitely controlled in time and space throughout the entire lifespan. The defining mechanisms governing such a balance constitute key unresolved issues in adult stem cell biology.

The connection between deregulated stem cell proliferation and tumor biology is one of the major discoveries of the last decade [1]. Seminal studies in *Drosophila* larval brains revealed that aberrant symmetric divisions and defective cell cycle exit of mutated neuroblasts suffice to generate massive tumor-like overgrowth [2,3]. In vertebrates, an equally clear demonstration that switching from asymmetric to symmetric cell divisions is sufficient to cause cancer is still lacking, likely due to technical difficulties in identifying correctly stem cells and studying their proliferation and differentiation potential, as well as to our limited knowledge of ACDs in vertebrates. Nonetheless, converging evidence indicates that in several human cancers, aggressiveness correlates with a stem cell signature, and expansion of stem cell compartments causes tissue disorganization and malignant overproliferation [4].

In this review, we summarize the principles underlying the execution of ACDs highlighting the specific aspects of *asymmetry* addressed by the assays most commonly used to study stem cells (see Box 1 and Fig 2 for a summary of assays used). In addition, we survey the experimental approaches used to uncover the molecular contribution of cancer stem cells (CSCs) to tumor progression, with particular emphasis on the role of mammary stem cell ACDs in breast cancer.

Operational definition of ACDs: basic lessons from flies

The concept of self-renewal as defined in the previous section implies that stem cells enter the cell cycle and divide, and that at least one of the progeny is an undifferentiated cell identical to the mother. Notably, short-term self-renewal has also been documented in progenitors, thus complicating the design of experimental assays aimed at studying stem cell ACDs based on their proliferation potential. Furthermore, stem cell divisions are accompanied by both periods of quiescence and maturation events occurring after completion of the self-renewing mitosis. These non-mitotic processes, which in a physiological context are strictly connected

Department of Experimental Oncology, European Institute of Oncology, Milan, Italy

^{*}Corresponding author. Tel: +39 02 57489831; E-mail: piergiuseppe.pelicci@ieo.eu

^{**}Corresponding author. Tel: +39 02 94375018; E-mail: marina.mapelli@ieo.eu

[†]These authors contributed equally to this work

Glossary

ACD	asymmetric cell division
AurkA	Aurora-A kinase
BCC	basal cell carcinoma
BMP	bone morphogenetic protein
Cre-ER	Cre recombinase
CSC	cancer stem cell
DP	dermal papilla
ECM	extracellular matrix
EGF	epidermal growth factor
GMC	ganglion mother cell
GSC	germline stem cell
HFSC	hair follicle stem cell
HTT	huntingtin
ISC	intestinal stem cell
IF	immunofluorescence
MaSC	mammary stem cell
MRU	mammary repopulating unit
NB	neuroblast
SCC	squamous cell carcinoma
SCD	symmetric cell division
SC	stem cell
SFE	sphere-forming efficiency
SHH	sonic hedgehog
SOP	sensory organ precursor
TA	transit amplifying
TEB	terminal end bud

with self-renewing mitoses of stem cells, will not be discussed in this review.

Unequal partitioning of polarity proteins and cell fate determinants

Much has been learnt in recent years on the molecular mechanisms of ACDs. Studies conducted in the early 1990 in *Drosophila* neuroblasts (NBs, the stem cells of the central nervous system), sensory organ precursors (or SOPs, precursors of the peripheral nervous systems), and male germline stem cells paved the way to the identification of fundamental principles of ACDs. Neuroblasts always divide asymmetrically to give rise to one neuroblast and one ganglion mother cell, the latter destined to differentiate into two neurons

or glia (Fig 1B). The remarkable ability of neuroblasts to undergo ACDs even in isolated cultures has made them the prototype of intrinsic ACDs, which are characterized by the ability of mitotic cells to cell-autonomously polarize the cortex, align the mitotic spindle along the polarity axis, and unequally partition cellular components, including the polarity proteins Par3/Par6/aPKC and the so-called fate determinants, that is, molecules able to confer a given fate on the cell that inherits them. In neuroblasts, this activity has been documented for the transcription factor Prospero, the endocytic Notch inhibitor Numb, and the tumor suppressor Brat [5,6]. The characterization of genetic lesions impairing asymmetry in neuroblasts also led to the identification of genes coding for proteins essential for mitotic spindle coupling with cortical polarity, such as the Par3-binding protein Inscuteable [7], the switch molecule Pins/Rapsinoid, the Dynein-adaptor Mud [3,8,9], the Aurora-A kinase [10–12], and the G α i subunit of heterotrimeric G-proteins [13,14] (see Table 1 for a description of their functions). Most notably, the asymmetric distributions of Par3, aPKC, Pins (LGN in vertebrates), and Numb with respect to the orientation of the division plane are often regarded as the distinguishing feature of ACDs. However, it is important to stress that it has never been formally proven that these molecules act ubiquitously as fate determinants.

In parallel, recent research consolidated the view that stem cell maintenance depends on specific master transcriptional regulators, whose activity is governed by asymmetrically segregating epigenetic marks. Using a photoconvertible dual-color method, it was possible to demonstrate that during male germline stem cell (GSC hereon) asymmetric divisions, the pre-existing pool of histone H3 selectively segregates into the GSCs thanks to selective phosphorylation on H3-Thr10 by the mitotic kinase Haspin [15], whereas the newly synthesized H3, incorporated into nucleosomes during DNA replication, partitions into the differentiating cell [16].

Unequal sister cell positioning with respect to the niche

In flies, also GSC ACDs occur with a stereotypical orientation with respect to cortical polarity. In this case, however, asymmetry is not cell autonomous as for NBs, but relies on the GSC microenvironment (Fig 1C). GSCs reside in a specialized niche, physically attached to somatic hub cells in an E-cadherin-dependent manner

Figure 1. Self-renewing symmetric and asymmetric divisions sustain tissue morphogenesis and homeostasis.

(A) Scheme of asymmetric versus symmetric self-renewing stem cell divisions. In ACD (left), self-renewal is attained by unequal partitioning of fate determinants and niche contacts, so that only one cell retains stemness (pale yellow), while the other one is committed to differentiation (gold). In SCD (right), stem cells proliferate by equally distributing cellular components between the two daughter cells, generating two stem cells. (B) Intrinsic ACDs of *Drosophila* neuroblasts delaminated from the neuroepithelium generating two differently sized daughters: one neuroblast and one ganglion mother cell (GMC). The larger neuroblast inherits the apical Baz/Par6/aPKC polarity complex (purple crescent), the spindle orientation proteins Pins, Mud, G α i, and Inscuteable (cyan crescent) and maintains stemness. The smaller GMC inherits fate determinants (brown dots), which activate a neuronal differentiation program, and the mother centrosome (red circle). (C) *Drosophila* male GSCs divide asymmetrically producing one stem cell contacting the niche (Hub) through adherens junctions (magenta rods), and a distal daughter differentiating into a gonoblast and positioned among somatic cyst cells. The mother centrosome (red circle) segregates into the stem cell. (D) During development, murine epidermal progenitors balance ACDs and SCDs to stratify the skin. Basal progenitors adhere to the basement membrane (niche) through β -integrins (green), and to neighboring cells through adherens junctions (magenta rods). These contacts and the apical localization of the Par complex Par3/Par6/aPKC (purple dots) define the progenitor apico-basal polarity. Vertical ACDs (left) occur with the spindle aligned to the apico-basal polarity axis, and generate a basal progenitor and a differentiating suprabasal cell inheriting Par3, Insc, LGN, and NuMA (cyan dots). Planar SCDs expand the basal progenitor pool (right). (E) During hair follicle (HF) morphogenesis (top panel), HFSCs originate by ACDs of epithelial placode cells. These cells divide perpendicular to the tissue basement membrane with LGN (cyan dots) partitioned into the suprabasal cell, and integrins (green) and Wnt components confined in the basal cell. In the adult hair follicle (bottom panel), mesenchymal cells lying beneath the placode condense in the dermal papilla (DP) with niche functions. HFSCs show a dual localization: quiescent HFSCs in the bulge and activated HFSCs in the hair germ in direct contact with the DP. Activated HFSCs divide perpendicularly to the niche, generating the inner differentiated layers (gray area), whereas undifferentiated HFSCs expand in the outer layer by oriented divisions. (F) The small intestine is formed by a monolayered epithelium folding into villi and crypts. At the crypt base, ISCs intercalate with Paneth cells (green) secreting Wnt ligands and thus acting as niche. Upon proliferation, ISCs move upward along the crypt wall, experience reduced Wnt signals, and differentiate into transit-amplifying (TA) progenitors. TA progenitors, in turn, differentiate into the variety of cells that populate the villi to replace the epithelial cells which are shed into the intestinal lumen at the villus tip.

Figure 1.

Box 1: Experimental assays employed to study asymmetric divisions**Imaging**

According to a mechanistic definition, ACDs deal with the unequal partitioning of fate determinants and niche contacts. Thus, monitoring the orientation of division and the partitioning of such determinants can be considered the best indication of asymmetry, when—and only when—the identity of the niche and the determinants is clearly defined for the system under investigation. Because mitosis is a dynamical process, the best insights into the mechanisms of ACD have been obtained by live imaging, in which the distribution of determinants and the position of daughter cells after cytokinesis can be followed both in space and in time [18,42,146].

Lineage tracing

In lineage tracing experiments, cells expressing a SC-specific promoter are tagged with a permanent and heritable genetic marker. Most commonly, reporter genes for the expression of GFP, RFP, YFP, mCherry genes are used. The distribution of the marker is monitored long term within a tissue or an entire organism, ideally *in vivo*. If all the differentiated lineages can be tracked back to a single cell, this cell can be considered a multipotent stem cell. Long-term generation of marked cell lineages indicates that the labeled cell has self-renewal capacity.

Sphere-forming assays

The ability of stem cells isolated from tissues and grown in non-adherent cultures to form clonal spheroids when plated in liquid or semi-liquid cultures has been used to identify the number of stem cells within a cell population. The assay is based on the notion that only cells with SC properties are able to form a spheroid composed of cells at different differentiation stages, as assessed by functional assays. Therefore, the number of spheres in culture reflects the number of stem cells. In the case of MaSCs, these spheres are called mammospheres. Mammospheres can be serially passaged and the sphere-forming efficiency (SFE) expressed as a measure of their self-renewal potential. SCs dividing mainly asymmetrically will progressively decrease their SFE until complete exhaustion in culture, whereas SCs dividing mainly symmetrically will aberrantly expand and propagate indefinitely.

SCs and progenitors within mammospheres can be isolated to near homogeneity using the PKH26-based label retention assay. PKH26 is a lipophilic dye that labels the cell membrane and gets segregated upon cellular divisions. Cells that are quiescent or slowly dividing are enriched in MaSCs (~1:4) and retain the label during the assay. PKH26 retention has been employed for determination of the mode of SC division, as ACDs generate one cell that remains quiescent, and therefore retains the label, and another that continues to divide. In a culture in which SCDs are prevalent, instead, the dye will be also rapidly diluted in MaSCs. Indeed, in this case, the PKH^{neg} fraction of cells will be able to initiate a mammosphere culture and re-form a mammary gland upon transplantation [66,70,72].

Organoids

Primary stem cells purified from a number of tissues including intestine, pancreas, liver, and brain can be expanded in clonal 3D organoids with architectural features resembling the organ of origin. The differentiation pattern sustaining organoid morphogenesis can be ascribed to self-renewing ACDs, which indeed have been proven experimentally in the case of cerebral organoids by lineage tracing experiments [147].

Organ reconstitution upon transplantation

The ability to reconstitute a tissue in a recipient host has been widely used to assess the presence of SCs in a given population. In the mammary system, cells are transplanted in the gland of pre-pubertal mice ablated of the rudimentary epithelial branches, and the presence of a fully differentiated mammary outgrowth is assessed 10–14 weeks after transplant.

The transplantation protocol in limiting dilution conditions requires injecting a serially diluted number of cells into the cleared fat pad of pre-pubertal recipient mice. This allows to measure the size of the stem cell pool in a given population and to assess whether one single stem cell might be sufficient to reconstitute the gland tissue. In this setting, it is indirectly assumed that rounds of ACDs and, eventually, SCDs are needed to balance self-renewal and expansion. The calculated frequency of SCs reflects the prevalence of one of the two modes of division [70].

Serial transplantation, instead, functionally defines stem cells for their ability to self-renew by generating new stem cells alongside a more differentiated progeny [148]. In theory, asymmetric stem cell self-renewal ensures maintenance of a stable number of stem cells. However, wild-type stem cells become functionally exhausted after six to seven divisions; therefore, a constant (in numbers) stem cell pool has limited organ reconstitution ability upon serial transplantation. If instead the stem cell pool expands through symmetric divisions, then an infinite number of *in vivo* passages can be achieved [70].

[17]. Hub cells secrete the ligand Unpaired that activates Jak-STAT signaling in the neighboring cells, in this way maintaining their stem cell identity. GSCs divide asymmetrically with the spindle perpendicular to the hub so that only one daughter retains contact with the hub cells, and hence stemness. Interestingly, upon stem cell loss, vacancies are replenished by proliferative symmetric divisions which are oriented parallel to the hub. Elegant live imaging studies revealed that GSC perpendicular divisions occasionally result in the production of two GSCs or two differentiating cells, and that upon injuries differentiated cells can revert to GSCs by migrating back to the hub [18], thus displaying a cell plasticity impossible to observe in fixed tissues. The fly GSC behavior well exemplifies the concept of stem cell niche, a “specific anatomic location that regulates how stem cells participate in tissue regeneration, maintenance, and

repair” [19]. It also highlights the paramount importance of sister cell positioning with respect to the niche for fate specification.

Alignment of the mitotic spindle with the polarity axis

Studies in neuroblasts and GSCs uncovered an intimate connection between the orientation of the division plane (and hence the spindle axis) and the asymmetry of fate specification, which is required for the asymmetric segregation of both determinants and adhesion molecules/cell–cell contacts. Elegant studies with molecular markers specific for mother and daughter centrioles revealed that in these systems, not only ACDs occur with the mitotic spindle aligned with the polarity axis and perpendicular to the niche, but also that mother and daughter centrosomes are specifically partitioned either in the stem or in the differentiating cell [20,21], suggesting that the

Figure 2.

Figure 2. Schematic representation of the assays used to study ACDs in mammary stem cells.

Details of the working principles and the kind of information provided by each of the assays are reported in Box 1. (A) Imaging: Visualization of the distribution of DNA and fate determinants with respect to a known cellular niche allows monitoring of asymmetric fate partitioning and daughter cell positioning. Live imaging of the mitotic process is most informative. (B) Lineage tracing: Transgenic mice expressing an inducible Cre recombinase (Cre-ER) under the control of a stem cell-specific promoter are crossed with a reporter model harboring a stop codon flanked by LoxP sites upstream of a reporter gene (e.g., GFP in the figure) under a constitutive promoter. Administration of 4-hydroxytamoxifen (4-OHT) allows the activation of the Cre in cells expressing the SC promoter. Cre mediates the recombination between the LoxP sites, causing the excision of the stop cassette and leading to the permanent expression of the reporter gene in the SC and its progeny. In order to minimize any adverse effects of the 4-OHT administration on the mammary gland, the Cre gene can also be expressed under a Tet-ON/OFF system of inducible regulation. (C) Top: Sphere-forming assay. Epithelial cells isolated from the mammary gland can be grown in anchorage-independent conditions allowing the formation of mammospheres. Mammospheres are clonal in origin, contain SCs and more differentiated cells (progenitors), and can be serially passaged. Sphere-forming efficiency (SFE) is calculated as a percentage of total number of cells plated (the number of spheres formed/the number of cells plated). Bottom: PKH26 assay. Epithelial cells are labeled with the lipophilic dye PKH26 and allowed to grow as mammospheres for label retention. Only the quiescent or slowly dividing cells will retain PKH26 at the end of the assay. In a culture in which SCDs are prevalent, the PKH^{neg} population will contain cells with SC features, able to form mammospheres and positive transplantations. (D) Organoids: Isolated and digested mammary epithelium is embedded in Matrigel supplemented with ECM components that allow branching. (E) Transplantation assay: Isolated epithelial cells are transplanted in the cleared fat pad of pre-pubertal recipients and their ability to reconstitute a mammary gland is assessed. Transplantation in limiting dilution conditions allows calculating SC frequency, while serial transplantation allows measuring SC lifespan. Both assays provide information regarding the size of the SC pool in a given population.

inherent centrosome asymmetrical maturation is involved in asymmetric fate choices.

Also, the asymmetric outcome of SOP divisions relies on tissue-derived external cues that instruct the anterior–posterior cortical polarity, though in a different flavor of environmental mitotic

regulation compared to niches as defined before [14]. SOP cells delaminate from a polarized epithelium, from which they inherit planar polarity that is used in two subsequent mitoses to asymmetrically segregate Notch regulators (including Numb) and to generate two outer and two inner sensory cells. Because of this simple lineage, the

Table 1. Names, functions, and interactions of proteins involved in ACD as discussed in this review.

<i>Drosophila</i>	Vertebrates	Protein type/epithelial localization/function
Bazooka	Par3/Par3L	Polarity protein/apical (TJs)/polarity establishment
Par6	Par6	Polarity protein/apical/polarity establishment
aPKC	aPKC ζ / ι / λ	Polarity protein, Ser/Thr kinase/apical/polarity establishment
Lkb1	Lkb1	Ser/Thr kinase/apical/proliferation
Scrib	Scrib	Scaffold protein/baso-lateral/polarity regulator
Dlg1	DLG1	Scaffold protein/baso-lateral/polarity regulator
Lgl	Lgl	Scaffold protein/baso-lateral/polarity regulator
Inscuteable	mInsc	SC adaptor molecule/apical/orientation; partner of Par3 and LGN
Pins	LGN	Scaffolding protein/apical (ACD), lateral (SCD)/orientation; partner of mInsc and NuMA
Mud	NuMA	Adaptor/apical (ACD), lateral (SCD) and at spindle poles/orientation; partner of LGN and Dynein
G α_i /G α_o	G α_i	Subunit of heterotrimeric G-proteins/plasma membrane/orientation; partner of LGN
Dynein	Dynein	MT motor/apical (ACD), lateral (SCD)/orientation; partner of NuMA
—	β 1-integrin	Collagen receptor/basal membrane/niche contact, orientation
Huntingtin	Huntingtin	MT-associating protein/spindle poles/orientation, partner of Dynein
Prospero	Prox1	Transcription factor/fate determinant
—	Lef1	Transcription factor/Wnt signaling effector, differentiation
Armadillo	β -catenin	Transcriptional co-activator and adhesion/Wnt signaling effector; partner of Lef1
—	p53	Transcription factor/apoptosis, senescence, DNA damage response
—	SOX-9	Transcription factor/SHH signaling effector, stemness
Numb	Numb	Endocytic Notch inhibitor/basal (<i>Dm</i> neuroblasts)/fate determinant
Brat	Trim32	Scaffolding protein/basal (<i>Dm</i> neuroblasts)/fate determinant
—	Musashi	RNA-binding protein/regulation of Numb translation
—	miR-34a	microRNA/Numb mRNA targeting
—	ErbB2/Her2	Tyrosine kinase receptor/lateral membrane/proliferation
Aurora-A	Aurora-A	Ser/Thr kinase/spindle poles/orientation, bipolar spindle assembly

Proteins are listed based on their role; from the top: polarity proteins, spindle orientation components, and fate determinants.

precise knowledge of fate determinants, and the easiness of imaging, SOPs have provided numerous insights into molecular mechanisms of asymmetry. Segalen *et al* [22] showed that the non-canonical Wnt pathway is involved in establishing correct SOP orientation by direct binding of the spindle motors to the Wnt-effector dishevelled. More recently, it was also shown that specialized endosomes, known as Sara endosomes, are inherited asymmetrically by the two SOP daughter cells, PIIa and PIIb, and mediate Notch/Delta signaling between the nascent sisters [23] via the action of antagonistic kinesins moving on the central spindle during cytokinesis [24]. Importantly, these latter studies revealed that organelle trafficking is implicated in ACDs.

In addition to these mechanistic insights into how ACDs are executed, fly neuroblasts served as a model tool to explore the effect of the aberrant switch between asymmetric and symmetric divisions on the stem cell proliferative potential. Seminal studies from the Gonzalez laboratory not only revealed that impairment of asymmetry-related genes causes massive expansion of the neuroblast compartment, but also that, upon allograft implantation, asymmetry-defective neuroblasts hyper-proliferate into wild-type fly abdomens and subvert tissue homeostasis in a malignant-like form [2]. Thus, these assays first highlighted the intimate causative connection between stem cell deregulation and cancer.

ACDs in vertebrates

Studies of ACDs in vertebrate systems lagged substantially behind the ones in flies, mainly because of imaging difficulties and lack of stem cell markers amenable to track asymmetric divisions. Contrary to fly neuroblasts, vertebrate stem cells balance SCDs versus ACDs in response to developmental programs and regenerative stimuli. Recent studies have revealed that in vertebrates, the symmetric or asymmetric outcome of each individual SC division is unpredictable. Nonetheless, the ratio between the two modes of division within a stem cell population is tightly regulated in order to maintain tissue homeostasis, and it plastically readjusts upon injuries in order to promote regeneration. Notably, this intrinsic stochasticity in differentiation may also account for clonal competition of mutant stem cells and therefore might contribute to cancer development [25].

Molecularly, extracellular cues instructing stem cell divisions are communicated as short-range morphogen gradients such as Wnt, Hedgehog (HH), EGF and BMP signaling [26]. For these reasons, studies of mitotic phenotypes of vertebrate stem cells require *in vivo* population statistics and greatly benefited from recent developments of intravital microscopy [27]. Often, experimental strategies addressing ACDs in vertebrates have been guided by working principles learnt in *Drosophila* stem cells, somehow biasing the discovery of novel and more specific mechanisms.

In this paragraph, we will review the approaches used to study ACDs in vertebrates, emphasizing whether asymmetry was approached from a mechanistic perspective, investigating the self-renewing mitosis, or by lineage tracing of daughter cells. In particular, we will discuss stem cells of the epidermis and intestinal crypts, which thanks to their fast cycling rates and accessibility have been easier to characterize. Breast stem cells will be discussed in more detail in following sections.

Epidermal stem cells: contact with the basement membrane defines stemness

Embryonic mouse epidermal progenitors reside in a single basal layer, kept adherent to the basement membrane (bona fide acting as niche) by integrins, and to each other via adherens junctions, with Par3/aPKC localized apically (Fig 1D) [28]. At early developmental stages, they divide parallel to the basement membrane to enlarge the progenitors' pool, while later they switch to vertical divisions that result in physical displacement of one of the daughters in the suprabasal layer [29,30]. Mechanistically, the effector pathway responsible for the change in orientation is the same of fly neuroblasts, impinging on mammalian Inscuteable (mInsc), LGN, NuMA, and Dynein, all of which polarize apically [31]. The kinase aPKC λ has also been implicated in controlling the balance between ACD and SCD both in the inter-follicular epidermis and in the hair follicle [32]. Imaging studies revealed that, upon ACD, suprabasally positioned cells activate a Notch-dependent differentiation program ultimately resulting in skin stratification [33]. Thus, as for invertebrates, in the developing murine skin, orientation perpendicular to the niche is readout of asymmetric fate specification. Consistently, impairment of orientation genes in the epidermis by *in utero* electroporation of lentiviral shRNAs prevents skin stratification [33], whereas overexpression of the apical adaptor mInsc transiently increases vertical ACDs with no long-term effects on epidermal architecture, likely due to compensatory feedback loops [29]. Interestingly, imaging of the asymmetric distributions of NuMA and Dynein during the first divisions of isolated primary keratinocytes in culture suggested that keratinocytes are a good model system to study ACDs at molecular resolution [34,35]. Studies in adult mice evidenced that the cells of origin of basal cell carcinoma (BCC) are long-lived inter-follicular epidermal stem cells carrying oncogenic *Smoothened* mutations (*SmoM2*), which undergo a profound reprogramming [36]. Transcriptional profiling of *SmoM2*-BCC uncovered a Wnt-dependent upregulation of the transcription factor Sox9. Sox9 in turn controls a gene regulatory network promoting symmetric planar divisions, represses normal differentiation, and drives the extracellular matrix, adhesion, and cytoskeleton remodeling required for tumor invasion [37]. Collectively, these findings well exemplify how subversion of both stem cell transcriptional activity and stem cell self-renewing synergize to cause BCC skin tumors.

Hair follicle stem cells: distinct positions for distinct functions

Tremendous insights into vertebrate stem cell biology came from studies of the hair follicles, which are uniquely accessible and endowed with a continuous pattern of regeneration [38]. Such peculiar properties coupled with a defined compartmentalization of the epithelial hair follicle components, whereby cell position defines cell identity, allowed *in vivo* studies of hair follicle formation, stem cell quiescence, activation, and proliferation in a complete mini-organ setting [39]. The growing hair follicle comprises two epithelial stem cell populations: the bulge, which surrounds the hair shaft, and the hair germ, in contact with the mesenchymal-dermal papilla (DP) and with niche functions (Fig 1E) [39]. In formed follicles, these distinct stem cell pools serve different purposes: the activated germ cells cyclically respond to growth stimuli to regenerate the hair, while the quiescent bulge stem cells confer long-term follicle homeostasis [40]. Remarkably, such a bipartite stem cell organization was reported for other rapidly regenerating tissues including the

intestine, the blood, and the brain [41]. Elegant intravital multi-photon microscopy of hair follicles pioneered in the Greco and Fuchs laboratories allowed single-cell imaging of proliferative events underlying hair follicle generation and re-growth. Lineage tracing coupled to live imaging and genetic manipulations revealed that hair bud stem cells develop by asymmetric division of cells within thickened epithelial platforms named placodes (Fig 1E). These divisions occur perpendicular to the basement membrane with apically polarized LGN [42]. Upon asymmetric cytokinesis, integrins and the Wnt signaling-dependent transcription factor Lef1 are confined into the basal cell, characterized by low proliferation rates, whereas the suprabasal daughters are positive for the SHH-induced stem cell marker Sox9 and divide rapidly and symmetrically generating hair follicle stem cells. Thus, during morphogenesis, hair follicle stem cells are generated by Wnt-driven asymmetric divisions, long before niche formation. Interestingly, in this mitotic process, the daughter destined to become stem is the one “exiting” the high-Wnt parent microenvironment, contrary to what observed for adult stem cells in most known systems. How stem cell proliferation turns into differentiation to restrict newly born stem cells in the bulge as follicle morphogenesis proceeds is an outstanding open question.

Intravital imaging of hair follicles conducted on transgenic mouse lines, with the epithelial compartment labeled with K14-H2B-GFP and the mesenchymal population labeled with Lef1-RFP, provided an ideal setting for lineage tracing experiments also during growth and regression of formed hair follicles [43]. They revealed that, at first, germ cells at the DP–niche interface divide perpendicularly to the niche, along the follicular long axis, to form the inner differentiated layers of the follicle, while the expanding basal layer (referred to as outer root sheath) is formed by basally restricted oriented divisions [27]. These oriented divisions are triggered by coordinated Wnt/SHH signaling, but further molecular information is needed to clarify whether they are also asymmetric. Constitutive Wnt/ β -catenin activation of bulge stem cells up-regulates Wnt ligand production inducing a non-cell-autonomous hyper-proliferation in the neighboring follicles [44]. These findings are consistent with the notion that squamous cell carcinomas (SCCs), aggressive skin cancers induced by oncogenic RAS mutations, accumulate tumor-initiating mutations in follicular stem cells that dysregulate the epithelial–mesenchymal crosstalk and favor Wnt-dependent over-proliferation. In summary, studies of inter-follicular stem cells and placode cells served to elucidate fundamental principles of vertebrate ACDs, including the role of molecular effectors common to invertebrates, and the critical Wnt gradient-dependent regulation of orientation and proliferation subtending asymmetric fate definition.

Intestinal stem cells: SCCs maintain tissue homeostasis

In the last decade, great insights into epithelial stem cell biology came from studies of the murine small intestine, a monolayer epithelium with the highest daily regenerative rate [45]. The continuous replenishment of the intestinal epithelium rests on the self-renewal ability of an adult stem cell population residing at the base of the crypts (Fig 1F). The true identity of intestinal stem cells has been long elusive, and only recently the discovery of intestinal stem cell markers combined with the establishment of clonal fate-mapping techniques allowed the understanding of the cellular principles of crypt homeostasis. The concomitant advent of organotypic *ex vivo* mini-gut cultures brought unique breakthroughs in

the field, allowing the study of crypt morphogenesis in a mesenchymal-free environment. Crypt structures develop postnatally from restricted pools of proliferative cells found at the base of embryonic villi. Lgr5-positive proliferating cells with stem cell-like characteristics, as monitored by mini-gut assays, are present in the E14 fetal intestine and persist throughout the entire lifespan [46,47]. Theoretical modeling and short-term lineage tracing suggested that these intestinal stem cells (ISCs) expand via symmetric division in the first 2 weeks of life and then switch to asymmetric divisions, generating one stem and one differentiated progeny in order to maintain a functional and properly sized intestinal epithelium [48]. In adult mice, intestinal homeostasis is maintained by Lgr5⁺ stem cells, which intercalate with post-mitotic Paneth cells at the crypt bottom where expression of Wnt-target genes (including Sox9, Ascl2, and EphB2) peaks. *In vivo*, Paneth cell-secreted Wnt3 is redundant with mesenchymally produced Wnt ligands, but, *in vitro*, it suffices to promote mini-gut formation [49], indicating that Paneth cells act as a niche for the ISC compartment. Multicolor lineage tracing experiments supported by mathematical modeling revealed that Lgr5⁺ ISCs undergo symmetric divisions generating either two ISCs or two transit-amplifying cells [50,51], which migrate from the crypt bottom toward the villus. This evidence implies that proper crypt size is maintained by a pattern of population asymmetry rather than individual ACDs, in which stochastic ISC loss is compensated by duplication events. As a result, ISC clones undergo neutral drift since the contraction of one clone, or just the displacement of the ISC from the Paneth cell niche, would be compensated by expansion of the neighboring one. By contrast, imaging of individual mitotic events seemed to suggest that Lgr5⁺ cells can undergo oriented divisions, perpendicular to the crypt monolayer and characterized by asymmetric segregation of the template DNA strand into the stem cell progeny [52]. Subsequent analyses of spindle orientation and DNA label distribution challenged these findings, suggesting that in ISCs orientation of divisions and fate inheritance can be uncoupled [53,54]. An interesting twist to the discussion comes from recent data reported by Farin and colleagues, who generated a functional HA-epitope-tagged Wnt3 allele to monitor how a Wnt3 gradient is sustained in the intestinal crypts [55]. Immunofluorescence (IF) and mini-guts analyses revealed that Wnt3 enriches at the basolateral membrane of ISCs, at contact sites with the adjacent Paneth cells, in a Frizzled-dependent manner. Most notably, the authors demonstrate that Wnt3 propagates in a cell-bound manner via cell divisions, and not by free diffusion. Thus, ISC membranes store Frizzled-bound Wnt proteins, which are spatially diluted by receptor trafficking and cell divisions. At a cellular level, these findings seem to imply that individual ISCs display an asymmetric distribution of surface Wnt3-engaged Frizzled receptors, with highest concentration at the Paneth cell boundary. The conclusion is that the ISC plasma membrane carries positional information that “could be involved in timing differentiation” [55]. Intriguingly, these data remind of recent findings by the Nusse laboratory showing that in cultured human embryonic stem cells, a polarized bioactive Wnt3a-coated bead applied to one side of the stem cell surface induces asymmetric division with β -catenin confined at the bead contact and the spindle orthogonal to the bead surface [56]. Most notably, *in vitro* paired-cell analysis of early-stage dividing colon cancer stem cells (CCSCs) showed that they divide asymmetrically, with the tumor suppressor microRNA miR-34a and Numb acting as stem fate

determinants, inhibiting Notch activity [57,58]. Thus, contrary to other systems, intestinal and colon stem cells might switch from symmetric to asymmetric divisions to reduce proliferation upon inflammation or oncogenic mutations. In conclusion, studies of crypt homeostasis in normal and oncogenic conditions uncovered a mechanism of clonal competition between symmetrically dividing ISCs that sustains crypt population asymmetry and reverts to asymmetric division mode upon inflammation or oncogene activation in order to prevent hyper-proliferation. Homeostasis is controlled by a Wnt gradient from the crypt base, spatially diluting from the secreting Paneth cells as a consequence of ISC divisions in which the Wnt3 ligand is polarized toward the Paneth cell surface.

Asymmetric cell divisions in the mammary gland

The mammary gland represents a unique system for the study of stem cell properties and behavior. The functional core of this organ resides in its epithelial component, organized in ducts and alveoli that are embedded in a vast stroma, mainly composed of adipocytes and connective tissue. The branched system of ducts terminates in lobular structures that are appointed to the secreting function of the gland. The cells specialized for the production and secretion of milk at pregnancy look onto the central lumen (luminal cells) and are characterized by the expression of cytokeratins 8 and 18 (K8/K18). These cells are surrounded by an outer layer of basal/myoepithelial cells, marked by cytokeratins 5 and 14 (K5/K14), which are able to contract facilitating the release of milk (Fig 3A).

The so composed epithelial tissue undergoes many remodeling events during the female reproductive cycle as summarized in Fig 3B. At puberty, the cells composing the terminal end buds (TEBs) proliferate and expand invading the fat pad and giving rise to primary and secondary branches. These structures are then further expanded and remodeled at every estrus cycle during adult life and constitute a putative stem cell transient niche [59]. At pregnancy, the alveolar compartment reaches its maximum development, yielding to the production of milk. After lactation, the gland undergoes an involution phase in which the alveoli undergo apoptosis and a simple ductal structure is restored. This complex series of rearrangements is repeated at every new estrus and pregnancy. While such an intense regenerative capacity suggests the existence of a cellular reservoir able to sustain it, the nature and localization of a mammary stem cell (MaSC)

population performing this function is the subject of a fervent debate.

Multipotent stem cells, self-renewing unilineage progenitors, or both?

The existence of multipotent MaSCs, also referred to as bipotent, that is able to give rise to both the lineages composing the mammary gland epithelium, has been demonstrated in mice via transplantation assays in the cleared fat pad of recipient hosts, by means of mammary fragments [60] or surface marker-sorted cell populations [61,62]. The success of transplantation has been ascribed to the ability of a particular cell population to undergo both symmetric and asymmetric divisions, leading to expansion of the MaSC pool and generation of daughter cells endowed with distinct differentiation fates (see Box 1). This assay led to the identification of a population that putatively resides in the basal compartment and is characterized by the expression of a combination of surface markers (CD24, Sca1, EpCAM, CD49f, CD29, and CD61; Fig 3C). It is important to emphasize, however, that transplantation studies do not prove the existence of a multipotent population of stem cells *in situ* but rather reflect the potential of the injected cells to survive and maintain regenerative capacity in a new environment. Moreover, transplantation assays have been biased by the different dissociation protocols used by the different research groups to isolate MaSCs. These differences have led to contrasting results, especially with respect to the definition of a bipotent cell population [63–66] able to give rise to both the basal and the luminal compartment.

Lately, genetic lineage tracing approaches, in which a fluorescent reporter is expressed in a given cell and its progeny, have provided further insight into the nature and behavior of putative stem cells *in situ* in the mammary gland tissue, challenging the existence of multipotent stem cells able to sustain the full differentiation of the gland and consequent morphogenetic rearrangements. To elucidate the cellular hierarchy of the mammary epithelium during development, adulthood, and pregnancy, Van Keymeulen and colleagues used the K14-rtTA/TetO-Cre/Rosa-YFP and K5-CreER/Rosa-YFP models to track basal cells and K8-CreER/Rosa-YFP and K18-CreER/Rosa-YFP models to track luminal cells. Their work led to the conclusion that only unipotent long-living progenitor cells (either basal or luminal), able to sustain the proliferation burst required during pregnancy and lactation and to survive over the involution time, exist in the adult mouse mammary gland [67]. The authors dispute the existence, *in vivo*, of a multipotent cell population, arguing that its identification in the past might have been driven by the

Figure 3. Asymmetric self-renewal in mammary gland morphogenesis.

(A) Schematic view of a post-pubertal fat pad in which the mammary epithelium is embedded. The ductal epithelium is composed by a basal/myoepithelial layer, which is in contact with the basement membrane, and a luminal layer with secreting function. Lineage tracing experiments have demonstrated the existence of bipotent MaSCs residing in the basal layer [69] and of unipotent basal and luminal SCs [67] (white cells). The ducts are surrounded by fibroblasts, collagen fibrils, and circulating cells of the immune system, all embedded in the adipose tissue. The invasion of the fat pad starts at puberty from the TEBs. The TEB is considered a SC niche where cap cells and body cells highly proliferate constituting the leading edge of the invasion front. The cap cell layer contains MaSCs (white cells) that are able to undergo symmetric and asymmetric self-renewal [72,75,95]. Cap cells' mitotic divisions taking place perpendicularly to the basement membrane are asymmetric, as one of the daughter cell abandons the contacts with the original niche (dividing cells at the bottom of the bud). Cap cells' mitotic divisions parallel to the basement membrane are considered symmetric and guarantee maintenance of SC identity and expansion of SC number (dividing cells at the top of the bud) [95,99,100]. It is not known whether parallel division could also result in asymmetric fate and lead to the generation of more differentiated cap cells (dividing cells on the right side of the bud). (B) Stages of mammary gland development. At birth, the mammary epithelium is only rudimentary and remains quiescent until puberty. During puberty, hormonal cues stimulate the formation of TEBs and the elongation, bifurcation, and side-branching of the mammary tree. The gland reaches its full development and functional specialization upon pregnancy when alveologenesis and lactogenesis take place. After lactation, the epithelium involutes and returns to a state that resembles that of the virgin gland. (C) Mammary epithelium cell hierarchy. Schematic summary of the available markers used to isolate distinct subsets of mammary cells at different differentiation stages.

Figure 3.

experimental setting of the transplantation assay, which induced the differentiation of basal MaSCs into both epithelial lineages.

However, a later study showed that traced progenitor cells expressing Wnt-responsive Axin2 can switch their fate depending on the developmental stage of the mammary glands, giving rise to either the basal or the luminal lineage [68]. Indeed, clonal experiments tracing Axin2⁺ cells through multiple rounds of pregnancy, lactation, and involution proved the existence of a bipotent stem cell and found clonal Axin2⁺ clusters containing adjacent labeled basal and luminal alveolar cells. These cells were long-lived and continued to give rise to both basal and luminal offspring in subsequent pregnancies. These data support a model in which Axin2 marks a bipotent adult stem cell, suggesting that unipotent and multipotent stem cells might co-exist in the mammary epithelium.

In line with these findings, taking advantage of 3D imaging techniques, Rios *et al* tracked bipotent MaSCs *in situ* and analyzed their clonal dynamics, showing that, in both the pubertal and the adult glands, cells expressing either the K5 or the K14 promoter are capable of generating both basal and luminal progeny and of surviving for long periods of time, coordinating ductal homeostasis. Furthermore, different clones of myoepithelial and luminal cells compose the alveolar structure upon induction of pregnancy. Of note, each alveolus contained unicolored luminal cells, indicating their origin from one stem cell [69]. This evidence implies that a MaSC undergoes rounds of asymmetric divisions in order to generate such a diverse progeny, as previously demonstrated by *in vitro* studies of individual asymmetric mitoses of isolated MaSCs [66,70,71]. Also, the fact that rare cells co-expressing markers of basal and luminal differentiation (K5, K14, and Elf5) can be tracked in the lineage tracing experiments, and that K5⁺ cells are observed in the basal layer adjacent to luminal cells, indicates the execution of an asymmetric cell division program in MaSCs [69]. ACD was formally visualized *in situ* only recently though, exclusively in the end buds of the pubertal gland (which are thought to be the site of the mammary gland self-renewal), by the localization of LGN and NuMA in a crescent structure on one side of the dividing cells, in both cap cells and body cells (Fig 3A) [72].

Regardless of the putative bipotency of MaSCs, ACD is required for both unilineage and multilineage differentiation, as progenitors at multiple developmental stages seem to co-exist in the mammary epithelium. Nevertheless, their precise characterization is still wanting and their definition greatly varies among research studies based on the adoption of different cocktails of surface markers (Fig 3C) or cell-based assays. This poses an important issue as the lack of a defined hierarchy complicates the possibility of correctly determining and technically showing ACDs right at the moment of generation of two cells with different cell fates.

Tissue polarity and cell polarity

In stratified epithelia such as those found in the mammary gland, where the maintenance of an apico-basal polarity is required for the proper functioning of the organ, the nature and function of MaSCs are clearly crucial. Disruption of such polarized architecture is one of the hallmarks of breast cancer development [73].

Tissue polarity starts within individual mammary epithelial cells that compartmentalize their plasma membrane in distinct functional and structural domains in order to establish an apical side, deputed to the secreting function of the luminal cells, and a basal surface,

which interacts with contacting epithelial or stromal cells and the extracellular matrix (ECM). Tight junctions ensure the separation between the two domains and direct intracellular signaling pathways and cytoskeleton dynamics. Furthermore, at the intracellular level, polarized epithelia orient their Golgi stacks toward the apical membrane, thus directing intracellular and vesicular traffic. This results in the regulation of the spindle orientation, which coordinates the plane of cell division and therefore determines the localization of the daughter cell after mitosis. Establishment of an apico-basal polarization in mammary cells requires the recruitment of Par proteins at the apical side and of the Scribble complex at the basolateral side [74].

Par3 regulates morphogenesis Par3 is a scaffolding protein deputed for the spatial localization of many signaling effectors and their recruitment to the apical side. In 2009, Macara *et al* used limiting dilution transplantation (see Box 1 for a definition) to analyze the relevance of Par3 for mammary gland development [75]. By these means, they proved that Par3 is essential for the complete reconstitution of the mammary gland by a population of mammary cells in a virgin host. In particular, the mammary outgrowths derived from cells interfered for Par3 exhibited defects in the process of TEB elongation [75]. Par3 depletion also leads to the accumulation of cells co-expressing both luminal (K8) and basal (K14) markers throughout the whole ducts; these cells might represent a population of immature bipotent progenitors [61,65,76]. This occurrence could be an indication of increased number of symmetric rather than asymmetric divisions, with the net result of an unbalanced differentiated progeny. Indeed, Par3 silencing reduces the number of myoepithelial cells and, at the histological level, leads to the formation of highly disorganized ducts at the basal layer [75].

Disruption of the Par3/aPKC complex leads to breast cancer Par3 binding to aPKC seems to be crucial for organ morphogenesis, as the Par3 splice variant lacking the aPKC-binding domain is not able to rescue the above-described phenotype. Indeed, deregulation or mislocalization of aPKC is responsible for epithelial mis-organization [77,78], as well as increased invasion of breast cancer cells and poorer prognosis [79]. The mechanism downstream of the Par3–aPKC axis that leads to tumor development seems to progress toward the activation of Stat3 and induction of ECM disaggregation [80]. Furthermore, in an *in vitro* system of cultured MCF10a acini, constitutive activation of the ErbB2 receptor, which mimics an event that occurs in 15–20% of human breast cancers [81], was shown to lead to severe disruption of apical polarity, as a consequence of the association of ErbB2 with aPKC and Par6 and decreased Par3–aPKC interaction [82].

Par3L loss impairs MaSCs' self-renewal via regulation of Lkb1 kinase Another member of the Par proteins that has been studied for its role in the self-renewal of mammary stem and progenitor cells is Par3-like (Par3L). *In vivo*, primary cells harboring Par3L-shRNA possess lower reconstitution abilities than WT cells in limiting dilution transplantation assays and, *in vitro*, form lower number of colonies [83]. Importantly, putative MaSCs interfered for Par3L and marked by GFP positivity, isolated on the basis of the expression of the SHIP promoter [84,85], were capable of forming colonies that retained a significantly lower number of GFP⁺

cells compared to WT MaSCs, suggesting that Par3L depletion causes loss of mammary stem cells. As also reported for Par3 RNAi [75], this failure of self-renewal was especially evident in the myoepithelial lineage, with a strong decrease in the generation of K14⁺ differentiated cells. In the case of Par3L-shRNA however, the authors do not report any accumulation of intermediate progenitors [83], indicating that loss of Par3L might not favor symmetric self-renewal. Interestingly, the mechanism proposed by the authors for the maintenance of MaSC functions involves the suppression of Lkb1 kinase (the human homolog of *C. elegans* Par4) activity. Lkb1 is the only member of the family of Par proteins with a recognized tumor suppressor role [86,87]. It appears that in securing the mechanisms preventing tumor development, many genes with a role in tumor suppression also preserve stem cell state. High levels of Lkb1, for instance, were shown to be deleterious for MaSCs which became unable to form colonies *in vitro*, and Par3L seems to be the factor that regulates the abundance of Lkb1 inside a cell [83].

The Scribble complex antagonizes polarization As regards the basolateral side of the cell, the Scribble complex (composed by Scrib, DLG, and Lgl) is in charge of antagonizing the apical complexes and restricting the apical domain. Of note, Scrib, DLG, and Lgl are lost or mislocalized in many types of cancer, and down-regulation of Scribble has been demonstrated to be an initiating event in the transformation of the mammary epithelium [88]. Furthermore, silencing of Scrib in murine mammary cells appeared to cooperate with c-Myc in fostering proliferation but had no effect on tumor latency, and did not induce metastasis [89]. However, its precise effect on MaSCs and on the regulation of ACD and polarity has not been addressed yet.

Altogether, these findings highlight that polarity proteins affect MaSCs' ability to correctly differentiate and give rise to the mature mammary epithelium after puberty (or upon transplantation in the cleared fat pad). Nonetheless, none of the evidence described here provides direct and mechanistic insights in the perturbation of the ACD program execution. The exhibition of peculiar differentiation markers, or impairment in the generation of the progeny, suggests, though, that the ACD machinery is profoundly involved in the morphogenesis of the mammary gland.

Guided by the knowledge available from model systems like *Drosophila*, many research groups have been trying to unravel the role of these polarity determinants in the regulation of ACD and tissue apico-basal polarity also in mammals, but the task has turned out to be extremely challenging, suggesting that the translation of these mechanisms in the mammalian system is not straightforward.

Spindle orientation

Proper execution of ACD depends on spindle coupling with epithelial polarity.

mInsc scaffolding role is essential for proper ACD The Par3/Par6/aPKC complex interacts with mInsc, which in turn binds to the microtubule-associated complex NuMA/LGN/G α i whose role is to contact and tether the spindle (see Table 1). Molecularly, in asymmetrically dividing stem cells, mInsc is believed to bring LGN to the

apical cortex, this way favoring ACD [90]. A recent paper showed that deregulation of mInsc in murine models leads to the skewing of the mode of division of mammary stem cells from asymmetric to symmetric [72]. In this work, the execution of ACD is demonstrated by the localization of LGN and NuMA in a crescent structure above one mitotic spindle pole and is visualized exclusively in the end buds (both cap and body cells), although it is not possible to appreciate whether these dividing cells are MaSCs or dividing progenitors. Intriguingly, in a murine model in which mInsc is overexpressed, the frequency of asymmetric divisions is significantly reduced in favor of the symmetric modality [72]. This was confirmed also *in vitro* through a label-retaining assay based on a membrane dye, the PKH26 (henceforth PKH), which allows tracking the proliferative fate of labeled cells (see Box 1). A PKH⁺ stem cell dividing asymmetrically generates one cell that remains quiescent, and therefore retains the label, and another cell that continues to divide. By these means, Ballard *et al* [72] were able to show that overexpression of mInsc leads to a prevalence of proliferative and likely symmetric divisions, and to larger mammary glands, as observed in the transplantation assay. Whether the mInsc-induced increase in SCDs is a consequence of its high overexpression that in turn regulates other LGN functions still remains an open issue. The balance between symmetric and asymmetric divisions is also regulated by extrinsic factors, as earlier mentioned in this review, and the mammary gland tissue does not represent an exception in this respect. Indeed, mInsc abundance seems to be dictated by the extent of SLIT2 ligand signaling (expressed by cap and body cells in the end buds), on its receptor ROBO1 [72], and on the downstream activation of Snail.

Huntingtin (HTT) controls localization of polarity proteins Similar to mInsc, HTT also functions as an adaptor between the spindle pole and the microtubule, being able to bind Dynein/Dynactin/NuMA/LGN and determining their apical localization [91]. Cre-mediated depletion of HTT in the basal compartment of the mammary epithelium (K5-expressing cells) led to proliferation defects in terms of colony formation and to an unbalanced ratio of basal to luminal cell fate determination [91,92]. This was accompanied by *in vivo* expansion of the K8-K14 double-positive compartment, a phenomenon already described for Par3 inhibition [75], which indicates the accumulation of potential bipotent progenitors generated by symmetric division. HTT depletion disrupts the apico-basal polarity of the mammary tissue by randomizing the orientation of the mitotic spindle in the basal cells. Interestingly, HTT loss is a frequent event in mammary carcinogenesis [93].

Aurora-A kinase (AurkA) associates with the mitotic spindle and orchestrates the localization of fate determinants In fly neuroblasts, AurkA contributes to ACD by phosphorylating the Par complex and determining asymmetric localization of Numb [94]. Its impairment causes mis-oriented symmetric divisions and results in aberrant expansion of the NB compartment [10,11]. Likewise, in vertebrates, AurkA has been reported to play a role in fate control mechanisms in stem cells. One study confirmed the pivotal role of AurkA in the correct execution of ACD in the normal mammary gland. When the mammary cells were transduced with a mutated form of AurkA, unable to associate with the mitotic spindle, their ability to proliferate and differentiate was impaired, with an

unbalanced proportion of basal versus luminal cells generated. Furthermore, the self-renewal potential of the MaSCs was severely diminished, since the cells expressing AurkA-mut were not able to survive serial transplantation [95] (see Box 1). As mentioned, AurkA is an important regulator of the spindle positioning in NBs. Regan and colleagues demonstrated that enhanced expression of WT AurkA favors mitotic divisions in the cap cells of the TEBs, with a perpendicular orientation with respect to the basement membrane resulting in an increased proportion of luminal cells [95]. Although also in this case we cannot determine the identity of the dividing cells (stem cells or progenitors), these findings suggest that the perpendicular divisions might be of the asymmetric type, with the daughter cells destined to different localizations in the multilayered epithelium and possibly diverse cell fates, even if this remains to be experimentally assessed. Concordantly, cells expressing a mutated AurkA mainly divide parallel to the basement membrane and only give rise to basal cells. The observed phenomena are clearly compatible with both unilineage and multilineage asymmetric divisions.

Altogether, these observations highlight the importance of regulators of spindle assembly and spindle orientation to determine cell fate in the mammary gland upon mitotic division. In a confined space such as the one in which the mammary epithelial cells need to co-exist, the localization of the daughter cell after mitosis is crucial, as the paracrine signaling from the surrounding niche has huge influence on cell fate decisions.

Niche-dependent signaling

All SC functions, including the ability to self-renew and generate differentiated progeny, reflect their capacity to comply with the changing demand of the tissues. The interaction between stem cells and the surrounding niche, the microenvironmental signals, constitutes *de facto* the functional unit of a tissue. The mammary epithelium is embedded in a vast stroma where the extracellular matrix (ECM) plays a great role in determining the epithelial polarity and in favoring the formation of lumens.

β 1-integrin bridges ECM stimuli and intrinsic cell polarity Integrins are the immediate sensors of the ECM signals. In the mammary cells, they are involved in a number of signaling networks, transducing the microenvironmental stimuli into intracellular responses [96]. Notably, β 1-integrin (CD29) and α 6-integrin (CD49f) are among the widely accepted surface markers utilized to isolate MaSCs by FACS [61], while the expression of β 3-integrin (CD61) labels luminal early progenitors [97]. Nonetheless, for most of them, a precise role in stem cell biology is not yet known.

β 1-integrin has been widely studied in the context of cytoskeleton regulation and polarity determination. Its specific deletion in the luminal compartment (WAP-expressing cells) impairs the capacity of mammary fragments to form outgrowths in a recipient's cleared fat pad [98], hinting at defects in the luminal progenitors' ability to self-renew. Conversely, genetic removal of β 1-integrin in basal cells, which are enriched in MaSCs, profoundly changes the orientation of TEB divisions, leading to severe perturbation of the balance between cell lineages [99]. More specifically, β 1-integrin-ablated glands fail to reconstitute a mammary tissue upon serial transplantation, exhibiting high impairment in both proliferation and self-renewal abilities. This phenotype is accompanied by an

aberrant TEB-like structure formation upon induction of pregnancy in β 1-integrin floxed/floxed (β 1^{fl/fl}) mice. Collectively, these findings suggest the presence of potential defects in the execution of ACD. Taddei *et al* addressed, mechanistically, the involvement of β 1-integrin in spindle orientation by measuring the angle of the spindle axis compared to the basement membrane in the TEB basal cells of a pregnant gland, and found that WT cells mainly divide parallel to the basement membrane, while the absence of β 1-integrin allows more perpendicular divisions [99]. These data indicate that, upon pregnancy and consequent expansion of the mammary epithelium, basal cells only rarely undergo perpendicular divisions [99]. This was confirmed also during normal maintenance of mature estrus-staged glands [100]. In contrast, in the β 1-integrin-depleted epithelium, basal cells significantly contribute to the luminal compartment as a result of the increased frequency of perpendicular divisions [99]. Thus, the expansion of WT mammary cells at pregnancy might mainly rely on the symmetric self-renewal of basal cells, which is consistent with the notion that only unipotent stem cells are responsible for gland rearrangements in adult age [67]. Similar findings were obtained by double depletion of the Timp1 and Timp3 metalloproteinases in the ECM of adult virgin glands, further underscoring the importance of the microenvironmental signals in influencing the symmetry of mitotic division [100].

In the context of microenvironmental stimuli, it is worth spending a few words on the hormone-dependent signals that primarily influence mammary cells' fate decisions. Indeed, ovarian hormones, and progesterone in particular, profoundly impact on MaSC homeostasis since both the basal and the luminal stem cell-enriched compartments are expanded at diestrus [101]. Excessive stimulation by progesterone seems to actively induce symmetric self-renewal and increase the size of the MaSC pool (as assessed by the use of CD49f, CD24, CD61 surface markers), likely via paracrine stimulation of Wnt4 and RankL signaling pathways [101]. Nevertheless, direct demonstration of an actual role of hormone stimuli in the regulation of the modality of mitotic division still remains to be provided.

Collectively, these data show how critical polarity is, and how the positioning of SCs and progenitors upon mitotic division has an impact on cell fate specification. At the intracellular level, the choice between ACD and SCD reflects the activation of signaling pathways that either maintain the mother cell identity or contribute to differentiation. In this respect, the Notch pathway appears to be crucial, as discussed more in detail in the next section. Notably, nearly all the fate determinants and niche signals described in this review have been demonstrated to directly or indirectly regulate the Notch pathway. For example, the Par3/aPKC/Par6 complex is known to interact with Numb, the best-characterized antagonist of Notch activation, determining its asymmetric enrichment at the basal site [102] and its function by phosphorylation in one of the daughter cells in *Drosophila* neuroblasts [103]. Furthermore, deregulation of the AurkA and HTT proteins promotes Notch activation in displaced suprabasal daughters, which acquire luminal cell fates [92,95]. Remarkably, different cell fate decisions can be induced in a context-dependent manner by the Notch ligand, Jagged1 [104], highlighting how much this pathway is sensitive to microenvironmental cues [99–101].

Notch/Numb/Musashi1

The effects of Notch functions are highly context-dependent The Notch pathway has a central role in the regulation of both self-renewal and differentiation of MaSCs in the normal mammary gland during development and morphogenesis [105]. Constitutive expression of active Notch4 has been shown to inhibit differentiation of mammary epithelial cells *in vitro* and normal gland morphogenesis *in vivo* [106,107]. Dontu and colleagues used the mammosphere assay, established by the same group [108], and 3D organoid cultures in Matrigel, to study the role of Notch specifically in stem cell self-renewal and the regulation of cell fate decisions in normal mammary epithelial cells. By implementing complementary approaches to activate or inhibit Notch signaling, they observed diverse effects on stem and more differentiated populations. Treatment with Notch agonists (DSL or recombinant Dll1) resulted in elevated MaSC self-renewal through symmetric divisions, as shown by the increasing number of mammospheres upon serial re-plating and their ability to subsequently give rise to multilineage progenitors in differentiation assays (see Box 1). Treatment of the cultures with Notch antagonists [Notch4 antibodies (N4Ab) or γ -secretase inhibitors (GSI)], instead, led to rapid mammosphere exhaustion. Notch activation had a proliferative effect also in the more differentiated progenitor populations, favoring, at the same time, commitment to the myoepithelial lineage [109].

Later studies, however, attributed to Notch signaling an anti-proliferative effect on MaSCs, and commitment to the luminal lineage [110–112]. Bouras *et al* reported that Notch homologs 1–3 are mainly expressed in the luminal cells, while Notch4 levels are overall lower and comparable among the different subsets. Knock-down of Cbf-1 or γ -secretase inhibition in the stem population (defined this time as CD29^{hi}CD24⁺, see Fig 3C) resulted in an expansion of the MaSC pool, which was documented by enhanced *in vivo* repopulation ability in limiting dilution transplantation assays, and increased clonogenic capacity in Matrigel cultures. On the contrary, upon exogenous constitutive expression of the Notch intracellular domain NICD1, the same cells appeared to lose their capacity to reconstitute a normal mammary gland, as they produced hyperplastic nodules with luminal cell characteristics (luminal marker expression—K8, K18, Stat5a, and E-cadherin) *in vivo*, albeit failing to reach terminal differentiation during pregnancy [110].

The outcome of Notch activation or inhibition is, overall, very much dependent on the cell context and developmental stage; in addition, discrepancies between *in vivo* and *in vitro* systems in different studies hamper the interpretation of seemingly contradicting results [113]. For instance, enforced expression of NICD1 in luminal progenitors (CD29^{low}CD24⁺CD61⁺) conferred mammosphere initiating capacity on this population and, conversely, knock-down of Cbf-1 or GSI had a restrictive effect on their proliferation in Matrigel assays [110]. Therefore, the initial observations by Dontu and colleagues in bulk mammosphere cultures could be attributed to the effect of Notch activation on luminal progenitors rather than on MaSCs. Nevertheless, as discussed in previous sections of this review, Notch signaling has been shown to respond to cell–matrix interactions and other microenvironmental cues that are involved in the regulation of ACD in mammary epithelial cells [95,99,100].

Numb is asymmetrically partitioned during ACDs and retained by the cell with SC identity Upstream of Notch is Numb, which prevents nuclear translocation of NICD and, thus, inhibits its transcriptional activity. In fly NBs, asymmetric partitioning of Numb leads to the generation of two daughter cells with distinct fates. Numb asymmetric localization during mitosis has also been used as a marker in mammary epithelial cells in order to define the modality of division [66,70], although the direct connection between the asymmetric distribution of Numb and the fate choice of daughter cells has not been yet addressed experimentally in MaSCs. Recently, Tosoni and colleagues reported that in PKH^{high} isolated MaSCs, Numb is inherited by the daughter cell that retains the stem identity [114]. This is in line with the above-described expression pattern of Notch homologs in mammary stem and progenitor cells, and the observations of luminal lineage commitment upon enforced activation of Notch signaling in CD29^{high}CD24⁺ cells [110]. Numb also seems to be involved in the regulation of MaSC ACDs through the modulation of p53, which will be discussed later [114,115].

Musashi regulates Numb and Notch expression Musashi1 (Msi1) is an RNA-binding protein that is known to regulate Numb gene expression at the translational level. It functions as a positive regulator of Notch, and its specific role in asymmetric cell division was first studied in *Drosophila* SOPs and the mammalian nervous system [116]. In breast, Msi1 was suggested as a putative marker of the MaSC compartment [117] and was shown to promote luminal progenitor cell expansion in the COMMA-1D cell line, through activation of the Notch and Wnt signaling pathways [118]. Notwithstanding the lack of a direct link to the regulation of asymmetric division in the context of normal mammary epithelial SCs, the decreased sphere-forming efficiency of the human breast cancer SUM159T cell line upon RNAi of Msi1 might be suggestive of a switch to the asymmetric mode of division [119]. In the latter study, the authors attributed the loss of self-renewal capacity of the breast CSCs to the upregulation of NF- κ B and the consequent increase in 26S proteasome activity, altogether promoting NICD degradation.

The Wnt pathway

Wnt proteins are extracellular glycosylated polypeptides acting on proximal cells that express transmembrane receptors of the Frizzled family. It is becoming increasingly clear that Wnt proteins are an additional facet in the interplay between SCs and the microenvironment by creating short-ranged signals (autocrine or paracrine) that regulate stem cell self-renewal and differentiation. Several Wnt members (i.e. Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, and Wnt10b) are expressed in the mammary glands at different developmental stages to promote growth or differentiation and to maintain tissue homeostasis [105]. However, there is no experimental proof yet of a clear implication of Wnt signaling in the regulation of ACDs in the mammary epithelium.

Wnt-1 constitutive expression perturbs MaSC functions Constitutive Wnt signaling *in vivo* has been generally associated with the formation of hyperplastic glands and/or the appearance of invasive carcinomas, due to the expansion of basal-type undifferentiated cells [61,120,121]. In particular, substantially higher numbers of “MRUs” (mammary repopulating units—defined as Lin[−]CD29^{hi}CD24⁺ cells)

were scored in the premalignant glands of a Wnt-1-driven tumor model as compared to wild-type glands. This premalignant CD29^{hi}CD24⁺ population further showed enhanced *in vivo* regenerative ability and increased tumorigenic potential (the outgrowths were profoundly hyperplastic) upon transplantation, implying a role for Wnt signaling in the self-renewal of MaSCs [61]. However, other studies have suggested that the mammary epithelial hierarchy could be perturbed in the mouse mammary tumor virus (MMTV) Wnt-1 model and that excessive Wnt-1 signaling might also elicit dedifferentiation of committed progenitors to a more stem cell-like state [122].

Axin2 is a target of the Wnt/ β -catenin pathway and affects MaSC self-renewal In contrast to the above-mentioned models, Axin2^{lacZ/lacZ} transgenic mice develop a milder phenotype of hyper-morphic, but not hyperplastic, mammary glands. The *Axin2* gene is a known target of Wnt/ β -catenin and, at the same time, a negative feedback regulator of the pathway. Thus, disruption of the gene by lacZ provides the possibility to elevate the Wnt signal in a ligand-dependent and cell-autonomous manner. Competitive transplantation assays of Axin2^{lacZ/lacZ} and wild-type MRUs revealed a correlation between responsiveness to Wnt signaling and repopulation ability, which was mirrored by the relative clonogenic capacity of the two cell types in Matrigel cultures [123]. Similarly, an increase in the absolute number of MaSCs was scored upon continuous exposure of wild-type MRUs to purified Wnt3a *in vitro*. An expanding number of colonies were formed at each successive passage, which, with the exception of GATA-3 downregulation, did not significantly differ from the untreated control in terms of size, cell composition, other differentiation markers, and proliferation and apoptosis indexes. Importantly, the Wnt-treated colonies retained their ability to generate a fully functional mammary gland upon transplantation even at late passages [123]. Despite the evidence pointing to the involvement of the Wnt pathway in the regulation of MaSC self-renewal, the possibility that the documented *in vivo* and *in vitro* growth potential might derive from a block in differentiation of the dividing cells cannot be formally excluded.

Lipoprotein receptor-related proteins (LRPs) are implicated in the canonical Wnt pathway as co-receptors of Frizzleds Dkk1, a Wnt inhibitor which functions by interfering with the Lrp5/6 receptor, was shown to limit MaSC (CD29^{hi}CD24⁺) self-renewal upon serial passaging in Matrigel [123]. Also, loss of Lrp5 was shown to confer resistance to Wnt-1-induced tumorigenesis in mice. In fact, in response to dysregulated Wnt signaling, *Lrp5*^{-/-} mice grow hypomorphic glands that are depleted of regenerative potential, as almost no positive outgrowths were scored even at high cell doses in limiting dilution transplantation assays. The authors of the study argue that, apart from the minimal growth of the mammary tree that limits, *per se*, stem cell numbers, these observations could be potentially attributed to a decrease in the number of symmetric divisions; however, another valid explanation could be that Wnt signaling is essential for MaSC and mammary progenitor survival [124]. Following up this study, Badders and colleagues further elucidated the role of Lrp5-mediated response to Wnt signaling. In brief, in mammary gland reconstitution experiments, they found that Lrp5-expressing cells possessed increased repopulation ability compared to bulk mammary epithelial suspensions, whereas Lrp5-negative cells were

depleted of stem cell properties upon transplantation. However, *Lrp5*^{-/-} glands were able to reach terminal differentiation and go through multiple rounds of lactation and involution without any apparent functional deficits. This observation, along with the elevated expression of p16^{Ink4a} and TA-p63 in *Lrp5*^{-/-} cells *in vitro*, led to the conclusion that the reported loss of regenerative potential could be associated, instead, with increased senescence [125]. In accordance with the findings based on the expression of Lrp5 [125], Axin2⁺ MRUs showed increased *in vivo* repopulation ability upon transplantation, maintaining a normal histology and the ability to undergo multiple rounds of pregnancy and involution [68,123]. Nonetheless, parallel lineage tracing experiments, using an Axin2^{CreERT2} model, pointed toward a unipotent basal stem cell identity for the Axin2⁺ cells, highlighting the discrepancy between regenerative ability and normal *in situ* developmental potential of Wnt-responsive cells [68].

p53 and downstream effectors

The tumor suppressor p53 interferes with cell cycle regulation through induction of apoptosis and senescence upon various stimuli, such as DNA damage and oxidative stress, and has also been involved in the maintenance of a quiescent stem cell pool [126].

p53 regulates stem cell number An additional tumor-suppressive function for p53 emerges from the control it exerts on the expansion of the number of SCs through regulation of the modality of cell division. In mammary epithelial cells, this was first proposed by Sherley and colleagues who reported a change in *in vitro* growth kinetics from exponential to linear upon p53 upregulation in an inducible non-tumorigenic mouse cell line. The authors reasoned that this shift in the growth pattern was compatible with asymmetric cell divisions accompanied by the generation of a quiescent stem cell pool [127]. Later studies placing Numb as an upstream regulator of p53 created further speculation on the potential role of p53 in the control of ACD in mammary epithelial cells [128].

The question was formally addressed by Cicalese and colleagues, who showed that p53 signal attenuation in the ErbB2 model of mammary tumorigenesis is responsible for the unlimited self-renewal potential of breast CSCs and their expansion in numbers through symmetric self-renewing divisions. The shift of the modality of mitoses from mainly asymmetric to symmetric was directly demonstrated by time-lapse microscopy and Numb immunostaining of dividing PKH^{high} isolated cells. The authors further documented an increased proliferative capacity of CSCs, which was shown through limiting dilution transplantation assays of both bulk and PKH subsets of WT and tumor epithelial cells. The same observations also held true for the bulk epithelial cells and mammospheres derived from the mammary glands of p53-null mice, suggesting that loss of p53 signaling is responsible for the increased frequency of symmetric divisions. Pharmacological restoration of p53 in ErbB2 cells by Nutlin-3 administration resulted in a reduction in the self-renewal potential of tumor mammospheres *in vitro* and tumor growth *in vivo*. Altogether, these data led to the conclusion that functional p53 signaling limits the expansion of MaSC numbers by imposing an asymmetric mode of division [70].

The role of p53 in governing ACD in MaSCs was further illustrated by studies from the same group, in which p53 levels were modulated in normal mammary epithelial cells using DNA damage

(DD) induction by X-ray irradiation. Under these conditions, MaSCs (PKH^{high}) exhibited a p21-dependent DD response that inhibited p53 activation. Similar to p53^{-/-} PKH^{high} cells, low levels of p53 activity in p53^{+/+} MaSCs (PKH^{high}) upon DD skewed the modality of division toward symmetric mitoses, resulting in increased self-renewal and expansion of the functional stem cell pool as seen in the mammosphere assay and upon transplantation *in vivo* [129].

The above studies established p53 as a key player in the regulation of ACD and, by extension, in the maintenance of tissue homeostasis. The control that p53 exerts on the size of the MaSC pool could be seen as part of its tumor-suppressive function, underlining the need to identify potential downstream effectors. Members of the miR34 family have been appointed as candidate p53 direct targets associated with tumor appearance and progression. Interestingly, *in vitro*, ectopic expression of miR34a or miR34c was found limiting breast CSC expansion in the mammosphere assay, while *in vivo*, restoration of miR34 in donor cells reduced tumor growth upon xenotransplantation. In both cases, the effects were mediated by Notch1 and Notch4, respectively [130,131]. Collectively, these studies implicate miR34 members in the suppression of breast CSC self-renewal capacity, albeit without a direct demonstration of their effect on the modality of division.

ACD deregulation in human breast cancers

In adult tissues, symmetric mitoses are essential to replenish the stem cell pool. However, a strict control over the number of cells with extended self-renewal capacity is crucial for the maintenance of homeostasis [132]. Perturbation of ACD regulation by genetic and/or functional alterations of the relevant molecular players and pathways may result in the expansion of the stem cell pool and/or the disruption of the polarized architecture of a given tissue and could therefore be involved in tumor initiation and progression. Indeed, stem cell content *per se*, and stem cell-related biological and molecular features have been associated with tumor aggressiveness and poor prognosis in breast cancer [63,66,133].

From a mechanistic point of view, there is evidence to support the implication of ACD dysregulation in breast tumorigenesis, but a direct demonstration of a causative link between the two has been challenging. Biological processes, including hyper-proliferation, apoptosis evasion, loss of tissue polarity, and EMT, are known to promote malignant phenotypes [88]. Decoupling *in vivo* these processes from the effect of deregulated ACDs is not trivial. In most cases, the relevance of ACD deregulation in tumorigenesis might be inferred on the basis of the physiological function of the players defining cell polarity, spindle orientation, or niche contacts. For instance, polarity proteins are frequently found amplified/overexpressed (e.g. aPKC, PARD6) or, alternatively, deleted/downregulated (e.g. Par3, Lkb1/Par-4) in breast cancer [80,88]. Besides gene expression deregulation, changes in the subcellular localization of some components of the polarity machinery (e.g. SCRIB) were also reported in several breast tumors [134]. Consistently, ErbB2 activation in the mammary epithelium was shown to result in loss of apico-basal polarity and unlimited growth *in situ*, through vertical stratification of luminal cells [135]. Similarly, mutant HTT was implicated in breast cancer progression, through EMT promotion, enhanced cell motility, and proliferation [93]. Finally, AurkA was found mutated or highly

expressed in human breast cancers [95], while β 1-integrin was associated with the growth and metastatic capacity of ErbB2 tumors [136].

The activation of the self-renewing pathways discussed in this review also leads to pleiotropic cancer-promoting effects, including ACD deregulation. The implication of Notch and Wnt genes in breast cancer development became evident already in the 1980s, during studies of insertional mutagenesis using MMTV [120,137,138]. Later, it was shown that both the ectopic expression of truncated forms of Notch receptors and the direct activation of the downstream effector (RBPj κ) alone were sufficient to transform normal mammary epithelial cells [139–141]. Similar observations were also reported for some Wnt family members, namely Wnt-1, Wnt-3a, and Wnt-7a [142]. In line with studies in mouse models [110], NICD accumulation and aberrant Notch signaling were observed in the luminal epithelium of primary tumors [141]. Importantly, exogenous Numb expression in human breast cancer cell lines exhibiting abnormal Notch activation, or in primary tumor cells deficient for Numb, leads to attenuation of the respective cancer-related phenotypes [141,143]. In line with these findings, Numb is frequently lost in the most aggressive human breast carcinomas, which exhibit poor prognosis in the clinic [143]. The oncosuppressor activity of Numb in breast tumors is not attributed, however, exclusively to the modulation of Notch signaling, but also to the stabilization of p53 [114,128,143]. Notably, loss of functional p53 signaling results in a higher rate of symmetric divisions in Numb-deficient and ErbB2 tumor stem cells (PKH^{high}). Consistently, restoration of p53 activity is sufficient, alone, to inhibit unlimited breast CSC expansion, *in vitro*, and to block tumor growth, *in vivo* [70,114].

Conclusions and perspectives

One of the most remarkable findings of the last decade in tumor biology is that tumors grow as abnormal tissues, where heterogeneous cell populations are organized hierarchically with specialized cells placed at the apex of the cellular hierarchy (CSCs or tumor-initiating cells). This concept was first established in hematological malignancies [144] and later confirmed in several solid tumors, including breast cancer [145]. CSCs share many characteristics with normal somatic stem cells, including their ability to generate tissue heterogeneity by the alternate use of self-renewing asymmetric divisions and proliferative symmetric divisions.

Great advances into stem cell biology and their mode of division have been recently obtained in vertebrates, with experimental strategies often guided by the knowledge previously acquired in invertebrate systems, including *Drosophila* NBs, SOPs, and GSCs. Consistently, *in vivo* and *in vitro* studies of MaSCs have revealed fundamental principles of their proliferation potential during development and the various stages of female adult life. However, our molecular knowledge on the *in vivo* execution of symmetric and asymmetric cell divisions of MaSCs is still at its infancy. We believe that the identification of mammary stem cell markers, specific niche signals, and unequally segregating fate determinants will be key to better dissect the mechanistic of MaSC ACDs *in vivo*.

Increasing evidence points at the notion that the same signaling pathways governing stem cell homeostasis in normal MaSCs also

Sidebar A: In need of answers

- (i) Do MaSCs divide asymmetrically in the mammary glands?
- (ii) Is orientation of division compared to the basement membrane linked to asymmetry in MaSCs?
- (iii) Which are good fate determinants for MaSC ACDs?
- (iv) Is the balance between symmetric versus asymmetric MaSC divisions involved in breast cancer initiation and progression? If so, would it be possible to devise therapeutical strategies targeting ACD deregulation in breast cancers?
- (v) Are there assays that can be used to readily identify ACD deregulation in clinical samples *ex vivo*?

work in cancer MaSCs, although their dysregulation in pathological conditions might alter the proportion of asymmetric versus symmetric mitoses occurring in physiological conditions. Most intriguingly, such differential regulation, if understood at a molecular scale, may provide a unique therapeutic window for the selective eradication of breast CSCs. As mentioned above, poorly differentiated breast cancers are intrinsically enriched in CSCs, due to a shift of the physiological asymmetric mode of division toward a symmetric one [66]. In this view, loss of ACD regulation could be envisioned as a key oncogenic event that, notwithstanding the limitations of the presently available assays, should be further elucidated in vertebrate model systems in order to reveal potentially druggable targets of a prominent tumor-promoting mechanism.

Acknowledgements

We are grateful to Paola Dalton for critical reading of the manuscript. This work is supported by the Italian Association for Cancer Research (AIRC) and the Italian Ministry of Health.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Knoblich JA (2010) Asymmetric cell division: recent developments and their implications for tumour biology. *Nat Rev Mol Cell Biol* 11: 849–860
2. Caussinus E, Gonzalez C (2005) Induction of tumor growth by altered stem-cell asymmetric division in *Drosophila melanogaster*. *Nat Genet* 37: 1125–1129
3. Bowman SK, Neumuller RA, Novatchkova M, Du Q, Knoblich JA (2006) The *Drosophila* NuMA homolog Mud regulates spindle orientation in asymmetric cell division. *Dev Cell* 10: 731–742
4. Beck B, Blanpain C (2013) Unravelling cancer stem cell potential. *Nat Rev Cancer* 13: 727–738
5. Betschinger J, Mechtler K, Knoblich JA (2006) Asymmetric segregation of the tumor suppressor brat regulates self-renewal in *Drosophila* neural stem cells. *Cell* 124: 1241–1253
6. Betschinger J, Knoblich JA (2004) Dare to be different: asymmetric cell division in *Drosophila*, *C. elegans* and vertebrates. *Curr Biol* 14: R674–R685
7. Kraut R, Chia W, Jan LY, Jan YN, Knoblich JA (1996) Role of inscuteable in orienting asymmetric cell divisions in *Drosophila*. *Nature* 383: 50–55
8. Izumi Y, Ohta N, Hisata K, Raabe T, Matsuzaki F (2006) *Drosophila* Pins-binding protein Mud regulates spindle-polarity coupling and centrosome organization. *Nat Cell Biol* 8: 586–593
9. Siller KH, Cabernard C, Doe CQ (2006) The NuMA-related Mud protein binds Pins and regulates spindle orientation in *Drosophila* neuroblasts. *Nat Cell Biol* 8: 594–600
10. Lee CY, Andersen RO, Cabernard C, Manning L, Tran KD, Lanskey MJ, Bashirullah A, Doe CQ (2006) *Drosophila* Aurora-A kinase inhibits neuroblast self-renewal by regulating aPKC/Numb cortical polarity and spindle orientation. *Genes Dev* 20: 3464–3474
11. Wang H, Somers GW, Bashirullah A, Heberlein U, Yu F, Chia W (2006) Aurora-A acts as a tumor suppressor and regulates self-renewal of *Drosophila* neuroblasts. *Genes Dev* 20: 3453–3463
12. Johnston CA, Hirono K, Prehoda KE, Doe CQ (2009) Identification of an Aurora-A/Pins/LINKER/Dlg spindle orientation pathway using induced cell polarity in S2 cells. *Cell* 138: 1150–1163
13. Yu F, Morin X, Kaushik R, Bahri S, Yang X, Chia W (2003) A mouse homologue of *Drosophila* pins can asymmetrically localize and substitute for pins function in *Drosophila* neuroblasts. *J Cell Sci* 116: 887–896
14. Knoblich JA (2008) Mechanisms of asymmetric stem cell division. *Cell* 132: 583–597
15. Xie J, Wooten M, Tran V, Chen BC, Pozmanter C, Simbolon C, Betzig E, Chen X (2015) Histone H3 threonine phosphorylation regulates asymmetric histone inheritance in the *Drosophila* male germline. *Cell* 163: 920–933
16. Tran V, Lim C, Xie J, Chen X (2012) Asymmetric division of *Drosophila* male germline stem cell shows asymmetric histone distribution. *Science* 338: 679–682
17. Yamashita YM, Jones DL, Fuller MT (2003) Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. *Science* 301: 1547–1550
18. Sheng XR, Matunis E (2011) Live imaging of the *Drosophila* spermatogonial stem cell niche reveals novel mechanisms regulating germline stem cell output. *Development* 138: 3367–3376
19. Scadden DT (2014) Nice neighborhood: emerging concepts of the stem cell niche. *Cell* 157: 41–50
20. Yamashita YM, Mahowald AP, Perlin JR, Fuller MT (2007) Asymmetric inheritance of mother versus daughter centrosome in stem cell division. *Science* 315: 518–521
21. Januschke J, Llamazares S, Reina J, Gonzalez C (2011) *Drosophila* neuroblasts retain the daughter centrosome. *Nat Commun* 2: 243
22. Segalen M, Johnston CA, Martin CA, Dumortier JG, Prehoda KE, David NB, Doe CQ, Bellaiche Y (2010) The Fz-Dsh planar cell polarity pathway induces oriented cell division via Mud/NuMA in *Drosophila* and zebrafish. *Dev Cell* 19: 740–752
23. Coumalleau F, Furthauer M, Knoblich JA, Gonzalez-Gaitan M (2009) Directional delta and Notch trafficking in Sara endosomes during asymmetric cell division. *Nature* 458: 1051–1055
24. Derivery E, Seum C, Daeden A, Loubery S, Holtzer L, Julicher F, Gonzalez-Gaitan M (2015) Polarized endosome dynamics by spindle asymmetry during asymmetric cell division. *Nature* 528: 280–285
25. Colom B, Jones PH (2016) Clonal analysis of stem cells in differentiation and disease. *Curr Opin Cell Biol* 43: 14–21
26. Xin T, Greco V, Myung P (2016) Hardwiring stem cell communication through tissue structure. *Cell* 164: 1212–1225
27. Rompolas P, Deschene ER, Zito G, Gonzalez DG, Saotome I, Haberman AM, Greco V (2012) Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration. *Nature* 487: 496–499

28. Fuchs E (2016) Epithelial skin biology: three decades of developmental biology, a hundred questions answered and a thousand new ones to address. *Curr Top Dev Biol* 116: 357–374
29. Poulson ND, Lechler T (2010) Robust control of mitotic spindle orientation in the developing epidermis. *J Cell Biol* 191: 915–922
30. Clayton E, Doupe DP, Klein AM, Winton DJ, Simons BD, Jones PH (2007) A single type of progenitor cell maintains normal epidermis. *Nature* 446: 185–189
31. Lechler T, Fuchs E (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature* 437: 275–280
32. Niessen MT, Scott J, Zielinski JG, Vorhagen S, Sotiropoulou PA, Blanpain C, Leitges M, Niessen CM (2013) α PKC λ^a controls epidermal homeostasis and stem cell fate through regulation of division orientation. *J Cell Biol* 202: 887–900
33. Williams SE, Beronja S, Pasolli HA, Fuchs E (2011) Asymmetric cell divisions promote Notch-dependent epidermal differentiation. *Nature* 470: 353–358
34. Seldin L, Poulson ND, Foote HP, Lechler T (2013) NuMA localization, stability, and function in spindle orientation involve 4.1 and Cdk1 interactions. *Mol Biol Cell* 24: 3651–3662
35. Le Roy H, Zuliani T, Wolowiczuk I, Faivre N, Jouy N, Masselot B, Kerkaert J-P, Formstecher P, Polakowska R (2009) Asymmetric distribution of epidermal growth factor receptor directs the fate of normal and cancer keratinocytes *in vitro*. *Stem Cells Dev* 19: 209–220
36. Youssef KK, Van Keymeulen A, Lapouge G, Beck B, Michaux C, Achouri Y, Sotiropoulou PA, Blanpain C (2010) Identification of the cell lineage at the origin of basal cell carcinoma. *Nat Cell Biol* 12: 299–305
37. Larsimont JC, Youssef KK, Sanchez-Danes A, Sukumaran V, Defrance M, Delatte B, Liagre M, Baatsen P, Marine JC, Lippens S et al (2015) Sox9 controls self-renewal of oncogene targeted cells and links tumor initiation and invasion. *Cell Stem Cell* 17: 60–73
38. Mesa KR, Rompolas P, Greco V (2015) The dynamic duo: niche/stem cell interdependency. *Stem Cell Rep* 4: 961–966
39. Rompolas P, Mesa KR, Greco V (2013) Spatial organization within a niche as a determinant of stem-cell fate. *Nature* 502: 513–518
40. Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E (2004) Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 118: 635–648
41. Li L, Clevers H (2010) Coexistence of quiescent and active adult stem cells in mammals. *Science* 327: 542–545
42. Ouspenskaia T, Matos I, Mertz AF, Fiore VF, Fuchs E (2016) WNT-SHH antagonism specifies and expands stem cells prior to niche formation. *Cell* 164: 156–169
43. Tumber T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E (2004) Defining the epithelial stem cell niche in skin. *Science* 303: 359–363
44. Deschene ER, Myung P, Rompolas P, Zito G, Sun TY, Taketo MM, Saotome I, Greco V (2014) β -Catenin activation regulates tissue growth non-cell autonomously in the hair stem cell niche. *Science* 343: 1353–1356
45. Barker N (2014) Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol* 15: 19–33
46. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ et al (2007) Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449: 1003–1007
47. Mustata RC, Vasile G, Fernandez-Vallone V, Strollo S, Lefort A, Libert F, Monteyne D, Perez-Morga D, Vassart G, Garcia MI (2013) Identification of Lgr5-independent spheroid-generating progenitors of the mouse fetal intestinal epithelium. *Cell Rep* 5: 421–432
48. Itzkovitz S, Blat IC, Jacks T, Clevers H, van Oudenaarden A (2012) Optimality in the development of intestinal crypts. *Cell* 148: 608–619
49. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ et al (2009) Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. *Nature* 459: 262–265
50. Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD et al (2010) Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 143: 134–144
51. Lopez-Garcia C, Klein AM, Simons BD, Winton DJ (2010) Intestinal stem cell replacement follows a pattern of neutral drift. *Science* 330: 822–825
52. Quyn AJ, Appleton PL, Carey FA, Steele RJ, Barker N, Clevers H, Ridgway RA, Sansom OJ, Nathke IS (2010) Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. *Cell Stem Cell* 6: 175–181
53. Bellis J, Duluc I, Romagnolo B, Perret C, Faux MC, Dujardin D, Formstone C, Lightowler S, Ramsay RG, Freund JN et al (2012) The tumor suppressor Apc controls planar cell polarities central to gut homeostasis. *J Cell Biol* 198: 331–341
54. Schepers AG, Vries R, van den Born M, van de Wetering M, Clevers H (2011) Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes. *EMBO J* 30: 1104–1109
55. Farin HF, Jordens I, Mosa MH, Basak O, Korving J, Tauriello DV, de Punder K, Angers S, Peters PJ, Maurice MM et al (2016) Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. *Nature* 530: 340–343
56. Habib SJ, Chen BC, Tsai FC, Anastassiadis K, Meyer T, Betzig E, Nusse R (2013) A localized Wnt signal orients asymmetric stem cell division *in vitro*. *Science* 339: 1445–1448
57. Bu P, Chen KY, Chen JH, Wang L, Walters J, Shin YJ, Goerger JP, Sun J, Witherspoon M, Rakhilin N et al (2013) A microRNA miR-34a-regulated bimodal switch targets Notch in colon cancer stem cells. *Cell Stem Cell* 12: 602–615
58. Bu P, Wang L, Chen KY, Srinivasan T, Murthy PK, Tung KL, Varanko AK, Chen HJ, Ai Y, King S et al (2016) A miR-34a-Numb feedforward loop triggered by inflammation regulates asymmetric stem cell division in intestine and colon cancer. *Cell Stem Cell* 18: 189–202
59. Joshi PA, Di Grappa MA, Khokha R (2012) Active allies: hormones, stem cells and the niche in adult mammapoiesis. *Trends Endocrinol Metab* 23: 299–309
60. DeOme KB, Faulkin LJ, Bern HA, Blair PB (1959) Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res* 19: 515–520
61. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat M-L, Wu L, Lindeman GJ, Visvader JE (2006) Generation of a functional mammary gland from a single stem cell. *Nature* 439: 84–88
62. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ (2006) Purification and unique properties of mammary epithelial stem cells. *Nature* 439: 993–997
63. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer C, Liu S et al (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 1: 555–567

64. Keller PJ, Arendt LM, Skibinski A, Logvinenko T, Klebba I, Dong S, Smith AE, Prat A, Perou CM, Gilmore H *et al* (2012) Defining the cellular precursors to human breast cancer. *Proc Natl Acad Sci USA* 109: 2772–2777
65. Villadsen R, Fridriksdottir AJ, Ronnov-Jessen L, Gudjonsson T, Rank F, LaBarge MA, Bissell MJ, Petersen OW (2007) Evidence for a stem cell hierarchy in the adult human breast. *J Cell Biol* 177: 87–101
66. Pece S, Tosoni D, Confalonieri S, Mazzarol G, Vecchi M, Ronzoni S, Bernard L, Viale G, Pelicci PG, Di Fiore PP (2010) Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell* 140: 62–73
67. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, Sharma N, Dekoninck S, Blanpain C (2011) Distinct stem cells contribute to mammary gland development and maintenance. *Nature* 479: 189–193
68. van Amerongen R, Bowman AN, Nusse R (2012) Developmental stage and time dictate the fate of Wnt/beta-catenin-responsive stem cells in the mammary gland. *Cell Stem Cell* 11: 387–400
69. Rios AC, Fu NY, Lindeman GJ, Visvader JE (2014) *In situ* identification of bipotent stem cells in the mammary gland. *Nature* 506: 322–327
70. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, Briskin C, Minucci S, Di Fiore PP, Pelicci PG (2009) The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* 138: 1083–1095
71. Smith GH (2005) Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. *Development* 132: 681–687
72. Ballard MS, Zhu A, Iwai N, Stensrud M, Mapps A, Postiglione MP, Knoblich JA, Hinck L (2015) Mammary stem cell self-renewal is regulated by Slit2/Robo1 signaling through SNAI1 and mINSC. *Cell Rep* 13: 290–301
73. Lelièvre SA (2010) Tissue polarity-dependent control of mammary epithelial homeostasis and cancer development: an epigenetic perspective. *J Mammary Gland Biol Neoplasia* 15: 49–63
74. Muthuswamy SK, Xue B (2012) Cell polarity as a regulator of cancer cell behavior plasticity. *Annu Rev Cell Dev Biol* 28: 599–625
75. McCaffrey LM, Macara IG (2009) The Par3/aPKC interaction is essential for end bud remodeling and progenitor differentiation during mammary gland morphogenesis. *Genes Dev* 23: 1450–1460
76. Woodward WA, Chen MS, Behbod F, Rosen JM (2005) On mammary stem cells. *J Cell Sci* 118: 3585–3594
77. Hao Y, Du Q, Chen X, Zheng Z, Balsbaugh JL, Maitra S, Shabanowitz J, Hunt DF, Macara IG (2010) Par3 controls epithelial spindle orientation by aPKC-mediated phosphorylation of apical Pins. *Curr Biol* 20: 1809–1818
78. Horikoshi Y, Suzuki A, Yamanaka T, Sasaki K, Mizuno K, Sawada H, Yonemura S, Ohno S (2009) Interaction between PAR-3 and the aPKC-PAR-6 complex is indispensable for apical domain development of epithelial cells. *J Cell Sci* 122: 1595–1606
79. Kojima Y, Akimoto K, Nagashima Y, Ishiguro H, Shirai S, Chishima T, Ichikawa Y, Ishikawa T, Sasaki T, Kubota Y *et al* (2008) The overexpression and altered localization of the atypical protein kinase C lambda/iota in breast cancer correlates with the pathologic type of these tumors. *Hum Pathol* 39: 824–831
80. McCaffrey LM, Montalbano J, Mihai C, Macara IG (2012) Loss of the Par3 polarity protein promotes breast tumorigenesis and metastasis. *Cancer Cell* 22: 601–614
81. Cancer Genome Atlas N (2012) Comprehensive molecular portraits of human breast tumours. *Nature* 490: 61–70
82. Aranda V, Haire T, Nolan ME, Calarco JP, Rosenberg AZ, Fawcett JP, Pawson T, Muthuswamy SK (2006) Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. *Nat Cell Biol* 8: 1235–1245
83. Huo Y, Macara IG (2014) The Par3-like polarity protein Par3L is essential for mammary stem cell maintenance. *Nat Cell Biol* 16: 529–537
84. Rohrschneider LR, Custodio JM, Anderson TA, Miller CP, Gu H (2005) The intron 5/6 promoter region of the ship1 gene regulates expression in stem/progenitor cells of the mouse embryo. *Dev Biol* 283: 503–521
85. Bai L, Rohrschneider LR (2010) s-SHIP promoter expression marks activated stem cells in developing mouse mammary tissue. *Genes Dev* 24: 1882–1892
86. Jansen M, Ten Klooster JP, Offerhaus GJ, Clevers H (2009) LKB1 and AMPK family signaling: the intimate link between cell polarity and energy metabolism. *Physiol Rev* 89: 777–798
87. McCarthy A, Lord CJ, Savage K, Grigoriadis A, Smith DP, Weigelt B, Reis-Filho JS, Ashworth A (2009) Conditional deletion of the Lkb1 gene in the mouse mammary gland induces tumour formation. *J Pathol* 219: 306–316
88. Huang L, Muthuswamy SK (2010) Polarity protein alterations in carcinoma: a focus on emerging roles for polarity regulators. *Curr Opin Genet Dev* 20: 41–50
89. Waghmare SK, Bansal R, Lee J, Zhang YV, McDermitt DJ, Tumber T (2008) Quantitative proliferation dynamics and random chromosome segregation of hair follicle stem cells. *EMBO J* 27: 1309–1320
90. Culurgioni S, Mapelli M (2013) Going vertical: functional role and working principles of the protein Inscuteable in asymmetric cell divisions. *Cell Mol Life Sci* 70: 4039–4046
91. Elias S, Thion MS, Yu H, Sousa CM, Lasgi C, Morin X, Humbert S (2014) Huntingtin regulates mammary stem cell division and differentiation. *Stem Cell Rep* 2: 491–506
92. Elias S, McGuire JR, Yu H, Humbert S (2015) Huntingtin is required for epithelial polarity through RAB11A-mediated apical trafficking of PAR3-aPKC. *PLoS Biol* 13: e1002142
93. Moreira Sousa C, McGuire JR, Thion MS, Gentien D, de la Grange P, Tezenas du Montcel S, Vincent-Salomon A, Durr A, Humbert S (2013) The Huntington disease protein accelerates breast tumour development and metastasis through ErbB2/HER2 signalling. *EMBO Mol Med* 5: 309–325
94. Wirtz-Peitz F, Nishimura T, Knoblich JA (2008) Linking cell cycle to asymmetric division: aurora-A phosphorylates the Par complex to regulate Numb localization. *Cell* 135: 161–173
95. Regan JL, Sourisseau T, Soady K, Kendrick H, McCarthy A, Tang C, Brennan K, Linardopoulos S, White DE, Smalley MJ (2013) Aurora A kinase regulates mammary epithelial cell fate by determining mitotic spindle orientation in a Notch-dependent manner. *Cell Rep* 4: 110–123
96. Glukhova MA, Streuli CH (2013) How integrins control breast biology. *Curr Opin Cell Biol* 25: 633–641
97. Guo W, Keckesova Z, Donaher JL, Shibue T, Tischler V, Reinhardt F, Itzkovitz S, Noske A, Zurrer-Hardi U, Bell G *et al* (2012) Slug and Sox9 cooperatively determine the mammary stem cell state. *Cell* 148: 1015–1028
98. Li N, Zhang Y, Naylor MJ, Schatzmann F, Maurer F, Wintermantel T, Schuetz G, Mueller U, Streuli CH, Hynes NE (2005) Beta1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli. *EMBO J* 24: 1942–1953
99. Taddei I, Deugnier MA, Faraldo MM, Petit V, Bouvard D, Medina D, Fassler R, Thiery JP, Glukhova MA (2008) Beta1 integrin deletion from

- the basal compartment of the mammary epithelium affects stem cells. *Nat Cell Biol* 10: 716–722
100. Jackson HW, Waterhouse P, Sinha A, Kislinger T, Berman HK, Khokha R (2015) Expansion of stem cells counteracts age-related mammary regression in compound Timp1/Timp3 null mice. *Nat Cell Biol* 17: 217–227
 101. Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, Stingl J, Waterhouse PD, Khokha R (2010) Progesterone induces adult mammary stem cell expansion. *Nature* 465: 803–807
 102. Goldstein B, Macara IG (2007) The PAR proteins: fundamental players in animal cell polarization. *Dev Cell* 13: 609–622
 103. Neumüller RA, Knoblich JA (2009) Dividing cellular asymmetry: asymmetric cell division and its implications for stem cells and cancer. *Genes Dev* 23: 2675–2699
 104. LaBarge MA, Nelson CM, Villadsen R, Fridriksdottir A, Ruth JR, Stampfer MR, Petersen OW, Bissell MJ (2009) Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. *Integr Biol* 1: 70–79
 105. Kalirai H, Clarke RB (2006) Human breast epithelial stem cells and their regulation. *J Pathol* 208: 7–16
 106. Smith GH, Gallahan D, Diella F, Jhappan C, Merlino G, Callahan R (1995) Constitutive expression of a truncated INT3 gene in mouse mammary epithelium impairs differentiation and functional development. *Cell Growth Differ* 6: 563–577
 107. Uyttendaele H, Soriano JV, Montesano R, Kitajewski J (1998) Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion. *Dev Biol* 196: 204–217
 108. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS (2003) *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* 17: 1253–1270
 109. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS (2004) Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res* 6: R605–R615
 110. Bouras T, Pal B, Vaillant F, Harburg G, Asselin-Labat M-L, Oakes SR, Lindeman GJ, Visvader JE (2008) Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. *Cell Stem Cell* 3: 429–441
 111. Buono KD, Robinson GW, Martin C, Shi S, Stanley P, Tanigaki K, Honjo T, Hennighausen L (2006) The canonical Notch/RBP-J signaling pathway controls the balance of cell lineages in mammary epithelium during pregnancy. *Dev Biol* 293: 565–580
 112. Raouf A, Zhao Y, To K, Stingl J, Delaney A, Barbara M, Iscove N, Jones S, McKinney S, Emerman J et al (2008) Transcriptome analysis of the normal human mammary cell commitment and differentiation process. *Cell Stem Cell* 3: 109–118
 113. Visvader JE (2009) Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev* 23: 2563–2577
 114. Tosoni D, Zecchini S, Cozzoli M, Colaluca I, Mazzarol G, Rubio A, Caccia M, Villa E, Zilian O, Di Fiore PP et al (2015) The Numb/p53 circuitry couples replicative self-renewal and tumor suppression in mammary epithelial cells. *J Cell Biol* 211: 845–862
 115. Faraldo MM, Glukhova MA (2015) Regulating the regulator: numb acts upstream of p53 to control mammary stem and progenitor cell. *J Cell Biol* 211: 737–739
 116. Okano H, Kawahara H, Toriya M, Nakao K, Shibata S, Imai T (2005) Function of RNA-binding protein Musashi-1 in stem cells. *Exp Cell Res* 306: 349–356
 117. Clarke RB, Spence K, Anderson E, Howell A, Okano H, Potten CS (2005) A putative human breast stem cell population is enriched for steroid receptor-positive cells. *Dev Biol* 277: 443–456
 118. Wang XY, Yin Y, Yuan H, Sakamaki T, Okano H, Glazer RI (2008) Musashi1 modulates mammary progenitor cell expansion through proliferin-mediated activation of the Wnt and Notch pathways. *Mol Cell Biol* 28: 3589–3599
 119. Lagadec C, Vlasi E, Frohnen P, Alhiyari Y, Chan M, Pajonk F (2014) The RNA-binding protein Musashi-1 regulates proteasome subunit expression in breast cancer- and glioma-initiating cells. *Stem Cells* 32: 135–144
 120. Nusse R, Varmus HE (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31: 99–109
 121. Teuliere J, Faraldo MM, Deugnier MA, Shtutman M, Ben-Ze'ev A, Thiery JP, Glukhova MA (2005) Targeted activation of beta-catenin signaling in basal mammary epithelial cells affects mammary development and leads to hyperplasia. *Development* 132: 267–277
 122. Vaillant F, Asselin-Labat ML, Shackleton M, Forrest NC, Lindeman GJ, Visvader JE (2008) The mammary progenitor marker CD61/beta3 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis. *Cancer Res* 68: 7711–7717
 123. Zeng YA, Nusse R (2010) Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. *Cell Stem Cell* 6: 568–577
 124. Lindvall C, Evans NC, Zylstra CR, Li Y, Alexander CM, Williams BO (2006) The Wnt signaling receptor Lrp5 is required for mammary ductal stem cell activity and Wnt1-induced tumorigenesis. *J Biol Chem* 281: 35081–35087
 125. Badders NM, Goel S, Clark RJ, Klos KS, Kim S, Bafico A, Lindvall C, Williams BO, Alexander CM (2009) The Wnt receptor, Lrp5, is expressed by mouse mammary stem cells and is required to maintain the basal lineage. *PLoS ONE* 4: e6594
 126. Liu Y, Elf SE, Miyata Y, Sashida G, Liu Y, Huang G, Di Giandomenico S, Lee JM, Deblasio A, Menendez S et al (2009) p53 regulates hematopoietic stem cell quiescence. *Cell Stem Cell* 4: 37–48
 127. Sherley JL, Stadler PB, Johnson DR (1995) Expression of the wild-type p53 antioncogene induces guanine nucleotide-dependent stem cell division kinetics. *Proc Natl Acad Sci USA* 92: 136–140
 128. Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, Pece S, Di Fiore PP (2008) NUMB controls p53 tumour suppressor activity. *Nature* 451: 76–80
 129. Insinga A, Cicalese A, Faretta M, Gallo B, Albano L, Ronzoni S, Furia L, Viale A, Pelicci PG (2013) DNA damage in stem cells activates p21, inhibits p53, and induces symmetric self-renewing divisions. *Proc Natl Acad Sci USA* 110: 3931–3936
 130. Park EY, Chang E, Lee EJ, Lee HW, Kang HG, Chun KH, Woo YM, Kong HK, Ko JY, Suzuki H et al (2014) Targeting of miR34a-NOTCH1 axis reduced breast cancer stemness and chemoresistance. *Cancer Res* 74: 7573–7582
 131. Yu F, Jiao Y, Zhu Y, Wang Y, Zhu J, Cui X, Liu Y, He Y, Park EY, Zhang H et al (2012) MicroRNA 34c gene down-regulation via DNA methylation promotes self-renewal and epithelial-mesenchymal transition in breast tumor-initiating cells. *J Biol Chem* 287: 465–473
 132. Clarke MF, Fuller M (2006) Stem cells and cancer: two faces of eve. *Cell* 124: 1111–1115
 133. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 40: 499–507

134. Zhan L, Rosenberg A, Bergami KC, Yu M, Xuan Z, Jaffe AB, Allred C, Muthuswamy SK (2008) Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell* 135: 865–878
135. Huebner RJ, Lechler T, Ewald AJ (2014) Developmental stratification of the mammary epithelium occurs through symmetry-breaking vertical divisions of apically positioned luminal cells. *Development* 141: 1085–1094
136. Huck L, Pontier SM, Zuo DM, Muller WJ (2010) beta1-integrin is dispensable for the induction of ErbB2 mammary tumors but plays a critical role in the metastatic phase of tumor progression. *Proc Natl Acad Sci USA* 107: 15559–15564
137. Gallahan D, Callahan R (1987) Mammary tumorigenesis in feral mice: identification of a new int locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. *J Virol* 61: 66–74
138. Gallahan D, Kozak C, Callahan R (1987) A new common integration region (int-3) for mouse mammary tumor virus on mouse chromosome 17. *J Virol* 61: 218–220
139. Dievert A, Beaulieu N, Jolicœur P (1999) Involvement of Notch1 in the development of mouse mammary tumors. *Oncogene* 18: 5973–5981
140. Robbins J, Blondel BJ, Gallahan D, Callahan R (1992) Mouse mammary tumor gene int-3: a member of the notch gene family transforms mammary epithelial cells. *J Virol* 66: 2594–2599
141. Stylianou S, Clarke RB, Brennan K (2006) Aberrant activation of notch signaling in human breast cancer. *Cancer Res* 66: 1517–1525
142. Wong GT, Gavin BJ, McMahon AP (1994) Differential transformation of mammary epithelial cells by Wnt genes. *Mol Cell Biol* 14: 6278–6286
143. Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V, Zurrida S, Maisonneuve P, Viale G, Di Fiore PP (2004) Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. *J Cell Biol* 167: 215–221
144. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 3: 730–737
145. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 100: 3983–3988
146. Rebollo E, Sampaio P, Januschke J, Llamazares S, Varmark H, Gonzalez C (2007) Functionally unequal centrosomes drive spindle orientation in asymmetrically dividing *Drosophila* neural stem cells. *Dev Cell* 12: 467–474
147. Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA (2013) Cerebral organoids model human brain development and microcephaly. *Nature* 501: 373–379
148. Orford KW, Scadden DT (2008) Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet* 9: 115–128